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Metabolism in Presymptomatic Mutation Carriers of familial Amyotrophic Lateral Sclerosis

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Abbreviations

AgRP	Agouti-related peptide
ALS	Amyotrophic Lateral Sclerosis
AMPK	5' adenosine monophosphate-activated protein kinase
BMI	Body mass index
BMR	Basal metabolic rate
C9orf72	Chromosome 9 open reading frame 72
CaMKIIα	Calcium/calmodulin-dependent protein kinase type II
cMRI	Cranial magnetic resonance imaging
cRMR	Calculated resting metabolic rate
DNA	Desoxyribonucleic acid
DTI	Diffusion tensor imaging
fALS	Familial Amyotrophic Lateral Sclerosis
FFM	Fat-free mass
FTD	Frontotemporal dementia
FUS/TLS	Fused in sarcoma/translated in liposarcoma
GPS-ALS	German PreSymptomatic ALS study
HC	Healthy controls
M	Median
m	Mean
MC	Mutation carriers of the GPS-ALS study
MR	Metabolism ratio
MRI	Magnetic resonance imaging
mRMR	Measured resting metabolic rate
n	Amount of
NC	Non-mutation carriers of the GPS-ALS study
NRF-1/2	Nuclear respiratory factor 1/2
OGTT	Oral glucose tolerance test
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

POMC	Proopiomelanocortin
PPAR-γ	Peroxisome proliferator-activated receptor γ
RMR	Resting metabolic rate
RNA	Ribonucleic acid
sALS	Sporadic Amyotrophic Lateral Sclerosis
SD	Standard deviation
SIRT	Sirtuin-1
SOD1	Superoxid dismutase 1
TARDP	Transactive response DNA binding protein 43 gene
TBK1	Serine/threonine-protein kinase
TDP-43	Transactive response DNA binding protein 43 kDa
VCO_2	Volume of carbon dioxide production
VO_2	Volume of oxygen consumption

1. Introduction

1.1. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting upper and lower motor neurons leading to fatal paralysis. In some ALS cases, a positive family history can be tracked down and several genetic mutations have been found to account for the disease [19]. Therefore, a distinction is drawn between familial ALS (fALS) and sporadic ALS (sALS).

1.1.1. Epidemiology

In Europe, the incidence of ALS is 2-3 out of 100 000 with a relatively low prevalence (~5 per 100 000) since the disease is progressing rapidly to death. The peak age of onset is between 58 and 63 years in sporadic ALS, and 47-52 years in familial ALS [100]. After diagnosis, the average time to death is 3-5 years. However, 10 % may survive for a decade or more [134]. Men tend to be affected more often (3:2) and have an earlier age of onset than women [111]. Although these numbers suggest ALS being a rather rare disease, it should be noted that about 1 in 350 men and 1 in 450 women eventually suffer from ALS throughout lifetime [100].

1.1.2. Clinical Profile

ALS is a clinical diagnosis based on symptoms, clinical examination and results from electrodiagnostic, laboratory and neuroimaging studies [25]. It is characterized by a combination of signs and symptoms of the upper motor neuron such as spasticity, hyperreflexia, pathological reflexes, cramps or pseudobulbar affect, and signs of the lower motor neuron such as muscle atrophy, paralysis, and fasciculations. The first symptoms appear focally and asymmetrically most frequently with either a spinal or bulbar dominance followed by continuous propagation. Extremities are initially affected in the spinal pattern (70 %). In patients with a bulbar onset (25 %), the cranial motor nuclei are primarily affected resulting in dysarthria and dysphagia [87]. A few patients have an atypical onset, such as

respiratory problems due to affection of the intercostal muscles [151] or develop ALS on top of frontotemporal dementia (FTD) [140].

ALS is not only a motoneuron disease but rather a multisystem degeneration [72]. Cognitive impairment is observed over the disease course as well as dysfunctions of the autonomous nervous system, paresthesia, and extrapyramidal symptoms. Motor symptoms classically remain predominant, and with disease progression patients suffer from severe muscle wasting resulting in reduced physical capacity, dysphagia, and respiratory insufficiency due to the loss of muscle innervation. These symptoms eventually lead to death by respiratory failure at an average of 3-5 years after diagnosis [141].

1.1.3. ALS and FTD

Emerging evidence proposes a strong connection between ALS and FTD. FTD is also a neurodegenerative disorder. It primarily affects neurons in the frontal and temporal lobe, which results in changes in behavior, social skills, and personality, as well as impaired language, thinking and memory functions [70]. Clinical, pathological, and genetical overlaps have been found between the two diseases. This led to the concept of a spectrum disease ALS-FTD with the typical phenotype of ALS, respectively FTD, presenting the extremes of the spectrum [102, 175].

About 50 % of ALS patients suffer from cognitive changes and in 15 % symptoms fully apply to the diagnosis of FTD [140, 179]. On the other side, 40 % of FTD patients show motor neuron symptoms besides their cognitive features and 15 % fulfill the criteria for ALS [101].

1.1.4. Genetics

In the last twenty-five years, a huge step has been made with discovering genetic mutations causing ALS. As stated above, ALS is classified as sporadic and familial ALS with approximately 5-10 % accounting for fALS [7]. However, this number is assumed to be higher due to incomplete penetrance or an oligogenic mode of inheritance [117]. There is no remarking clinical difference between sALS and fALS despite an earlier mean age of onset in fALS [100]. The positive family history of ALS and/or FTD is determining. Many of the mutations found in fALS patients have also been found in apparently sporadic ALS.

According to Andersen and Al-Chalabi (2011), genes of Mendelian inheritance account for 75 % of fALS and for 14 % of apparently sporadic ALS [7]. ALS is less frequently inherited in an autosomal recessive pattern or in an x-linked dominant pattern [5]. De novo mutations do not seem to have a major impact on the risk of suffering from ALS [166].

So far more than 30 different gene mutations have been identified. The affected genes are involved in different cellular processes such as ribonucleic acid (RNA) processing, protein quality control, cytoskeletal dynamics and deoxyribonucleic acid (DNA) damage response [175]. The four most common gene mutations account for over 50 % of fALS, are usually of autosomal dominant inheritance, and should be shortly mentioned here.

In 1993, the first gene to be implicated in ALS was the superoxide dismutase 1 gene (SOD1) encoding the copper/zinc superoxide dismutase-1 [145]. Over 170 ALS causing mutations of the SOD1 gene have been identified so far, accounting for about 20 % of fALS and 2 % of sALS cases [160]. A novel toxic function is likely to cause motoneuron and glial cell degeneration, but no consensus has emerged so far about the common primary damaging effect of the mutations [160]. Alterations in proteostasis and protein accumulation are common features [18]. The clinical phenotype of the mutations varies widely from rapid, early onset to slow progressive disease courses; FTD is not typically associated with SOD1 mutations [22].

The transactive response DNA binding protein 43 (TDP-43) has been found to be a common denominator for many ALS and FTD cases [119]. Pathological aggregates were found in most cases of ALS and there is a typical distribution and spreading of TDP-43 inclusions over the course of the disease [17]. Hence, it was not surprising to find pathogenic mutations in the TARDP (transactive response DNA binding protein 43 gene) gene encoding the TDP-43 protein, where mutations have been found in 3-4% of fALS patients [7]. 4-5 % of fALS patients have a mutation in the gene encoding the predominantly nuclear protein fused in sarcoma/translated in liposarcoma (FUS/TLS). TDP-43 and the FUS/TLS have structural and functional similarities; both contain RNA-recognition motifs and seem to play an important role in transcription, RNA splicing and transport, as well as being involved in the processing of small regulatory RNAs (microRNA) [92, 94, 155].

