Dissertation

Individual metabolic phenotyping in females of different energy status

Adipose tissue, carotenoid status, leptin levels, inflammation and oxidative stress markers in the ESAN (Energy Sensing in Anorexia Nervosa) cohort

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

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Disclosures

Part of this thesis has been published in Lackner S et al. "Novel approaches for the assessment of relative body weight and body fat in diagnosis and treatment of anorexia nervosa: A cross-sectional study", Clinical Nutrition, 2019 [1]
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Abbreviations and definitions

Agrp	Agouti related peptide
AN	Anorexia nervosa
ANOVA	Analysis of variance
ARE	Antioxidant response element
AT	Athletes
atRA	All-trans-retinoic acid
BAT	Brown adipose tissue
BCO 2	β-carotene-9´,10´-oxygenase
BIA	Bioelectrical impedance analysis
BMI	Body mass index
B-mode	Brightness mode
BOC 1	β-carotene-15,15´-oxygenase
BR	Brachioradialis
C/EBPs	CCAAT-enhancer-binding proteins
CCK	Cholecystokinin
CD	Cluster of differentiation
CK	Creatin kinase
CRP	C-reactive protein
CVD	Cardiovascular diseases
DC	Dentritic cells
D_{ES}	Embedded structures
D_{EXCL}	SAT thicknesses sum without embedded tissues
D_{INCL}	SAT thickness sum of the eight measured body sites
d_M	Mean SAT thickness of the eight sites
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Health Disorders
DT	Distal triceps
DXA	Dual energy X-ray absorptiometry
ECLIA	Electro chemi-luminescent immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
EO	External oblique
ES	Erector spinae

ESAN	Energy sensing in Anorexia nervosa
FFM	Fat free mass
FM	Fat mass
FT	Front thigh
FTND	Fagerström test for nicotine dependence
GH	Growth hormone
GIT	Gastrointestinal tract
GLP-1	Gucagon-like peptide 1
H_2O	Water
H_2O_2	Hydrogen peroxide
HDL	High-density-lipoprotein
HPA	Hypothalamo-pituitary-adrenocortical- axis
HPLC	High performance liquid chromatography
ICD	International classification of diseases
IG	Immunoglobulins
IGF	Insulin growth factors
IL	Interleukin
IOC	International Olympic Committee
IPAQ	International Physical Activity Questionnaire
IQR	Interquartile ranges
ISAK	International Society for the Advancement of Kinanthropometry
Keap-1	Kelch-like ECH-associated protein 1
LA	Lower abdomen
LAP	Lipid accumulation product
LDL	Low-density-lipoprotein
LT	Lateral thigh
MAMC	Mid arm muscle circumference
MAPK	Mitogen-activated protein kinase
MC	Medial calf
MC4R	Melanocortin 4 receptors
Mdn	Median
MET	Metabolic equivalent of task
MI	Mass index
MRI	Magnetic resonance imaging

NF-κB	Nuclear factor -κB
NHANES	National Health and Nutrition Examination Survey
NIST	National Institute of Standards and Technology
NK cells	Natural killer cells
NO°	Nitric oxide
NPY	Neuropeptide Y
Nrf2	Nuclear factor erythroid 2-related factor 2
NW	Normal weight
$O_2^{\circ-}$	Superoxide anion
°OH	Hydroxyl radical
OB	Obese
OSI	Oxidative stress index
OW	Overweight
PA	Phase angle
PAL	Physical activity level
PG	Prostaglandin
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome proliferator-activated receptors γ
PRR	Pattern recognition receptors
PUFA	Polyunsaturated fatty acids
PYY	Peptide YY
R	Resistance
Rald	Retinaldehyd
RAR	Retinoic acid receptor
RED	Relative energy deficiency in sport
RED-S	Relative energy deficiency in sports syndrome
ROS	Reactive oxygen species
RXR	Retinoid X receptor
SAT	Subcutaneous adipose tissue
SD	Standard deviation
SOD	Superoxide dismutase
SRM	Standard reference material
T3	Triiodothyronine
TAC	Total antioxidant capacity

TBF	Total body fat
TCR	T-cell receptor
Th	T-helper cells
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor-α
TOC	Total oxidative capacity
Treg	Regulatory T cells
TSF	Triceps skin fold
UA	Upper abdomen
UCP1	Uncoupling protein 1
US	Ultrasound
UV	Ultraviolet
VAI	Visceral adiposity index
VAT	Visceral adipose tissue
WAT	White adipose tissue
WHO	World Health Organization
WHR	Waist to hip ratio
Xc	Reactance

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Zusammenfassung

Einleitung: Der individuelle metabolische Phänotyp wird stark durch das Körperfett und das Adipokin Leptin bestimmt. Beiden wurden modulierende Effekte auf Entzündungsprozesse und oxidativen Stress zugeschrieben. Nutritive Faktoren wie Carotinoide sind mit potentiellen regulatorischen Effekten in Adipozyten assoziiert. Das Ziel dieser Arbeit war es, Zusammenhänge von Körperfett, Leptin, Carotinoiden, Entzündungsmarkern und oxidativem Stress in einer weiblichen Kohorte unterschiedlicher Energiestatusgruppen zu evaluieren.

Methoden: In diese Querschnittsstudie wurden 107 Teilnehmerinnen entsprechend des Body Mass Index (BMI) und Aktivitätsgrads in die fünf Gruppen Anorexia nervosa (AN), Normalgewicht, Übergewicht, Adipositas und Athletinnen eingeteilt. Subkutanes Fett (SAT) wurde an acht Messpunkten mittels einer standardisierten Ultraschallmethode gemessen. Plasma-Carotinoide wurden durch Hochleistungsflüssigkeitschromatographie bzw. Carotinoide der Haut über Resonanz Raman Spektroskopie bestimmt. Leptin und oxidative Stress Marker wurden mittels Enzyme-linked Immunosorbent Assays analysiert. Die Analyse der Entzündungsmarker erfolgte im Rahmen der klinischen Labordiagnostik.

Ergebnisse: Die SAT-Summe und alle SAT-Messpunkte unterschieden sich signifikant zwischen den Gruppen ($p<0.001$). Das Plasma-Leptin korrelierte stark mit SAT ($r_s=0.895$, $p>0.001$). Einige Carotin-Werte zeigten eine negative (z.B. Haut-Carotinoide: $r_s=-0.498$, $p<0.001$, β-Carotin: $r_s=-0.563$, $p<0.001$), die Entzündungs- (CRP: $r_s=0.617$, $p<0.001$, IL-6: $r_s=0.557$, $p<0.001$) und oxidative Stressmarker (totale oxidative Kapazität: $r_s=0.379$, $p<0.001$) eine positive Korrelation mit SAT. Eine multiple lineare Regressionsanalyse ergab kollektive Signifikanz zwischen den Parametern ($p<0.001$). Auffällig waren signifikante Unterschiede der SAT-Summe ($p<0.001$) und aller SAT-Messpunkte bei AN-Patientinnen mit ähnlichem BMI. Diese Unterschiede zeigten sich ebenfalls in den Laborwerten.

Diskussion: In dieser Studie wurde die SAT-Topographie zur metabolischen Phänotypisierung herangezogen. Dabei stand die SAT-Dicke mit Plasma-Leptin, Entzündungs- und oxidativen Stressmarkern und dem Carotinoid-Status in Zusammenhang. Das metabolische Profil könnte die regulatorische Antwort auf modifizierte Fettspeicher sein. Auf Basis der beobachteten Unterschiede im SAT der AN-Patientinnen sollte die Adaptierung von Therapiekonzepten diskutiert werden. Hinsichtlich der Rolle von Phytonährstoffen auf das komplexe Regelsystem des Adipozyten bedarf es noch

weiterführender klinischer Studien, um potentielle klinische Implikationen ableiten zu können.

Schlüsselwörter: subkutanes Fettgewebe, Carotinoide, Leptin, Entzündung, oxidativer Stress, Anorexia nervosa, Athletinnen

Abstract

Background: The individual metabolic phenotype is strongly determined by body fat that is associated with the expression of the adipokine leptin. Both have been shown to alter metabolism including inflammation and oxidative stress. Nutritive factors like carotenoids are supposed to impact adipocyte properties. This thesis aimed to evaluate the association of body fat, plasma leptin, carotenoid levels, inflammation and oxidative stress in females of different energy status.

Methods: In this cross-sectional study, 107 female participants were assigned to the five body mass index (BMI) and activity level groups anorexia nervosa (AN), normal weight, overweight, obesity, and athletes. Subcutaneous adipose tissue (SAT) was measured by a standardized ultrasound method at eight body sites. Plasma and skin carotenoids were analyzed by high performance liquid chromatography and resonance Raman spectroscopy, respectively. Furthermore, leptin and oxidative stress parameters were analyzed by enzyme-linked immunosorbent assays and inflammation markers were determined according to standard clinical chemistry.

Results: SAT sums and each of the measured sites differed significantly between the groups ($p<0.001$). Plasma leptin was highly correlated to SAT ($r_s=0.895$, $p<0.001$). Some carotenoid values were correlated negatively (e.g. skin-carotenoids: $r_s=-0.498$, $p<0.001$, β-carotene: $r_s=-0.563$, $p<0.001$) while inflammation (CRP: $r_s=0.617$, $p<0.001$, IL-6: $r_s=0.557$, $p<0.001$) and oxidative stress markers (TOC: $r_s=0.379$, $p<0.001$) were correlated positively to SAT. Multiple linear regression analysis revealed collective significance between these factors ($p<0.001$). Striking differences in SAT amounts of AN patients with similar BMI ($p<0.001$) and at each SAT-site were detected. These variations corresponded with the clinical markers.

Conclusion: This study used SAT topography for metabolic phenotyping. Thereby, an association between SAT amount, plasma leptin, inflammation and oxidative stress indicators, and carotenoid status was observed. Alterations in fat depots may result in homeostatic disturbance and determine specific metabolic profiles. Based on the observed differences in AN patients, therapeutic adoptions are discussed. The potential role of phytonutrients in the complex adipocytes' regulation system needs to be further investigated in humans to derive potential clinical implications.

Keywords: subcutaneous adipose tissue, carotenoids, leptin, inflammation, oxidative stress, anorexia nervosa, athletes

1. Introduction

The individual human metabolic phenotype is strongly altered by genetic predisposition, external lifestyle factors such as diet and eating behavior, physical activity and exercise, and environmental factors including external and internal stressors [2]. The organisms' exposure to certain environmental stressors like enhanced or reduced dietary energy availability or a deficiency or overload on micronutrient level may challenge phenotypic flexibility and lead to modified metabolic responses, and adjusted regulatory processes including the immune system and energy-sensing pathways. Additionally, certain phenotypic characteristics of an individual such as excessive or severely reduced amounts of body fat as can be observed in obesity and anorexia nervosa, respectively, may challenge the organisms' individual phenotypic flexibility and may lead to severe metabolic disturbances. Chronic inflammation, enhanced immune response, and alterations in the energy metabolism are some examples of potential influences of the individuals' physical condition on metabolically altered response.

The adipokine leptin is directly linked to the amount of fat stored in adipocytes and modulates energy-sensing pathways. Additionally, it also influences the expression of immune cells [3-5]. Interestingly, dietary factors such as plant-derived nutrients like carotenoids and polyphenols impact adipocyte quality and thus body composition. Furthermore, they interact with the immune system through their anti-oxidative and anti-inflammatory properties and influence leptin secretion [6,7]. Consequently, a connection of plant nutrient intake, body composition, leptin levels, and immunomodulation is indicated. However, many aspects regarding the interaction of leptin with inflammation and the immune system remain unclear [5]. Most of the proposed interactions have been investigated isolated using in-vitro (cell) and/or in-vivo (animal) models. The complex interaction has not been investigated in humans under free living conditions.

This thesis aimed to evaluate elective parameters to distinguish phenotypic metabolic characteristics of female participants of different energy status groups in a cross-sectional setting. The results contribute to a deeper understanding of metabolic phenotyping in groups of different body composition.

1.1 Energy physiology and energy regulation

To maintain physiological function, the human organism is dependent on exogenous energy and nutrient supply. In a well-balanced system, the amount of energy provided meets the energy demand of the organism. However, if the energy availability exceeds or does not achieve the requirement the organism is forced to store the excess energy or to mobilize energy stores, respectively, to keep the system balanced. This mechanism is called homeostasis and describes the organism's preference to keep conditions stable [8,9]. However, the internal state is not only maintained in its original form, it is maintained at its current status that adopts over time according to the availability of nutritional energy and environmental factors. This regulatory mechanism is called homeorhesis [8]. Concerning energy storage and regulation this means that a long-term overload or a lack of energy supply leads to altered physiological conditions and thus altered response in energy balance. The modified body weight is set as a new reference in the brain, and metabolic regulation mechanism aim to maintain it [9].

Three major energy consumers are predominant in humans: the brain, muscles and the immune system [10]. The energy requirement is therefore not only dependent on the maintenance of physiological functions (basal metabolic rate) and the energy expenditure due to physical activity and exercise; it is additionally strongly influenced by external and internal determinants and health conditions. Several nutritional factors including body composition and thus energy storages, as well as macronutrient composition, and nutrient timing are proposed to influence energy homeostasis and eating behavior [8] and the distribution of energy supply within the three energy expenditure systems [10]. During acute and chronic – even mild - inflammatory conditions energy expenditure pathways are upregulated; however, the continuous use of inflammation-driven energy expenditure is supposed to be highly unfavorable.

Several neuroendocrine signaling molecules contribute to fat storage and fat utilization. However, they also affect inflammatory conditions [10] which are explained in more detail in the following paragraphs.

1.1.1 Energy sensing mechanisms

The term energy sensing describes the complex mechanisms of communication processes between the organism's nutritional status (the energy and nutrient supply level as well as their available stores) and the regulation system. Since energy-providing molecules are macronutrients, nutrient sensing is another terminus that can simultaneously be used for energy sensing [11]. Importantly, different stimuli of energy sensing can be distinguished. Specific afferent hormones deliver information on the current status and changes in the supply level to the brain (hypothalamus) where further energy regulation pathways are induced. Via this process, the periphery is linked to the central nervous system [8]. However, not only the availability of nutrients triggers the release of signaling molecules, but also storage conditions of energy supplying molecules take over a relevant part in energy sensing. Besides body composition – especially the amount of fat stored in the adipocytes, and the storage level of glycogen in muscle tissue – also health conditions are proposed to activate communication cascades [10].

1.1.1.1 Body composition, perception, and nutritive factors

There are several approaches to how energy sensing mechanisms may influence energy intake and eating behavior. The demand for energy due to energy expenditure may regulate dietary intake by central hunger modulation via hormonal and neuronal regulation [12]. Increased energy expenditure may lead to elevated hunger and food-seeking behavior and promote overeating and thus weight gain. Moreover, energy expenditure is dependent on body composition. Particularly the fat-free mass (FFM) is the metabolically active and energy consuming body component. It highly determines energy expenditure [12]. However, weight gain due to excess of dietary energy is associated with body fat gain, which alters body composition and thus energy expenditure. Body composition itself induces energy sensing signals via expressing adipokines and myokines according to the physical status of the organism.

Furthermore, also non-homeostatic factors influence energy intake. Energy homeostasis can easily be overruled with dietary modifications like an increase in caloric density of the consumed food, e.g., by consuming a high-fat diet. Thereby, the food's palatability plays an essential role in this shift from energy homeostasis towards increased energy intake which is also related to the hedonic reward system [8]. There again, the central regulation and recognition of the food's palatability play an important role. The perception of food includes

its cognitive, visual, and olfactory characteristics [13,14]. However, the perception itself is proposed to be influenced by the central action of hormones such as leptin [14]. In addition to these cognitive impulses also circadian rhythms affect the decision to consume food as well as the amount of food consumed. Even this decision-making is suggested to be centrally regulated by metabolic and endocrine factors [13].

1.1.1.2 Endocrine mediators

Several endocrine messengers are involved in the complex regulation system of energy homeostasis [8] and are responsible for energy expenditure, energy allocation, and mobilization, or storage in the organism [10]. Mainly, two critical factors play a role in the secretion of endocrine energy sensing mediators such as hormones, neurotransmitters and neurons: the physical state of the organism (e.g. expressed in the abundance of body fat and thus body composition) and the gastrointestinal (GIT) filling state (e.g. the amount and volume of the digested food influences GIT receptors). However, the secretion of signaling molecules is also altered by perception and cognition [8], as well as sleep duration and circadian rhythms [9] which may be dysregulated in psychiatric disorders.

Regarding the fullness of fat stores, specific hormones – adipokines - are secreted by adipocytes to report on the fat storage of the organism. Thereby, adipokines operate systemically and induce appetite regulation, and energy and nutrient homeostasis [11]. Leptin is an vital adipokine that is secreted from the adipocytes to indicate the current status of energy stores [15,16]. Circulating leptin levels directly correspond to lipid storage. Thus, leptin is a surrogate indicator for the organismal fat storage [11]. It is a crucial energy sensing molecule since it reports on the adequacy of lipid storage in terms of adipocyte volume and provides the signal for reducing energy intake. Leptin exerts an anorexigenic signal in the hypothalamus. Thus, in energy metabolism leptin is a signaling hormone for satiety. In energy deficiency conditions lipids are mobilized from their storage sites. Consequently, the adipocyte volume decreases, which leads to a reduction in leptin secretion, inducing appetite-stimulating pathways, hunger, and energy acquisition behavior [11].

Besides leptin, which is of major interest for this thesis and will be explained in more depth in the following sections, other important adipokines and hormones impact energy sensing. However, they cannot be dealt with here in much detail, but for the sake of comprehensiveness further energy sensing messengers should at least be mentioned:

Adiponectin, an adipokine, is inversely correlated with lipid storage [11]. Adipokines in general act systematically by modifying glucose and lipid metabolism, energy expenditure, insulin sensitivity, and appetite. They act as key hormones in energy homeostasis and have immunomodulation properties [17].

The counterpart of leptin is ghrelin. It is a gastrointestinal hormone, which is excreted by gastric oxyntic glands of the stomach. It stimulates hunger and exerts orexigenic signals. For exerting its function, ghrelin requires acylation after binding to its receptor, that is – besides peripheral tissues – mainly located in the feeding center of the hypothalamus [18]. The stimulation effect on food intake of ghrelin is related to the activation of the hypothalamic neurocircuits and is associated with activation of neurons in the hypothalamus that excrete neuropeptide Y (NPY) and Agouti-related peptide (Agrp) [19]. Also, ghrelin promotes gastric emptying and peristaltic and activates gastric acid secretion. Further, it plays a role in food reward and taste and olfactory perception [19]. Circulation ghrelin levels are negatively correlated with the organism's lipid storage and react on fasting via enhanced plasma levels [18,20].

Furthermore, the gut-brain-axis represents a neuroendocrine regulation of energy balance. The response to information of hormones released in the GI-tract is centrally regulated via the activation of receptors in the hypothalamus. Thereby, the vagal nerve and the metabolic products of the gut microbiota are important energy-sensing messengers [21].

Last but not least, hormones of other peripheral organs such as the thyroid hormone triiodothyronine (T3) is associated with energy expenditure, the pancreatic hormones insulin and glucagon are responsible for cellular energy supply and mobilization, respectively, and insulin growth factors (IGF), mainly secreted in the liver after growth hormone stimulation (GH) impact energy metabolism [10]. Glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) act anorexigenic and are released from ileum and colon and contribute to glucose metabolism. Cholecystokinin (CCK) is another intestine hormone contribution to anorexigenic effects [8].

Similar to adipokines, myokines are secreted in muscle cells and contribute to energy homeostasis. Interestingly, interleukin 6 (IL-6) is an important crucial myokine that enhances lipid oxidation in the organism and elevates cortisol levels [22].

Also, stress biology and energy metabolism are linked via glucocorticoids. Glucocorticoids act via the hypothalamo-pituitary-adrenocortical (HPA) axis and support gluconeogenesis

in the liver, proteolysis in the muscle and lipolysis in fat and thus enhancing glucose abundance and energy availability [23].

In summary, afferent signals communicate with the brain, adipose tissue, peripheral organs, and the GI-tract in order to adopt energy and food intake and thus regulate energy storage over time. For energy homeostasis, it is vital that these hormonal mediators act synchronized and mirror the organism's energy requirements adequately [13].

1.1.1.3 Immunometabolism

The above-described energy sensing mechanisms occur under normal physiological conditions. However, it has been shown that pathophysiological conditions influence energy-sensing pathways. The appropriate function of both, the immune system and energy-related processes, are interconnected and rely on each other [24]. Overlapping pathways possibly regulate both nutrient-related metabolic and immune functions. Interestingly, adipose tissue and the liver - both extremely important for energy sensing – contain immune cells (Kupffer cells, macrophages, lymphocytes, and dendritic cells) and blood vessels (compare to Figure 3), that enable them to communicate with other organismic sites such as pancreas or muscle tissue [24].

Chronic and low-grade inflammation is often metabolically triggered and can mainly be observed in correlation with increasing body fat [24] and thus unfavorable body composition. This condition is also called meta- or sterile-inflammation due to the absence of inflammation-triggering pathogens. An energy overload leads to a metabolic surplus which results in a nutrient triggered inflammatory response that is comparable to infection response pathways [13]. However, in this case, inflammatory response aims to regulate nutrient metabolism and nutrient intake. This coordination of metabolism with immunity is referred to as immunometabolism [13].

Interestingly, inflammation – even smoldering inflammation that is defined by elevated interleukin 6 (IL-6) and C-reactive protein (CRP) levels – has been associated with increased energy expenditure due to increased basal metabolic rate [9]. It has been hypothesized, that energy demanding metabolic processes are connected to immune defense mechanisms and pathogen sensing. Thus, appropriate energy homeostasis is thought to be essential to maintain effective immune function. This function may be

disturbed due to prolonged energy overload or energy deficiency as it occurs in obesity or anorexia nervosa, respectively, and may finally lead to aberrant immune responses [13,24]. Overall, energy sensing, on the one hand, delivers the current nutritional and energy status from the periphery to the brain, on the other hand, it additionally regulates hunger and satiety mechanisms to achieve energy homeostasis. However, these complex regulatory processes are influenced by several factors (such as environmental factors like stress, psychological factors, the physical health condition, and adaptive processes due to changed physical conditions in homeorhesis) that may lead to dysregulation of this tightly controlled sensing pathways and result in unfavorable energy status conditions.

1.1.2 The human energy status

The term energy status describes energy availability for physiological functions including the current status of energy stores and the ability to mobilize energy stores adequately. An excess of energy is either stored in the form of triglycerides in adipocytes or in the form of glycogen in muscle or liver tissue, depending on the physiological status of the organism and the amount of energy excess. Thus, excessive energy storage leads to body weight increase. For a first classification of the individual's energy status, the World Health Organization (WHO) recommended body mass index (BMI) classification [25] can be applied, however, additional factors need to be assessed and considered for a more precise characterization of the energy status. The BMI classification has known limitations in defining the actual metabolic state of the individual since no distinction between the amounts of different body compartments is possible [26]. Thus, in addition to endocrine biomarkers for the energy status, body composition assessment – especially the determination of the body fat content and muscle mass – are essential for the determination of the human energy status and are part of deeper phenotyping of the individual's metabolic profile.

1.1.3 Phenotyping

The phenotype of an organism comprises all aspects of its appearance including the morphological and physiological characteristics. These characteristics are based on genetic predisposition on the one hand and are also modified by environmental factors such as lifestyle behavior including diet and physical activity, and a great variety of stressors on the other hand [27]. For example, the level of physical activity strongly influences muscle hyper- or hypotrophy, energy dense diet promotes access of body fat storage, or malnutrition impairs physiological functions and development processes. The organism's phenotype can be separated into partial phenotypes that describe specific foci in detail (e.g., the genotype, metabotype, fat patterning, etc.). The metabolic phenotype is part of the individual's whole phenotype. It displays the interaction of genetic determinants, lifestyle and environmental exposure at a molecular level, and thus mirrors the individual's metabolic state [28]. Metabolic processes result in specific metabolites that for their part influence further metabolic outcome since they may trigger pathways differently. Thus, a complex interaction of various metabolic pathways determines the metabolic outcome. The entirety of metabolites in the organism is considered in the metabolome. Many thousands of metabolites contribute to the individual's metabolome. The diet's food metabolome plays an important role in the metabolic outcome and is also a relevant biomarker for nutritional intake [29]. For metabolic profiling, various biological materials such as blood serum and plasma, urine, stool, saliva, and others can be used [30]. Importantly, the metabolites from these sources differ metabolically from each other and reveal different information. For example, plasma and serum metabolites (as used for this study) give information on the bioavailability of nutrients, whereas metabolites derived from stool samples give insides into the interaction of the gut microbiota with food [30].

Deep phenotyping involves various aspects of the individual, such as the metabolome, the gut microbiota composition, genetic factors and lifestyle parameters like diet, eating behavior and habitual physical activity. It is a comprehensive analysis of all phenotypic characteristics of the individual [31].

Information derived from metabolic phenotyping contribute to a deeper understanding of the system biology and can be used for personalized medicine approaches.

In this study, individual metabolic phenotyping was performed in a cohort of female participants that in a first approach had been differentiated by phenotypic and lifestyle associated parameters such as the body mass index and the activity level. For metabolic

profiling, nutrition-related parameters had been elected. Thereby, body fat was chosen as primary phenotypic descriptor. In addition to body fat, further nutrition-related parameters were defined for an in-depth phenotype characterization: the inflammatory status, the level of leptin in plasma, the carotenoid status, and oxidative stress levels. All determined factors – beside other factors that cannot be considered in depth in this thesis[1] - contribute to the individual metabolic phenotype of the individual that is codetermined by dietary choices and physical activity behavior.

1.1.4 Challenges for the energy metabolism

1.1.4.1 Phenotypic flexibility – an organismic concept to cope with metabolic challenges

The individual phenotypic flexibility describes the ability to adopt physiological response under challenging conditions to maintain the system's homeostasis. Thereby, the whole system with all its interconnected physiological and biochemical processes is involved [32]. Challenges for the organism occur continuously in daily life. The organism needs to adapt constantly to environmental changes, whereby nutrition has major impact. Food intake causes metabolic responses and is accompanied by oxidative and inflammatory reactions. An overload or a lack of nutrient supply stresses the flexibility regulation. The system is flexible enough when it can react adequately to external challenges like an extreme digestive burden due to dietary exposures or other external stressors [2]. However, certain physiological conditions support unfavorable physiological pathways and may trigger metabolic disturbances that challenge the immune response and lead to inflammatory conditions. In obese, athletes, or patients with anorexia nervosa the physiological response may be affected negatively due to the exposure to extremely challenging conditions. The internal coordination of metabolic response may be disturbed by challenges from caloric overload, excessive exercise, or severe energy restriction, respectively. There are inter- and intra-individual ranges in the physiological response to metabolically stressful events. The failure of one regulation system can be compensated by others to preserve the systems' homeostasis. However, over time, also the phenotypic flexibility may be altered since the organism is no longer capable of maintaining homeostasis. This may result in

[1] This thesis is part of the larger cross-sectional Energy Sensing in Anorexia Nervosa (ESAN) study. Within this study, additional parameters that contribute to deep and metabolic phenotyping have been investigated such as the gut microbiome and metabolomics. However, this thesis focusses on elected phenotypic characteristics of the study population as described above.

modified metabolic response and adapted regulatory processes including the immune system and alter energy sensing mechanisms. The system's loss of flexibility indicates the onset of disease [33].