Approximately one third of fALS and 10 % of sALS cases with Caucasian origin can be explained by a pathogenic hexanucleotide repeat expansion in the noncoding part of the chromosome 9 open reading frame 72 (C9orf72) [20, 37, 138]. Instead of having 2-30 repeats, mutation carriers (MC) have hundreds or even thousands of repeats. The C9orf72 mutation is an essential link between ALS and FTD causing different phenotypes of the ALS-FTD spectrum, even within the same pedigree [31]. Over 80 % of cases with a positive FTD and ALS family history can be explained with C9orf72. To date, it is unclear whether anticipation occurs in families with C9of72 mutations [167].

1.2. Metabolism in ALS

There is strong evidence that energy homeostasis is dysregulated in ALS patients [47, 55]. Epidemiological and clinical studies, as well as studies in animal models, support this finding as will be explained in the next chapter. What remains a central question though is the character of the relationship between metabolic alterations and ALS – whether they are causative or a compensatory reaction, and in which way they are pathogenic [45, 55].

1.2.1. Energy homeostasis

Maintaining energy homeostasis within an individual can be achieved when there is a balance between energy intake and expenditure. This balance is challenged in ALS by different developments during the disease process: first of all, ALS patients have a decreased energy intake due to numerous reasons such as swallowing difficulties, reduced muscle strength and dexterity of the upper limb extremities, psychological stress, and impaired salivary secretion [83]. This puts them at risk of malnutrition. Insufficient energy intake itself already causes muscle atrophy and weakness [85] contributing to ongoing pathophysiological mechanisms. Secondly, they suffer from severe skeletal muscle atrophy and a reduced physical activity level resulting in a significant decrease of metabolically active body mass. Considering this, a reduced energy expenditure in ALS patients could be assumed. However, studies have paradoxically demonstrated a hypermetabolic state throughout the disease [15, 41]. Decreased intake paired with increased needs explains the common phenomenon of weight loss in ALS patients [164]. However, it is crucial to maintain

energy homeostasis, since nutritional status has been found to be a significant and independent prognostic factor in ALS patients [21, 39, 107, 121]. The enforcement of an adequate high-calorie diet is well tolerated and associated with an increased survival [44, 180], whereas low BMI at time of diagnosis and rapid weight loss are connected to a more rapid disease progression [108, 126]. In transgenic SOD1 mice, it was shown that a high-energy diet led to weight gain, increased survival, and even improved motoneuronal function [45].

1.2.2. Body composition and nutritional habits

1.2.2.1. Body weight

Several studies have examined body weight development before disease onset and during the course of ALS. In population-based studies, low premorbid body mass index (BMI) was associated with increased risk of ALS, whereas normal or obese people were found to be at lower risk to develop ALS [74, 122]. Patients with motoneuron diseases were more likely to report “having always been slim” and recalling lower premorbid BMI [146]. A stable or decreasing BMI instead of increasing with age was also associated with an elevated risk of suffering from ALS [109]. Other studies reported a higher BMI in ALS patients, decades or years before symptom onset compared to healthy controls (HC) [118, 126]. Interestingly, a change occurred years ahead of clinical manifestation: by the time of diagnosis mean BMI in ALS patients was found to be lower compared to HC [118, 126]. These longitudinal observations might explain controversial findings concerning premorbid BMI. Weight loss after symptom onset and throughout the disease has been reported by several studies and correlates with survival [108, 126].

1.2.2.2. Nutritional habits

Assessing the question what causes the weight loss and whether there are compensatory mechanisms such as increased energy intake or nutritional habits over time, are of great interest: in questionnaires asking ALS patients for presymptomatic eating habits a higher total energy intake as well as fat intake compared to HC has been reported [74, 118]. In Japan, a higher carbohydrate intake combined with lower fat intake was associated with increased risk of ALS [123]. After disease onset, the energy intake of ALS patients was low

in relation to recommended dietary allowances [83, 152], and with an average of 21 kcal/kg/d low compared to clinical standards estimating needs with 25-30 kcal/kg/d [164]. High energy diets are beneficial in mouse models [45] and a carbohydrate-based high-calorie diet in patients was associated with better outcome [180], although evidence is still limited for the actual effect of high energy diets on survival besides weight stabilization [84]. A recent study examining an ALS-FTD spectrum cohort demonstrated a change in eating behavior in the past six months being associated with prolongated survival [4]. In ALS patients and those with cognitive impairments an increased intake of saturated fats was observed.

1.2.3. Pathological glucose tolerance and dyslipidemia

Metabolic laboratory parameters such as pathological glucose tolerance or dyslipidemia are mainly known as cardiovascular risk factors. In ALS however, the presence of dyslipidemia and high triglycerides is found to prolongate survival [43, 46] and is associated with reduced risk of suffering from ALS [89, 91]. An increased peripheral lipid clearance was observed in mutant SOD1 mice, most likely triggered by muscle hypermetabolism, which might be an underlying process of hyperlipidemia being associated with increased survival [54]. However, in other studies, a rather favorable or basically unremarkable cardiovascular profile in ALS patients had been found [30, 157]. No association with prolongated survival was found [124, 136]. Cultural differences in eating habits might add up to these divergent findings with the studies having been conducted in different countries.

An association between pathological glucose tolerance, insulin resistance, and ALS has already been found and discussed decades ago [76, 139]. A more recent study with sALS patients affirmed higher incidence of pathological glucose tolerance in patients, as well as an impaired fatty acid metabolism [130]. It was ruled out that the pathological glucose tolerance was the effect of a reduction in muscle mass and therefore a reduction in glucose transporters [139]. A retrospective analysis of data from 2371 ALS patients revealed a later disease onset and slower disease progression in patients with premorbid diabetes mellitus type II [80]. Controversial findings were published not affirming ALS being associated with alterations in glucose metabolism [67], and a recent review critically stated the limitations of the existing data on the association between ALS and diabetes so far [95].

The antidiabetic pioglitazone emerged as a candidate drug for ALS via multiple protective mechanisms stimulating the peroxisome proliferator-activated receptor gamma (PPAR-γ) involved in the control of glucose and lipid metabolism [38]. Besides the desired reduction of peripheral insulin resistance and decreased blood sugar levels in diabetes therapy, it has been shown to have an anti-inflammatory effect as well as leading to substantial weight gain [27, 104]. In SOD1 mice improved motor function and prolongated survival were observed [86, 147]. However, in a clinical trial, no effects on survival were seen in ALS patients leading to early study termination [48]. Interestingly no weight gain was observed, which will be discussed later (1.2.6).

1.2.4. Physical activity

Physical activity has been controversially discussed as an exogenous risk factor of ALS for years [103]. Due to famous sportsmen suffering from ALS like Lou Gehrig or Krzysztof Nowak [49], and increased incidence of ALS in Italian soccer players [29], physical activity has been under suspicion as a risk factor. Some studies found an increased risk for ALS having been exposed to work-related or leisure-related physical activity [12, 73] with and without a dose-response relationship [29, 73]. In contrary, no association between physical activity and an increased risk of ALS was found [53], or even a protective connection between physical activity and ALS was claimed in a Level I study [133]. Actually, reviews also come to differing results: in a recent review looking at 26 case-control and cohort studies an increased risk was found [93], whereas Hamidou and colleagues reviewed the inconsistent findings of 37 studies and concluded, that physical activity is not an exogenous factor associated with ALS [65]. A commonly favored hypothesis was stated by Huisman and colleagues of a genetic profile determining both - physical fitness and an increased susceptibility to ALS [73].

1.2.5. Energy expenditure

Total energy expenditure is the amount of energy an individual's body needs for maintaining body function every day. It is composed of three components: basal metabolic rate (BMR), diet-induced thermogenesis and physical activity. BMR represents the amount of energy needed to maintain basal vital functions in an awake, though physical and

psychological resting state, surrounded by a neutral environment in a post-absorptive state, in the morning after having slept overnight and without any activity ahead. It accounts for 60-70 % of total energy expenditure in sedentary individuals and about 50 % in active persons. Resting metabolic rate (RMR) is 3-10 % higher than BMR due to minimal effects of diet-induced thermogenesis and recent physical activity [42, 129]. For measuring BMR or RMR indirect calorimetry is an accurate and practicable method [69]. Fat-free mass (FFM) includes the metabolically active mass of the body and is a major determinant of BMR [173]. Organs account for approximately 60-70 % of BMR, although contributing to less than 6 % of body weight, whereas skeletal muscle accounts for 20-30 % of BMR with 40-50 % of body weight [79]. Many individual factors influence BMR such as weight, height, sex, age, ethnicity, stress, smoking, menstruation, physical activity, hormones, organ mass, diseases as well as family traits [178]. There are numerous prediction equations for estimating BMR of which the equation by Harris and Benedict taking gender, age, height and weight into account is commonly used in scientific matters [68].

Due to severe muscle wasting a decrease in FFM is found during the disease process of ALS [15, 164], consequently suggesting a corresponding decrease in RMR in ALS patients. Paradoxically several studies having examined RMR in sporadic ALS patients reported a hypermetabolic state. This was defined by putting measured RMR (mRMR) in relation to calculated RMR (cRMR). Values greater than 1.1 were stated as hypermetabolic. A stable hypermetabolic state was found in 50-68 % of sALS patients, even when normalized for FFM [15, 40, 41, 81]. Additionally, in 315 patients an adverse correlation between hypermetabolism and survival was found [81]. Funalot and colleagues [62] examined energy expenditure in 11 patients with fALS compared to 33 sALS patients. All fALS patients were in a hypermetabolic state, whereas only 52 % sALS patients were hypermetabolic, suggesting that energy homeostasis in ALS might be genetically impaired. The underlying mutations had not been reported, except for no one having an SOD1 mutation. In 33 sALS patients and 33 age and sex-matched controls, normometabolic conditions were found [164]. However, after normalizing for FFM a significant elevation of mRMR was found in the ALS group. Hypometabolism has also been found in ALS patients [150]. However, all patients had been mechanically ventilated. The average was BMI of 15 kg/m^2 and suggested severe malnutrition being an important factor affecting energy homeostasis in this study group.

Animal models of SOD 1 and TDP-43 transgenic mice corroborate the findings of a hypermetabolic state and reduced body weight in ALS [28, 45].

The reasons for an increased energy expenditure in ALS patients are still matters of research. Fasciculations, cramps, spasticity and increased energy needs for functional movements and respiratory muscular work had been discussed accounting for the increased needs [82]. However, an increase in respiratory muscle work is not sufficient to explain the hypermetabolism as studies have shown [41, 149]. An intrinsically elevated RMR is most likely, the underlying pathophysiology is still unknown [47]. Mitochondrial dysfunction in the nervous system and in peripheral tissue such as skeletal muscle is discussed as an important factor involved [45, 114, 153, 169].

1.2.6. Hypothalamus

The hypothalamus is a part of the brain involved in regulatory processes of the autonomous nervous system. Besides maintaining homeostasis of body temperature and osmolarity, its several nuclei are responsible for regulating immune defense, sexual behavior, circadian rhythms, sleep, food intake, and energy expenditure by affecting hormonal axes producing hormones themselves [78]. The hypothalamic melanocortin system is responsible for regulating food intake, energy expenditure, lipid metabolism as well as glucose metabolism and insulin response. Peripheral organs such as the liver or the adipose tissue communicate with the hypothalamus via hormones like leptin or via nutrients for maintaining energy homeostasis [88]. All the named targets of the melanocortin system are altered in ALS, supporting the idea of a dysfunctional hypothalamic axis.

An indication for this hypothesis gave a study mentioned above with the antidiabetic pioglitazone (Chapter 1.2.3), which amongst other things, increases food intake and reduces energy expenditure via a hypothalamic axis. Surprisingly, it did not have any effect on the body weight in ALS patients. This led to the idea that hypothalamic function itself could be disturbed as part of the disease process [48]. Based on this hypothesis, further studies were conducted in mouse models of ALS. Hereby functional and molecular alterations of the melanocortin system were observed: Agouti-related-peptide (AgRP) level was elevated and proopiomelanocortin (POMC) level was decreased in transgenic mice;

fiber density of AgRP was increased and less POMC neurons were present in the hypothalamus [168]. AgRP physiologically stimulates energy intake and inhibits POMC neurons, whereas POMC promotes satiety amongst other effects. Overall this observed constellation suggested an increased food intake, which was confirmed in the transgenic ALS mice. These alterations might be triggered by decreased energy intake, increased energy needs as one would expect in hypermetabolism, or the other way around with hypothalamic neurons being a target or part of the neurodegenerative process of ALS causing the metabolic alterations stated above. Gorges and colleagues analyzed MRI data regarding hypothalamic morphology and found a severe global atrophy of the hypothalamus in sporadic and familial ALS patients compared to HC [64]. Additionally, TDP-43 accumulation was found in some patients in the hypothalamus. The presence of TDP-43 pathology was associated with a significantly lower body mass index compared to those patients without deposition of TDP-43 [34].

1.3. Presymptomatic phase

1.3.1. The presymptomatic phase in neurodegenerative diseases

Nowadays it is widely recognized that the degeneration in neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease begins years or even decades before the typical clinical manifestation appears. One distinguishes between a preclinical phase where no symptoms can be detected and a presymptomatic phase where the patient does not recognize any symptoms, but the physician can already detect abnormalities [50, 90]. Studying the presymptomatic phase can give essential information about pathophysiological mechanisms as well as insights into compensatory mechanisms for maintaining normal brain function. Furthermore, the relevance and relationship of environmental risk factors can be studied; biomarkers, early therapeutic interventions and possibly even strategies of disease prevention. In Alzheimer's disease and Parkinson's disease pathological changes occur years before clinical manifestation [16, 26] and correspondingly the characteristics of the preclinical and presymptomatic stages have been discussed in ALS as well [14, 36, 158].

There are two main options for studying the presymptomatic phase of ALS: firstly, examining those people, who are at an increased risk of suffering from ALS regularly over the years until disease onset. These are people carrying a mutation known to cause ALS. Besides that, several animal models of ALS have been established and contribute to our understanding of the presymptomatic phase and the pathophysiology of the disease in general.

1.3.2. Studies of mutation carriers

1.3.2.1. Anamnestic and clinical measures

There is some retrospectively acquired data from sporadic ALS cases addressing the questions of premorbid BMI, nutritional habits and level of physical activity as described above (Chapters 1.2.2 and 1.2.4). Most information is based on self-reports in questionnaires or interviews, indicating the limitation of these data.

Addressing the question of early cognitive and clinical alterations a recent study compared the Revised ALS Functional Rating Scale, the Frontal Behavioral Inventory and letter fluency in 33 C9orf72 MC over 18 months [59]. Eight of them were asymptomatic and did not develop any clinical symptoms during follow-up. They had a stable performance in all measurements over time.

1.3.2.2. Electrophysiological and imaging data

More data is available for electrophysiological and imaging data. Electrophysiological parameters such as motor unit number estimation and cortical excitability did not reveal any differences between SOD1 MC and controls in cross-sectional analysis [2, 171]. In a longitudinal perspective though, several months before symptom onset an increased cortical excitability and reduced motor unit number estimation had been found in two, respectively three MC, who turned symptomatic during the three-year follow up [3, 170].