1.1.4.2 Anorexia nervosa

Anorexia nervosa (AN) is a serious psychiatric disease. Two established diagnostic tools provide criteria for the diagnosis of AN: The WHO's International Classification of Diseases (ICD-10) [34] and the Diagnostic and Statistical Manual of Mental Health Disorders (DSM-5) of the American Psychiatric Association [35]. Both diagnosis guidelines contain a certain BMI cutoff (below 17.5 and below 17.0 kg/m², respectively) indicating a severe reduction of energy and nutritional status. Furthermore, altered body self-perception [36], concerns about the body shape [37], and the fear of gaining weight are essential characteristics of the disease.

The low body weight in AN is most commonly induced and maintained by restrictive eating behavior and severely reduced energy intake; however, also purging behavior occurs. The malnutrition and energy-undersupply lead to life-threating conditions that are caused by the loss of body structure including body fat, muscle mass, the loss of bone mass, and even organ mass [38,39] as well as a series other health consequences [40,41]. Among AN patients the mortality rate is high [42].

Another result of the insufficient nutrient supply are metabolic disturbances that alter brain function [43]. The appropriate function of the brain and neurons is dependent on adequate energy supply in the form of glucose. In addition, the availability of fat is necessary for proper myelin synthesis, and a lack of protein supply is associated with reduced ability of neurotransmitter and hormone synthesis. The production of serotonin and dopamine is dependent on adequate availability of the amino acids tryptophan and phenylalanine. Mood and motivation are altered by a lack of these signaling molecules [44]. The food restriction induces neuroendocrine processes that activate the reward system: The hypothalamus releases orexins and melanin-containing hormone which leads to dopamine increase in the nucleus accumbens. This mechanism supports the restrictive eating behavior of AN patients since they get reward from food restriction [45].

Regarding the palatability of food, the AN patients' perception is altered, as well as the sensibility for hunger and satiety [46]. The endocrine response is affected on multiple levels

in AN patients [47], and thus, also energy sensing mechanisms are altered. Especially hypoleptinaemia, corresponding to the reduced body fat amount, and ghrelin response seem to be changed in AN and impact appetite-regulation. Most of the alteration in the endocrine response can be seen as an adaptive response to chronic starvation [48].

The causes of AN are multidimensional and complex, which is a challenge for effective therapeutic strategies [49-54]. However, besides the stabilization of vital function, the therapies' leading aim is a rapid weight restoration [52-54]. Therefore, treatment plans commonly include two main approaches that are supposed to stimulate weight restoration and energy metabolism [41]. First, nutritional treatment focuses on increased energy intake [52,55,56] under consideration of possible refeeding complications [57]. High caloric diet is commonly combined with artificial nutrient enriched dietary supplements [40] to achieve the energy requirements for weight gain [52,54]. Second, physical activity and exercise are restricted to reduce energy expenditure [40,52-54]. Over-exercise [58], hyperactivity [59] and the problem of hypermetabolism [52] are common among AN patients. Moreover, physical activity in AN patients is associated with compulsive [59] and compensatory behavior [60] and an increased risk for adverse physiological outcomes [38,58,59,61]. Nevertheless, adequate physical activity is of great importance for the individual's wellbeing [38,59,62,63] and influences body composition positively [41,64] which contributes to a more sustainable therapy [38,41,58]. Therefore, some therapeutic concepts support supervised exercise instead of total restriction [38,41,63]. This may reinforce weight gain and does not necessarily influence it negatively [41,65-67].

The nutritional status is commonly assessed by the BMI [64], and the speed of weight gain [68] or the increase of BMI [69] are used as indicators for therapy progress. Thereby, the patients' body composition remains unconsidered [64], although the gain of abdominal body fat is a known side effect of fast weight gain [41,70-74] and a major risk factor for relapse [52,70,73].

1.1.4.3 Obesity

Obesity is defined as a condition of increased body weight due to elevated body fat mass. The WHO established BMI cutoff values for the categorization of obesity in three subclasses, namely obesity grad I – III. People with a relative body weight from above 30 kg/m² are considered to be obese [75]. The main problem of the increased body weight can be seen in the accumulation of body fat since it has been shown that adipocytes are

metabolically active cells that detrimentally affect physiology and lead to systemic disturbances. Obesity is associated with many chronic diseases (e.g., diabetes mellitus type II, coronary heart diseases, liver diseases) and can be seen as a major factor in the development of non-communicable diseases [76]. It goes along with severe metabolic disturbances and is associated with chronic inflammatory conditions that challenge the immune response and thus energy homeostasis [77,78]. Indeed, the metabolic effects of obesity impact all biological systems (e.g., endocrine function, physiological homeostasis, immune response, etc.) that are eventually interrelated with each other. Thus, obesity must be seen as a systemic challenge and dysfunction [9].

The causes of obesity have been discussed on multiple levels [79]. Many different reasons are taken into consideration ranging from genetic, environmental and epigenetic factors [76], to unfavorable dietary choices, sedentary lifestyle and lack of physical activity [8,12], to endocrine involvement in homeostatic dysregulation [14] and also the gut microbiota is blamed for contributing to a positive energy balance [80]. However, some sources also classify obesity as an eating disorder [34,35], indicating a psychiatric connection to this condition.

Regarding energy homeostasis leptin plays an important role. Obesity impacts regulation mechanisms due to enhanced levels of adipocyte size and numbers, leading to elevated leptin levels. In obesity, leptin resistance contributes to fat accumulation. However, many details regarding leptin resistance and leptin signaling remain uncertain [79]. Glucose and lipid metabolism are additionally affected, contributing to insulin resistance and impaired energy usage for enhanced muscle force levels [9,22]. Energy homeostasis is affected systemically in obesity, e.g., the excess of lipids stored in adipocytes also affects the topography of other tissues and leads to liver, blood vessel wall and muscle changes [9]. However, energy balance is still an essential factor to maintain homeostasis, although some studies suggest the contribution of hormones and the microbiota to influence energy harvest [79]. It has been shown that a low physical activity level (PAL) in obese individuals as an important part of energy balance impairs energy homeostasis. Exercise-induced energy expenditure is not necessarily compensated via increased energy intake if it remains under a particular activity volume. Thus, it contributes to increased energy expenditure and regulates body weight. Possible involvements in this regulation process of gut peptides (GLP-1, PYY) and acylated ghrelin, as well as increased meal-intervals (probably due to increased free fatty acids after exercising), have been discussed [8].

One major challenge in obesity is the chronical low-grade inflammatory condition which leads to increased energy expenditure on the one hand, however, also to elevated oxidative stress response on the other hand [10]. In adipose tissue, immune cells such as macrophages are located directly beside adipocytes and blood vessels [24], secreting proinflammatory cytokines that also support insulin resistance [76]. This condition may additionally lead to malnutrition in obese people; tough, poor dietary choices are known problems for many obese people.

For the clinical assessment of obesity, the BMI is still used. However, as already highlighted, the estimation of body fat by the BMI is weak on an individual level [9]. In addition to the determination of body composition, fat patterning, and anthropometric indicators like waist circumference, the measure of biochemical parameter such as the lipidic blood profile are proposed for deep phenotyping in order to characterize the patients more precisely, which may allow classifying the individual regarding potential health impacts of its phenotype with higher precision.

1.1.4.4 Athletes

Athletes (AT) represent an energy group with high energy turnover rates induced by enhanced levels of physical activity. Due to training overload, the organism's energy balance may be extremely challenged. Increased energy expenditure can often not be compensated through nutrition alone, leading to a problematic condition in high-performance AT called relative energy deficiency in sports (RED) [81]. Due to a chronic negative energy balance, the organism is forced to change energy supply mechanisms, leading to endocrine dysregulation on various axes and thus unfavorable metabolic outcomes. A prominent example of chronically severe energy deficiency in females is the female triad, resulting in osteoporosis and amenorrhea [82].

Additionally, regular training on enhanced levels leads to fundamental changes in body composition. Athletes are supposed to have higher muscle mass and lower body fat mass compared to inactive normal weights. Exercise induces fundamental metabolic changes that shift the metabolic participation of energy delivering substrates from storage to oxidation which leads to the release of free fatty acids from adipose tissue. Thus the organism's fat depots are shifted to a catabolic condition [8]. For the female athletes' triad, the term "anorexia athletica" had been synonymously used [83]. It described a preclinical form of an eating disorder. However, the International Olympic Committee (IOC) introduced

a broader and more comprehensive term for this occurrence among athletes: the term relative energy deficiency in sports syndrome (RED-S) includes a broader spectrum of health consequences due to reduced energy availability. RED-S also refers to health consequences in male athletes, since the physiological and metabolic consequences of energy depletion are not limited to women [84]. Besides the above-described consequences of decreased energy availability in athletes, several other endocrine consequences occur in the athletic organism affecting metabolism detrimentally [82]. Figure 1 provides an overview of possible health consequences as a result of RED-S.

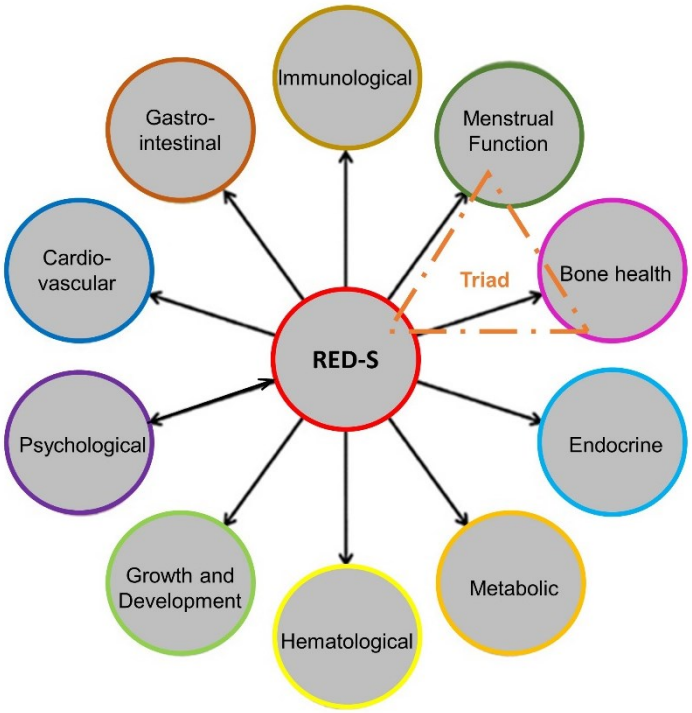


Figure 1: Health consequences related to relative energy deficiency in sports
RED-S: relative energy deficiency in sports syndrome adopted from [81].

Even in AT, immune response and inflammation are key issues. Post-exercise enhanced inflammatory markers indicate muscle damage and the involvement of the immune system in the recovery phase [85,86]. Due to post-exercise induced protein-synthesis, energy metabolism is additionally challenged. Protein supply is suggested to be crucial for appropriate immune response as well as for recovery.

1.2 The interplay of adipocytes, phytonutrients, leptin and the immune system

An important essential human phenotypic characteristic is the individual's amount of adipose tissue which is directly linked to energy homeostasis and influences metabolism in various ways [77]. Besides muscle mass, adipose tissue is a surrogate for the organism's energy status. Excessive fat storage is a known risk factor for inflammatory conditions and induces oxidative stress, which is responsible for the onset of various diseases (such as cancer, atherosclerosis, arthritis, etc.) [78]. Notably, plant-derived nutrients have been found to influence adipocyte biology positively. The two most abundant pigments in fruits and vegetables – namely carotenoids and polyphenols – affect adipocyte properties and fat accumulation and exert antioxidant, anti-inflammatory and immunomodulatory effects [6,87]. Both are associated with a decrease in adiposity, especially in the abdominal area. An important physiological link between the organismic fat stores and immunomodulation is the adipokine leptin. It acts as a signaling molecule in energy sensing and modulates the immune response conspicuously [5].

However, to our best knowledge, so far these interconnections have been posed independently from each other, and only limited data of clinical studies focusing on the effects of diet-related phytonutrients on fat accumulation and fat patterning, and immunometabolism in humans are available [7,88]. The impact of many misguided pathways on cellular regulation processes concerning adipose tissue and leptin, inflammation and carotenoid metabolism have often been investigated in obese. This thesis attempts to evaluate this possible interconnection comprehensively in a cross-sectional setting in various energy status groups.

A more detailed insight into the proposed cellular regulation mechanisms will be provided in the following sections. Figure 2 summarizes the complex interplay of these four components.

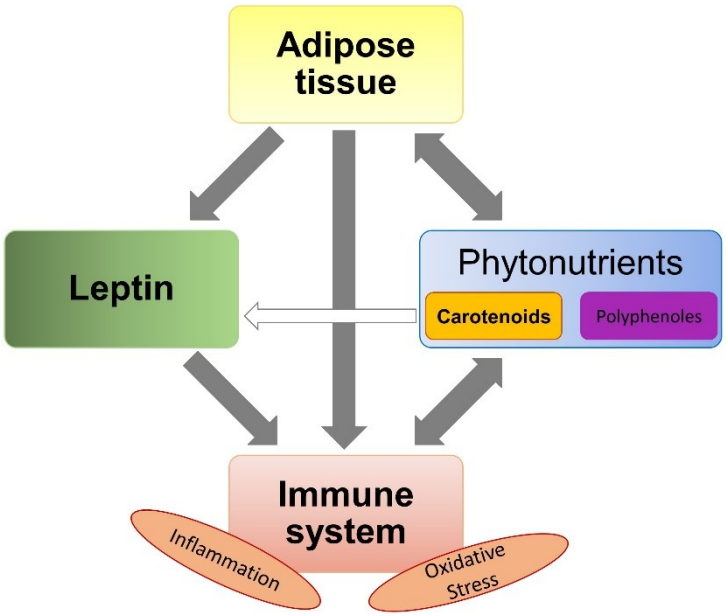


Figure 2: The interplay of adipose tissue, carotenoids, leptin and the immune system

1.2.1 The immune system

The human immune system is a natural and tightly regulated defense regime against exogenous pathogens and infections. It can be divided into two major forms: First, the innate immune system protects the organism unspecifically as a first defense reaction against harmful pathogens by producing a series of immune cells. It comprises the complement system, Toll-like receptors and phagocytotic cells that together respond quickly to unwanted intruders. These response mechanisms are inherited, and a crucial part of keeping the organism balanced since they also maintain normal tissue function and structure by identifying and repairing tissue damage and removing dead cells [89]. Mast cells, neutrophils, macrophages, natural killer (NK) cells, eosinophils, and dendritic cells belong to the innate immune system [5].

Second, the adaptive immune system fights pathogens targeted after a first contact with the pathogen, the so-called sensitization phase. B and T lymphocytes are the responsible cells for producing immunoglobulins (Igs; also referred to as antibodies) and T-cell receptors (TCRs), respectively. They have specific receptors for antigen identification on their surface that are developed after the first antigen contact. Importantly, when encountering an antigen, lymphocytes proliferate exponentially to increase their defensive power. T-lymphocytes differentiate to helper and effector cells and secrete specific cytokines that

mediate the defense response. They develop a specific memory in order to defend the organism at the second encounter of the pathogen more efficiently [89].

Importantly, the innate and the acquired immune systems communicate with each other. The link to this communication is that innate immune cells that prepare antigens for antigen-presentation to the lymphocytes. The most important antigen-presenting cells are dendritic cells [89].

1.2.1.1 Inflammation

Inflammation is an integral part of the innate immune system. It is induced in order to protect the organism against detrimental processes that may be caused by microbial or viral invasion, hypersensitivity reactions or tissue damage. In general, inflammation can be differentiated in an acute and chronic form. Acute inflammation follows a strict procedure mainly regulated by mast cells that secrete histamine, prostaglandins, and serotonin at the site of inflammation. After this first response to an inflammatory stimulus, blood vessels expand and granulocytes invade to remove the unwanted aggressors. This form of inflammation is helpful for the organism and protects it against exogenous and endogenous damage. If the inflammatory condition persists over an expanded period it is entitled chronic inflammation which is detrimental to the organism since physiology is affected negatively leading to dysregulation on a systemic level as well as tissue damage. Macrophages and T-lymphocytes and their cytokines and enzymes are mainly involved in this mechanism [90,91].

Toll-like receptors (TLRs) belong to the family of pattern recognition receptors (PRRs) and are capable of detecting exogenous as well as endogenous inflammation inducers. Due to the stimulation of TLRs, inflammatory cytokines (tumor necrosis factor-α (TNF-α), IL-6), chemokines and interferons are produced and determine the expression of inflammation by inducing phagocytosis and tissue repair. However, dysregulation of TLRs leads to chronic inflammation [91].

There are certain pathways associated with the regulation of inflammation. The two essential transcription factors nuclear factor -κB (NF-κB) and nuclear factor erythroid 2-related factor 2 (Nrf2) are activated by the mediators of inflammation (e.g. cytokines, chemokines, and prostaglandins) and the increased number of macrophages [92].

NF-κB is involved in the expression of pro-inflammatory genes (such as cytokines, chemokines) by acting in the nucleus after stimulation. Unstimulated, it is bound to the cytoplasmic protein IκB that inhibits its activity. The NF-κB-pathway can be induced by inflammation stimulation signals such is TNF-α, interleukins, oxidative stress and UV radiation [93,94]. After stimulation IκB releases NF-κB which further moves to the nucleus where it activates the transcription of immune-regulatory and inflammatory genes [92].

Importantly, inflammation leads to oxidative stress. Macrophages and leucocytes are produced and infiltrate the site where the inflammatory stimulus occurs. This leads to overproduction of reactive oxygen species (ROS) and thus oxidative stress [92]. Nrf2 is bound to kelch-like ECH-associated protein (Keap1), a cytosolic protein, under normal physiological conditions. When this complex encounters oxidants, Nrf2 is released and migrates to the nucleus where it binds to the antioxidant response element (ARE). This induces the expression of antioxidant enzymes [92]. The Nrf2/ARE pathway is widely accepted for its crucial role in inflammation regulation and links inflammation to the oxidative stress response. Nrf2 induces the production of proteins that suppress pro- and support anti-inflammatory activities, and detoxification enzymes [91].

1.2.1.2 Oxidative stress

Many physiological processes are dependent on the availability of oxygen, making it essential for the organism. However, due to biochemical processes and the impact of environmental factors (such as pollutants or radiation), reactive oxygen species (ROS) are formed. They are characterized by unpaired electrons leading to increased reactivity and thus causing potentially detrimental and damaging effects. To reach more stability, ROS aim to donate or access electrons from other molecules. Since most biomolecules are non-radical within this reaction, new radicals are formed, leading to a reactivity chain. Notable examples of ROS that naturally occur in the organism are the hydroxyl radical (°OH) as the most radical form that induces chain reactions, the superoxide anion ($O_2^{\circ-}$) that is produced as a byproduct of energy metabolism in the respiratory chain and a rather weak oxidant, and nitric oxide (NO°) as a radical that fulfills physiological functions in the control of vascular tone [95]. Examples of detrimental oxidative processes in the organism are lipid oxidation, especially the oxidation of low-density lipoprotein (LDL) as an atherogenic particle and link to immune activation, protein damage, and DNA oxidation. These processes cause

severe damage in the organism and are responsible for many pathological processes like vascular damage, carcinogenesis, as well as aging [95,96].

Besides the detrimental effects of ROS, they also have essential signaling function. E.g., $O_2^{\circ-}$ is formed in the mitochondria, the endoplasmic reticulum or by specific enzymes and is neutralized by the enzyme superoxide dismutase (SOD) by converting it into hydrogen peroxide (H_2O_2) which is less reactive and is further converted into water (H_2O) by the antioxidant enzymes catalase and glutathione peroxidase. H_2O_2 acts as a second messenger [96].

The organism is capable of neutralizing ROS and maintaining a balance between pro- and anti-oxidant processes if sufficient antioxidants that scavenge radicals are accessible. The antioxidant defense includes enzymatic (e.g., catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic (micronutrients) mechanisms. Importantly, α-tocopherol (vitamin E), a chain-breaking antioxidant in lipid peroxidation, synergizes with ascorbate (vitamin C) in the antioxidant defense. Ascorbate regenerates the reduced α-tocopherol. Furthermore, carotenoids and polyphenols exhibit antioxidant properties [97].

The disturbance of the balance between the production of ROS and the antioxidant defense is referred to as oxidative stress [95].

1.2.2 Adipose tissue

1.2.2.1 Characteristics of distinct adipose tissues

Adipose tissue is mainly composed of adipocytes that can primarily be distinguished in white adipocytes and brown adipocytes. Concerning the predominance of white or brown adipocytes, adipose tissue is either termed white adipose tissue (WAT) or brown adipose tissue (BAT). The two cell types differ substantially in their morphology and physiological function. White adipocytes are the most abundant fat cells in the adult human organism. They contain a narrowed nucleus and small mitochondria. However, around 90% of their volume is comprised by a large lipid droplet that is necessary for the main function of those cells, namely to store the excess of dietary energy in form of triglycerides for metabolic needs in stages of starvation. The lipid droplet is capable of expanding following the amount of triglycerides stored [98].

On the contrary, brown adipocytes are polygonal cells with a roundish nucleus and several smaller lipid droplets. Related to their main physiological purpose, they contain several relatively large mitochondria that express uncoupling protein 1 (UCP1), an inner membrane protein which is necessary for burning energy for thermogenesis [98]. Besides these two predominant forms also intermediate forms of both, the so-called “beige” adipocytes also referred to as “bright” or “paucilocular” adipocytes, exist. These intermediate forms comprise the morphology of white and brown adipocytes and exhibit anti-adiposity effects due to increased fat oxidation. There are several factors that are suggested to influence the development of beige adipocytes (also referred to as “browning” of WAT). Prolonged cold exposure as a thermogenic stimuli is proposed to be a major determinant for browning [99], however, further endogenous (e.g. myokines and adipokines, thyroid hormones, sympathetic activators), pharmacological and nutritional (e.g. carotenoids, long chain polyunsaturated fatty acids, polyphenols) browning factors have been observed [100,101]. Anatomically, BAT is primarily located at the neck and interscapular region of the adult human, whereas WAT occurs all over the body. Importantly, both also comprise adipocytes of all variants in varying amounts.

Besides adipocytes, adipose tissue also comprises blood vessels and immune cells. This close proximity of immune cells and blood supply enables local interaction and fast communication between the cells and access to distribute mediators to the central nerve system and the periphery to induce further regulation processes [24]. Adipose tissue involves complex cytology and characterized by high physical plasticity which is of great importance for the physiological purpose. With respect to the physical state of the adipose tissue, it acts as an endocrine organ and secretes a series of hormones that contribute to energy homeostasis (e.g., leptin, adiponectin), influence lipid and glucose metabolism and modulates immune response (via enhancing oxidative stress and secreting inflammatory cytokines).

Figure 3 depicts the typical structure of the three different adipocyte subtypes as well as the composition of adipose tissue.

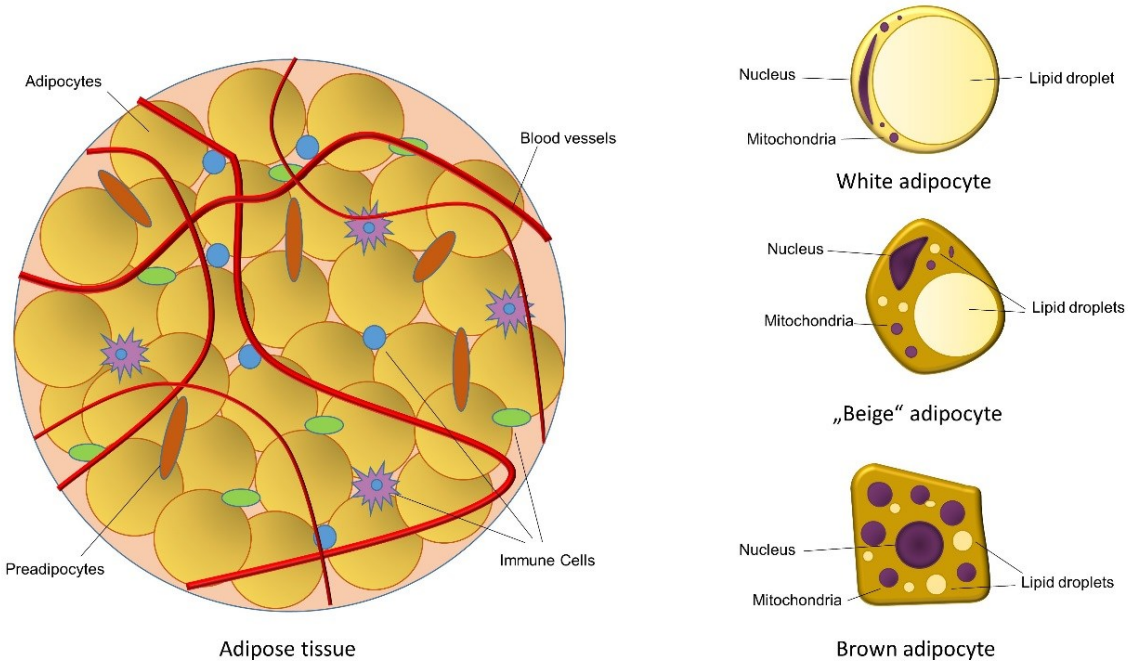


Figure 3: Composition and morphology of adipose tissue and adipocytes

adopted from [24,98].

1.2.2.2 Anatomical distribution of adipose depots

Adipose tissue is distributed all over the body and can anatomically be divided into two regional forms: visceral adipose tissue (VAT) that is located in the trunk and surrounds organs in order to protect them against mechanical burden, and subcutaneous adipose tissue (SAT) that constitutes a layer between skin and muscle necessary for thermoregulation and fat storage. They differ in their metabolic profiles such as endocrine function, lipolytic properties, hormone response and presence of inflammatory cells. Importantly, VAT has a direct connection to the liver via the portal vein. Thus, free fatty acids and adipokines are delivered straightly to hepatic cells where they activate immune response (e.g., induction of CRP), whereas messengers released from SAT are distributed via systemic veins [102].

About 80% of total body fat amounts are SAT, whereas the main storage sites are located in the abdominal and subscapular area as well as in the gluteal-femoral area. There are gender differences regarding VAT contribution: in females, VAT amounts to approximately 5-8%, in males up to 20% of total body fat [102].

Certain factors that contribute to specific fat distribution are assumed. For example, increased cortisol levels (e.g., due to chronic stress or due to the gluconeogenetic function of cortisol in starving conditions as it may occur in AN patients) lead to accumulation of VAT [102].