MRI imaging techniques revealed controversial results in cross-sectional analysis. No differences were detected using diffusion tensor imaging (DTI) between SOD1 MC and age-matched controls [171], whereas a Chinese study reported abnormalities with DTI in SOD1

MC in the posterior limb of the internal capsule [120]. An increase of functional connectivity between the cerebellum and the precuneus-cingulate-middle frontal network was found without structural alterations in 12 presymptomatic MC with different autosomal dominantly inherited mutations (SOD1 and C9orf72). This increase was in-between the results of the HC and symptomatic group [113]. A study comparing symptomatic and asymptomatic C9orf72 MC with healthy age-matched controls detected greater ventricular volume loss and thalamic atrophy in the symptomatic participants. No differences between asymptomatic MC and HC were reported [58]. As aforementioned a global hypothalamic atrophy was found in ALS patients, and interestingly this atrophy was already detectable in presymptomatic MC [64]. Neurometabolic abnormalities measured by magnetic resonance spectroscopy were detected in 24 presymptomatic SOD1 MC compared to a symptomatic and a HC group in the absence of clinical or electrophysiological signs of disease manifestation [24].

1.3.3. Presymptomatic phase in animal models

Several animal models of ALS are established especially in rodents [112]. They offer the detection of early alterations and pathomechanisms on different levels during the disease process which is essential for further understanding of the pathophysiology and onset of ALS. Eisen and colleagues [50] developed a model of the preclinical and presymptomatic process based on these data. An early determination of suffering from ALS in the future are genetic mutations present from the day of conception. Besides genetic conditions, there are different sorts of stress factors impacting the nervous system and compromising cellular function throughout a person's life, which could increase an individual's susceptibility to ALS. These are mainly neuroinflammation, excitotoxicity, mitochondrial dysfunction, excessive oxidative stress, and environmental risk factors [10, 13, 35, 98, 127, 153] which might lead to epigenetic processes increasing an individual's vulnerability [56]. Continuation and accumulation of these factors cause protein dysfunction and aggregation over time, which are counterbalanced by several compensatory mechanisms for years or decades. At some point, these mechanisms might fail and the presymptomatic stage starts with the first alterations detectable with electrophysiological or imaging techniques as stated above (Chapter 1.3.2.2). From then on, an irreversible progression into a clinical

manifest state can be observed. Looking at the development of ALS like that, it is obvious that neuroprotective therapies might be a lot more effective in early stages.

1.3.3.1. Metabolism in ALS mouse models

The metabolic alterations described above (1.2) occur weeks before disease onset in mutant SOD1 mice. An increased energy expenditure with skeletal muscle hypermetabolism is present in presymptomatic mice as well as reduced adipose tissue accumulation and less body weight [45]. A high-fat diet reducing this energy deficit is associated with later disease onset, improvements in motor neuron survival and prolongated survival [45]. Examining the utilization of substrates in glycolytic muscles of mutant SOD1 mice a metabolic shift was revealed: during the asymptomatic stage glycolytic muscles lost their ability to utilize glucose as their primary fuel. Thereupon a switch occurred to primarily utilizing lipids [125]. Additionally, an increased aerobic capacity and a worse anaerobic capacity was found in asymptomatic mutant mice compared to wildtype mice [125].

1.4. Study objectives

Alterations in metabolism are common findings, however controversial reports are to be found in nearly all topics. In mouse models, results are more consistent and even give evidence for dysfunctional energy homeostasis in the presymptomatic phase. The next step is to examine the presymptomatic phase in future patients in a longitudinal manner and see if, and when metabolic alterations occur in MC. Metabolic parameters such as body composition, energy expenditure or laboratory findings should be monitored throughout the presymptomatic phase up to disease onset. Essential for robust findings and observing intra-individual changes, is a longitudinal perspective.

In this baseline cross-sectional analysis, the following points were addressed:

(1) Are there differences in body composition in asymptomatic MC compared to healthy individuals?
(2) Is there a difference in resting metabolism in asymptomatic MC compared to healthy individuals?
(3) Are there any mutation or sex-specific effects?

2. Methods

This work is based on data acquired within the framework of the German PreSymptomatic ALS Study (GPS-ALS). The purpose of the study is to examine and characterize people carrying one of the mutations causing ALS to get information about the presymptomatic phase of fALS. The study is designed to be longitudinal with follow-ups every second year. The present analysis is limited to the cross-sectional data set from base-line testing.

2.1. Study Design

The GPS-ALS Study is set in an ethically complex situation examining potential presymptomatic MC of the fatal disease ALS. This required that all participants fully understood the study, were well informed and able to make informed choices at every stage of the study process.

Potential participants were identified either with files from the ALS network Süd-Württemberg or via affected relatives. They were contacted by the study nurse, doctors, patients, or relatives, and informed about the existence of the study. If they were interested, they were provided with information about the study, ALS in general and specifically the genetic form with the along going risk.

Time was given for consideration. If participants agreed to participate they had to give informed consent and were invited to come to the study site in Ulm for a two-day assessment. The study center was the neurological clinic in Ulm, with participants coming from all over Germany and other European countries.

During the two study days, all examinations were performed. This included imaging (cranial magnetic resonance imaging (MRI), oculomotor exams, body fat MRI), metabolic assessments (calorimetry, bioelectrical impedance, oral glucose tolerance test, BMI, body circumferences) and tissue markers (serum, liquor, muscle, skin, hair follicle stem cells). Participants could decide specifically if, and which of the invasive examinations they wanted to be done (skin/muscle biopsy, lumbar puncture).

During the whole study process, no information about the mutation status was given to the participants at all. Participants asking for genetic counseling were referred to human genetics. They had to go through the process of predictive genetic testing according to the guidelines [1].

The GPS-ALS study was approved by the ethical review committee of the University of Ulm (20/12).

For this work looking merely at metabolic processes with indirect calorimetry and anthropometric data HC and symptomatic ALS patients were asked to participate in the study as control groups. Having given informed consent, they participated solely in the indirect calorimetric measurements.

2.2. Study Population

All participants of the GPS-ALS study must have had a family history of ALS and a first-degree relative suffering or having suffered from fALS. No participants were included being underage. The underlying mutation had to be of autosomal dominant inheritance. Further exclusion criteria for this analysis were GPS-ALS participants with typical ALS symptoms, metabolic diseases such as diabetes as well as unclear or autosomal recessive mutations. For calorimetric analysis, those taking metabolism affecting medication such as L-Thyroxin or ß-blockers had to be excluded as well. HC must not have been suffering from any neurological or metabolic disease. sALS patients were diagnosed at the neurological department of the University hospital in Ulm.

2.3. Examinations

2.3.1. Resting metabolic rate (RMR)

Energy expenditure can be measured with calorimetric techniques. In clinical and research settings, indirect calorimetry is usually used, being an accurate and more easily accessible method than direct calorimetry. It is reliable, reproducible and highly accurate [69]. The

heat production can be calculated from measuring the amount and pattern of oxygen consumption (volume of oxygen (VO_2)) and byproduct use (volume of carbon dioxide (VCO_2)) by analyzing respiratory gases. The modified Weir equation is used for calculating the resting metabolic rate per day in kilocalories (kcal) [174].

Calculating the respiratory quotient (VCO2/VO2) gives information about the sort of substrate burned and is a tool to control correct measurement [32]. The respiratory quotient (RQ) is physiologically in a range between 0.67-1.2 [69]. The complete oxidation of glucose results in an RQ of 1.00, whereas a lower/higher RQ reflects fat/protein oxidation.



Figure 1: Indirect calorimetry with a canopy.