1.2.2.3 Adipose tissue response to energy excess

Adipose tissue can either respond to energy excess via increasing the size of adipocytes (hypertrophy) or via developing new adipocytes from precursor cells (adipogenesis leading to hyperplasia). The adipocyte number is a major factor for the amount of fat mass in adults. There are different hypothesis on the stability of adipocyte number during adulthood. One established hypothesis is, that the number of adipocytes remains stable even after severe weight changes, indicating that the cell number is determined in childhood and adolescence. Around 10% of adipocytes is renewed within a year to compensate the natural cell loss due to apoptosis [103].

1.2.2.4 Adipose tissue and immunometabolism

Adipose tissue is a recognized metabolically active endo- and paracrine organ that exhibits important immunological activity. Especially overloaded fat storages as a result of prolonged energy excess lead to immunological response and chronic low-grade inflammation, which results in changes of energy sensing pathways [104]. This condition is also referred to as “metainflammation” [105]. Adipose tissue contains a series of innate (macrophages, neutrophils, dentritic cells, mast cells, and eosinophiles) and adaptive (lymphocytes such as B- and T-cells) immune cells (Figure 3). Their occurrence is associated with nutritional factors. E.g., high fat-diet has been shown to enhance the presence of these cells within relatively short time-periods ranging from 3 days to 4 weeks [106].

Macrophages have a unique role in the occurrence of obesity-associated inflammation. Their proportional content increases in obese adipose tissue and exhibits their function by phagocytosis of dead adipocytes. Thereby, several macrophages form a crown-like structure around the dead cell. Importantly, macrophages can exhibit pro-inflammatory (M1, classically activated) as well as anti-inflammatory (M2, alternatively activated) function, depending on their activators [105,107,108].

In obese people, adipose tissue can amount up to 50% of total body mass and thus represents a critical immunomodulating organ that influences systemic inflammation. This meta inflammatory condition contributes to many obesity-associated comorbidities, especially insulin sensitivity is affected [106]. Thereby, the elevated glucose levels induce a vicious circle by promoting and activating pro-inflammatory immune cells since many of them are dependent on glycolysis (e.g., T-helper cells 17 (Th17), IL-1β). Insulin resistance modulates the shift from anti- to pro-inflammatory immune cell production and a loss of regulatory T-cells in the adipose tissue. Moreover, certain macrophages expressed in adipose tissue are supposed to regulate lipid metabolism and influence energy expenditure via expressing catecholamines. These changes in cell composition affect the adipose tissues' fat storage ability and affects glucose metabolism and metabolic homeostasis systemically [106,109].

Adipose tissue secretes a series of adipokines such as leptin, adiponectin, pro- and anti-inflammatory mediators such as tumor necrosis factor (TNF) α, and interleukins (IL). Studies on the metabolic health of obese have revealed higher levels of inflammatory markers in plasma (complement C3, C-reactive protein CRP, IL-6, interferon-γ and TNF), reduced levels of adiponectin and higher levels of cluster of differentiation 68 (CD68) marker of macrophages in the adipose tissue in the metabolically altered group [106,110].

1.2.3 Leptin

Leptin is a circulating protein that is produced almost exclusively in adipocytes and mainly occurs in WAT. It exhibits important signaling and controlling properties in the organism; thus it has hormone status and belongs to the group of adipokines - signaling molecules secreted by adipocytes. Its best-described features are the regulatory function in energy homeostasis and the delivery of the satiety signal from the periphery to the hypothalamus. It has an anorectic effect [15]. However, further important functions of leptin have been identified. Most importantly, leptin is involved in the immune response and stimulates inflammation and thus links the nutritional status to the immune system [16]. Figure 4 provides an overview of the currently known actions of leptin in the human organism.

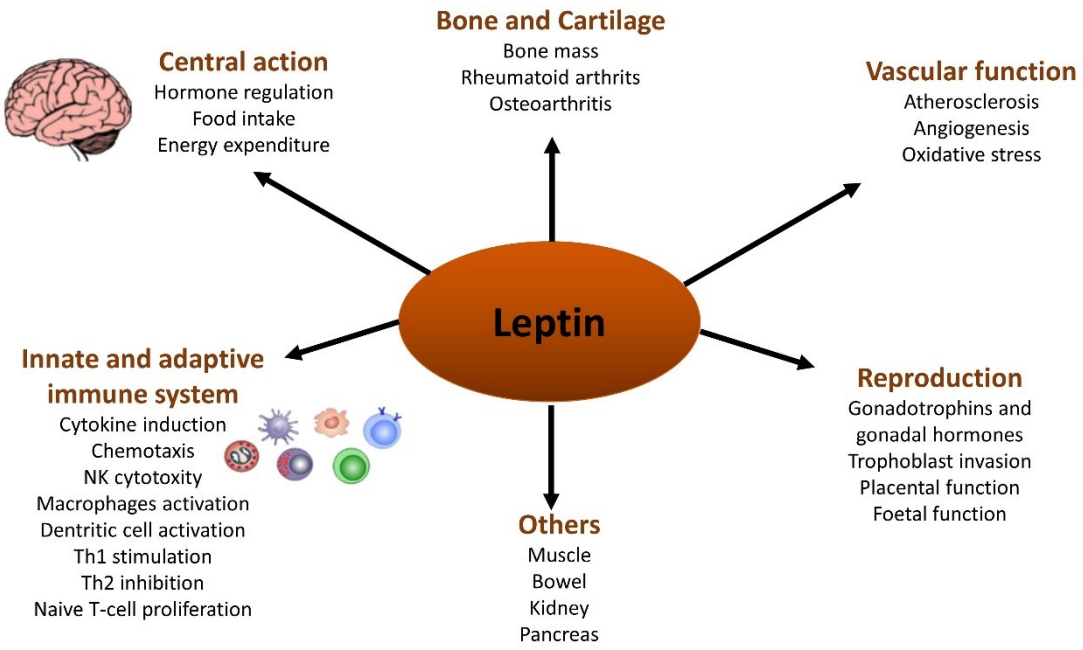


Figure 4: Leptin's function in the human organism

adopted from [111]

1.2.3.1 Energy regulatory function of leptin

As already pointed out in the section 1.1.1 Energy sensing mechanisms leptin holds important functions in the regulation of energy homeostasis. In the following, the most important functions will be summarized shortly.

Leptin's main effect in the organism is to deliver the information on the quantity of fat stored in the adipocytes to the brain. Its core site of action is the hypothalamus, where a cascade of hunger suppressing and energy expenditure increasing signals is induced. It binds primarily to receptors in the hypothalamus. However, leptin-receptors are also present in peripheral tissues. Leptin receptors belong to the class 1 cytokine receptors family [16]. Circulating leptin levels are proportional to body fat mass in humans and thus reflecting the amount of energy storages. In other words, the leptin levels in obese are usually higher compared to normal weight controls [5].

Leptin is supposed to be a vital regulator of the human energy homeostasis since it exhibits remarkable effects on food intake and energy expenditure [16]. The anorectic response of leptin is connected to the neuronal release of pro-opiomelanocortin (POMC) in the arcuate nucleus of the hypothalamus. This leads to the production of melanocortins that mediate

the appetite reducing response by acting as melanocortin agonists. Accordingly, the orexigenic response induced by leptin is mediated by the melanocortin antagonists Agouti-related peptide and neuropeptide Y. Both pathways induce the neuronal expression of melanocortin 4 receptors (MC4R), leading to interactions with other brain regions and the regulation of appetite, expression of efferent signals regulating peripheral metabolism and energy expenditure [16].

1.2.3.2 Effect of leptin on the immune response

Leptin links nutritional status and the immunological response. It has been discovered that leptin plays a key role in immunometabolism. It contributes to the inflammatory condition in obesity and the immunosuppressive state in undernutrition. Leptin receptors are expressed all over the immune system, and leptin exhibits regulatory function regarding both, the expression of cells of the innate and the adoptive immune system [5].

Regarding the innate immunity, leptin influences mast cell activities by increasing their ability to migrate as well as their survival rate. Furthermore, leptin affects the neutrophil properties due to stimulation of elevated secretion of ROS (such as H_2O_2, O°_2) and increased chemotaxis. Next, leptin's impacts on macrophages involve the enhanced secretion of TNF-α which is supposed to be responsible for the mediation of leptin's activity, as well as increased phagocytosis, and elevated expression of pro-inflammatory mediators. The proliferation of natural killer (NK) cells is increased by leptin as well as their cytotoxic capacity and secretion of cytokines (e.g., IL-2). The migration and survival rate of eosinophils is increased due to the activity of leptin. Importantly, leptin also stimulates the production of inflammatory cytokines and chemokines (e.g., IL-1β, IL-6, IL-8, and monocyte chemotactic protein-1, respectively). Last but not least, leptin promotes changes in dendritic cell (DC) structure and function. The production of Th1 is encouraged, DC survival is enhanced, and their mature, as well as the capacity for antigen presentation, is improved [5,111,112].

Concerning the adaptive immune system, leptin affects T cells apoptosis rate, leading to increased proliferation, maturation and survival rates. Additionally, leptin supports the differentiation of T helper 1 (Th1; produces mainly pro-inflammatory cytokines and activate macrophages) and Th17 cells. On the contrary, leptin may be involved in a reduced expansion of regulatory T cells (Treg). Th1 cells are capable of secreting leptin which maintains this process and promotes Th1 and suppresses Th2 (secrete mainly anti-

inflammatory cytokines, that activate B cells and basophils) proliferation. Eventually, leptin activates the secretion of cytokines by B cells (e.g., TNF-α, IL-6, and IL-10) as well as the production of antibodies (e.g., IgG) [5].

Interestingly, leptin itself acts as a pro-inflammatory cytokine. It has been shown that serum leptin levels, together with IL-6, TNF-α and others, increase during acute inflammation [5]. Thus, a loop is induced that promotes chronic inflammation.

In conclusion, leptin influences a large number of immune cell types of the innate and adoptive immune system by elevating their function, proliferation and survival rate. Thus, leptin stimulates inflammatory responses, T-cell proliferation and T-helper (Th)-1 cytokine production [16]. A summary of leptin's immunoactivational properties is provided in Figure 4.

1.2.4 Phytonutrients

A plant-based nutrition including at least five portions of fruits, vegetables and legumes a day is highly recommended by nutrition societies since these natural food sources are high in nutrient density and thus beneficial for health. Dietary patterns including several servings of plant food contain high contents of secondary nutrients that fulfill a valuable and protective function in plant physiology. However, they also exhibit numerous health benefits to the human organism due to their antioxidant, anti-inflammatory and immunomodulatory properties [113,114]. Polyphenols and carotenoids belong to this group of non-essential nutrients, more detailed they are plant pigments. Their intake is inversely associated with blood inflammation markers such as CRP, IL-6 and adhesion factors [115]. Both have shown to have an impact on adipocyte biology and fat accumulation and are associated with a decrease in adiposity. This implicates a connection of phytonutrient intake, body composition, leptin secretion, and immunomodulation.

1.2.4.1 Carotenoids

Carotenoids are lipophilic C-40 based isoprenoid polyene molecules. Many hundreds of different carotenoids exist but only around fifty occur in the human diet of which six - namely α-carotene, β-carotene, β-cryptoxanthin, lutein, lycopene, and zeaxanthin – occur in

relevant concentrations in the human organism. Since carotenoids cannot be synthesized in the human body, they need to be taken up from the diet [116]. Their primary dietary sources are fruits and vegetables which account for about 90% of their intake. Carotenoids are – besides polyphenols - the most abundant pigments in fruits and vegetables and responsible for their red, orange and yellow color. Additionally, other food products like algae, eggs, milk, and oils contribute to carotenoid supply [117].

The absorption and transport of carotenoids in the organism is directly linked to lipid metabolism and lipoproteins, respectively. Carotenoids are stored in different tissues such as adipose tissue as a main compartment of storage as well as in the skin and subcutaneous tissue but also the liver and retina are storage and utilization sites [118]. It has been shown that the concentration of carotenoids in plasma correlates with the skin concentrations, whereas β-carotene and lycopene are the most abundant ones [119]. Plasma, as well as skin carotenoid levels, are supposed to reflect fruit and vegetable intake.

The bioavailability of carotenoids is strongly influenced by the food matrix. Especially dietary fiber and the presence of fat sources reduce or enhance the intestinal utilization since the absorption of the carotenoids into enterocytes is inhibited or enabled, respectively. Additionally, thermally or mechanically processed food releases carotenoids more easily due to the denaturation of chloroplasts, where carotenoids are primarily located [120]. However, not only food and meal properties influence the bioavailability of the carotenoids, but also host related factors like genetics, nutritional status, body composition, gender, and diseases influence their bioaccessibility [118,121,122]. Also, carotenoids affect the metabolism of adipocytes and immune pathways and thus control host related factors. This interaction will be in the focus of the next chapters.

1.2.4.1.1 **Carotenoids in adipose tissue biology**

The carotenoids' effects on adipose tissue are mainly described for β-carotene and strongly related to its provitamin A function. However, they are not restricted to β-carotene and its conversion products. Results of animal and cell studies suggest a carotenoid impact on adipocyte differentiation, their fat storage capacity, fat oxidation and thermogenesis, and the inflammatory and oxidative stress response of adipose tissue by interacting with transcription factors, modulating signaling pathways and independent genomic actions. Also, human data support the anti-adiposity effect of carotenoids [6,88].

1.2.4.1.1.1 Adipogenesis

Adipogenesis is induced by nutritional (e.g., high-fat diet, availability of vitamin D, folic acid, vitamin B12) and hormonal signals (such as insulin, IGF-1, glucocorticoid, triiodothyronine) [123]. During cell differentiation CCAAT-enhancer-binding proteins (C/EBPs) which trigger the expression of peroxisome proliferator-activated receptor γ (PPARγ) have an essential role. The latter is considered as the master regulator in adipogenesis: PPARγ is a ligand-activated nuclear receptor. It is necessary for adipocyte differentiation, survival, function, and maintenance of the adipocyte phenotype (white, brown, beige), but also involved in insulin sensitivity regulation and lipogenesis. Several lipid metabolites including polyunsaturated fatty acids (PUFAS), eicosanoids and a certain prostaglandin have been shown to activate PPARγ. However, they occur in low concentrations in adipocytes and have low affinity to PPARγ. Thus, the actual endogenous relevant ligands remain uncertain [124].

To form an active heterodimer PPARγ needs retinoid X receptor (RXR) as an obligate dimerization partner (PPAR:RXR). However, retinoic acid receptor (RAR) acts as a ligand in this complex. Dietary β-carotene is converted to retinal (retinaldehyd, Rald) via the enzyme β-carotene-15,15´-oxygenase (BCO1) and β-carotene-9´,10´-oxygenase (BCO2) which is then further oxidized to retinoic acid or retinol [6].

In cell models, β-carotene inhibited the differentiation from preadipocytes to adipocytes, most likely because of the conversion of β-carotene into all-trans-retinoic acid (atRA). atRA acts as an inhibitor on PPARγ and promotes apoptosis. Cell models have shown dose and adipogenesis-stage dependency of the atRA effects on adipocyte differentiation: Higher doses at early stages had a inhibitory impact, whereas lower doses promoted adipocyte development. Additionally, animal studies observed pro-obesogenic effects of high vitamin A intake in early life by promoting the proliferation status of adipocytes [6].

Therefore, it can be summarized that mainly β-carotene impacts the conversion of preadipocytes to adipocytes because its cleavage to atRA (all-trans retinoic acid) which inhibits adipogenesis due to the suppression of PPARγ. However, also other carotenoid metabolites have shown antagonistic effects on PPARγ activity, namely retinaldehyd (Rald), astaxanthin and β-apo-14´carotenal [6]. That means that sufficiently available carotenoids may limit the number of adipocytes. Figure 5 highlights critical suppression and promotion factors in adipogenesis.

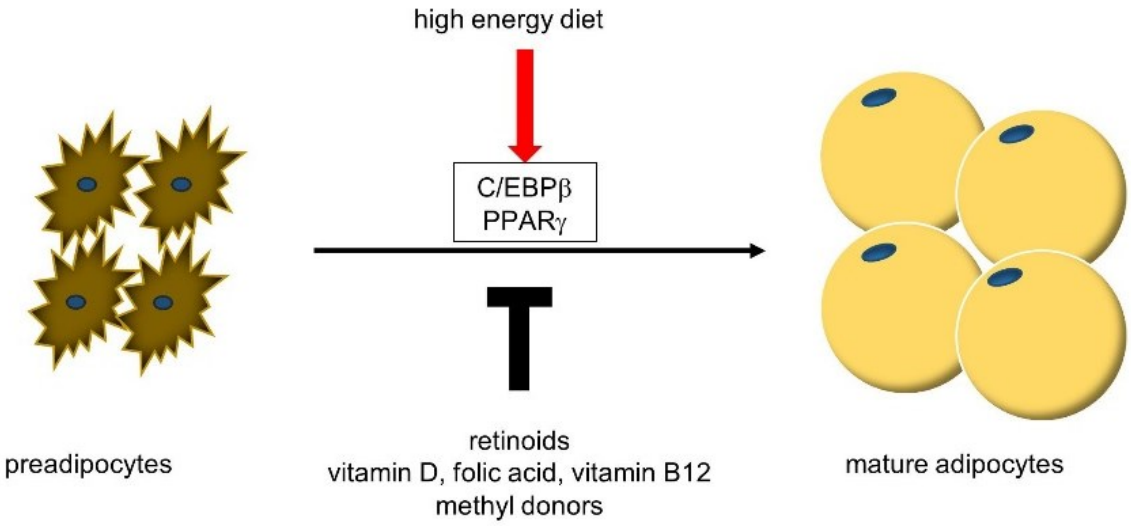


Figure 5: Nutrigenomic regulation of adipocyte differentiation
C/EBPβ: CCAAT-enhancer-binding protein β, PPARγ: peroxisome proliferator-activated receptor γ
adopted from [123]

1.2.4.1.1.2 Fat storage capacity

Carotenoids and their conversation products impact the fat storage capacity of adipocytes in two ways: First, due to their PPARγ inhibitory effect: PPARγ mediates adipocyte hypertrophy that is induced by a high fat-diet [125] and a reduction in PPARγ results in fat mobilization. Moreover, it contributes to the maintenance of the adipocytes phenotype (such as white and brown). It induces triglyceride synthesis and lipid droplet formation via specific pathways and thus enhances the adipocyte's fat storage capacity [126]. β-carotene is cleaved to retinoic acid by the cytosolic enzyme BCO1. All-trans retinoic acid (atRA) exhibits its PPARγ suppressing function as described above which leads to fat mobilization and decreased lipid content in the adipocyte. Results of animal and cell studies support that PPARγ expression in WAT correlates with the vitamin A status. Similarly, β-cryptoxanthin, astaxanthin and fucoxanthin showed anti-adiposity effects with reduced lipid accumulation [6].

Second, carotenoids and their conversion products enhance energy utilization by increasing fat oxidation and energy dissipation: They induce uncoupling protein 1 (UCP1) expression in brown adipocytes (most likely due to atRA). UCP1 is an inner-membrane protein of the mitochondria and involved in the fat oxidation process. atRA has been shown to activate thermogenesis in BAT and to reduce body fat and body weight in rodents. Vitamin A status is considered to be an important regulator of BAT thermogenesis since animal studies

showed vitamin A-dependent effects on BAT oxidative capacity. Retinoids have also shown to stimulate browning of WAT. atRA and Rald led to increased UCP1 and browning related gene expression in adipocytes in cell culture and mice. However, these effects could not be observed in human cells, and there is also a lack of evidence for β-carotene [6].

β-carotene and probably other provitamin A precursors may scavenge reactive oxygen species in the adipocyte. Together with the ability to repress hypertrophy this antioxidant property may help to avoid the pathological expansion of adipose tissue under persistent obesogenic influences and may support to keep the individual lean [88]. However, β-carotene is used up by these protective processes. In case of ongoing adiposity promoting conditions and a lack of dietary carotenoid supply, the adipose β-carotene stores will be consumed by the reactive oxygen species (ROS) scavenging process. Concerning the proposed anti-obesogenic effects of β-carotene, this results in a detrimental condition for the development of obesity [6] (Figure 6).

In summary, the retinoid signaling represses adipocyte hypertrophy and enhances energy utilization. Thus, carotenoids as precursors of vitamin A have anti-adiposity effects. Studies support the role of carotenoids in the prevention of excess adiposity and suggest that carotenoid requirements may be dependent on body composition [121]. Figure 6 provides an overview about the impact of β-carotene and its conversion products on the adipocyte's abilities to store fat and to scavenge ROS, as well as elected cellular pathways of cellular β-carotene degradation that are of importance for the anti-adiposity effect.

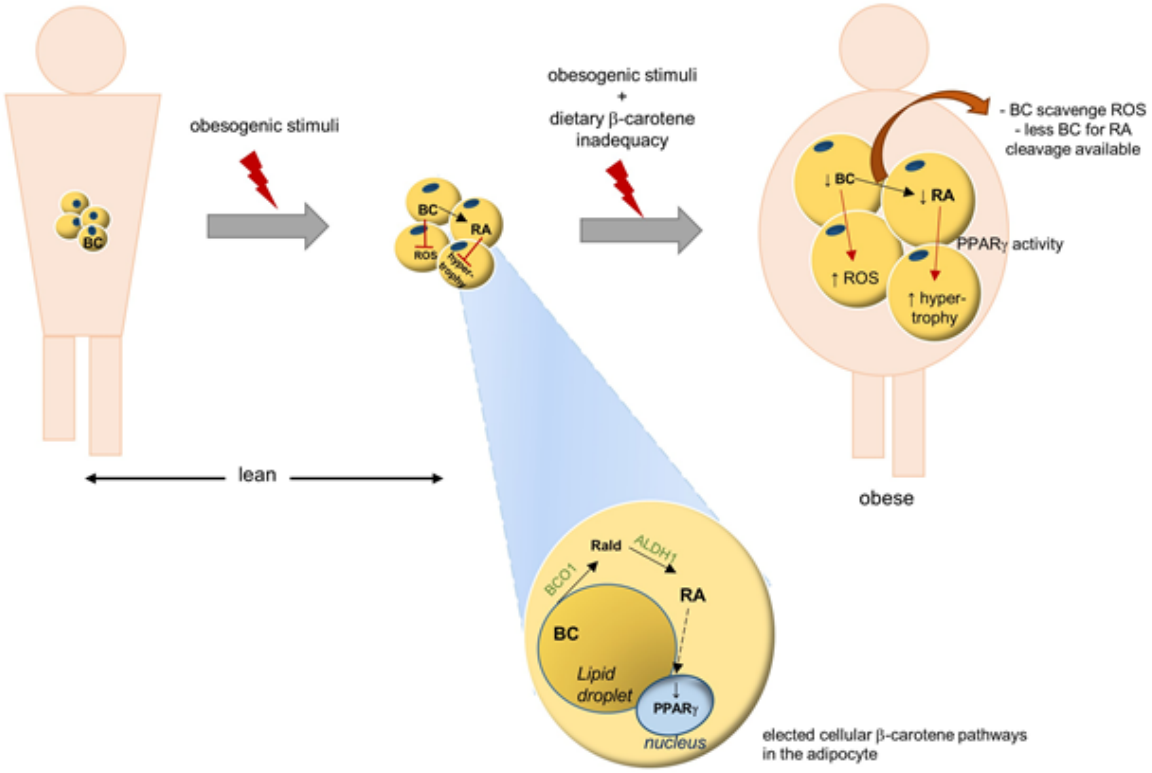


Figure 6: Impact of β-carotene on adipocyte fat storage capacity
ALDH1: aldehyd dehydrogenase 1, BC: β-carotene, BCO1: β-carotene-15,15´-oxygenase, PPARγ: peroxisome proliferator activated receptor γ, RA: retinoic acid, Rald: retinaldehyd, ROS: reactive oxygen species adopted from [6,88]

1.2.4.1.2 **Carotenoids, inflammation and oxidative stress**

The anti-inflammatory properties of carotenoids are the result of both their decreasing effect of oxidative stress and the suppressive effect on inflammatory pathways. These effects have been shown in a variety of tissues including adipose tissue [88].

Regarding the inhibition of inflammatory pathways, carotenoids suppress the activity of NF-κB (a nuclear factor that activates inflammatory pathways in the nucleus) and thus inhibiting the NF-κB pathway, which induces the downstream of pro-inflammatory cytokines (such as IL-8 and prostaglandin PG E2) [92]. In adipocytes, the carotenoids lycopene and fucoxanthin exhibit anti-inflammatory function due to the reduction of TNFα mediated cytokine and chemokine expression [6].

Regarding oxidative stress, carotenoids scavenge ROS (O°_2, peroxyl radicals) and influence the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway: The transport of Nrf2 to the nucleus is increased, and thus the expression of antioxidant and cytoprotective enzymes is activated (e.g., glutathione-S-transferases). Furthermore, mitogen-activated protein kinase (MAPK) is inhibited. These anti-inflammatory mechanisms are proven for different tissues. However, the evidence for this effect, especially in adipocytes, is rare [6,92].

Because of the conjugated double-bond structure of carotenoids (extended conjugated pi-electron system), they are capable of scavenging O°_2 (singlet oxygen radical) and peroxyl radical. To neutralize O°_2, physical quenching is common, which transfers energy between two molecules. However, also further scavenging mechanisms for free radicals are possible such as electron acceptance or donation, or hydrogen abstraction or acceptance [92].

The number of conjugated double-bonds is responsible for the antioxidant properties. Thus carotenoids exhibit different anti-oxidative capacity with regard to their chemical structure. E.g., lycopene quenches O°_2 more effectively than α- or β-carotene [97,127]. During the process of quenching carotenoids, radicals can be formed and may result in the pro-oxidative activity. Carotenoids occur in cell membranes as well as in the mitochondria, the nucleus and in rather low concentrations in the cytosol. Thus they have an important role in the protection of cellular membranes and lipoproteins against peroxide radicals [92]. E.g., β-carotene protects low-density lipoproteins (LDL) against oxidation [127].

Importantly, the oxidative properties of carotenoids are dose-dependent. Thus, carotenoids can also act pro-oxidative in high cellular concentrations and an oxidative environment. Conversion products like Rald are highly reactive, and epoxides that are formed during the anti-oxidative action may increase oxidative stress and impair mitochondria function. An excess of carotenoid breakdown products is accumulated in mitochondria, since the degradation is limited to BCO2 activity, and results in mitochondria dysfunction and induction of apoptotic mechanisms [128]. This has mostly be shown in hepatic cells [6].