Indirect calorimetry was performed with the Quark RMR calorimeter from COSMED using a canopy (Figure 1). Several preliminaries had to be fulfilled [32] to make sure that only metabolic processes in rest are measured to obtain RMR. We performed all measurements in the morning between 7:30-9:00 o'clock at the study center. Participants had to have been fasting for at least five hours including abstinence from nicotine (>2 hours) and caffeine (>4 hours). No medication should have been taken in the morning. Furthermore, the participants must not have been participating in physical activity for at least 2 (moderate exercise) to 14 hours (vigorous training). When arriving at the study center in the morning,

participants had to rest for 10-20 minutes, before the 16 minutes of measurement in a supine position could start [163]. Room temperature was in a comfortable zone and we advised the participants to relax, but not to fall asleep.

2.3.2. Equations predicting resting metabolic rate

Besides measuring RMR, it is also possible to estimate RMR based on regression equations referred to as cRMR. Well established prediction equations are the Harris and Benedict equation for male and female [68] which were used in this work having been examined to provide acceptable results in ALS patients [51].

Male: $RMR = 66.46 + 13.75 \cdot m + 5 \cdot l/100 - 6.75 \cdot t$

Female: $RMR = 655.09 + 9.56 \cdot m + 1.84 \cdot l/100 - 4.67 \cdot t$

2.3.3. Glucose metabolism

As part of the GPS-ALS study, subjects underwent an oral glucose tolerance test (OGTT). It is a standardized test for assessing pathological glucose metabolism [128]. After a fasting baseline sample is drawn, subjects have to drink a solution in which exactly 75 mg of glucose are dissolved. Two hours later another blood sample was collected. At this point a blood sugar level of < 140 mg/dl is normal. Blood sugar levels between 140 and 200 mg/dl are considered to reflect a pathological glucose tolerance, whereas a manifest diabetes is to be diagnosed with the blood sugar level being over 200 mg/dl.

2.3.4. Others

During the study days, anthropometric data were collected. Weight and height were assessed in a standardized manner. Hence we could calculate BMI for each participant (BMI=weight in kg/(height in meter)^2).

2.4. Analysis

2.4.1. BMI

Since the respective groups were not matched in age and BMI is known to increase with age [126], the BMI data for all subjects were age-adjusted using a linear regression model and subjected to further statistical analysis.

2.4.2. Calorimetry

The protocol of the QUARK RMR took 15:45 minutes. According to the review of Compher and colleagues [32], the steady-state was defined as follows: the initial five minutes of measuring were discarded. Within the next 10 minutes, a period of five minutes displaying the achievement of steady-state had to have less than 10% of variation in mRMR, VO_2 and VCO_2. These five minutes of steady-state measuring were used for analysis. In Figure 2 an exemplary output of the QUARK RMR software is shown, after having chosen the timeframe for steady-state analysis.

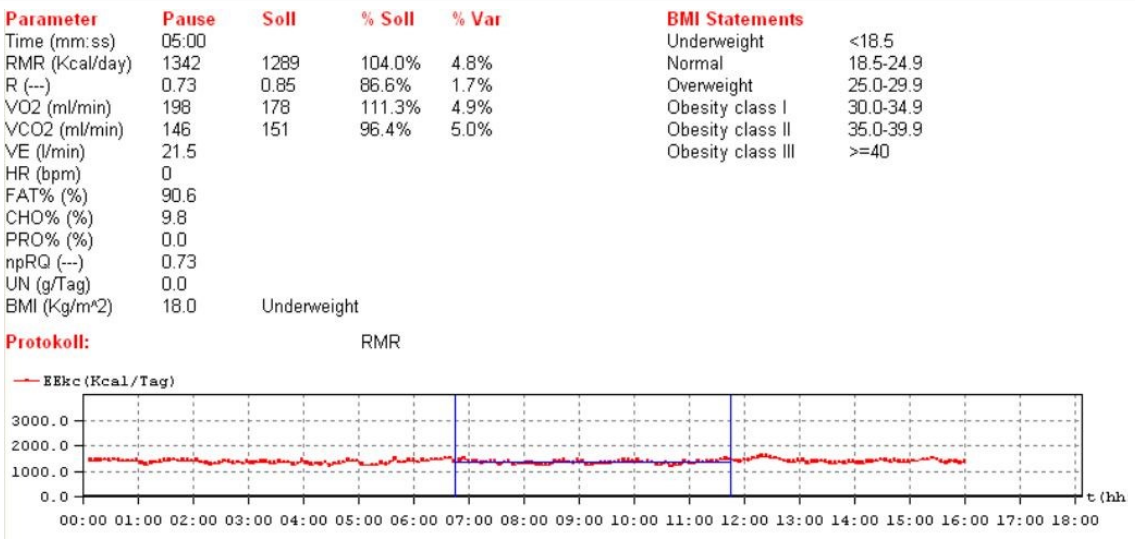


Figure 2: Exemplary output of calorimetric results presenting measured resting metabolic rate in the chosen timeframe. Abbreviations: RMR: resting metabolic rate.

2.4.3. Estimated time to disease onset

Estimated time to disease onset (ETTO) was calculated by using the age of onset of the index person (first degree relative with fALS) and the effective age of the study participant at the study days. This method has been used in research of other neurodegenerative disorders before [116, 177].

> ETTO = (age of participant at the study days) - (age at ALS diagnosis of first degree relative of the participant)

2.4.4. Statistics

Statistical analysis was done with IBM SPSS Statistics 21. Data were tested on normal distribution using the Kolmogorov-Smirnov-Test. The Levene-test was used for testing equality of variances. For differences in means, Student's t-test was used with $\alpha = 0.05$. If preliminaries for parametric testing were violated and non-parametrical test procedures were required, the Mann-Whitney-U test was used to question differences in means. The Chi-squared test or Fisher's Exact test was used for testing contingency. Bivariate correlations were calculated for evaluating relationships between certain variables.

3. Results

3.1. GPS-ALS study population

About 120 potential study participants have been identified and asked to participate in the study so far. From January 2013 until August 2017, 90 persons could be included and took part in the two study days. For this analysis, a cut was made here and the data of these 90 participants were used, with the study still being continued.

Of the 90 participants having participated in the GPS-ALS study, 45.6 % were male (n = 41), 53.3 % female (n = 48) and one participant was transsexual. They were between the age of 18 and 77 years (m = 43.3). Mean BMI was 25.6 ± 4.9 kg/m². All participants were of Caucasian origin.

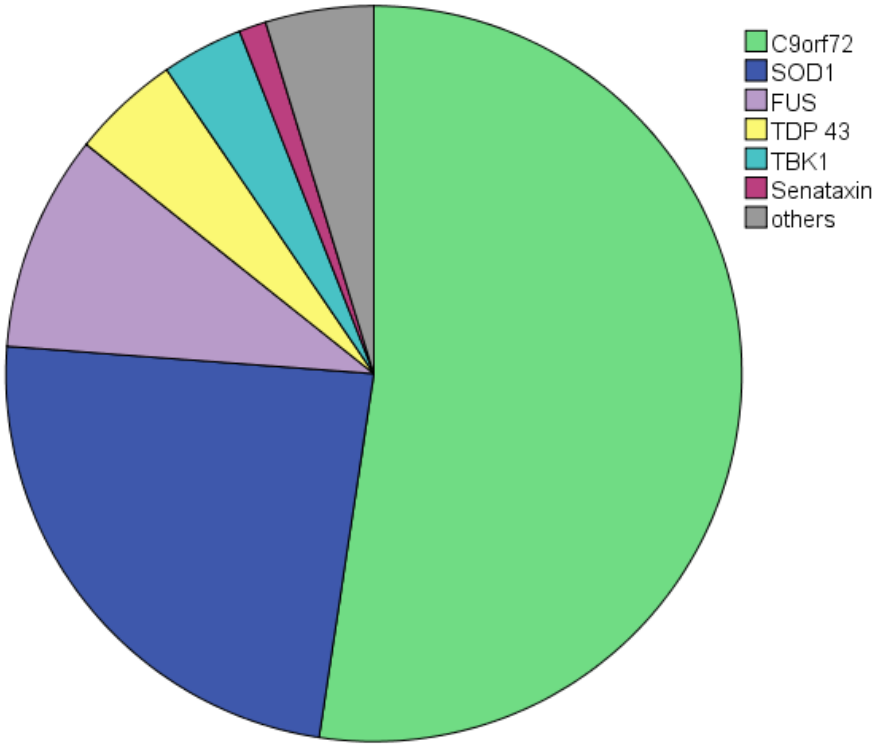


Figure 3: Percental distribution of mutation loci (n = 84). Abbreviations: C9orf72: chromosome 9 open reading frame 72; FUS: fused in sarcoma; SOD1: superoxide dismutase 1; TBK1: serine/threonine-protein kinase; TDP43: transactive response DNA binding protein 43kDa.