1.2.4.1.3 **Influence of carotenoids on leptin production**

It has been shown in cell and in-vivo models that the exposure of all-trans retinoic acid (atRA), the conversion product of pro-vitamin A carotenoids, suppresses leptin production in adipose tissue by influencing RAR-and RXR- pathways. atRa serves as a ligand in RAR

transcription which interacts with RXR and PPAR nuclear receptors. Thus, atRA may enhance RXR and RAR levels. Additionally, PPARγ activation has been shown to limit leptin production. [129,130]

1.2.4.1.4 Clinical relevance: Hypercarotenemia in anorexia nervosa

Host-related factors influence the bioprocessing of carotenoids. Besides the body composition also the presence of diseases belongs to these influencing factors [118]. AN impacts lipid metabolism [131] and thus the transport and storage of carotenoids. The phenomenon of hypercarotenemia is a recognized occurrence in some AN patients [132-134]. Hypercarotenemia is characterized by high β-carotene plasma concentrations and the dermal accumulation of carotenoids which leads to obvious changes in skin color [134]. There are several possible explanations for this occurrence in AN patients such as relatively high intake of carotenoid-containing food or decreased demand, hypercholesterinemia or acquired disturbances in carotenoid metabolism like impaired lipoprotein degradation and altered storage ability. However, the underlying pathophysiological mechanisms are not clear yet [135].

1.2.4.2 Polyphenols

For the sake of completeness, a summarizing overview of the impact of polyphenols in the above described context should be provided. However, since polyphenols are not of major interest in this thesis, they will not be dealt with in more detail here.

Polyphenols comprise a large group of complex molecules and occur in plants. Besides their already mentioned coloring function – they are primarily responsible for darker colors like black, purple and intense red (anthocyanins) as well as yellow (flavonoids), they additionally fulfill protective functions concerning defense against microorganisms and pathogens, UV radiation and oxidative stress in the plant. Polyphenols occur quite frequently in the human diet. Around one gram of polyphenols is ingested within a regular diet each day. The primary dietary sources of polyphenols are fruits, especially berries, green and black tea, coffee, red wine, and chocolate, but also vegetables, legumes, and cereals contain specific amounts of polyphenols. They can roughly be divided into two major groups: Flavonoids and phenolic acids [136].

Polyphenols have a positive impact on diverse health parameters, and a protective function in the generation of degenerative diseases is presumed. They have the ability to scavenge ROS and thus exhibit antioxidant properties. A positive impact on the progression of inflammation (inactivation of NF-κB and MAPK) has been described. Additionally, it has been indicated that polyphenols have an anti-obesity effect. They interact with adipose tissue similarly as has been described for carotenoids. Especially the inhibition of preadipocyte differentiation and elevation of preadipocyte apoptosis, the suppression of adipocyte proliferation by down-regulation expression genes (PPARγ, C/EBPα) and lipogenesis as well as the increase in lipolysis and β-oxidation of fatty acids can be pointed out in this context. However, also hunger-suppression has been shown to be induced due to the release of CCK which leads to a reduction of food intake [7].

Human intervention studies dealing with the immunomodulatory effect of polyphenols are rare and revealed inconsistent results, mainly because of profound differences in the study design, the sources of polyphenols, and the polyphenol concentrations. Investigations showed improved antioxidant capacity after the intervention phase with polyphenol-rich products [115], however, others could not observe effects on inflammatory markers and the immune system [137]. Polyphenols are strongly metabolized by microbes of the intestinal tract. Hence, all metabolites derived from polyphenol-digestion need to be considered in the evaluation of changes in polyphenol status, making analysis complex [136,138].

1.3 Aims and hypothesis

The main aims of this thesis were:

(1) To measure subcutaneous adipose tissue thicknesses by a novel ultrasound technique in different female energy status groups to distinguish phenotypic profiles of the study population in more depth.

(2) To investigate associations between the SAT thicknesses, plasma and dermal carotenoids, inflammation and oxidative stress markers, plasma leptin levels, nutritional intake data, and laboratory parameters.

We hypothesized that:

(1) SAT thicknesses would be significantly different between groups.

(2) SAT thicknesses would vary strikingly in individuals with the same BMI.

(3) SAT thickness would be correlated to the parameters investigated: carotenoid levels, inflammation, and oxidative stress markers and leptin levels.

(4) Leptin levels would be associated with oxidative stress and inflammation markers.

(5) Leptin levels would be negatively correlated to carotenoid levels.

2. Material and methods

2.1 Study population

This dissertation is part of the “Energy Sensing in Anorexia nervosa (ESAN)”-study that was conducted at the Division of Immunology and Pathophysiology at the Medical University of Graz and had a cross-sectional design. A total of 107 female Caucasian participants aged from 18 to 40 years have been investigated for the study.

2.1.1 Group allocation

According to the widely established body mass index (BMI) classification [26] that has been published by the World Health Organization (WHO) subgroups were formed: a normal weight group (NW, n = 27) with BMI values ranging from 18.5-24.9 kg/m², an overweight group (OW, n = 22) with BMI values from 25.0-29.9 kg/m² and an obese group (OB, n = 20) which included individuals with BMI values ≤ 30 kg/m². In addition, a group of anorexia nervosa patients (AN, n=18) diagnosed with AN according to the WHO international statistical classification of diseases and related health problems (ICD)-10 diagnostic criteria F.50.0. [34] and a group of athletes (AT; n = 20) defined by a minimum of seven hours of regular training per week and the regular participation at competitions were assigned.

2.1.2 Recruitment

The majority of the study participants (groups NW, OW, and OB) was recruited from the universities of Graz and via social media. Local sports teams were asked to widespread the information on the study among their members with the result that the members of local level volleyball and water polo teams met the study requirements and were enrolled to the AT study group. The cooperation with three psychiatric clinics (Department of Psychiatry and Psychotherapeutic Medicine, Medical University of Graz; State Hospital Graz South-West, Location South; Department of Psychiatry, Hospital of the Brothers of St. John of God) enabled the recruitment of the AN patients. The recruitment of the study population as well as the conduction of the investigation setting at the study center was performed by the author of the thesis.

2.1.3 Selection criteria

We selected the study population by age and gender. Since mainly young women are affected by AN [42], solely female persons within the age range of 18 to 40 years were included in our study. The group of AN patients had to meet the criteria for AN diagnosis according to ICD-10, and the diagnosis had to be made by a clinical psychiatrist. The minimum training time per week required for being included to the AT group was seven hours in addition to the regular participation at sports-specific competitions.

Besides the above described inclusion criteria, several exclusion criteria were listed in the study protocol: Persons who suffered from acute or chronic diseases or infection (including metabolic disorders such as diabetes mellitus or hyperlipidemias, etc.), alcohol or drug abuse, and major cognitive deficits were not included. Also, gastrointestinal complaints such as a history of digestive diseases (e.g., inflammatory bowel diseases and irritable bowel syndrome) or a history of gastrointestinal surgery were exclusion criteria. The treatment with pro-, pre- or antibiotics within the last eight weeks was not permitted. Neither pregnant nor currently breastfeeding women were included, and AN-patients under life-threatening conditions were not enrolled.

The study was carefully conducted in accordance with the Helsinki Declaration, and the ethics committee of the Medical University of Graz approved the study protocol (MUG-26-383ex13/14). All participants have signed the written informed-consent and agreed on the anonymous use of their data. First results of this study have already been published [1,139-141].

2.2 Physique assessment

The measurement setting for the determination of the physique constitution included a series of different anthropometric parameters to obtain a comprehensive anthropometric profile of the participants. The anthropometric measures and body composition methods described below were performed by the author of this thesis.

2.2.1 Anthropometric measures and calculations

Body height [m] and **body weight** [kg] were measured with the integrated measurement station seca 764 (Seca GmbH, Hamburg, Germany), which combines a calibrated electronic scale and an electronic height measurement in one device. **Sitting height** [m] was measured with a stadiometer. Thereby the participants were asked to sit upright on a platform with the spine most possibly extended. For the measurement of the body and sitting height, the participants' head was kept in the Frankfort plane, an imagined vertical line between the lower border of the eye socket and the upper border of the external auditory canal. In this position, the vertex is the highest point of the skull. The above mentioned anthropometric measurements were performed according to the guidelines of the International Society for the Advancement of Kinanthropometry (ISAK) [142].

The circumferences of the participants' waist and hip, and the circumference of the upper arm, as well as the triceps skinfold thickness, were measured, whereas WHO- [143] and ISAK-[142] guidelines were applied. For the determination of the circumferences, a flexible tape that was non-extensible and calibrated in centimeters with a gradation in millimeters was used. Since the position of the participant while the measurement is taken affects the accuracy of the measurement, the subjects were asked to stand in a relaxed position with their feet close together and the arms hanging relaxed at the sides of the trunk. The **waist circumference** was measured in a neutral breathing position of the thorax at the narrowest point of the trunk which is typically between the lower edge of the costal arch and the iliac crest. For the determination of the **hip circumference**, the greatest girth at the height of the buttocks was identified and measured parallel to the ground. The **waist to hip ratio (WHR)** was calculated to roughly distinguish between gynoid and android fat distribution and estimate abdominal fat accumulation [143].

The circumference of the **upper arm** was measured at the middle between the acromion and the olecranon. This height was projected horizontally to the posterior site to measure the **triceps skinfold thickness (TSF)** with a calibrated slim guide skinfold caliper. The **mid-arm muscle circumference (MAMC)** was calculated according to: MAMC = upper arm circumference [cm] – (0.314 x TSF [mm]). This measure is based on data of the National Health and Nutrition Examination Survey (NHANES) studies of the United States and presents a rough estimate of the persons lean muscle reserves. It is primarily used for detecting malnutrition concerning somatic protein reserves in clinical settings since it is easy to apply without burdening the patients and is related to lean muscle mass [144].

2.2.1.1 Body mass index

The body mass index (BMI) assesses the body weight of a person by relating it to the person's body height. The BMI is calculated by dividing the body weight in kilograms through the body height in meters squared: BMI = body weight [kg] / body height [m]² [26]. The BMI assesses nutritional status indirectly and assumes higher body fat amounts in those individuals with higher BMI values and vice versa. According to this assumption, the nutritional status of individuals is classified into the following categories:

Table 1: Nutritional status assessed by the WHO BMI-classification

Body mass index BMI = body weight [kg] / body height [m]²		
BMI	Nutritional status	
< 18.5	Underweight	
18.5 - 24.9	Normal weight	
25.0 – 29.9	Pre-obesity	Overweight
30.0 – 34.9	Obesity class I	Obesity
35.0 - 39.9	Obesity class II	
> 40.0	Obesity class III	

adopted from [25]

Based on these BMI-categories, our study participants were allocated to subgroups for the first estimate of nutritional and energy status. However, since the BMI cannot consider the individual's body composition appropriately, additional measures for further profiling and in-depth analysis of nutritional and body composition status have been applied.

2.2.1.2 Mass index

The mass index (MI) has been developed in order to improve shortcomings of the BMI. The expert committee of the WHO stated in a technical report that the BMI formula leads to questionable results in adult individuals whose body proportions deviate from the average, especially in individuals with longer or shorter legs than expected for their body height [26]. Müller et.al. [145-147] developed the mass index (MI) which is a leg length corrected equation for the calculation of relative body weight that considers the individual's sitting height (and thus indirectly the leg length). Consequently, it counters the WHO's remark on body proportions and determines relative body weight by a differentiated approach. The MI equation is as follows:

Table 2: Calculation of the mass index (MI)

Mass index
MI = 0.53 body weight [kg]/(body height [m]/ sitting height [m])

The factor 0.53 is the so-called cormic index. It is derived from dividing the sitting height through the body height and thus characterizes the relative leg length. In different population the cormic index ranges from 0.50 to 0.55; thus, for the MI equation a value of 0.53 has been chosen since it represents the middle of this range [147]. The mass index uses the same categories for classifying weight rages as the BMI. In persons with long legs, the MI is higher than the BMI and the other way around. For people with average leg length, the MI is as high as the BMI.

2.2.1.3 Body surface area and subcutaneous fat mass calculation

In order to calculate a rough estimate of the SAT mass based on the ultrasound measurement and the measured D_{INCL} values (SAT thickness sum of the eight measured body sites as explained at the following section 2.2.2.1), the body surface area (S) was determined. S was calculated according to the Du Bois formula [148]. The SAT mass was calculated by means of S, the mean SAT thickness of the eight sites (d_M), the density of fat (ρ) [149] and a calibration factor derived from comparative measurements at 216 randomly distributed sites in a set of test persons in order to provide an estimate of the whole body fat distribution [150]. Notably, it has to be considered that the mean of the eight standardized

sites does not represent the mean of the overall body fat since most of the eight standardized sites were chosen at typical fat depot areas on purpose.

Table 3: Calculation of the body surface area and the mass of subcutaneous adipose tissue

Body Surface Area [m²]
$S = 0.20247\ h^{0.725}\ m^{0.425}$
h… body height, m… body mass
Subcutaneous Adipose Tissue Mass [kg]
SAT mass = $S\ d_M\ \rho\ 0.65$
$d_{M...}$ the mean SAT thickness of the eight ultrasound sites (see chapter 2.2.2.1)
ρ …density of fat = 0.92 kg dm^{-3}
0.65… calibration factor

2.2.2 Body Composition

To characterize the metabolic phenotypes in depth, we determined body composition parameters. Importantly, we measured the subcutaneous adipose tissue (SAT) patterning by a recently introduced standardized ultrasound measuring technique and additionally applied a bioimpedance analysis to compare the measurement results of both methods.

2.2.2.1 Ultrasound measurement of subcutaneous adipose tissue

The fat patterning of the study population was measured at eight clearly defined and standardized body sites by the recently established ultrasound method [151,152]. Additionally, the sum of these eight sites was calculated and represented in the value D_{INCL} to gain a value of the overall SAT thickness of a patient.

Brightness mode (B-mode) ultrasound (US) is an emerging measurement technique for the determination of body fat in body composition assessment [151,153,154]. This method has been found to have high accuracy and reproducibility for people of diverse body composition ranging from extremely lean to obese [152], and thus US measures SAT layers precisely. The main principle of the method comprises the measurement of the eight standardized

sites upper abdomen (UA), lower abdomen (LA), erector spinae (ES), distal triceps (DT), brachioradialis (BR), lateral thigh (LT), front thigh (FT), and medial calf (MC) for determination of SAT-layers [151]. However, a previous version of the measurement protocol included the site external oblique (EO) instead of the later introduced LT. Because this study was conducted before LT was defined, EO was used here instead of LT [155,156]. This needs to be considered in the interpretation of the preliminary reference values, which comprise LT in the assessment of SAT thicknesses [157]. The eight defined body sites represent the body parts trunk, arms, and legs that are the major storage sites of fat. To make the sites comparable between individuals and ensure reproducibility within individuals, a standardized procedure for marking has been defined, which allows an anatomically clear identification of the sites. This standardized marking procedure includes the application of distances that are relatives to the person's body height and ensure interpersonal comparability of the body sites. Importantly, for the ultrasound measurement, a thick gel layer between the probe and the skin at the marked sites is applied to prevent the compression of SAT by avoiding the exertion of pressures on the skin surface. At the US image, the borders of the SAT-layer are clearly visible. The upper border is constituted by the epidermis and the lower border by the muscle fascia, both visible as a consistent line in the US image. A typical example of an ultrasound image including the SAT region and the described borders is shown in Figure 7.

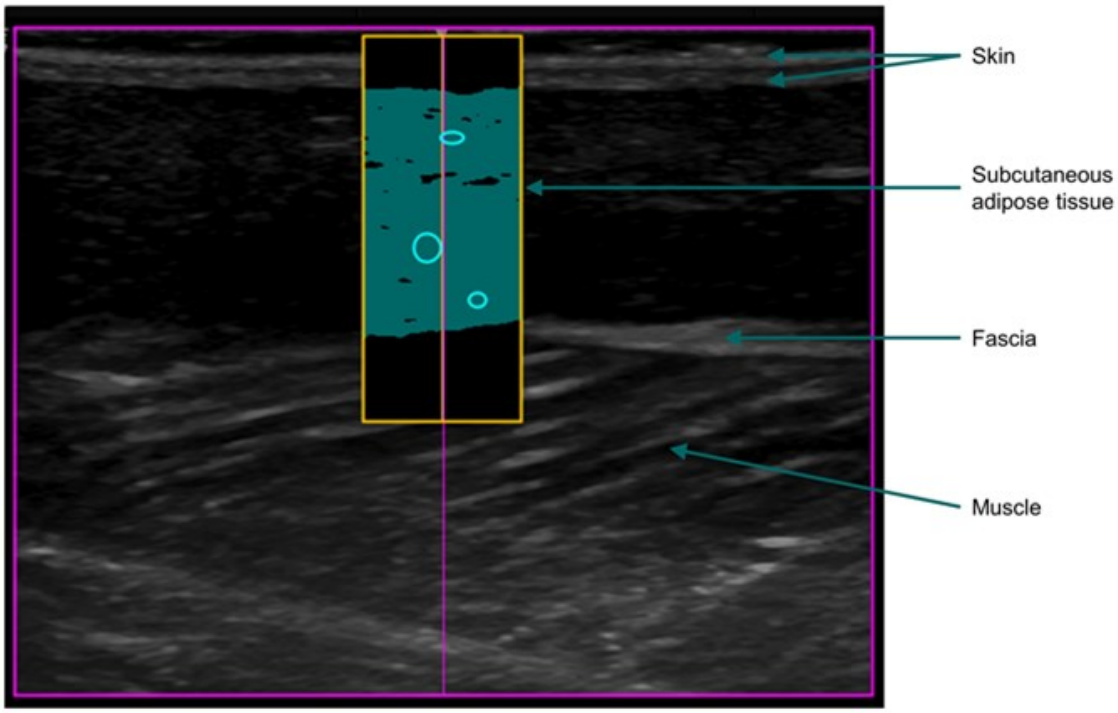


Figure 7: Explanation of an ultrasound image

For ultrasound imaging, a conventional US system (GE Logiq-e, General Electric) with linear probes (L8-18i RS and 12L RS) operated at 8 to 16 MHz was used. The US images were evaluated by using a specific software (the USTissue-FAT 3.2, Rotosport, Stattegg, Austria, rotosport.at), which had been developed for semi-automatic evaluations of SAT-layer thicknesses. The software provides information on D_{INCL} (the sum of eight measurement sites) and D_{EXCL} (the sum of SAT thicknesses without embedded tissues such as fibrous structures). Embedded structures were calculated as follows: $D_{ES}=D_{INCL}-D_{EXCL}$.

Based on D_{INCL} the SAT mass was calculated according to the above mentioned formula that comprises the mean SAT thickness, the body surface area, the density of fat, and the calibration factor. Importantly, the SAT mass calculation includes model assumptions whereas the SAT thicknesses are accurately measured values. Thus, they should be used preferentially. The SAT mass values were calculated here to present a rough estimate of SAT in terms of fat mass [kg|.

For the assessment of SAT values, preliminary reference values have been published recently [157]. Table 4 provides an overview of these reference values.

Table 4: Preliminary reference values for the assessment of subcutaneous adipose tissue (SAT).

Sum of SAT [mm]	Valuation	Comment
Competitive athletes: Female		
Below 25	Extremely low	Medical surveillance recommended
25-35	Very low	Surveillance recommended
35-50	low	Desirable range
50-70		Noticeable ballast weight
Above 70		Considerable ballast weight
General public: female		
Below 25	Extremely low	Medical surveillance strongly recommended
25-35	Very low	Medical surveillance recommended
35-80	Low	Desirable range
80-110		Noticeable ballast weight
110-140		Considerable ballast weight
140-180	High	Medical surveillance recommended
Above 180	Very high	Medical surveillance strongly recommended

adopted from [157]

2.2.2.2 Bioelectrical impedance analysis

The bioelectrical impedance analysis (BIA) is generally based on the deviating electrical conductivity of different body compartments. Tissue without fat has relatively higher water and electrolyte content compared to fat mass. Electrical impedance, which is defined as the flow of electrical current through human tissues, is used for the determination of total body water and subsequently for the calculation of fat-free mass. The amount of body fat is indirectly calculated, by subtracting FFM from body weight. Currant, voltage, and phase angle are measured [158].

In this study, single frequency BIA (BIA 101 – Body Impedance Analyzer Akern) was conducted at 50 kHz following recommended procedures [159,160]. The BIA measurement was performed in a lying position with the extremities not touching the trunk or each other. While the patients rested for at least 10 minutes in this position in order to allow the body water to distribute homogenously, four self-adhesive electrodes were located on the dorsal sides of the patient's right hand and foot. The impedance was measured from the wrist to the ankle. The participants were instructed to fast overnight before performing BIA. The obtained results of resistance (R), reactance (Xc) and phase angle (PA) were further analyzed by a commercially available software (BodyComposition – Professional v9.0.14325) which applies the equations of Sun et.al. [161] for calculation of fat free mass (FFM) and total body water (TBW), and Sergie et.al [162] for calculation of extracellular water (ECW). To calculate total body fat (TBF), FFM was subtracted from body weight: TBF [kg] = body weight - FFM.

In addition to the software evaluation, other BIA equations have been applied in the subgroup of AN patients, since these formulas had been proposed to be more appropriate for this patient group. The equations of Deurenberg [163] and Kushner [164] were suggested by Matter et al. [165] for the assessment of FM and FFM in AN patients and have been applied for comparison reasons.

Table 5: BIA equations for calculation of fat-free mass (FFM)

BIA equations for the calculation of fat-free mass (FFM)	
Sun [161]	FFM [kg] = -9.529 + 0.696 (h^2/R) + 0.168 m + 0.016 R
Kyle [166]	FFM [kg] = -4.104 + 0.518 (h^2/R) + 0.231 m + 0.13 Xc
Deurenberg [163]	FFM [kg] = -12.44 + 0.34 (h^2/R) + 0.1534 h + 0.273 m - 0.127 age
h… body height, m… body mass, R… resistance, Xc… reactance	

2.3 Lifestyle parameters

2.3.1 Nutritive assessment

To provide an estimate on the dietary supply of energy and nutrients, structured and interviewer guided 24 hour-recalls were performed. The interviews were completed twice and included detailed questions on the consumed food of the previous day [167]. The first interview was done on the day of the investigation; the second interview was performed at a randomly chosen day within a maximum timeframe of four weeks after the investigation took place. The interviews analyzed and evaluated by the national specific nutritional software nut.s® (www.nutritional-software.at, Vienna, Austria) that comprises an Austrian specific food and nutrient database [168]. The nutritive assessment was conducted by the author of this thesis.

2.3.2 Physical activity

The participants' physical activity level was determined by the International Physical Activity Questionnaire (IPAQ) [169]. The IPAQ is a standardized questionnaire that had been validated for various populations. It is used to determine the habitual level of physical activity in different parts of daily life. In total, it can be divided into five subparts namely, physical activity related to occupational work, physical activity for transport purposes, physical activity related to household activities, leisure-time actives including exercise, and sitting time. The IPAQ is a self-administered, self-assessment questionnaire and comprises 27 questions that are related to the previous seven days. The activities are categorized into vigorous and moderate actives. This allows a further weighting of the activities in the assessment of the physical activity level. The results are presented in "metabolic equivalent of task" (MET)-minutes per week.

2.3.3 Smoking behavior

Since smoking influences energy expenditure and oxidative stress status, it was a central factor to be assessed and considered in the interpretation of the results. The Fagerström test for nicotine dependence (FTND) was applied to assess the smoking behavior of the study population [170]. This questionnaire comprises six questions that address different

parts of addiction and thus enables a distinction of dependent and non-dependent smoking as well as the classification of low, moderate, strong or severe smoking behavior. The evaluation of the questionnaires was done by the author.

2.4 Laboratory parameters

2.4.1 Clinical chemistry

Standard blood values were determined in accordance with standard procedures. Blood draws were conducted in overnight fasted participants. The blood was taken by the study physician and the author of the thesis was involved in the whole bio-sampling and preparative analytical procedures, and the sample logistics. Further analyses of clinical parameters if not stated otherwise were done in cooperation with Harald Mangge and Sieglinde Zelzer from the Clinical Institute for Medical and Chemical Laboratory Diagnostics of the Medical University of Graz. Especially, plasma lipids were primarily relevant for this thesis since carotenoids transport is strongly related to lipid metabolism.

Plasma lipids such as total cholesterol, triglycerides, and HDL-cholesterol were measured by enzymatic photometric transmission measurement (Roche Diagnostics, Mannheim, Germany). The concentrations of LDL-cholesterol were calculated by the Friedewald's formula [171].

2.4.2 Inflammation markers

In addition to routine clinical values, markers of inflammation such as C-reactive protein (CRP) and Interleukin (IL)-6 were analyzed at the clinical institute for laboratory diagnostics of the Medical University of Graz in accordance with the standard procedures. A particle-enhanced turbidimetric assay and an electrochemiluminescent immunoassay (ECLIA), respectively, were applied on a Cobas 6000 chemical routine analyzer (Roche Diagnostics, Mannheim, Germany) for the determination of these parameters. The immune-turbidimetric test for CRP based on reaction enhancement via latex particles. Anti-CRP-antibody-latex formed an antigen-antibody complex with CRP from the sample. After agglutination, its concentration was determined turbidimetrically [172]. For ECLIA, which is a sandwich principle, IL-6 specific monoclonal antibodies were used and together with microparticle

form an antigen-antibody-complex with the IL-6 of the sample. The microparticles were fixed via magnetic forced on electrodes where chemi-luminescence emission was induced due to an electrical current and measured by a photomultiplier. The results were then evaluated by a calibration curve [173].

2.4.3 Oxidative stress markers

To assess overall oxidative stress in the study population, two comprehensive parameters were chosen. Those parameters comprise the amount of free radicals and the organism's capacity to counteract those. The parameters total oxidant capacity (TOC) and total antioxidant capacity (TAC) were determined in serum samples by specific enzyme-linked immunosorbent assays (ELISA).

With the parameter total oxidative capacity (TOC) the amount of total peroxides in serum samples is determined, whereas the parameter total antioxidant capacity (TAC) comprises the sum of all antioxidant enzymes and scavengers that are available to counteract ROS and thus neutralize oxidative stress. The total peroxide concentrations and total antioxidant capacity were determined by an enzymatic diagnostic assay (TOC®, TAC® assay; Labor Diagnostic Nord, Nordhorn, Germany). This assay uses tetramethylbenzidine (TMB) as a substrate for a peroxide-peroxidase reaction. Thereby, TMB is oxidized and changes its color. The reaction can be determined by a colorimetric approach. The results were calculated by using the linear standard curve for hydrogen peroxide. Peroxide levels were specified as µmol hydrogen peroxide equivalents [174]. Oxidative stress markers were analyzed in cooperation with Willibald Wonisch from the Otto Loewi Research Center, Division of Physiological Chemistry of the Medical University of Graz.

Based on these two parameters, the oxidative stress index (OSI) was calculated which is expressed as the ratio of TOC/TAC. It combines the pro- and anti-oxidant status and is comparably high in cases with a predominance of ROS. In other words, if TAC levels are low, OSI is elevated, indicating higher oxidative stress.

2.4.4 Leptin

Leptin was determined in plasma samples by applying an ELISA (BioVendor, Brno, Czech Republic). Thereby, the plasma was first incubated with polyclonal anti-human leptin antibodies. After washing a conjungated solution (consisting of polyclonal anti-human leptin

antibodies conjungated with horseradish peroxidase) was again added to the previously captured leptin. After another incubation and washing step, TMB is added as a substrate to the remaining conjugate and induces an oxidation process. The reaction is stopped by adding an acidic solution, resulting in a yellow color. The concentration of leptin, which is proportional to the absorbance, is determined by a photometric measurement using standard curves [175].

2.4.5 Carotenoids

2.4.5.1 Resonance Raman spectroscopy

Dermal carotenoids were assessed at the palm via resonance Raman spectroscopy [176,177]. This method is a non-invasive quantitative optical option for the detection of carotenoid concentration in the skin. It is a form of laser spectroscopy that determines the energy level of a molecule. The Raman effect describes the scattering of light at molecules and the related energy transmission. Due to the scattering of monochromatic light at the molecule a shift in the light's frequency is detectable. Thus, resonance Raman-spectroscopy measures the spectrum of scattering. The chemical structure of carotenoids, especially the strongly absorbing conjugated carbon double bonds, the methyl side chain and distinct end groups, make them in particular suitable for resonance Raman spectroscopy. For the excitation of the molecules, blue laser light with a wavelength of 473 nm is used. Due to the scattering at carotenoids, the wavelength shifts to 510 nm. Skin carotenoids are preferentially measured at the palm since the stratum corneum is relatively thick at this site and thus the interaction of the laser light with other tissues is avoided [178].

For this study, the Pharmanex®BioPhotonic Scanner S2 (NuSkin, Provo, Utah) was used for the determination of skin carotenoids. The measurements were performed at the palm of the right hand and conducted by the author. According to the manufacturers manual the carotenoids were measured at the stratum corneum in 0.1 mm depth. The scanner emits light from 471.3 to 473 nm and measures the scattered light at 507.8 to 509.8 nm. This detected shift is represented as skin carotenoid score (SCS) with a possible range from <19.000 (low carotenoid concentration) to >50.000 (high carotenoid concentration). The results of this measurement comprise the total of all carotenoids stored in the skin and does not differentiate between single carotenoid subtypes.

2.4.5.2 High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography (HPLC) allows the fractionation of a solution containing various substances. The analyte molecules are interacting with a stationary and a mobile phase, thus leading to differences in retention time through the separation column. For the separation of carotenoids, which are non-polar molecules, a reversed phase HPLC was applied, meaning that a polar mobile phase (also referred to as eluent) and a non-polar stationary phase (the column) was used for analyte separation. For the detection, a UV-vis detector was used, which is best suitable for carotenoids due to their light absorbing properties. The peak shown in the chromatogram represents the difference between the absorbance of the eluent and the samples (response/ intensity) after the appropriate retention time [179]. For quantification, the internal standard method with a known calibrator and external standard curves were applied. The standard reference material (SRM) 968e from the National Institute of Standards and Technology (NIST) was used to ensure the accuracy of the measurements. In this study, the sample preparation was done by the kit ClinRep® for HPLC analysis of β -carotene in plasma (Recipe, Munich, Germany).

α-carotene, β -carotene, β -cryptoxanthin, and lycopene were analyzed via HPLC in plasma samples, and the results of the concentrations and group comparisons have been published in the master thesis of Nathalie Meier-Allard [180], which was part of this study and this doctoral thesis. Since β-carotene was the most crucial carotenoid that influences adipose tissue biology, this thesis solely includes this parameter for analysis.

2.5 Statistical analysis

The software SPSS Statistics v23 (IBM, Armonk, NY, USA) was used for the statistical analysis. Shapiro–Wilk tests were applied to determine the normal distribution of the variables. Since this test is suitable even in small sample sizes, it was chosen for distribution assessment in the five subgroups. This test revealed that not all data were distributed normally. Descriptive statistics are presented as mean and standard deviation (SD) when data were normally distributed. Otherwise the median (Mdn) and interquartile ranges (IQR) were used. Levene's test was applied for the assessment of the data's heterogeneity. For normally distributed data analysis of variance (ANOVA) with post hoc Turkey-test were used to examine group differences between the five energy groups. Student's t-test was applied for the identification of significant differences in the comparison of two groups.

Additionally, covariates were considered for the correction of possible confounding effects. For non-normally distributed data, Kruskal-Wallis and Mann-Whitney u-tests were applied. Chi-squared-test was used for qualitative variables. Spearman's rank correlation coefficient (r_s) was used for bivariate correlations, and multivariate regression analyses were chosen for interconnection analysis of several independent variables. P-values below 0.05 were considered statistically significant.

3. Results

3.1 Descriptive statistics and group comparisons

3.1.1 Study population characteristics

A total of 107 participants was enrolled in the study. The participants' group allocation was as follows: 18 AN, 27 NW, 22 OW, 20 OB and 20 AT. The BMIs of the whole study population ranged from 13.24 to 46.89 kg/m². A detailed description of the group characteristics is shown in Table 6. The medians of the MI were similar to the BMI values. The age median of the studied cohort was 23 years (IQR: 6 years). Although a narrow age range was applied for recruiting, OB participants were significantly older compared to AN (U = 98, p = 0.016) and AT (U = 104.5, p = 0.009). The other groups did not differ significantly in age.

Table 6: Study population characteristics

Main characteristics		AN	NW	OW	OB	AT	p-value
n=		18	27	22	20	20	
Age [years]**		22	24	24	26	21	0.002
		6	5	6	10	3	
BMI [kg/m²]**		15.5	21.8	27.0	33.0	21.6	<0.001
		2.0	3.4	1.3	4.4	2.5	
BMI range	min.	13.24	19.02	25.12	30.44	19.09	
	max.	17.19	24.89	29.34	46.89	25.88	
MI [kg/m²]**		15.9	21.5	27.2	32.4	21.4	<0.001
		1.7	2.8	2.0	4.4	2.4	
* Mean (SD) ** Median (IQR)							

The group of AN patients was composed of the subtypes purging (n = 6) and non-purging (n = 12). The duration of the illness, which refers to the first documented diagnosis, was up to 13 years (Mdn = 2, IQR = 7). Regarding nutritional treatment, ten of the 18 investigated patients reported on having received high energy supplements during their treatment. The patients were investigated after an average of 16.5 (IQR = 46) days of treatment.

The AT group was composed of water polo player (n = 5), volleyball player (n = 11), and handball player (n = 4). Thus, the AT group could be further distinguished into water ball athletes (n = 5) and land ball athletes (n = 15).

3.1.2 Anthropometry

Except for body height, the study groups differed significantly in their anthropometric characteristics, whereas expectantly the OB group had the highest and the AN group the lowest values. The results of the anthropometric measurement are provided in Table 7.

Although the BMI of AT did not differ significantly from NW, AT had significantly lower TSF ($U = 100.5$, $p < 0.001$) and higher MAMC ($U = 41.5$, $p < 0.001$) values, indicating comparably higher muscle mass.

Presumably, due to severe malnutrition, the TSF was not measurable in one AN patient. Therefore, only 17 AN patients could be considered for TFS and MAMC values.

Table 7: Anthropometric group characteristics

Anthropometry	AN	NW	OW	OB	AT	p-value
Body height [m]*	1.66	1.69	1.67	1.68	1.71	0.204
	0.069	0.057	0.057	0.066	0.055	
Body mass [kg]**	41.7	60.8	75.0	97.7	64.6	<0.001
	7.5	10.4	7.6	13.1	6.8	
Sitting height [m]**	0.855	0.895	0.887	0.899	0.909	0.001
	0.047	0.055	0.046	0.031	0.048	
Body surface area [m²]**	1.43	1.71	1.82	2.02	1.79	<0.001
	0.15	0.17	0.16	0.17	0.15	
Waist circumference [cm]**	60.0	70.0	78.5	95.3	71.0	<0.001
	5.3	7.0	6.3	17.5	6.7	
Hip circumference [cm]*	79.3	93.3	106.6	121.8	97.9	<0.001
	4.1	6.4	6.6	8.0	5.8	
Waist-to-Hip-Ratio**	0.76	0.77	0.74	0.76	0.72	0.032
	0.04	0.06	0.08	0.12	0.06	
Upper arm circumference [cm]*	19.8	26.7	31.5	36.3	28.0	<0.001
	1.8	1.7	2.1	3.2	1.6	
Triceps Skinfold [mm]**	7	19	27	35	14	<0.001
	4	3	9	5	6	
Mid-Arm Muscle Circumference [cm]**	17.6	20.7	23.5	25.1	23.7	<0.001
	1.5	2.0	2.5	2.0	1.9	
* Mean (SD) ** Median (IQR)						

3.1.3 Body composition assessment

3.1.3.1 Ultrasound measurement of subcutaneous adipose tissue

Overall group comparison by Kruskal-Wallis-test and ANOVA revealed that SAT-thicknesses of the groups differed significantly in all measured sites and thus also in the calculated site-sum and SAT-mass. However, AT did not differ significantly in SAT-thickness from NW at sites BR, UA, LA, EO, FT, ES, D_{INCL} and D_{excl} although all mean values were lower in AT, and from AN at site UA, EO, MC, ES although all mean values were higher in AT, indicating SAT-thicknesses of AT between AN and NW. Fat patterning of all other groups and sites differed significantly. The results of SAT-thicknesses measured by the ultrasound technique are shown in Table 8.

Except for the site FT, data of at least one group per parameter was not normally distributed. Thus, data are presented as median and IQR. For the analysis of the ultrasound images, three participants (two NW, one OW) could not be considered due to technical issues. Thus, for further analysis related to D_{INCL} only 104 of the 107 participants could be considered.

Table 8: Subcutaneous adipose tissue thicknesses measured by ultrasound

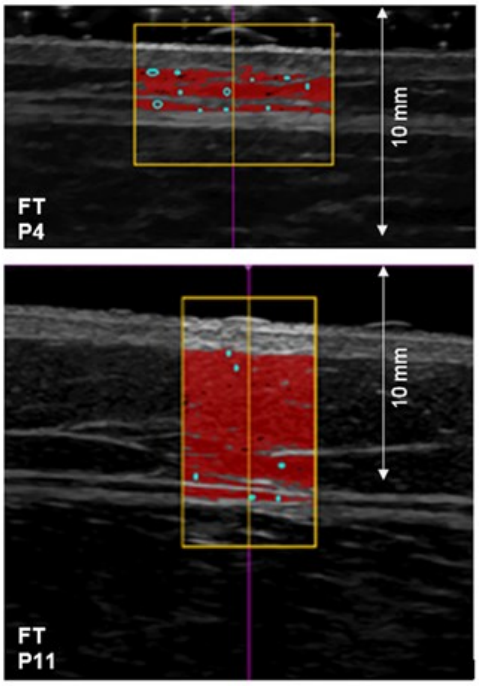
US measurement of SAT	AN	NW	OW	OB	AT	p-value
D_{INCL} [mm]**	30.17	83.62	140.78	196.94	58.42	<0.001
	36.11	33.62	43.75	42.76	43.59	
D_{EXCL} [mm]**	26.58	80.15	136.75	192.03	53.39	<0.001
	34.53	34.56	40.43	40.74	37.06	
SAT mass [kg]**	3.42	9.95	19.00	30.94	7.51	<0.001
	3.68	5.29	6.31	7.86	6.34	
Brachioradialis [mm]**	1.10	4.21	6.56	7.58	3.02	<0.001
	2.28	1.93	2.43	1.78	2.11	
Upper Abdomen [mm]**	3.97	12.16	24.55	44.93	7.01	<0.001
	3.50	7.76	13.85	20.03	7.82	
Lower Abdomen [mm]**	5.74	17.32	33.04	52.73	15.04	<0.001
	8.45	7.63	14.19	16.16	10.05	
External Oblique [mm]**	1.68	6.00	14.65	22.21	4.74	<0.001
	2.73	5.53	7.23	11.04	6.91	
Front Thigh [mm]*	4.99	12.60	19.23	24.06	10.33	<0.001
	3.35	2.93	4.05	7.86	3.31	
Medial Calf [mm]**	2.91	7.88	12.41	15.65	5.67	<0.001
	4.57	4.55	6.35	6.06	2.93	
Distal Triceps [mm]**	3.88	10.21	14.77	17.94	7.65	<0.001
	5.20	5.71	6.38	6.14	3.86	
Erector Spinae [mm]**	2.88	6.62	13.09	23.12	5.51	<0.001
	3.42	2.73	4.71	8.54	6.76	
* Mean (SD) ** Median (IQR)						

D_{EXCL}: SAT thicknesses sum without embedded tissues, D_{INCL}: SAT thickness sum of the eight measured body sites, IQR: interquartile range, SD: standard deviation

3.1.3.1.1 Intragroup comparison of anorexia nervosa patients - Identification of two body composition groups in AN patients

Based on the observation of extremely divergent D_{INCL} results in AN patients with similar BMI, the group was divided by the median of D_{INCL} into two subgroups. Compared to the recently published preliminary reference values, the group with the lower SAT values (mean D_{INCL} = 13.66 mm, SD = 9.55 mm) could be classified as persons with extremely low SAT and thus medical surveillance is recommended, whereas the group with the higher SAT thicknesses (Mean D_{INCL} = 44.59 mm, SD = 10.62 mm) belongs to the reference group with low and desirable SAT ranges [1,157]. Figure 8 highlights this enormous ranges by the direct comparison of the measurement point FT in two AN patients with a BMI of 14.5 kg/m² and 14.7 kg/m², respectively. The patient depicted at the upper picture had D_{INCL} of 9.0 mm and the SAT thickness at FT was 1.7 mm, whereas the patient at the lower image had D_{INCL} of 33.3 mm and SAT thickness at the same measurement site was 6.6 mm.

The percentages of fibrous structures D_{ES} embedded in the SAT were higher in the group with lower body fat (Mdn = 2.11 mm, IQR = 1.75 mm; 14% of D_{INCL}) compared to the group with higher body fat (Mdn = 3.75 mm, IQR = 0.96; 9% of D_{INCL}).



	BMI [kg m^{-2}]	D_{INCL} [mm]	d_{FT} [mm]
P4	14.5	9.0	1.7
P11	14.7	33.3	6.6

Figure 8: Subcutaneous adipose tissue variation in anorexia nervosa patients. An example of an ultrasound measurement.
BMI: body mass index, d_{FT}: subcutaneous adipose tissue thickness at the site front thigh, D_{INCL}: Sum of subcutaneous adipose tissue thicknesses of eight defined body sites, FT: front thigh, P: patient

3.1.3.1.2 **Intragroup comparison of athletes**

Since athletes of different sports were recruited, they were compared regarding their SAT thicknesses. When grouped into water ball and land ball groups (including volleyball and handball athletes) the groups differed significantly in their SAT-thicknesses ($t(18) = 2.204$, $p = 0.041$) despite comparable BMIs. Thereby, water ball athletes had a significantly higher mean D_{INCL} (90.9 mm, SD = 43 mm) compared to land ball athletes (Mean D_{INCL} = 58.9 mm, SD = 22.1 mm).

3.1.3.1.3 **Intergroup comparison of anorexia nervosa patients and athletes**

When comparing the two derived groups of AN patients and athletes, interestingly, no significant deviation between the group of AN patients with normal fat amounts and the group of land ball athletes could be observed ($t(22) = -1.812$, $p= 0.084$). ANOVA revealed significant differences of D_{INCL} between the four subgroups ($F(3, 34) = 15.494$, $p < 0.001$), however, those differences were derived from the above described intragroup deviations. Figure 9 depicts the striking group differences and includes p-values derived from ANOVA post-hoc Turkey analysis.

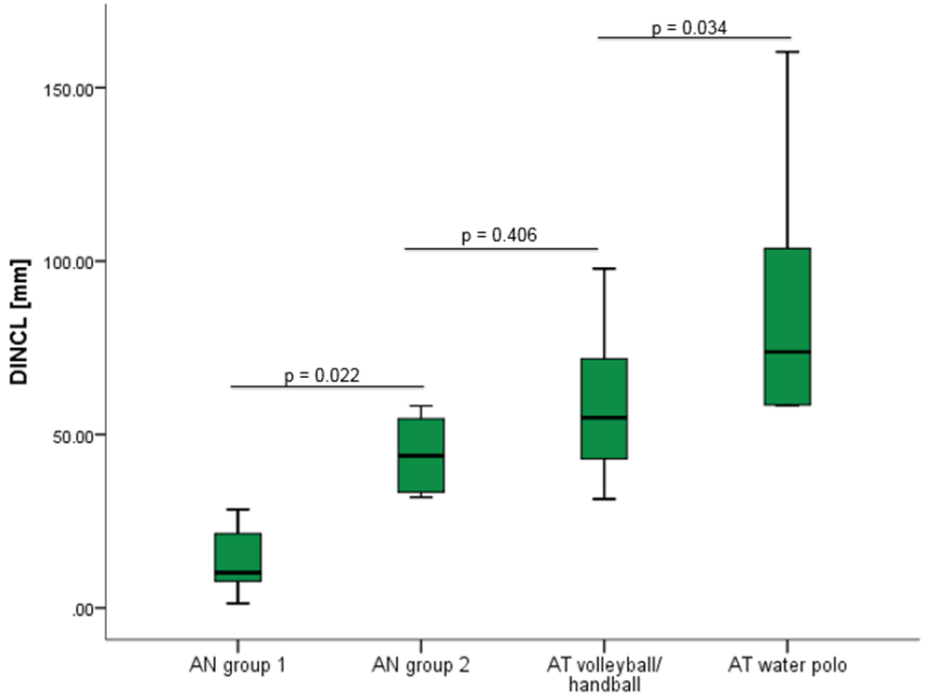


Figure 9: Intergroup comparison of subcutaneous adipose tissue thicknesses between the two anorexia nervosa and the two athletes groups.

AN: anorexia nervosa, AT: athletes, D_{INCL}: SAT thickness sum of the eight measured body sites

3.1.3.2 Bioelectrical impedance analysis

The results of the bioelectrical impedance analysis are shown in Table 9. The values for FFM and FM deviated depending on the formula used for calculation. However, according to ANOVA and Kruskal-Wallis-test, the six studied groups differed significantly in the calculated parameters. Thereby, AN patients had the lowest and obese participant the highest FFM and FM, as could be expected. The groups NW and AT differed significantly in their FFM derived from all the applied equations ($t(45) = -3.837$, $p < 0.001$, $t(45) = -4.222$, $p < 0.001$, $t(45) = -3.410$, $p = 0.001$, respectively), however, interestingly they did not differ significantly in FM although the median values were slightly lower in AT.

Table 9: Results of the bioelectrical impedance analysis

Bioelectrical impedance analysis	**AN**	**NW**	**OW**	**OB**	**AT**	**p-value**
Resistance R [ohm]*	728.6	667.5	621.7	548.9	582.9	<0.001
	68.7	50.3	38.2	77.2	46.9	
Reactance Xc [ohm]*	64.4	67.4	64.2	58.2	65.4	0.009
	13.0	7.4	6.5	8.8	6.1	
Phase angle PA [°]*	0.17	0.08	0.09	0.15	0.12	<0.001
	0.74	0.41	0.44	0.69	0.53	
FFM_{SUN} [kg]*	35.9	41.4	44.5	51.9	45.6	<0.001
	3.1	3.6	3.1	6.4	3.8	
FFM_{KYLE} [kg]*	33.9	41.2	45.0	52.9	45.3	<0.001
	2.6	3.1	2.7	6.1	3.5	
$FFM_{DEURENBERG}$ [kg]*	34.8	41.8	45.9	54.1	45.6	<0.001
	3.3	3.9	3.4	6.2	3.6	
FM_{SUN} [kg]**	6.9	19.7	30.4	43.2	18.8	<0.001
	5.0	7.6	3.9	9.1	4.8	
FM_{KYLE} [kg]**	8.9	20.3	30.5	42.4	18.2	<0.001
	3.2	6.4	4.3	8.3	4.6	
$FM_{DEURENBERG}$ [kg]**	7.7	20.1	28.9	41.7	18.4	<0.001
	4.5	6.3	2.9	8.3	4.2	
* Mean (SD) ** Median (IQR)						

FFM: fat free mass, FM: fat mass, IQR: interquartile range, SD: standard deviation

3.1.4 Lifestyle parameters

3.1.4.1 Nutritive assessment

A series of nutrients was derived from the analysis of repeated 24 hour recalls. Table 10 provides an overview of important elective nutrient intake values.

The groups did not differ significantly in their energy intake, and either in the composition of the main macronutrient intake, however, differences were recorded regarding the dietary content of fat qualities. Saturated fat uptake was significantly higher in the OB group compared to AN ($U = 76$, $p = 0.002$) and OW ($U = 140$, $p = 0.044$), and cholesterol uptake was significantly higher in the OB group compared to AN ($U = 112$, $p = 0.48$), NW ($U = 132$, $p = 0.03$), OW ($U = 119$, $p = 0.011$), indicating higher intake of food of animal origin in the OB group. Additionally, the diets fibre content differed significantly between the groups, whereas OW participants reported the lowest fibre intake while AT had the highest.

Regarding dietary micronutrient consumption, significant group differences were derived for vitamin C – which is important for antioxidant physiology. AT had by far the highest vitamin C intake. Also, AN showed high vitamin C intake. However, that is mainly the result of dietary supplements which were part of the therapeutic treatment. The nutrient content of these supplements is included in this analysis. Additionally, B-vitamin intake including vitamin B12 was significantly different between the studied groups.

Regarding vitamin A, retinoic acid equivalents and carotene dietary intake the groups did not differ significantly. The comparatively high minerals consumption of AN patients is again result of the dietary treatment strategy.

Table 10: Nutrient intake of the study groups

Nutritional Assessment	AN	NW	OW	OB	AT	p-value
Energy [kcal]	1918.5	1893.8	1749.2	2064.6	2010.9	0.187
	1471.7	895.6	327.4	615.5	585.1	
Energy [MJ]	8.02	7.94	7.32	8.65	8.37	0.187
	6.13	3.75	1.43	2.58	2.47	
Macronutrients						
Carbohydrate [g]	232.8	200.9	188.8	209.6	233.4	0.240
	215.4	107.0	61.4	71.1	104.0	
Sugar [g]	121.7	87.2	76.8	110.0	100.7	0.139
	118.1	59.9	46.3	68.1	86.3	
Protein [g]	78.6	62.1	60.5	73.9	73.7	0.072
	51.5	32.9	20.0	32.3	41.5	
Fat [g]	79.0	80.1	66.6	78.9	76.0	0.460
	39.7	48.8	20.6	33.4	34.5	
Saturated Fat [g]	24.6	31.1	29.7	36.9	30.8	0.033
	18.3	20.9	12.4	8.7	16.1	
Omega 3 fatty acids [g]	2.2	1.3	1.5	1.6	1.4	0.810
	1.4	1.4	0.6	1.1	2.6	
Omega 6 fatty acids [g]	10.3	13.8	9.0	10.2	14.7	0.165
	8.0	5.9	6.8	11.2	14.6	
Fibre [g]	19.4	20.7	16.6	17.4	23.8	0.050
	12.8	8.4	6.6	6.6	14.7	
Alcohol [g]	0.1	0.5	0.2	0.0	0.0	0.009
	0.6	9.2	7.5	0.1	0.7	
Water [l]	2.8	3.0	3.5	3.0	3.8	0.059
	1.3	1.5	1.3	1.2	1.8	

Micronutrients						
Elected water soluble vitamins						
Vitamin C [mg]	125.7	80.5	68.0	87.2	133.2	0.013
	134.7	74.6	42.2	150.9	115.0	
Vitamin B12 [µg]	4.3	3.0	3.0	4.3	3.6	0.010
	2.7	2.5	1.6	2.6	1.9	
Elected fat soluble vitamins						
Vitamin A, retinol [mg]	0.4	0.3	0.3	0.5	0.4	0.61
	0.2	0.2	0.2	0.3	0.4	
Vitamin A, retinol equivalents [mg]	1.3	1.0	0.9	1.1	1.2	0.281
	1.2	0.8	0.9	0.8	0.9	
Vitamin A, carotene [mg]	5.2	4.3	3.2	3.4	5.0	0.266
	8.4	3.5	5.4	4.3	4.7	
Vitamin E [mg]	13.1	13.5	10.5	12.2	14.0	0.324
	19.1	5.4	6.5	10.1	11.9	
Vitamin D [µg]	5.1	1.8	1.8	2.5	2.3	0.026
	11.4	1.2	2.2	2.7	2.8	
Minerals						
Iron [mg]	17.0	11.3	10.5	9.7	11.7	0.046
	15.4	3.4	4.3	7.2	8.4	
Zinc [mg]	10.7	9.0	7.6	9.7	9.8	0.061
	8.0	3.9	1.4	5.1	5.0	
Sodium [g]	2.3	2.4	2.7	3.0	2.6	0.136
	1.2	1.1	1.2	1.1	1.0	
Calcium [mg]	1162.1	786.8	868.4	978.4	936.4	0.174
	751.6	424.0	371.4	325.1	384.3	
Magnesium [mg]	373.0	318.7	302.6	304.1	322.9	0.333
	232.1	136.3	109.2	143.7	254.7	
All values are presented as median and IQR						

3.1.4.2 Physical activity and smoking behavior

Physical activity was determined by IPAQ scores and represented by (MET) minutes per week. IPAQ scores were significantly different between the groups ($p = 0.018$), whereas AT unsurprisingly had the highest (Mdn = 6012, IQR = 4139) and AN patients (Mdn = 2200, IQR = 5008) the lowest IPAQ scores.

28 of 107 study participants stated being smokers. Chi-squared test revealed that smokers were not equally distributed between the groups (χ^2 (4, N = 107) = 14.533, $p = 0.006$). Nine AN patients, five NW, eight OW, six OB participants, and none of AT were smokers. AN patients reported the highest nicotine dependency with a median Fagerström score of 4 (IQR = 5) followed by OB participants (Median = 3, IQR = 4). The scores of both groups can be classified as mild nicotine dependency. NW (Median = 0, IQR = 2) and OW (Median = 1, IQR = 1) participants had no or mild nicotine dependency.