All participants were first degree relatives of ALS patients with a known fALS. 48.9 % (n = 44) had C9orf72 mutations in their family, 20.0 % (n = 22) an SOD1 mutation, 8.9 % (n = 8) FUS, 4.4 % (n = 4) TDP43, 3.3 % (n = 3) TBK1, 1.1 % (n = 1) Senataxin and 4.4 % (n = 4) had autosomal recessive mutations (Alsin, SOD1 D90A) (Figure 3). In 6 participants the regarding mutation was still unknown, having an autosomal dominantly inherited family history though. In this study population 52.2 % (n = 47) were negative concerning the respective mutation, 41.1 % (n = 37) were MC. In 6.7 % no clear specification could be made carrying an autosomal recessive mutation (n = 2) or so far unknown mutation loci with an autosomal dominant inheritance pattern (n = 4). 8 participants (8.9 %) were already symptomatic at the baseline assessment.

3.2. Control groups – healthy subjects and symptomatic ALS patients

To have an independent control reference to these data from the GPS-ALS study we included calorimetric and anthropometric data from HC as well as data from symptomatic ALS patients with sporadic occurrence. 40 HC were measured with 17 males and 23 females being aged 33 years on average (between 19 and 66 years old). 19 symptomatic ALS patients were measured, 13 males and 6 females. Mean age was 60 years with a range between 40 and 76 years of age. 16 had a spinal onset, 2 patients presented with flail-arm-syndrome and one patient had a bulbar onset. Time between symptom onset and diagnosis was on average 14 months.

3.3. Body composition

3.3.1. Body composition – characterization of groups

Looking at body composition all GPS-ALS study participants were included except those having mutations with autosomal recessive inheritance (n=4), unknown mutation loci and therefore unknown mutation status (n=4), and one being transsexual. Additionally, eight participants were already symptomatic at the first study date and therefore had to be excluded. 73 participants of the GPS-ALS study remained with 44 healthy and 29 mutation

carrying GPS-ALS participants. 40 controls and 19 patients with sporadic ALS could be included. The respective groups are characterized in Table 1.

3.3.2. Body composition - results

In HC (n = 40), the BMI significantly increases by about 1.1 kg/m² per decade (r = 0.36, p = 0.022).

The mean of age-adjusted BMI was 24.9 ± 3.8 kg/m² in HC, 26.9 ± 5.5 kg/m² in NC, 24.2 ± 3.9 kg/m² in MC and 22.2 ± 3.6 kg/m² in sALS patients. They differed significantly from each other ($F(3,128) = 5.6$; $p = 0.001$) (Figure 4). Student's t-test revealed a significant difference between MC and NC of the GPS-ALS cohort ($t(71) = 2.21$; $p = 0.031$). A significantly lower mean of age-adjusted BMI was found in sALS patients compared to HC ($t(57) = 2.6$; $p = 0.012$) and NC ($t(61) = 3.45$; $p = 0.001$). MC and sALS patients did not differ significantly from each other ($t(46) = 1.94$; $p = 0.059$). NC and HC barely differed from each other significantly ($t(82) = 1,99$; $p = 0.05$).

These differences were present in male (n = 58): $F(3,55)=4.33$; $p = 0.008$) and female participants (n = 72) ($F(3,69)=2.87$; $p = 0.043$)) however more distinctive in men.

Table 1: Characteristics of the groups included in body composition analysis. Abbreviations: ALS: Amyotrophic Lateral Sclerosis; BMI: body mass index; HC: healthy controls; max: maximal value; M: median; m: mean; MC: mutation carriers; min: minimal value; n: amount of; NC; not mutation carrying; sALS: sporadic Amyotrophic Lateral Sclerosis; SD: standard deviation; WHO: World Health Organization.

n = 132	NC n = 44	MC n = 29	HC n = 40	sALS n = 19
Age (years): m ± SD	38.9 ± 11.5	41.1 ± 11.6	33 ± 13	60.2 ± 9.5
M [min; max]	38 [20; 63]	43 [18; 60]	28 [19; 66]	57 [40; 76]
Sex (n): male/female	20/24	9/20	17/23	13/6
BMI (kg/m²): m ± SD	26.6 ± 5.6	24.2 ± 4.1	23.8 ± 4.1	24.2 ± 3.6
Age-adjusted BMI: m ± SD	26.9 ± 5.5	24.2 ± 3.9	24.9 ± 3.8	22.2 ± 3.6
M [min, max]:	24.7 [17.6; 42.2]	23.5 [17.5; 34.8]	23.0 [17.8, 36.2]	23.8 [17.8; 30.1
WHO BMI classification (n):	Underweight: 1 Normal: 24 Pre-obese: 9 Adipose I: 5 Adipose II: 3 Adipose III: 2	Underweight: 1 Normal: 18 Pre-obese: 7 Adipose I: 2 Adipose II: 1 Adipose III: 0	Underweight: 2 Normal: 28 Pre-obese: 5 Adipose I: 4 Adipose II: 1 Adipose III: 0	Underweight: 2 Normal: 9 Pre-obese: 6 Adipose I: 2 Adipose II: 0 Adipose III: 0
Gene Mutation (n):	C9orf72: 26 SOD1: 9 FUS: 5 TDP43: 3 TBK1: 1 Senataxin: 0	C9orf72: 15 SOD1: 9 FUS: 3 TDP43: 1 Senataxin: 1	-	-