3.1.5 Laboratory parameters

Table 11: Results of laboratory chemistry

Laboratory chemistry	AN	NW	OW	OB	AT	p-value
Plasma lipids						
Cholesterol [mg/dl]	169.0	170.0	173.0	190.5	176.5	0.598
	56.8	57.0	45.3	60.8	48.8	
HDL-cholesterol [mg/dl]	73.0	80.0	75.0	58.0	81.5	0.001
	14.5	20.0	23.0	23.8	19.5	
LDL-cholesterol [mg/dl]	78.0	80.0	82.0	106.5	81.0	0.113
	43.0	41.0	44.3	46.5	19.0	
Triglycerides [mg/dl]	78.5	66.0	78.5	105.0	75.5	0.034
	53.8	39.0	61.3	74.8	49.5	
Inflammation marker						
CRP [mg/l]	0.6	1.3	1.5	5.3	0.7	<0.001
	1.0	1.9	3.6	6.2	1.4	
IL-6 [pg/ml]	1.5	1.5	2.0	3.6	1.5	<0.001
	0.4	0.6	1.1	1.9	0.7	
Oxidative stress marker						
Total oxidant capacity (TOC) [mmol/l]	0.07	0.07	0.08	0.16	0.15	<0.001
	0.05	0.07	0.18	0.17	0.17	
Total antioxidant capacity (TAC) [mmol/l]	1.00	0.98	0.87	0.97	1.01	0.138
	0.48	0.16	0.14	0.31	0.68	
Oxidative stress index (OSI)	0.07	0.07	0.10	0.18	0.15	0.001
	0.09	0.07	0.22	0.35	0.17	
Leptin						
Leptin [ng/ml]	1.6	10.9	23.9	49.1	8.6	<0.001
	2.8	7.9	21.1	15.6	7.8	
Carotenoids						
Skin carotenoid score (SCS)	34500	32000	26000	20000	36500	<0.001
	20500	18000	13250	14000	19000	
β-carotene [µg/l]	563.4	346.4	249.4	196.7	392.5	<0.001
	678.5	307.0	144.7	147.6	373.4	
All values are presented as median and IQR						

3.2 Analysis of correlations and multiple regression analysis

Subcutaneous adipose tissue thicknesses showed a high correlation with the participants BMIs ($r_s(104) = 0.942$, $p < 0.001$). However, participants with similar BMIs were distributed over several classification categories of D_{INCL} as can be seen in Figure 10. For example, the SAT thicknesses of AN patients with BMIs of around 15 kg/m² could be classified as extremely low up to low [1], which is considered to be the desirable SAT amount [157]. The SAT thicknesses of the NW participants with BMIs of around 22 kg/m² were distributed over the categories low to considerable ballast weight, and the OW participants with BMIs of around 27 kg/m² had SAT values distributed over three categories such as noticeable ballast weight, high and very high SAT values.

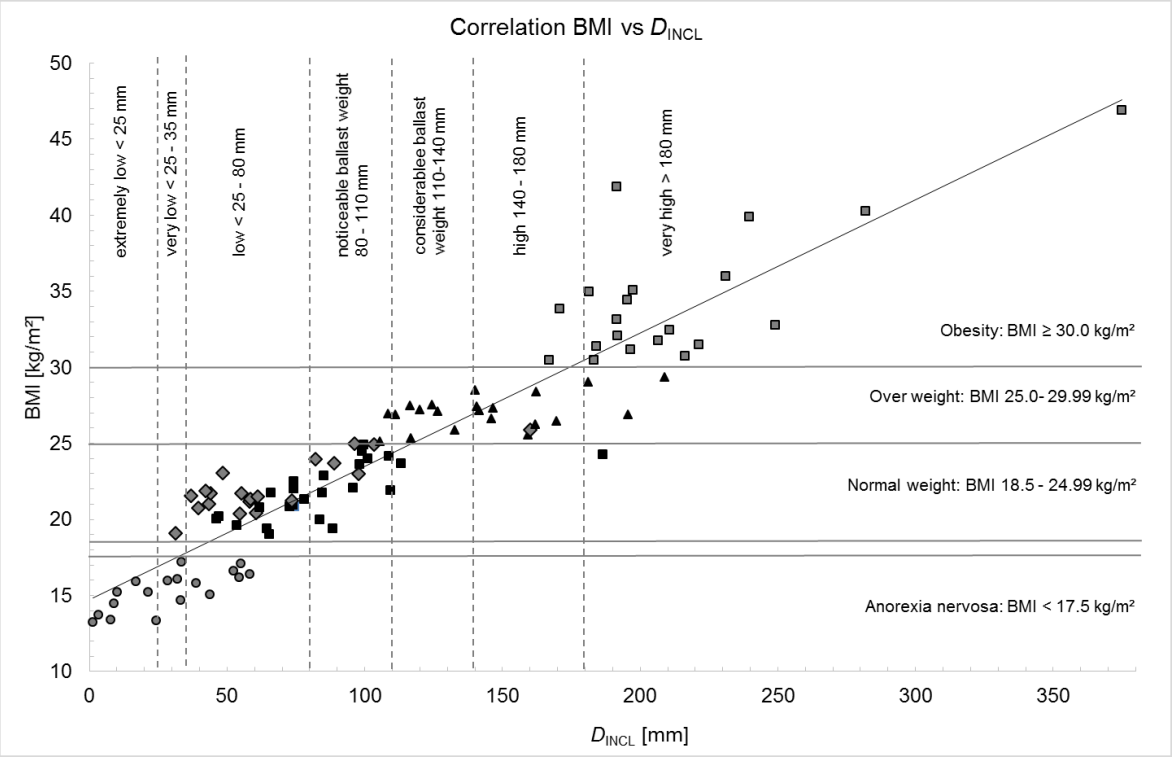


Figure 10: Correlation of body mass index and subcutaneous adipose tissue

The five study groups are highlighted with different symbols to distinguish them more easily: anorexia nervosa patients are marked as gray dots, athletes are depicted as gray diamonds, normal weights are shown as black squares, overweight participants are depicted as black triangles, and obese are represented as gray squares. The participants with similar BMIs are distributed over several SAT assessment categories. BMI: body mass index, D_{INCL}: SAT thickness sum of the eight measured body sites, SAT: subcutaneous adipose tissue.

In addition to this strong association of SAT and BMI, D_{INCL} was highly positively correlated to plasma leptin levels ($r_s(104) = 0.895$, $p < 0.001$), underpinning the physiological response to the observed SAT amount.

Regarding the physiological state of carotenoids in skin and plasma, D_{INCL} was negatively correlated to skin carotenoid counts ($r_s(104) = -0.498$, $p < 0.001$), α-carotene ($r_s(104) = -0.293$, $p = 0.003$), β-carotene ($r_s(104) = -0.563$, $p < 0.001$), and β-cryptoxanthin ($r_s(104) = -0.285$, $p = 0.003$), whereas lycopene was positively correlated to D_{INCL} values ($r_s(104) = 0.346$, $p < 0.001$). The strongest association was observed for total skin carotenoids (Figure 11) as well as for β-carotene, which accounts for the major part of plasma carotenoids and has been reported to influence adipose tissue biology most prominently [6].

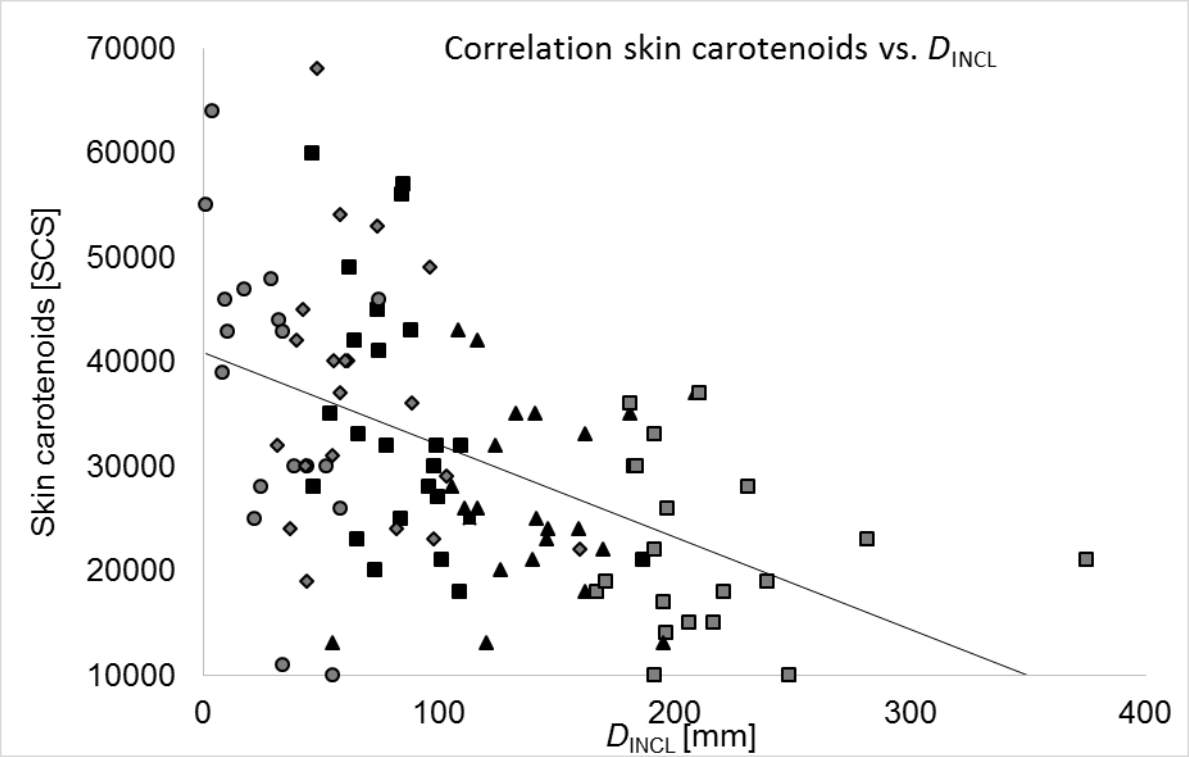


Figure 11: Correlation of subcutaneous adipose tissue and carotenoids
The study groups are depicted as follows: anorexia nervosa: gray dots, athletes: gray diamonds, normal weight: black squares, overweight: black triangles, obese: gray squares. Carotenoids are negatively correlated to subcutaneous adipose tissue. $D_{INCL:}$ subcutaneous adipose tissue thickness sum of the eight measured body sites, SCS: skin carotenoid score

Regarding oxidative stress parameters, D_{INCL} was significantly correlated to TOC ($r_s(104) = 0.379$, $p < 0.001$) and the oxidative stress index ($r_s(104) = 0.331$, $p = 0.001$), however, Spearman correlation did not detect a significant correlation of SAT subcutaneous adipose tissue thicknesses and total antioxidant capacity. Additionally, both investigated inflammation markers such as CRP ($r_s(104) = 0.617$, $p < 0.001$) and IL-6 ($r_s(104) = 0.557$, $p < 0.001$) were positively correlated to subcutaneous adipose tissue thicknesses.

Additionally, the correlations of leptin to oxidative stress (TOC: $r_s(107) = 0.387$, $p < 0.001$, OSI: $r_s(107) = 0.330$, $p < 0.001$), inflammation (CRP: $r_s(107) = 0.576$, $p < 0.001$, IL-6: $r_s(107) = 0.522$, $p < 0.001$) and carotenoid levels (Skin Carotenoids: $r_s(107) = -0.434$, $p < 0.001$, α-carotene: $r_s(107) = -0.280$, $p = 0.003$, β-carotene: $r_s(107) = -0.575$, $p < 0.001$, β-cryptoxanthin: $r_s(107) = -0.223$, $p = 0.021$, lycopene: $r_s(107) = 0.289$, $p = 0.002$) were determined.

To determine the statistical interconnection of these parameters, multiple regression analysis was applied with setting D_{INCL} as the primary factor. Based on the results of the Spearman correlation as a pre-evaluation, leptin, skin carotenoids, β-carotene, TOC, OSI, CRP, and IL-6 were chosen as co-variables. The results of this analysis adjusted for age indicate a collective significance between these factors ($F(8, 95) = 34.564$, $p < 0.001$, $R^2 = 0.744$). Thereby, leptin and carotenoid counts were the strongest predictors of D_{INCL} in this model. Both were significant predictors ($p < 0.001$ and $p = 0.048$, respectively) in this model. The other factors did not contribute significantly to the prediction of D_{INCL} in this linear regression model. Interestingly, when only considering carotenoids in the linear regression model for the prediction of SAT ($F(5, 98) = 8.581$, $p < 0.001$, $R^2 = 0.304$), besides total carotenoid counts ($p = 0.005$) only lycopene remained significant for the prediction of D_{INCL} ($p = 0.014$). The scatter plot matrix of Figure 12 visualizes the regression model and data distribution of the important D_{INCL} descriptors.

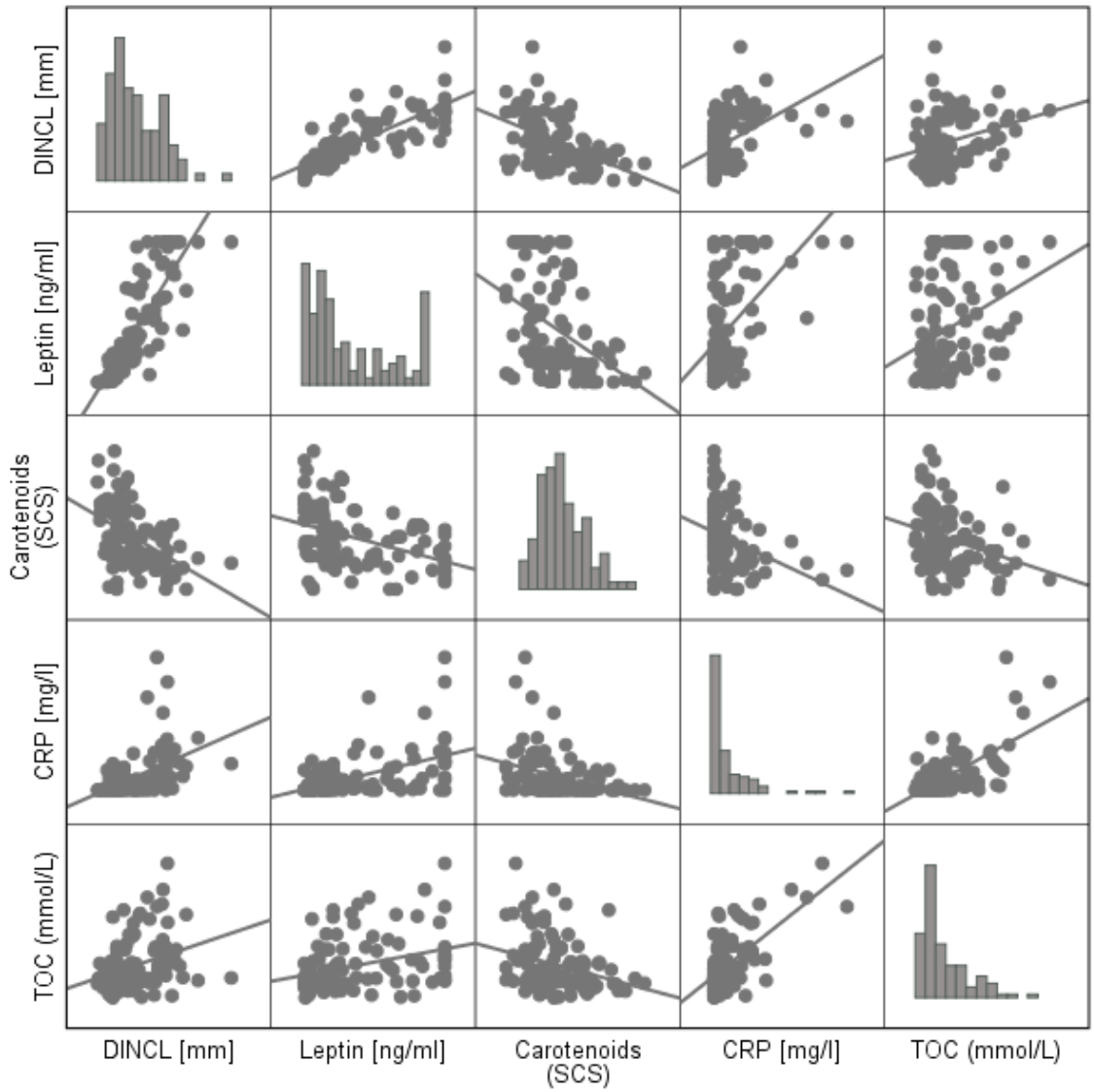


Figure 12: Scatter plot matrix depicting the distribution of interconnected variables
CRP: C-reactive protein, $D_{INCL:}$ subcutaneous adipose tissue thickness sum of the eight measured body sites, SCS: skin carotenoid score, TOC: total oxidative capacity.

4. Discussion

4.1 Summary of the findings

The primary focus of this thesis was set on the evaluation of the correlation of adipose tissue, leptin, oxidative stress, and inflammation, and the carotenoid status in a human cross-sectional model as this connectedness had been identified previously only in animal and cell models. The relation of those parameters had been evaluated separately in humans. However, to our best knowledge, the complexity in a personalized system biological approach had not yet been targeted.

In brief, the main results of this study can be summarized as follows: The application of a recently described standardized ultrasound measurement technique for the determination of subcutaneous adipose tissue thicknesses revealed considerable differences in fat patterning and total SAT amount among the investigated groups. However, striking differences within the assigned groups of AN and AT in total SAT and fat patterning despite comparable BMIs of the subjects could be observed [1]. Thus, it is indicated that the use of SAT is more convincing in the description of the metabolic phenotype of the participants than the BMI alone.

SAT values were highly correlated with investigated parameters of the oxidative stress, inflammation, carotenoid levels, and circulating leptin values. Regarding the interconnection of the foursome immune-modulating parameters of adipose tissue, leptin, oxidative stress, inflammation, and the nutritional component carotenoids, strong associations have been observed. Thereby, leptin and dermal carotenoid concentrations showed the strongest connection to SAT thickness among the investigated parameters.

4.2 Answers to the research questions

This thesis primarily aimed to examine the following research issues:
(1) To measure subcutaneous adipose tissue thicknesses by a novel ultrasound technique in different female energy status groups in order to distinguish phenotypic profiles of the study population in more depth.
(2) To investigate associations between the SAT thicknesses, plasma and dermal carotenoids, inflammation and oxidative stress markers, plasma leptin levels, nutritional intake data, and laboratory parameters.

The hypotheses were outlined in section 1.3 Aims and hypothesis of this thesis. Generally, we could verify our hypothesis:
(1) SAT thicknesses are significantly different between the investigated groups.
(2) SAT thicknesses vary strikingly in several individuals with the same BMI.
(3) SAT thickness is positively correlated with inflammation markers (CRP and IL-6), oxidative stress (TOC and OSI) markers and leptin levels, and SAT values were negatively correlated to carotenoids (α-carotene, β-carotene, and β-cryptoxanthin) and positively correlated to lycopene.
(4) Leptin levels are correlated to oxidative stress (TOC and OSI) and inflammation markers (CRP and IL-6).
(5) Leptin levels are negatively correlated to carotenoid levels (α-carotene, β-carotene, and β-cryptoxanthin) and positively correlated to lycopene.

4.3 Contribution to the current state of knowledge and innovatory value

A common strategy to determine the nutritional status of a person is to evaluate its BMI and classify the relative body mass in accordance with the WHO recommended weight categories [25]. However, the BMI is not capable of capturing body composition, which is also true for the other proposed equation for calculation of relative body weight: the mass index (MI). Thus, this approach is rather weak in estimating the metabolic consequences and health risks of increased or drastically reduced body weight and unfavorable fat distribution [181].

In recent years, research has focused on the complex interactions of metabolic pathways in systems biological approaches, integrating the response of the whole organism to certain physiological conditions or environmental stressors [2]. Within this complex machinery of the physiological regulatory processes, body composition, and body fat distribution take over a crucial role, since body fat has been shown to influence metabolism on various levels [76,182]. Thus, the determination of body fat is an interesting part of metabolic phenotyping. Moreover, plant nutrients and thus nutritional habits have been shown to influence adipocyte biology profoundly [6,183].

There is a huge number of papers dealing with health consequences and metabolic profiling in obesity available, and also the interest of metabolic profiling in athletes is noteworthy. However, only a few studies are evaluating the metabolic profiles of patients suffering from AN. Our data contribute to sharpening our knowledge on AN phenotypes by revealing different body composition types [1], suggest the adoption of therapeutic strategies in accordance with the body composition of the patients and highlights potential metabolic consequences with respect to oxidative stress and the clinical phenomenon of hypercarotenemia. In addition, we discuss the importance of body composition assessment in athletes with respect to clinical parameters.

Body fat acts as a metabolically active organ in the human organism [77]. Additionally, our data suggests the implementation of easy to apply, non-invasive, and reliable and accurate body composition assessment in clinical practice in addition to the BMI in the sense of deeper phenotyping. However, established field methods often lack reliability in individuals [153]. Thus, our findings support the application of a recently standardized ultrasound

method for measurement of SAT that has been shown to be accurately and reliably in individuals ranging from extremely lean to obese [152].

4.3.1 The necessity of evaluating body composition in addition to the BMI

Although the BMI is widely used for the assessment of the relative body weight, this approach has known limitations. BMI is not capable of predicting body composition adequately. Thus, additional factors need to be considered for the classification of the metabolic profile. Even the WHO, who disseminated the use of this classification, points out problems in adult individuals whose body characteristics differ from the average. Especially longer or shorter legs and thus differences in body proportions may lead to inadequate results [26]. Moreover, sex, age, and race differences in healthy body composition remain unconsidered [184].

Based on the statement of the WHO expert committee regarding the issue of unexpected body proportions, Müller et al. [145-147] developed the mass index (MI) in order to introduce a more sensitive equation which also considers individual leg lengths. In the population studied here, we compared BMI and MI values. Generally, the mean and median values did not differ strikingly for the studied groups. However, when focusing on individuals, values varied substantially, indicating the importance of considering body proportions in assessing relative body weight.

Regarding AN patients, the MI exceeded the BMI in 13 cases and was lower in two cases. This may be of importance for the diagnosis of AN since both – the ICD-10 criteria [34] as well as the diagnostic criteria of DSM-V [35] for the diagnosis of AN - use certain BMI cut-offs (17.5 kg/m² or 17.0 kg/m², respectively) as one of the essential diagnostic values. Applying the MI would shift the relative body weight of some AN individuals beyond the diagnostic BMI cut-off. For example, in persons with unexpected body proportions such as relatively long or short legs, BMI and MI could easily differ by a full unit or more. This issue has already been highlighted in the publication associated with this thesis [1]. Two concrete examples are provided in order to highpoint this consideration: One AN patient had a BMI of 13.3 kg/ m² and a MI of 14.4 kg/m^2 which means that the MI was 1.1 kg/m² larger than the BMI. In this case the severity of AN cannot be adequately assessed by the BMI. In another case, the BMI was 17.1 kg/m² whereas the MI was 17.8 kg/m² which means when using die MI this patient would not have been classified as AN patient.

However, as already criticized previously, the determination of relative body weight (neither by applying the BMI nor the MI) without additional accurate and reliable assessment of body fat does not predict healthy weight sufficiently [26,38,185].

4.3.2 Selection of appropriate body composition assessment methods

Currently, there is a lack of reliable threshold values for minimum fat necessary for healthy living. The acceptable minimum of body fat may be dependent on genetically determined factors [181]. Additionally, especially when focusing on the extreme body composition groups of AN and AT, sufficiently accurate field methods for the detection of body fat were missing until recently. Critical reviews of widely applied field methods for body composition assessment in sports and clinical fields exist [153,186]. The US method has been shown to provide accurate and reliable results in the measurement of SAT thicknesses [151]. On the contrary, broadly applied methods like dual-energy X-ray absorptiometry (DXA) [153], magnetic resonance imaging (MRI), skin folds [156] or BIA [158] have been criticized for insufficient accuracy for such extreme body composition groups.

Regarding DXA, which is often considered as a reference method in body composition determination and widely used in research, some striking issues occurred when measuring very lean athletes. One noteworthy occurrence is that DXA based fat determination failed to determine fat mass appropriately in very lean athletes. The measurement led to the paradox result of “negative fat” at the trunk region. This may be explained due to the underlying algorithms used in DXA models. DXA fat determination is based on model assumptions which may lead to profound measurement errors. This limits the applicability of DXA in extreme patient groups with extremely thin fat layers as can be observed in AN and AT. Regardless of the known measurement errors in these people, DXA should cautiously be used on repeated occasions because of the cumulative effect of the low-grade radiation. Moreover, the longitudinal detection of small changes in body composition may be limited due to the error of measurement [153]. However, there exist clinical practice guidelines for performance and analysis of DXA measurements, and also the detection of visceral fat is possible [187,188].

MRI scans also have inherent limitations in detecting extremely low body fat layers. They are not capable of gathering the extremely low layer thicknesses of subcutaneous fat expected and partially found in AN patients with sufficient accuracy. The main limitation is the pixel size that is used in MRI total body scans. Pixel size is typically between 1.3 and 2

mm, and this is much larger than the thickness of SAT to be measured in AN patients [153,189].

The main limitation of skinfold measurements can be summarized as follows. Although a constant closing compression of the calipers used for skinfold measurement is required for this technique, the results are strongly depended on the tissues' compressibility which cannot be considered as constant between individuals and either between body sites. In addition, the composition of the adipose tissue is not homogeneous, and either is the fat patterning [153,190].

BIA is widely used to determine body composition in clinical settings as well as for the assessment in sports. Additionally, BIA is also often used in research settings. However, the BIA method has substantial limitations. Several different equations for the evaluation of body composition exist. BIA equations are often validated with comparisons of DXA results. However, as stated above even DXA has known shortcomings, in particular when measuring lean persons. Instead of this comparison, the use of multi-component models should preferentially be applied as the reference method [191]. Multi-component models are the established state-of-the-art method for determination of total body fat on the molecular level [153]. The parallel use of multiple equations in the field makes it difficult to compare results between studies and individuals. In this study, also different equations were applied for comparison reasons. The results were comparable for the whole study groups. However, when focusing on the individual, the results deviated drastically. BIA is a common method for body composition determination in AN patients [192-194], but its reproducibility [195], accuracy [153], and validity [196] are low. Especially in AN patients, this is partly attributed to the impaired hydration and electrolyte status in AN patients [159,160].

Regarding total body fat assessment with BIA in AN patients, additionally, equations considered to be appropriate for AN patients have been applied in our study. For example, Matter et al. [165] suggested the use of the equations of Deurenberg [163] and Kushner [164] for the assessment of body composition in AN patients. The calculation of fat mass using these additional formulas also revealed deviating results from the values derived by ultrasound. In clinical practice, this erroneous assessment of body fat may lead to incorrect decisions in the selection of appropriate treatment and thus may influence therapeutic success negatively.

However, regardless of the applied algorithm, the BIA method has further fundamental shortcomings and associated errors [152]. Besides the fact, that it is built on an extremely

simplifying model of the human body, its compartments, and their electrical conductivities, there are several further factors such as hydration and electrolyte status (which is essential in AT as well as in AN), exercise, and food and fluid intake shortly prior the measurement that influence measurement outcome. [158]

Kerr and Hume [158] who critically reviewed this method stated that BIA generally could be applied for the assessment of groups and for the monitoring of individual changes over time. However, they concluded that the assessment of body composition in individuals at a single time point should not be recommended. Our observations support this statement. As shown in the result section, the mean BIA values for the group of AN patients derived from different equations deviated notably from each other.

The advantages of ultrasound as a field method for body fat determination have already been reviewed. The standardized ultrasound measurement points of SAT are distributed all over the body. Three sites are measured at the trunk, two sites are determined on arms and three on legs [151,152]. Those body sites can be considered as representative for the major fat storages at the human body and thus represent total subcutaneous fat appropriately [150]. In addition, a calibration factor is applied in order to estimate total SAT which is the major part of total body fat [102]. However, the exact amount of visceral fat is not captured by this method.

We applied US for the first time in AN patients with the result that pronounced differences in fat patterning and body composition could be observed in AN patients with the same BMI. This observation was additionally associated with the other parameters this thesis focusses on such as carotenoid levels, oxidative stress, inflammation, and leptin levels.

4.3.3 Body composition variations and metabolic consequences

4.3.3.1 Variations in body composition in anorexia nervosa patients

Based on the considerations above, the recently developed and standardized ultrasound method was applied for the measurement of SAT thickness in the whole study population. In the subgroup of AN patients, pronounced variation of SAT thicknesses in AN patients with the same or similar BMI was observed. Thus, we decided to divide the patient group into two subgroups in accordance with their body composition: The median of SAT thicknesses (D_{INCL} = 30.2 mm) was used for group assignment. Group 1 had extremely low

SAT values and group 2 had higher SAT values. Recently, preliminary reference values for D_{INCL} have been published [157]. According to this publication, the D_{INCL} values of female athletes with high physical activity levels and thus higher energy turnover rates (and assumed higher muscle mass) may vary from 35 to 50 mm. This range of SAT thickness is considered to be the "desirable range" of subcutaneous body fat in female athletes. For all other women D_{INCL} values from 35 to 80 mm are suggested to be the desirable range. Despite the low BMI of AN patients, the SAT amount found in group 2 was comparable with SAT values of healthy, sporty active and normal weight females [151,152,157].

The D_{INCL} median of the AN patient group was nearby the bottom range of the category "desirable range". According to the suggested classification system, six of the AN patients could be classified as females with desirable SAT thicknesses. We used the two assigned groups for further comparing the differences of fat amounts (D_{INCL}), fat patterning and the differences of relative body weights in terms of BMI: the median BMI values of the groups varied by 12%, whereas the differences in D_{INCL} values amounted to 330%. This further indicates the enormous difference in SAT between these two groups [1]. Consequently, also great differences in TBF can be assumed since SAT is known to comprise the major part of TBF and amounts to about 90% in females [102]. Thus, SAT cannot be captured by BMI (and neither by the MI – as both are only measures of relative body weight, but not of body composition).

Additionally, plasma values associated with body fat have been compared between the two AN body composition groups. As expected, plasma leptin levels were low in the whole AN group [197,198], however, the observed significant differences in body composition could also be shown regarding the clinically relevant leptin levels. This association further underlines the body composition differences with a known biomarker of body fat [15].

The comparison of the two groups showed remarkable differences not only in the sum of SAT layers but also in the fat patterning. Large variations in the individual SAT thicknesses at comparable BMIs were observed. The obtained data show, that the BMI (or MI) can only be used to assess relative body weight, but not body composition of AN patients.

It is assumed, that the loss or the extreme reduction of SAT are consequences of the reduced body weight in AN patients. Thus, low SAT is considered as a common sign and symptom of AN [40]. However, we could not confirm reduced body fat in all AN patients. The reduced body weight in the AN patients investigated in this study did not correspond with low SAT in all patients. The extremely low body weight of AN patients of group 2 where

D_{INCL} was larger than 30 mm must have been caused by the degradation of other body components. In particular, muscle mass, organ structure and bone mass [199-201] may have been degraded for energy supply. This may be even more dramatic than the loss of SAT. For example, cachexia of the heart muscle may be an irreversible consequence of the severely reduced loss of body weight [202].

Four of the patients had a sum of SAT thicknesses even larger than 50 mm which is beyond the amount of SAT of healthy and physically active women. According to the preliminary reference values for competitive female athletes such amounts of SAT would be considered as “ballast fat” [151].

Currently there are no reliable threshold values for minimum fat necessary for healthy living available. This may partly be explained by the lack of sufficiently accurate measurement techniques. Additionally, the suitable minimum of fat on an individual level may strongly be dependent on genetically determined factors [181]. As already mentioned in the section “4.3.2 Selection of appropriate body composition assessment methods” the novel ultrasound method provides reliable and accurate measurement of SAT also in extremely lean people [151]. On the contrary, other widely applied methods like DXA [153], skin folds [156] or BIA [1,153] are not capable of reaching the accuracy necessary for such an extreme group of patients that are diagnosed with AN or extremely lean athletes due to the methodological limitations.

However, differences in body composition at the baseline of the treatment have already been described in AN patients previously. These difference were at least partly explained by differences in the physical activity level and/or diet before hospitalization [39,203,204]. These authors stated that a decrease in skeletal muscle and internal organ mass was associated with a reduction in fat mass. In this study, we observed large differences in SAT despite the same or at least similar BMI. Consequently, the masses of muscle and other organ mass must have diminished without being affected by the level of fat mass depletion. Based on this consideration, treatment approaches need to consider the current physiological state of the patient to enhance treatment quality and effectivity.

For instance, one investigated AN patient had a body weight of 40.0 kg and her BMI was 15.1 kg/m^2. However, the sum of the eight SAT thicknesses amounted to a D_{INCL} of 43.9 mm. Here, it was not necessary to focus on an increase in body fat in this patient because this value can be found in many healthy and normal weight women, too. According to the described equation for calculating the SAT mass based on D_{INCL} (see section 2.2.1.3 Body

surface area and subcutaneous fat mass calculation), the fat mass corresponded to 4.5 kg [149]. Thus, the SAT mass accounts for 11.4% of her body mass without including the mass of other fat depots such as visceral fat, intramuscular fat or fat embedded in organs.

On the contrary, another AN patient of our study collective had almost the same BMI of 15.2 kg/m^2, a body mass of 45.5 kg; and D_{INCL} of merely 10.2 mm which resulted in SAT mass of only 1.2 kg and the alarmingly low percentage of 2.5% of her body weight. For this patient, the increase of body fat is urgently necessary in order to reestablish physiologically reasonable conditions. Obviously, there is a need for different therapeutic approaches to treat these patients adequately, in order to enhance treatment success and provide personalized medical surveillance. The considerations described above have been published in the appendix of the paper associated with this thesis [1].

In clinical practice as well as for research purposes, BIA is often applied for the assessment of body composition [192-194]. However, as already described above, BIA has certain limitations in cross-sectional detection of body composition in individuals. Thus, it is not recommended to apply this method for the assessment of the person's physical state at a single time point [158]. We compared the estimated fat mass derived by BIA and the results obtained by ultrasound and found high inaccuracy of the results obtained by BIA. For example, BIA estimated total body fat of several AN individuals at only 1 kg. However, ultrasound revealed SAT-thickness ranges at the eight sites (D_{INCL}) in those individuals from 1.3 to 24.4 mm which corresponds to 0.1 to 2.6 kg SAT. Another example: BIA revealed total body fat content of 1 kg and 8.2 kg in two patients, respectively. However, D_{INCL} values of ultrasound measurement were 24.4 and 33.4 mm, resulting in 2.6 and 3.7 kg of SAT, respectively [1].

Thus, the use of BIA in clinical practice for the assessment of body composition in patients that are affected by body disturbance disorders and altered perception regarding their body image as it occurs in AN patients should be avoided since incorrect and varying results may negatively influence compliance and therapy progress.

Evaluation of reported energy intake revealed significant differences between the two groups whereby group 1 with lower body fat reported higher energy intake. This difference may be partly explained by wrongly estimated amounts of consumed food. Since the presented data is based on reported food intake, AN patients may have overestimated portion sizes. It is known, that AN patients are often affected by altered perception [205,206] and that may have influenced the assessment of appropriate portion sizes. Additionally,

patients of group 1 (n=7) received high-calorie sip food more often than the group 2 patients (n=3) which is also considered in the total amount of energy consumed.

Furthermore, we assessed other factors that are connected to the patient's lifestyle, disease and treatment history that may have affected body composition in these patients. No significant differences regarding the duration of the diseases (determined by years since the diseases had been diagnosed for the first time), duration of the treatment and duration of the hospital stay and either the level of physical activity have been observed.

4.3.3.2 Variations in body composition among athletes

In our study, we observed pronounced differences in body composition among the investigated sports groups. The group of water polo players had significantly higher levels of SAT compared to the group of hand- and volleyball players. Differences in body composition variables between athletes of different sports have already been described previously [207-209]. Additionally, Field et al. [208] described differences concerning the sport-position in elite collegiate athletes. The observed differences in body composition across sports and sport-position were attributed to the specific physiological demands. However, due to the sample size in our population, we could not differentiate between single sports positions. Moreover, seasonal variations and longitudinal changes in body composition over training phases have been described in accordance with training strategies and training load [209,210].

Importantly, in the review on physical specifics of water polo players, Cox et.al. [211] highlighted the necessity and functionality of higher body fat in female water polo players for their performance compared to elite swimmers. They additionally pointed out differences in body fat levels in accordance with the playing position. Higher body fat may potentially provide certain physical benefits for the performance in terms of buoyancy. However, for female players, it was reported that difficulties occur in the achievement and maintenance of a high body mass without expanding body fat content. Cox et al. report on training strategies aiming to increase body mass, fat mass and strength in accordance with the playing position [211]. Though, this physical requirement may be conflicting for some individuals since gaining body fat may be negatively associated with the ideal body image of light-weight and lean body that is widely established in society. This needs to be taken into consideration when designing training plans in order to avoid negative pressure on the athletes and to minimize the risk for the development of misguided eating behavior and

eating disorders [212]. Moreover, the adverse metabolic effects of elevated body fat mass needs to be considered.

Variations in body composition may be of major importance in competitive sports for athletic performance. For example, an increase in fat mass through the phase of competition may alter performance drastically. Ballast weight impairs movements like jumping and increases energy needs for these activities. Thus, unfavorable body fat content during competitive stages is likely to be a competitive disadvantage. The monitoring of body composition changes over the course of training may allow assessment of training plan effectiveness, may contribute to assess nutritional status non-invasively, and potentially indicates the necessity for exercise and nutritional adaptions. Thus, it is suggested to implement body composition assessment as a valuable component in training assessment and predictor of competitive success [213,214].

The performance of athletes of certain sports such as weight sensitive (like boxing, judo, body building etc.) and gravitational sports (like ski jumping, sprinting, cycle racing, etc.) as well as aesthetic sports (like synchronic swimming, ballet, gymnastics etc.) is primarily affected by unfavorable body weight including misbalanced body composition [146,181]. Athletes of these sports are simultaneously endangered of exhibiting disordered eating behavior due to training and competitive requirements on their physical properties [215-217]. Thus, trainers are encouraged to implement sport and training specific nutritional plans in their training plans since the clinical relevance of body composition assessment in sports is indicated [81,211-213,218].

4.3.3.3 Subcutaneous fat and variations in fibrous structures

Another interesting aspect of body composition are fibrous structures that are embedded in SAT [151]. In the AN group with low SAT layers the percentage of fibrous structures was higher compared to group 2. This means that the fibrous structures seem to accumulate in the reduced fat layer and are not degraded in accordance with the reduced fat layer. This further reduces the amount of pure fat in the subcutaneous region of group 1 [1].

Interestingly, the role of fibrous and collagenous structures in adipose tissue has been studied within the context of energy homeostasis. The extracellular matrix of adipocytes and adipose tissue is suggested to exhibit a functional role in the physiology of the tissue [219]. It has been observed that metabolically demanding states such as obesity and

diabetic states are associated with an upregulation of some extracellular matrix components. However, the weakening of this matrix leads to improvement of the metabolic response in obese models. Thus, a reduction of collagenous fibers was associated with improved energy homeostasis, a stress-free expansion of adipocytes and reduced cell death, and improved inflammatory response [220].

However, to our knowledge so far the role of adipose tissue architecture in energy homeostasis has neither been investigated in AN patients nor in AT. Thus, our observation may initiate further research in this field.

4.3.3.4 Body fat, leptin, and endocrine function

The reproductive system is closely related to the availability of nutrients and energy. Thus, undernutrition as well as overnutrition leads to reproductive dysfunctions due to central and peripheral alterations [221].

It is well known that AN is associated with various endocrine alterations that are consequences of chronically severe malnutrition and additionally may contribute to a vicious circle that maintains or impairs the disease state [48,222]. Thus, endocrine disturbances are one of the diagnostic criteria of AN [34,35].

Especially the endocrine dysregulation of the hypothalamus-pituitary-gonadal-axis is associated with the reduced body fat in AN patients; however, even in athletes this phenomenon occurs frequently and is associated with reduced energy availability [82]. For instance, hypothalamic amenorrhea may be one of the consequences of reduced energy availability, reduced estrogen production and reduced body fat [223,224]. Also, hypothalamic amenorrhea persuades further weight loss and persists even after recovery [225]. Moreover, the endocrine disturbances in energy depletion are connected to unfavorable bone metabolism and are a major risk factor for osteoporosis [199-201,226]. In total, this leads to a detrimental physical condition due to reduced body fat content that alters the appropriate production of steroid hormones and thus endocrine function.

Since leptin is closely connected to the body fat content, several associations of the endocrine system and leptin have been investigated, especially with respect to its influence on puberty, pregnancy, and hypothalamic amenorrhea. The administration of leptin in hypo-leptin stages has been suggested to potentially exhibit therapeutic effects on metabolic and

reproductive health. For example, the attempts to restore the menstrual cycles of AN patients and athletes by leptin administration succeeded in some patients [224,227,228]. Even in athletic women with reduced body fat and hypothalamic amenorrhea, the substitution of leptin increased the hormonal release of the luteal hormone, leading to enlarged ovaries, more follicles and enhanced estradiol plasma levels with the result of the re-achieved menstrual cycle in amenorrhea states [224].

In AN patients and AT suffering from RED-S an acceptable body composition and a reasonable fat mass in terms of enabled adequate physiology are crucial for the reappearance of the regular menstrual cycle. However, further factors may additionally play a role in the recovery of the menstrual cycle. For example, the secretory patterns of leptin and its relation to adrenal function have been highlighted recently within this context [229].

Interestingly, decreased leptin levels have also been associated with the phenomenon of hyperactivity that frequently occurs in AN and could also be interconnected with athletes [230-232]. Thus, extensive and compulsive physical activity may not only be explained by the intention to increase energy expenditure. In animal models, reduced energy availability has been shown to increase hyperactive behavior [233,234]. This could possibly be explained in an evolutionary context since elevated physical activity may support food-seeking behavior. However, not all studies could verify the activity enhancing effects of reduced leptin levels. [197]

Besides the proposed impacts of the reduced body fat content and leptin expression on the hypothalamus-pituitary-gonadal axis, its endocrine alteration and consequences for bone metabolism [235], leptin may influence recovery in AN. The low leptin levels observed in AN patients have been reported to be detrimental for weight recovery, sine weight gain induces elevated leptin levels and thus exhibit inhibitory effects on appetite [225].

Due to the cross-sectional study design of our investigation, our data confirm the relatively low leptin levels in AN and AT in accordance with their body fat status. Though, we did not assess the menstrual status of the patients. However, the role of leptin and thus body fat status within the context of system biology approaches could be highlighted.

4.3.3.5 Association of carotenoid levels and body fat

In our study, we observed an inverse association of carotenoid levels and body fat. Obese participants had generally decreased carotene levels whereas AN patients had the highest levels of skin carotenoids and plasma β-carotene. Negative associations of body fat and carotenoids were found except for lycopene which was positively associated with body fat.

Previous findings revealed similar or inconsistent results. E.g., Nuss et al. [236] found a negative correlation of α-carotene, however, other investigated carotenoids such as β-carotene, lutein, and lycopene were not associated with body fat. Although we can confirm a negative correlation of α-carotene with body fat, we observed by far the strongest correlation for β-carotene. This is in line with the findings of Yeum et al. [237] who found a significant inverse correlation of the BMI and plasma β-carotene. They proposed that fat mass may negatively influence circulating carotenoid concentrations in certain populations, especially in women with high body fat mass [237]. Also, the study of Wallström et al. revealed a negative correlation of serum β-carotene and body fat in a mixed gender population [238]. Additionally, Kabat et al. who investigated antioxidant concentrations in serum of postmenopausal females observed the most prominent relation for waist circumference and serum β-carotene, indicating an association of abdominal adiposity and carotenoid status [239]. Bovier et al. [240] could confirm a negative association of lutein and zeaxanthin with body fat mass. However, we did not assess these two carotenoids.

Regarding lycopene, our results showed positive associations with body fat. Previous findings also showed independent effects of lycopene on body fat [241] and no correlations of lycopene and body fat [121], respectively. However, Grolier et al. found an inverse correlation of lycopene with fat mass in a gender-mixed population [242].

As described above, several studies indicate a negative correlation of several carotenoids such as α -carotene, β -carotene, and β –cryptoxanthin with fat mass [121,243]. There are some possible explanations for the alteration of serum carotene levels with respect to body fat. First of all, lower concentrations of carotenoids in the blood may be the result of poor dietary choices and habits. Some studies report on lower ingested carotenoids per kilogram body weight in individuals with higher BMI. This may lead to differences in the dilution of carotenoids within the organism's carotenoid storage sites [121]. To assess differences in nutritive uptake of carotenoids, we evaluated the dietary intake of vitamin A. We could not find significant differences between the study groups and between nutrient intake and serum carotenoid levels. However, the applied method for nutritive assessment was not

capable of differentiating between single carotenoids. Thus, only vitamin A intake could be assessed which possibly limits the value of this statement.

Next, the antioxidant properties of carotenoids and the elevated oxidative processes in subjects with increased fat mass may contribute to an enhanced carotenoid expenditure and their depletion within the organism. The inverse relation of carotenoids with obesity may reflect the higher antioxidant turnover due to obesity-associated inflammatory processes and elevated clearance of carotenoids through free radical oxidation [239]. Within this context, our results also confirm higher oxidative stress levels and inflammation markers with respect to higher body fat mass. Levels of skin carotenoids and β-carotene were negatively correlated to the oxidative stress index and total oxidative capacity, however, lycopene was positively correlated to TOC. Additionally, the inflammation marker CRP was negatively correlated to all evaluated carotenoid values except for lycopene a positive association was observed, and IL-6 showed negative correlations with skin carotenoids, α- and β- carotene and a positive correlation with lycopene. These positive correlations with lycopene are surprising since they contradict other findings. In literature, tendencies of beneficial effects of lycopene on inflammation and adipocyte biology are reported [244,245]. However, these findings are primarily based on animal models. Tomato juice, which contains large amounts of lycopene has been shown to reduce systemic inflammation in obese females [246]. A meta-analysis by Cheng et al. found reductions of IL-6 and other risk factors for cardiovascular diseases in human due to lycopene supplementation [247].

Last but not least, the negative correlations of carotenoids and body fat could be explained by enhanced absorption and breakdown of carotenoids within adipocytes themselves and thus directly due to the biological processes within the adipocytes. Especially β-carotene is supposed to exhibit regulatory effects on adipocyte biology and may influence adiposity in obese individuals with respect to gene function [248]. However, results in humans are lacking and animal studies lead to contradictory observations. Especially β-carotene as a precursor of vitamin A is proposed to be a dietary regulator of body fat depots. Although there are some studies describing the correlation of serum carotenoid levels and body fat, the hypotheses could not be verified in clinical studies. Supplementation studies with high doses of β-carotene failed to show correlations between the dietary carotene intake and BMI [248]. Hence, also other processes may be involved. The biological significance of these processes in human still needs to be further determined [121].

Interestingly, in AN patient's certain skin signs associated with carotene metabolism occur [134]. Thus, we evaluated the carotenoid status of the two AN subgroups separately. Due

to the observed differences in carotenoid status in accordance with body fat of the patients, the clinically relevant phenomenon of hypercarotenemia will be discussed in more detail in the following section.

4.3.3.6 Hypercarotenaemia

In addition to the observed negative correlation of carotenoids and body fat, significant differences in carotene levels within the two AN body composition groups could be observed [1]. The group of patients with extremely low SAT had significantly higher concentrations of plasma and skin carotenoids compared to the group with more SAT.

In the clinical field, hypercarotenaemia occurs from time to time in AN patients [134,249-251]. Thereby, hypercarotenemia is described to be accompanied by “carotenodermia” – a yellowing of the skin especially at the palm or sole region - and high concentrations of plasma carotenoids [135]. There are some possible mechanisms behind this occurrence described in the literature that may contribute to hypercarotenaemia. First, the accumulation of carotenoids in skin tissue may be linked to an elevated uptake of carotenoid-rich food sources. These food sources, primarily fruits and vegetables, are known to be low in energy and may thus be consumed in unproportioned amounts from AN patients in relation to total energy intake [252,253]. In our study, we could not observe differences in carotenoid intake due to the above described limitations of our nutritive intake data.

Secondly, the appropriate degradation of carotenoids is strongly connected to lipid metabolism [254]. In disorders associated with hyperlipidemia the relation between β-lipoprotein and β-carotene may cause hypercarotenaemia. The cleavage of β-carotene to vitamin A may be impaired in certain diseases such as hypothyroidism and diabetes mellitus. Anorexia nervosa is often associated with altered lipid metabolism [131,255]. Thus, catabolism of β-lipoprotein may be decreased due to the altered lipid metabolism, leading to an accumulation of carotenoids in the skin and adipose tissue, and liver tissue but may also result in elevated circulating levels. The AN patients investigated in our study had plasma lipid values within the reference ranges. No striking differences in their lipid values and no significant group differences could be observed.

Last but not least, it has been hypothesized, that the amount of body fat plays a crucial role in the availability of circulating carotenoids [256]. Due to the reduced amount of adipose tissue in some of the AN patients the organism’s ability to store carotenoids, is reduced.

Consequently, circulating carotenoid concentration increase and carotenoids are stored in skin tissue alternatively.

Our observation supports the latter hypothesis since pronounced differences in carotenoid plasma and skin concentrations have been observed in AN patients in accordance with their SAT thickness levels. However, Robboy et al. found a decrease in serum carotene levels after weight loss [132], contradicting this hypothesis. Interestingly, hypothalamic amenorrhea (and thus altered body composition and leptin levels) have also been associated with hypercarotaenemia [257].

Even though carotenoids have high antioxidant properties, they may have adverse effects in high concentrations that exceed physiological levels. Intervention studies using high concentrations of dietary supplemented β-carotene revealed adverse effects on the development of lung cancer in male smokers. In addition, clinical trials in humans using β-carotene in high concentrations concluded that there was no evidence of an advantageous effect and observed higher risk for the development of lung cancer in heavy smokers and workers that were exposed to asbestos. This might indicate, that under certain conditions; carotenoids – especially when administered in doses that would not occur in conventional diet might exhibit oxidant function [258]. However, these findings still may be confounded by the influence of smoking and the exposure of asbestos themselves on the pathogenesis of lung cancer. In general, epidemiological studies primarily have shown positive associations between a high dietary carotenoid intake and the reduction of certain cancer risks such as reduced risk of breast, cervical, ovarian, colorectal cancers, and cardiovascular as well as eye diseases and cardiovascular diseases [116,259-261].

4.3.3.7 Body composition and metabolic regulation of immune function

The observed differences in body fat content and carotenoid concentrations in the AN patients were further associated with oxidative stress and inflammation indices. This may further indicate various metabolic alterations as a result of misguided metabolism based on body fat content.

In general, the study group of AN patients had raised oxidative stress values, indicating the stress induced by the catabolic metabolism. Solemi and colleagues reviewed possible connections of oxidative stress and antioxidant levels in AN patients [262]. They identified possible inherent attributes of the disease that may contribute to this condition. Among

these factors, a lack of antioxidant compounds is suggested. This lack may be attributed due to inadequate intake of micronutrients in combination with enhanced physical and psychological stress. Additionally, long periods of starvation may contribute to elevated production of ROS and AN patients are known to smoke more frequently than healthy controls [263]. Also our group of AN patients had the greatest proportion of smokers among our study population which may have contributed to the elevated oxidative stress parameters. Studies evaluating inflammatory markers in AN observed elevated levels of IL-6 besides other enhanced pro-inflammatory cytokines [264]. In our study population IL-6 levels were not elevated with the exception of one outlier. It is also stated that it has not been clear whether oxidative stress would be a cause or result of AN [265].

We observed that the oxidative stress parameters of AN patients with lower SAT were higher compared to the other group. Thus, oxidative stress levels also corresponded with dermal carotenoid and β-carotene plasma concentrations underpinning the potentially adverse effects of high doses of circulating carotenoids and their pro-inflammatory properties. The observed variances in oxidative stress and carotenoid concentration in accordance with body fat content may point out diverse metabolic alterations due to the amount of body fat. Thus, the assessment of body fat content in clinical practice may be of special interest for phenotypic profiling.

Importantly, altered gut microbiota as it can be observed in AN patients may contribute to elevated inflammatory processes. Within the ESAN study also the gut microbiome and zonulin levels of the study population were investigated. The results of our observations have already been published elsewhere [139-141] and were also part of another doctoral thesis [266]. Although we could not find significantly altered zonulin levels in AN we observed a broad range within this study group. Zonulin is a gastrointestinal protein associated with dysbiosis and enhanced gut permeability, leading to inflammation and activation of the immune system [267-269]. We found significant associations of zonulin and body fat, indicating an additional role of body fat in the occurrence of inflammation. However, we could not support the association between dysbiosis and zonulin levels [140].

The potential role of dysbiosis in the etiology of AN is currently discussed in some publications [270,271]. Within this context, the main metabolic products of gut bacteria the short chain fatty acids are thought to impact endocrine secretion of certain hormones, acting on the vagal nerve and thus modulating appetite and eating behavior on the gut-brain- axis. Furthermore, immune cells are capable of detecting the neuroactive substances produced by the microbiota metabolites due to specific receptors. Thus, a direct link via nutritive

factors on host immune activation via modulation of the microbiota is suggested [270]. In one of our previous publications, derived from data of the study associated with this thesis, we could confirm reduced microbial diversity in AN patients and obese participants [139].

The impact of obesity on the interconnection between antioxidants and inflammation has been described previously [78]. For example, Mazidi et al. [272] found a modest impact of high body weight due to increased body fat on the interplay of antioxidants and CRP levels. They proposed that the level of inflammatory load in adiposity was negatively associated with antioxidant levels, meaning that the higher CRP levels were, the lower antioxidant levels. This is in line with our findings of inflammation and oxidative stress level as well as the nutritive antioxidant level defined by the carotenoid status. Additionally, the level of antioxidants was described to be inversely related to the risk of cardiovascular disease (CVD) development. The authors concluded, that lowered levels of antioxidant acting vitamins may increase CVD risk predisposition [272].

4.3.3.8 Oxidative stress in athletes and clinical value

During intense exercise, the production of reactive oxygen and nitrogen species is induced in skeletal muscle. These oxidative stress products are releases as a result of mitochondrial oxidative phosphorylation and lead to elevated levels of physical stress in the organism. Therefore, intense exercise induces muscle damage that promotes oxidative processes [273]. Thus, oxidative stress and inflammation markers have repeatedly been reported to be elevated in athletes. In our study population, we observed increased oxidative stress levels in terms of total peroxides of the AT group in comparison to normal weight controls. However, carotenoid levels were also higher in this group, indicating potential nutritive compensation of free radicals. Inflammation markers were not elevated in the AT group.