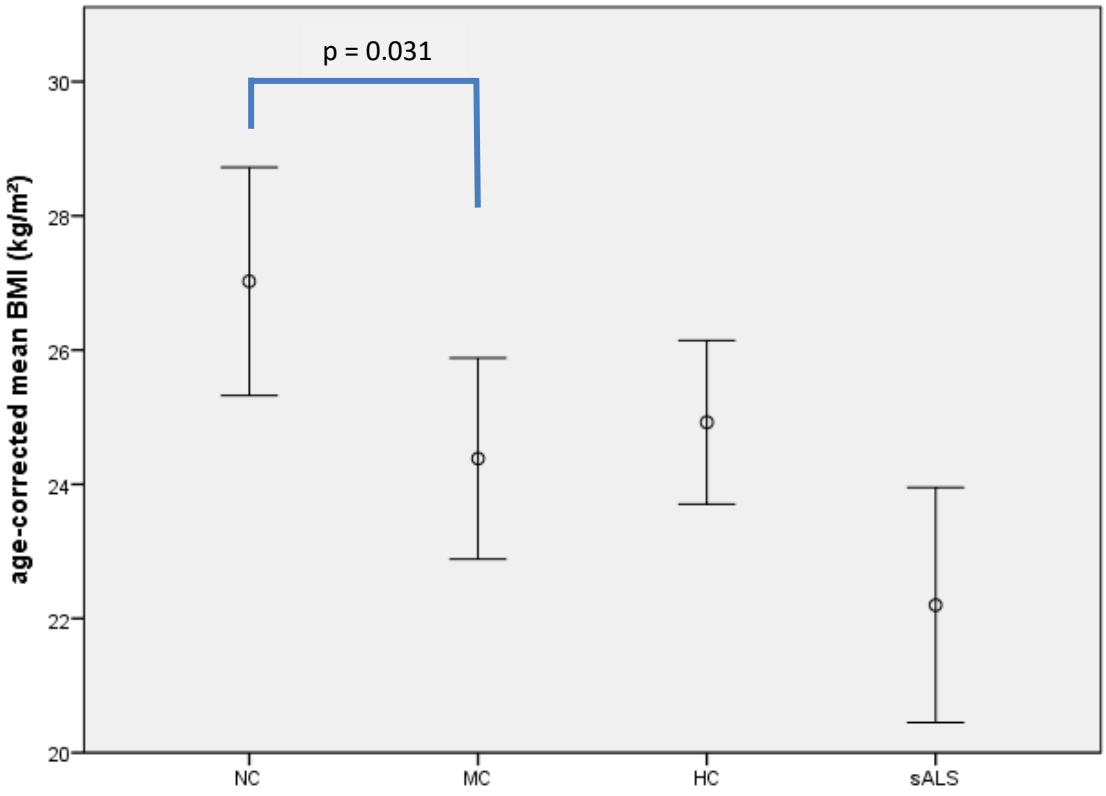


Figure 4: Age-adjusted mean BMI (kg/m²) differentiated between controls (HC; n = 40), mutation carriers (MC; n = 29), healthy GPS-ALS participants (NC; n = 44), and patients with sporadic ALS (sALS; n = 19). The circle is marking the mean and the error bars are marking the 95% confidence intervals. The blue lines are marking a significant difference of MC with the respective p-value. Abbreviations: BMI: body mass index; HC: healthy controls; MC: mutation carriers; n: amount of; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis.

Binning BMI values in six groups according to the WHO classification (underweight BMI < 18.5; normal BMI = 18.5-24.9; pre-obese BMI = 25 - 29.9; obese grade I BMI = 30 - 34.9; obese grade II BMI = 35 - 39.9; obese grade III BMI > 40), a visual analysis of the distribution of BMI values for the different groups is given in Figure 5. Fisher's Exact Test was used to examine the association between the different groups and the BMI classification. A significant connection was found (Fisher´s exact (15) = 21.52; p = 0.033).

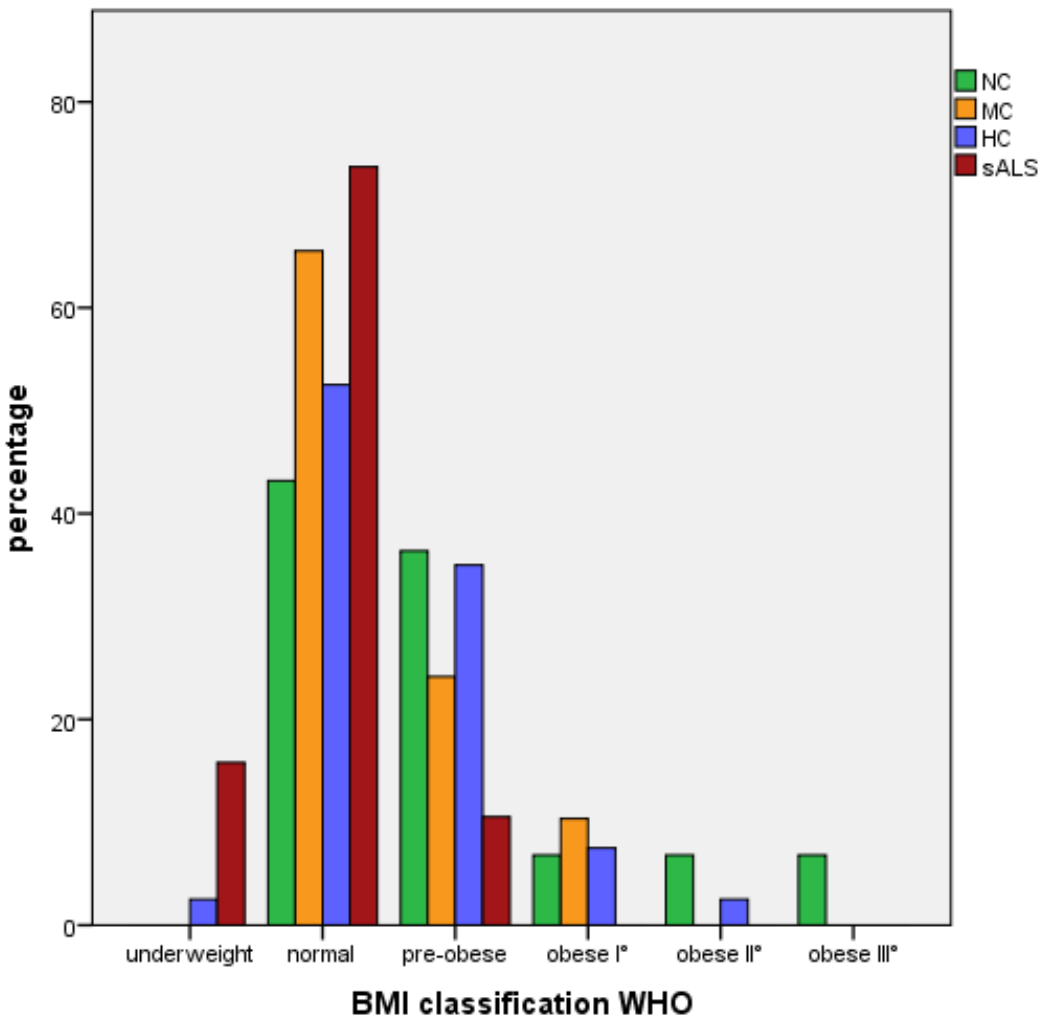


Figure 5: BMI classification according to the WHO guidelines differentiating between the different groups (n = 132). Whole percentages are shown for each legend variable category. Abbreviations: BMI: body mass index; HC: healthy controls; MC: mutation carriers; n: amount of; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis; WHO: World Health Organization.

3.3.1.1. Mutation-specific analysis

In a subanalysis of those participants with a C9orf72 mutation in their family history (Table 2) a significantly lower mean BMI was observed in MC compared to NC ($t(39) = 2.995$; $p = 0.005$) as well as to HC ($t(53) = 2.243$; $p = 0.029$). To sALS patients however, no significant differences in mean BMI could be found ($t(32) = 0.095$; $p = 0.77$). These results are visualized in Figure 6. Gender-specific differences could not be statistically analyzed due to small and highly divergent group sizes.

Table 2: Subanalysis of participants with a C9orf72 family history. Abbreviations: BMI: body mass index; C9orf72: chromosome 9 open reading frame 72; max: maximal value; M: median; m: mean; min: minimal value; MC: mutation carrier; n: amount of; NC: non-mutation carriers; SD: standard deviation.