The alterations in redox homeostasis occur due to aerobic and anaerobic exercise regardless of the training level (ranging from untrained to well trained). However, ROS have also been reported to exhibit beneficial effects on adaptive processes muscle tissue via acting as signaling molecules. Thus, low to moderate ROS as well as reactive nitrogen levels support adoptive processes in muscle tissue, however, an overload of these oxidative molecules induces detrimental physiological conditions such as elevated cell death, fatigue, and immunosuppression leading to reduced performance [274,275]. Additionally, it has been reported that the elevation of CRP levels is strongly depended on individual metabolic

processes. One determining factor may the individual response to creatin kinase (CK), a hallmark of exercise induced muscle damage [276].
Since oxidative stress promotes inflammatory processes, alterations in the redox homeostasis may affect the health status of the athletes detrimentally. In order to avoid negative health consequences, the longitudinal monitoring of biomarkers for redox homeostasis alterations has been suggested which should contribute to an optimization of health and performance in athletes [274].

4.4 Discussion of limitations

Some limitations need to be considered in the interpretation of our study results. In the following section these limitations will be discussed briefly.

4.4.1 Critical reflection of the methods

For the assessment of adipose tissue, we measured subcutaneous adipose tissue thicknesses as a surrogate for whole body adiposity. However, as described in the introduction, especially visceral body fat is supposed to be metabolically active and produces proinflammatory cytokines and signaling molecules that influence energy homeostasis. Thus, the amount of VAT is proposed to be of great importance for the intercorrelation of the observed parameters.

For the assessment of VAT, several methods such as CT, MRI, and DXA exist. It is reported, that CT and MRI are often considered being the method of choice in terms of applicability and measurement accuracy [277]. However, since these methods have known limitations for clinical and field operations such as high costs, exposure to radiation, prolonged scan times, accessibility and availability, the application of these methods is impractical for clinical purposes and larger clinical studies [277]. Several studies are reflecting the applicability and accuracy of dual energy X-ray absorptiometry (DXA) for the determination of whole body fat and the differentiation into SAT and VAT, and the assessment of lean body mass [187,278,279]. The advantages of this method stated are that DXA is broadly available and the radiation dose should be limited. In comparison to the other methods it is supposed to be relatively inexpensive. Nevertheless, the ability to determine body fat regions via DXA was reported to be limited, especially in extreme body composition groups

like AN patients and obesity [277]. However, body composition extremes of obesity and anorexia nervosa (AN) may impact DXA accuracy. As already stated in the discussion, the paucity of fat in the abdominal area is also documented to influence DXA-derived fat estimations leading to inaccurate results. Bredella et al. [277] showed that an estimation bias of DXA abdominal fat assessment (which algorithms rely on assumptions) increases with increasing body weight. Likewise, a study published by Goldberg et al. [278] revealed, that VAT determination by hologic DXA predicts fat levels satisfactorily in normal weight individuals (BMI of above 18.5 kg/m²). They stated, that accuracy was dependent on VAT amount and gender [278]. Fourman et.al. [279] showed that DXA underestimated VAT in comparison with CT in male individuals with high visceral fat amounts, whereas Bredella et.al. [277] who assessed the abdominal fat mass of individuals suffering from AN reported on higher abdominal fat mass obtained by DXA compared to CT results. They observed a reduction of this estimation bias with increasing weight and suggested the use of this method in obese individuals.

Besides these methods for VAT determination, approaches for assessing VAT indirectly are in use. For example, the WHO recommends the measurement of waist circumference for the estimation of increased risk of cardiovascular diseases due to elevated abdominal body fat [143]. The cut off values for waist circumference are also part of established assessment criteria of the metabolic syndrome and predict metabolic risk factors. This measure is easy to apply without a burden for the patients. In addition to the measurement of SAT, we also measured waist circumferences of the participants in our investigation.

However, the measure of waist circumference has of course also certain limitations since it does not consider the individual's body shape and frame size. In a study of Oh et.al. [280] the metabolic risk of individuals with normal waist circumference was assessed by calculating the ratio of SAT to VAT in individuals with normal waist circumferences. Additionally, for indirectly assessing VAT some novel clinical surrogate markers such as the lipid accumulation product (LAP) which combines triglyceride levels and waist circumference, and the visceral adiposity index (VAI) which includes anthropometric (BMI and waist circumference) and functional parameters (HDL-cholesterol and triglycerides) have been suggested. These measures are supposed to contribute to a more sensitive evaluation of CDV risk due to VAT’s metabolic activity. [281]

However, since we investigated a group of young females and excluded known acquired or inherited metabolic disorders, we decided to use SAT values, measured by the most reliable method currently available as a surrogate for whole body adiposity. Moreover, the

mentioned limitations of VAT determination in individuals presenting extreme body compositions, results may have been inaccurate too. As highlighted in the review of Ibrahim et.al. [102] SAT in females amounts to approximately 90% of whole body fat. Thus, SAT as a marker for whole adiposity may have been a reasonable surrogate for this study. Nevertheless, the determination of VAT and the development of other reliable measurement methods of VAT would be of great interest for further research questions.

Regarding metabolic phenotyping several different approaches to analyze metabolites exist. Generally, metabolites that contribute to the metabolic profile of an individual are tiny molecules that are most commonly identified via targeted or non-targeted metabolomics approaches, depending on the research question [182,282-287]. We also analyzed the whole metabolome in our cohort using a non-targeted approach. However, data derived from this analysis have not been used for this theses and will be further analyzed in a next step.

4.4.2 Critical reflection of the results

We conducted a cross-sectional study. The metabolome of an individual is highly variable and may strongly be influenced by environmental factors. Primarily when focusing on dietary biomarkers, they may be confounded by factors like genetics, physiological status and health status of an individual, and lifestyle factors such as smoking, or alcohol abuse. Although we screened for those factors and evaluated dietary intake of the day before the blood was drawn, possible alterations of the diet cannot be completely excluded. Additionally, individual pharmacokinetics or interaction of nutrients and medication may have altered the results [118,256]. Thus, the monitoring of intraindividual changes and variations in the individual metabolic profile over a certain period of time would be interesting to filter environmental influences.

Regarding the group of athletes, seasonal and longitudinal changes in body composition could not be considered due to the cross-sectional setting. Additionally, due to the sample size, we could not differentiate between certain sports-positions within the team [207,209]. Regarding the AN patients, differences in treatment duration and duration of the illness may have affected the observed differences. Although we checked for and evaluated these parameters, longitudinal observations may reveal different results. Finally, possible latent infections may have influenced our results.

In general, we used self-reported information in dietary intakes, smoking behavior and physical activity. There are known limitations of self-assessment data, most commonly the bias of over- or underreporting due to different problems (e.g., wrong estimation of dietary portion sizes, limited memory, or altered perception) occurs [167,288].

Most importantly, we observed pronounced differences in body composition in AN patients that were associated with leptin levels, carotenoid status, and oxidative stress indicators. The information on disease onset and weight cycling history as well as nutritive intake data and physical activity information were based on self-assessment of the patients.

Although we included a total of 107 participants, the sample size of each investigated group was limited to 18 to 27 participant per group, respectively. Thus, the observations described here are based on a group of 18 AN patients. For confirming our findings, larger cohorts should be included. Longitudinal studies will be necessary to test the suggested modifications of treatment practices that emphasize physical strength training at low energy turn-over rates for those AN-patients that have a sufficient amount of fat. The groups investigated here received treatment already; thus, the amount of fat and SAT patterning may be different in patients who have not received any treatment. Significant differences in the physical activity level of AN patients were not to be expected as most participants were inpatients and the applied IPAQ assesses physical activity of the previous seven days. This might have been different when most patients would have been outpatients.

Since most of AN patients are female [289], we decided to include solely females in our study. Thus, possible sex differences in the investigated parameters could not be considered. Especially the results with regards to body fat are strongly affected by sex since body fat percentage and fat patterning are enormously different between male and female [290,291].

Metabolic outcome parameters may be altered by hormonal status. Thus, the phase of the menstruation cycle may be of importance for analysis and interpretation of the metabolomics results [292]. However, we did not evaluate the current status of menses at the time point of the investigation.

Last but not least, AN patients received psychopharmacological medication within their treatment plans. Mostly, they received antidepressants. All other groups did not take regular medication. Thus, we cannot exclude any influences in metabolism due to pharmaceutical drugs.

4.5 Implications for further research

Based on the results obtained by this study, the following hypothesis for further research can be generated:

First, regarding the observed differences in body composition of AN patients, interventional studies should focus on the suggested adoption of treatment plans in accordance with the patients' body composition. We suggest the implementation of targeted exercises in order to increase muscle mass in those patients with higher amounts of subcutaneous adipose tissue. More precisely, we suggest testing the effectiveness of isometric strength training with few repetitions at low energy turnover rates [1,149,293].

We hypothesize that the implementation of individualized muscle training in AN patients with higher fat mass and consequently lower muscle mass may lead to increased treatment adherence and thus better long-term recovery.

Second, regarding elevated carotene status in AN patients, we hypothesize, that carotenoid levels of patients with low subcutaneous fat will decrease to normal ranges in accordance with the regain of fat mass.

The role of carotenoids in adipocyte biology has been described in detail within the context of fat storage capacity within the adipocyte and adipogenesis [6,88,294]. Thereby, carotenoids are supposed to suppress these metabolic features on a nuclear level. This factor may additionally contribute to reduced success in weight gain efforts in AN. However, to our best knowledge, there are currently no clinical studies in human focusing in the role of carotenoids in undernutrition and weight gain. Thus, basic research and clinical questions regarding possible inhibiting effects of elevated carotenoid status in recovery of exhausted fat stores may be generated.

Third, differences in embedded fibre structures in AN and AT have been observed. In the literature a potential role of adipocyte architecture in energy homeostasis has been identified [220,295,296]. However, the role of adipose tissue architecture in energy homeostasis in AN patients and either in AT has not been investigated so far. Thus, further research may focus on the role of adipose tissue composition in energy homeostasis and health.

Fourth, the described differences in body composition are based on observations on an anatomical level. However, since many features of adipose tissue are suspected on a cellular level, further clinical research regarding functional adipocyte measurement in in-vivo human studies are indicated. There are already methods targeting this issue [297-301], however, there is still a lack of studies confirming their applicability in clinical and field settings. Thus, further research should focus on the development and establishment of techniques capable of determining fat qualities and functions in humans.

4.6 Implications for clinical practice

Our data suggest the following implications for the clinical practice:

4.6.1 Implementation of body composition assessment in clinical routine

Firstly, the implementation of body composition assessment for the appropriate classification of body weight should be established in clinical routine. For the clinical assessment of obesity the BMI is commonly still used. However, as already highlighted, the BMI is not capable of assessing body fat – the major risk determinant in obesity [9]. In clinical practice, bioelectrical impedance analysis is widely used for body composition determination. However, besides the already published criticism on the accuracy and reliability of BIA results [153,158] our data clearly, show that more precise methods need to be applied in the clinical field. In addition to the determination of body composition, fat patterning, and anthropometric indicators like waist circumference, the measure of biochemical parameter such as the lipidic blood profile are proposed for deep phenotyping in order to characterize the patients more precisely, which may allow to classify the individual regarding potential health impacts of its phenotype with higher precision.

4.6.2 Adoption of treatment strategies for AN patients

Secondly, an adoption of treatment approaches for the therapy of AN patients is indicated. Current treatment guidelines recommend rapid weight recovery in AN patients by means of high caloric diets including energy supplements and restricted physical activity to reach a positive energy balance [53,54,302]. Of course, to aim at rapid weight restoration is

essential in the treatment of AN [49-54], but too fast weight gain at may be detrimental for later weight maintenance [52] and thus long-term recovery that is not achieved through weight restoration alone [58,64]. The need for monitoring the nutritional status beyond BMI was identified [64], and the implementation of body composition assessment in the standard care of AN patients was proposed previously [204], but no adequate monitoring tools for therapy progress have been established in therapy guidelines [53,54,302].

A common side effect of fast weight gain is the abdominal fat accumulation [41,70,71,74]. Besides the possible negative metabolic effects of an excess of visceral body fat [70], AN is associated with body image disturbance [36] and concerns about the body shape [37]. Despite this potentially negative influence on compliance, El Gouch et.al. [303] could not support any connection between body fat mass and its influence on long-term recovery. Additionally, Mayer et.al. [72,73] reported on the disappearance of the accumulated body fat after long-term-weight restoration. Still, it needs to be mentioned that the relapse rate among recovered AN patients is high [42] and this limits the number of long-term weight restored patients. It has been hypothesized previously that physical activity during refeeding would influence body fat distribution [41]. However, clinical data on body composition monitoring during therapy progress are rare. Weight gain strategies including interventions to reach an adequate amount of muscle mass could probably increase therapeutic success. Strength training with few repetitions or isometric training that are associated with low energy turn-over may be advantageous interventions to maintain and increase muscle mass and avoid an excessive gain of fat mass [304]. The restoration of lean body mass is supposed to be a major factor for outcome and quality of life [41]. As already supposed by Yamashita et.al. [39] a more individualized therapeutic approach according to the level of malnourishment in terms of fat mass and BMI would probably have benefits on therapy outcome. The implementation of body composition testing in standard care of AN patients was also already suggested previously [204]. Further, strict caloric intake requests should be reconsidered with a focus on nutritional quality. In addition, body composition assessment using highly reliable and accurate methods should be implemented in clinical routine.

Regarding the treatment of AN, even recently revised guidelines, e.g. the new evidence-based guideline of Germany for the treatment of AN does not consider body composition as central for therapeutic outcome [305].

4.6.3 Body composition assessment in athletes

The implementation of body composition assessment in regular training schedules has already been suggested previously [81,212,218] to detect potentially detrimental health conditions for the athletes. Weight depletion may be forced by trainers and athletes due to assumed competitive benefits in weight-sensitive sports or may occur unconsciously when the energy requirements cannot be met regularly and energy deficiency occurs. RED-S leads to severe health consequences as highlighted in the introduction and may have adverse effects on training and competition. However, reducing weight deliberately for assumed advantages in competition may be a precursor of developing a disordered eating behavior that may lead to clinical relevant conditions [215]. Thus, screening for RED-S in vulnerable groups of athletes may be necessary, though, mostly reported to be difficult in practice. According to the clinical assessment tool for screening of RED-S [213], the evaluation of body composition takes over a crucial part in risk assessment. However, due to the lack of applicable field methods, the suggestion is limited to annual DXA determinations. Thus, we suggest the implementation of non-invasive, easy to apply field methods like ultrasound in training concepts of vulnerable sports.

4.6.4 Carotenoids as potential food consumption and health biomarkers

The non-invasive determination of carotenoids in the skin could be a potential indicator of health status in terms of oxidative stress and inflammation as well as a food biomarker of fruit and vegetable intake [29]. However, although our study results support an association of carotenoid status and inflammatory and oxidative stress conditions, further research needs to address important questions regarding the applicability of novel devices.

5. Conclusions and outlook

The human metabolism comprises a complex machinery of highly controlled mechanisms that are sensibly coordinated. Multiple influences of daily life challenge homeostatic processes in the short-term, however, the combination of environmental and genetic factors as well as daily influences may alter the metabolic outcome for the long term and contribute to the metabolic phenotype. To define the human metabolic phenotype, a comprehensive

approach is required. However, due to the complexity of the human metabolism, certain perspectives need to be selected for analysis and description.

In this study, we focused on a deeper description of the interconnection of four selected factors that contribute to energy homeostasis and determination of the metabolic outcome. Thereby, subcutaneous adipose tissue was chosen as the primary determinant for metabolism and phenotype characterization and set into an inter-correlation with plasma leptin, dermal and plasma carotenoids and other immune related factors.

We observed enormous differences in subcutaneous fat among AN patients despite almost the same BMI by applying a novel standardized method for SAT measurement that has been shown to be highly reliable and accurate. The investigated biochemical parameters (such as leptin, oxidative stress values and carotenoid levels) were associated with this finding. Based on this observation we concluded, that the BMI is an insufficient predictor of body fat in AN patients and the group with higher SAT must have had extremely low muscle mass. Thus, we suggest the adoption of treatment strategies in accordance with the patients' body composition and the monitoring of body composition throughout the therapy progress in order to assess nutritional status and recovery more sophisticatedly.

In general, the field of personalized nutrition and medicine is emerging. Our findings may provide further insights into the influence of certain nutrients on a system biological level and contribute to show the proposed effects in real life conditions. However, further research needs to replicate our results and test the clinical implications.

In order to characterize the individual metabolic phenotypes of the study population, the results of the non-targeted metabolomics analysis will be evaluated in a next step. We will further set these results in relation to the gut microbiome data that was obtained from further examination of the ESAN study population [139,140,266] to gain a comprehensive view on the metabolism of the individuals and the groups.

Currently, the conception of the suggested intervention studies is in progress.

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7. Appendix

7.1 Original reports originated from this thesis

Lackner, S; Mörkl, S; Müller, W; Fürhapter-Rieger, A; Oberascher, A; Lehofer, M; Bieberger, C; Wonisch, W; Amouzadeh-Ghadikolai, O; Moser, M; Mangge, H; Zelzer, S; Holasek, SJ. Novel approaches for the assessment of relative body weight and body fat in diagnosis and treatment of anorexia nervosa: A cross-sectional study. Clin Nutr. 2019; https://doi.org/10.1016/j.clnu.2018.12.031

Mörkl, S; **Lackner, S**; Meinitzer, A; Gorkiewicz, G; Kashofer, K; Painold, A; Holl, A; Holasek, S. [Pilot study: Gut microbiome and intestinal barrier in anorexia nervosa]. Fortschr Neurol Psychiatr. 2019; 87(1):39-45

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7.2 Public presentations originated from this thesis

Lackner, S; Mörkl, S; Müller, W; Mangge, H; Zelzer, S; Wonisch, W; Holasek, SJ. The immunomodulatory property of leptin: Linking plasma leptin status with oxidative stress and inflammation marker in young females of divergent energy states. Nutrition 2019. 18. Dreiländertagung. May 16.-18. 2019; Bregenz, AUSTRIA. [Oral Communication]

Lackner, S; Meier-Allard, N; Mörkl, S; Müller, W; Moser, M; Poncza, B; Mangge, H; Holasek, SJ. Subcutaneous adipose tissue thicknesses, plasma carotenoid levels and inflammation marker in females of various energy status. 26th European Congress on Obesity (ECO2019), April 28- May 01 2019, Glasgow, SCOTLAND. [Poster]

Mörkl, S; **Lackner, S**; Wonisch, W; Painold, A; Holl, AK; Gorkiewicz, G; Kashofer, K; Holasek, S. Oxidative stress in anorexia nervosa - a possible role of the gut microbiome? DGPPN; NOV 28- DEZ 1, 2018; Berlin. 2018. [Poster]

Meier-Allard, N; **Lackner, S**; Mörkl, S; Moser, M; Poncza, B; Holasek, SJ. Carotenoids in individuals of different body composition. Ernährung aktuell. 2018; (4):21--ÖGE Jahrestagung 2018 - Ernährungstrends, Lebensstil und Sporternährung; NOV 15-16, 2018; Vienna, AUSTRIA. [Poster]

Oberascher, A; Mörkl, S; Moser M; **Lackner, S**; Holasek, SJ; Vagal tone, microbiome diversity and the Microbiota-Gut-Brain Axis Theodor Escherich Symposium; NOV 8-9, 2018; Graz, AUSTRIA. 2018. [Poster]

Lackner, S; Mörkl, S; Müller, W; Holasek, SJ. Ultrasound Measurement of Subcutaneous Fat for the Assessment of Fat Patterning in Overweight and Obese Females. Abstractband. 2018; -11th International Symposium on In Vivo Body Composition Studies Body Composition Analysis (Structural, Functional, Kinetic): Technologies and Models for Biomedical Research and Clinical Application; JUN 25-27, 2018; New York, USA. [Poster]

Lackner, S; Mörkl, S; Müller, W; Hammerl, K.; Holasek, SJ. Ultrasound measurement of subcutaneous adipose tissue thicknesses in patients with anorexia nervosa and in athletes. Abstractband Deutscher Olympischer Sportärztekongress. Gemeinsam für einen gesunden Sport. 2018; - Deutscher Olympischer Sportärztekongress; MAI 24-26, 2018; Hamburg, GERMANY. [Poster]

Mörkl, S; **Lackner, S**; Meinitzer, A; Gorkiewicz, G; Kashofer, K; Painold, A; Holl, AK; Holasek, S. Darmmikrobiom bei Anorexia nervosa: Diversität oder Permeabilität? 18. Jahrestagung der ÖGPP- Psychiatrie zwischen Vision und Realität; APR 25-28, 2018; Gmunden, AUSTRIA. 2018. [Poster]

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Mörkl, S; **Lackner, S**; Meinitzer, A; Blesl, C; Painold, A; Kashofer, K; Gorkiewicz, G; Oberascher, A; Holasek, S. Gut microbiota diversity is not related to intestinal permeability measured by serum zonulin in women. Theodor Escherich Symposium 2017; OCT 12-13, 2017; Graz, AUSTRIA. 2017. [Poster]

Lackner, S; Mörkl, S; Müller, W; Moser, M; Blesl, C; Amouzadeh-Ghadikolai, O; Holasek, S. Differences in skin carotenoid level with regard to subcutaneous adipose tissue thickness in patients with anorexia nervosa. Scripta Scientifica Pharmaceutica. 2017; 4(supp.1): 26-26. [Oral Communication]

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Lackner, S; Tatzber, F; Wonisch, W; Mörkl, S; Meier-Allard, N; Blesl, C; Holasek, SJ. Assessment of polyphenol content in fruit and vegetable juices and human plasma via the application of a novel measurement technique. Journal of International Society of Antioxidants in Helath & Nutrition - Abstracts Book. 2017; 3(4):-11th World Congress on Polyphenols Applications; JUNE 20-21, 2017; Vienna, Austria. [Poster]

Mörkl, S; Meinitzer, A; **Lackner, S**; Blesl C; Painold A; Kashofer K; Gorkiewicz G; Oberascher A; Holasek, SJ. Kynurenine-pathway and microbial diversity in anorexia nervosa-relationship or independency?-preliminary data http://www.cpo-media.net/WFSBP/2017/Final_Programme/. 2017; -13th World Congress of Biological Psychiatry; JUN 18-22; Copenhagen, DENMARK. [Poster]

Lackner, S; Mörkl, S; Müller, W; Oberascher, A; Holasek, SJ. Subcutaneous adipose tissue in patients with eating disorders: Application of a standardized ultrasound measurement technique in extremely lean and obese patients. 24th European Congress on Obesity (ECO2017), Porto, Portugal, May 17-20, 2017: Abstracts. Obes Facts 2017;10(suppl 1):1-274. 2017; -European Congress on Obesity; MAY 17-20, 2017; Porto, PORTUGAL. [Poster]

Mörkl, S; **Lackner, S**; Meinitzer, A; Blesl, C; Painold, A; Kashofer, K; Gorkiewicz, G; Oberascher, A; Holasek, S. Der Tryptophan-Kynureninstoffwechsel bei Anorexia nervosa-gibt es einen Zusammenhang mit der Alpha-Diversität des Mikrobioms? 17. Jahrestagung der Österreichischen Gesellschaft für Psychiatrie, Psychotherapie und Psychosomatik; APR 26-29, 2017; Gmunden. 2017. [Poster]

Lackner, S; Mörkl, S; Müller, W; Oberascher, A; Holasek, SJ. Body Composition Types in Patients with Anorexia Nervosa: Do we need to reconsider dietary treatment? Doctoral Day 2016 -Ventures into new realms - Abstract Book. 2016; -Doctoral Day 2016; DEZ 14, 2016; Graz, AUSTRIA. [Poster]

Mörkl, S; **Lackner, S**; Gorkiewicz, G; Kashofer, K; Oberascher, A; Amouzadeh-Ghadikolai, O; Holasek, SJ. Genus Lactobacillus and anthropometric measurements in different BMI groups and athletes. Doctoral Day 2016; DEZ 14, 2016; Graz, Austria. [Poster]

Mörkl, S; **Lackner, S**; Gorkiewicz, G; Kashofer, K; Oberascher, A; Amouzadeh-Ghadikolai, O; Holasek, SJ.Dietary effects on gut microbiota in different BMI-groups. Ernährung aktuell. 2016; 4/2016: 30--ÖGE Jahrestagung 2016 - Hot Spots in der Ernährung; NOV 24-25, 2016; Vienna

Oberascher, A; **Lackner, S**; Mörkl, S; Holasek, SJ; Moser, M. Autonomic regulation of patients with anorexia nervosa, athletes, obese, overweight and normal weight controls. Ernährung aktuell. 2016; 4/2016: 29--ÖGE Jahrestagung 2016 - Hot Spots in der Ernährung ; NOV 24-25, 2016; Vienna, AUSTRIA. [Poster]

Tmava, A; Mörkl, S; **Lackner, S**; Oberascher, A; Amouzadeh-Ghadikolai, O; Blesl, C; Wurm, W; Gorkiewicz, G; Kashofer, K; Holasek, S. Mikrobiom und Anorexia Nervosa - mehr als sein Bauchgefühl? vorläufige Daten. DGPPN-Kongress; NOV 23-26, 2016; Berlin, GERMANY. [Poster]

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Mörkl, S; **Lackner, S**; Gorkiewicz, G; Kashofer, K; Oberascher, A; Wallner-Liebmann, SJ; Dissecting the female gut microbiota- 106 participants, 115 measurements and millions of bugs. Phenotypes and prevention- the interplay of genes, life-style and gut environment-NUGO Week 2016. 2016; -13th Nugo Week- Phenotypes and prevention- the interplay of genes, lifestyle factors and gut environment; SEP 5-8,2016; Copenhagen, DENMARK. [Poster]

7.3 Doctoral and Master-thesis associated with this thesis

Dissertation

Mörkl, Sabrina. Energy Sensing and Eating Behavior in Patients with Anorexia Nervosa, Athletes, Obese, Overweight and Normal Weight Controls: The Gut Microbiome. Dissertation. Medical University of Graz. 2017

Master Thesis

Fitzek, Katharina. Evaluation of oxidative and nitrosative stress in young females of diverse nutritional status and lifestyle behavior. Diploma Thesis. Medical University of Graz. *In progress*.

Meier-Allard, Nathalie. Carotenoids detection in context of nutritive assessment in female groups of different body composition. Master thesis. FH JOANNEUM - University of Applied Sciences. 2018

Oberascher, Andreas. Autonome Regulation bei Patientinnen mit Anorexia nervosa, Adipositas, Übergewichtigen, Athletinnen und normalgewichtigen Kontrollpersonen. Diplomarbeit. Medizinische Universität Graz. 2016

Hammerl, Katharina. Ernährungszustand und subkutane Fett-Topographie von Athletinnen im Vergleich zu adipösen Frauen bei ähnlicher Energieaufnahme – eine Ist-Zustands-Analyse. Masterarbeit. UMIT – Private Universität für Gesundheitswissenschaften, Medizinische Informatik und Technik. 2015