	C9orf72 NC n = 26	C9orf72 MC n = 15
Age (years): m ±SD	40 ± 11.4	41 ± 10.8
M [min; max]	40.5 [20; 62]	43 [18; 57]
Sex (n): male/female	12/14	2/13
BMI (kg/m²): m ± SD	27.5 ± 6.5	22.4 ± 2.4
Age-adjusted BMI: m ± SD	27.9 ± 6.6	22.5 ± 2.8
M [min; max]	25.6 [17.6; 42.2]	22.5 [17.5; 26.5]

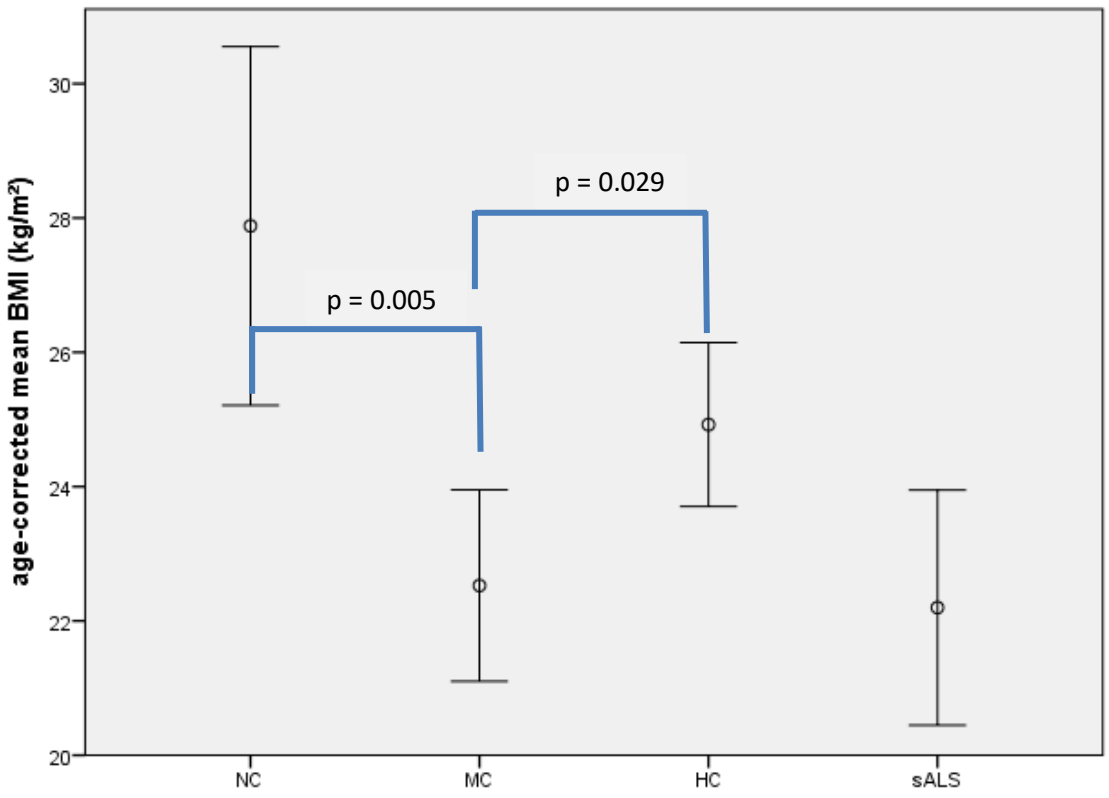


Figure 6: Age-adjusted mean BMI (kg/m²) in participants with a C9orf72 family history carrying the respective mutation (MC; n = 15) or not (NC; n = 26) compared to healthy controls (HC, n = 40), and sporadic ALS patients (sALS; n = 19). The circle is marking the mean and the error bars are marking the 95% confidence intervals. The blue lines are marking significant differences involving the group of MC with the respective p-value. Abbreviations: BMI: body mass index; C9orf72: chromosome 9 open reading frame 72; HC: healthy controls; MC: mutation carriers; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis.

In 18 participants a family history of an autosomal dominantly inherited SOD1 mutation was present with nine being MC themselves. Subanalyzing and comparing those GPS-ALS participants with the control groups revealed no significant differences between the MC, NC, and HC. A graphic presentation can be found in Figure 7.

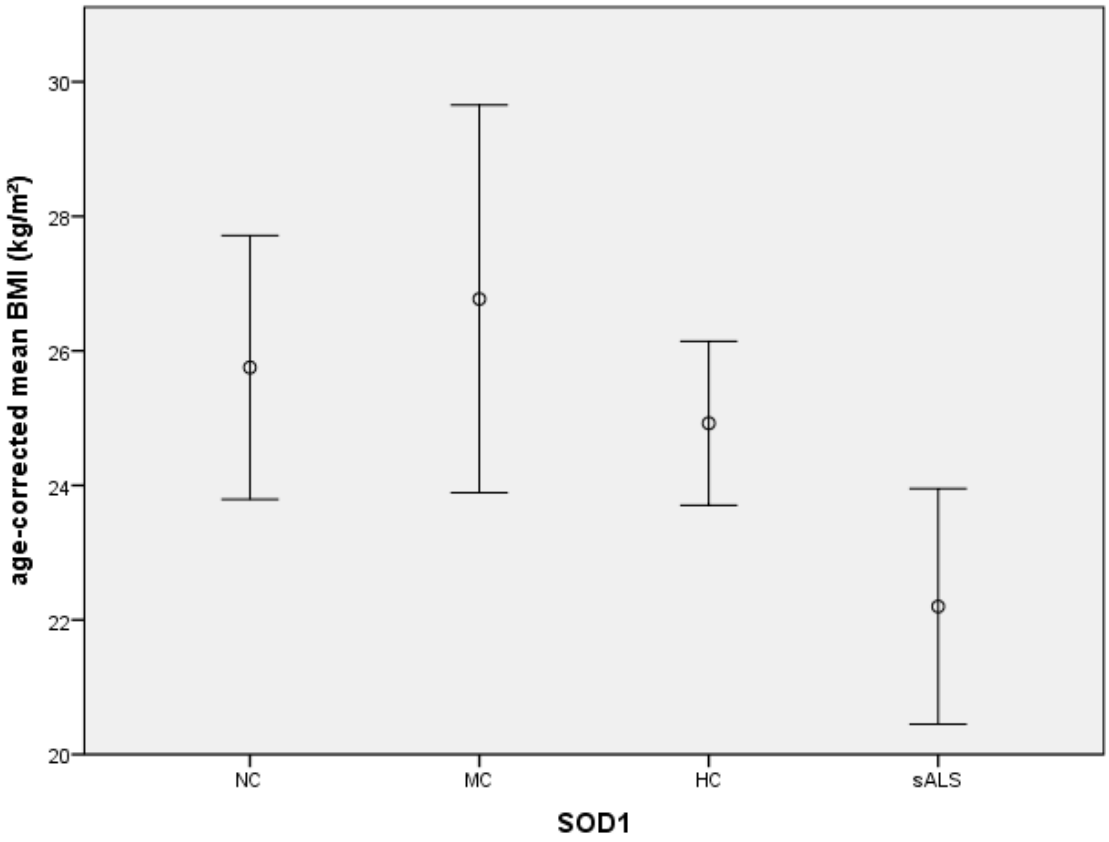


Figure 7: Mean BMI in participants of SOD1 families (n = 9 mutation carriers (MC) and n = 9 (non-mutation carriers (NC)) compared to healthy controls (HC; n = 40), and sporadic ALS patients (sALS; n = 19). The circle is marking the mean and the error bars are marking the 95% confidence intervals. Abbreviations: BMI: body mass index; HC: healthy controls; MC: mutation carriers; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis; SOD1: superoxide dismutase 1.

3.4. 3.4. Calorimetry

3.4.1. Calorimetry – characterization of the study groups

For calorimetric analysis, the same exclusion criteria had been applied as stated above (2.2 Study population and 3.3 Body composition). Of the remaining 73 subjects, fifteen persons had not gone through calorimetric assessment or had been tested with an incomparable old device. Additionally, six measurements did not fulfill steady-state criteria (as stated in 2.4.2 Analysis of Calorimetry) and two persons had to be excluded taking metabolism affecting medication. Eventually, 50 participants of the GPS-ALS study remained for analysis having participated in the calorimetric measurement and fulfilled steady-state criteria (Figure 8). 30 of them are not carrying the respective mutation, 20 carry the respective mutation. 33 (out of 40) control measurements met the steady-state criteria and 13 (out of 19) measurements of patients with sporadic ALS. In Table 3 and Table 4 each group is characterized.

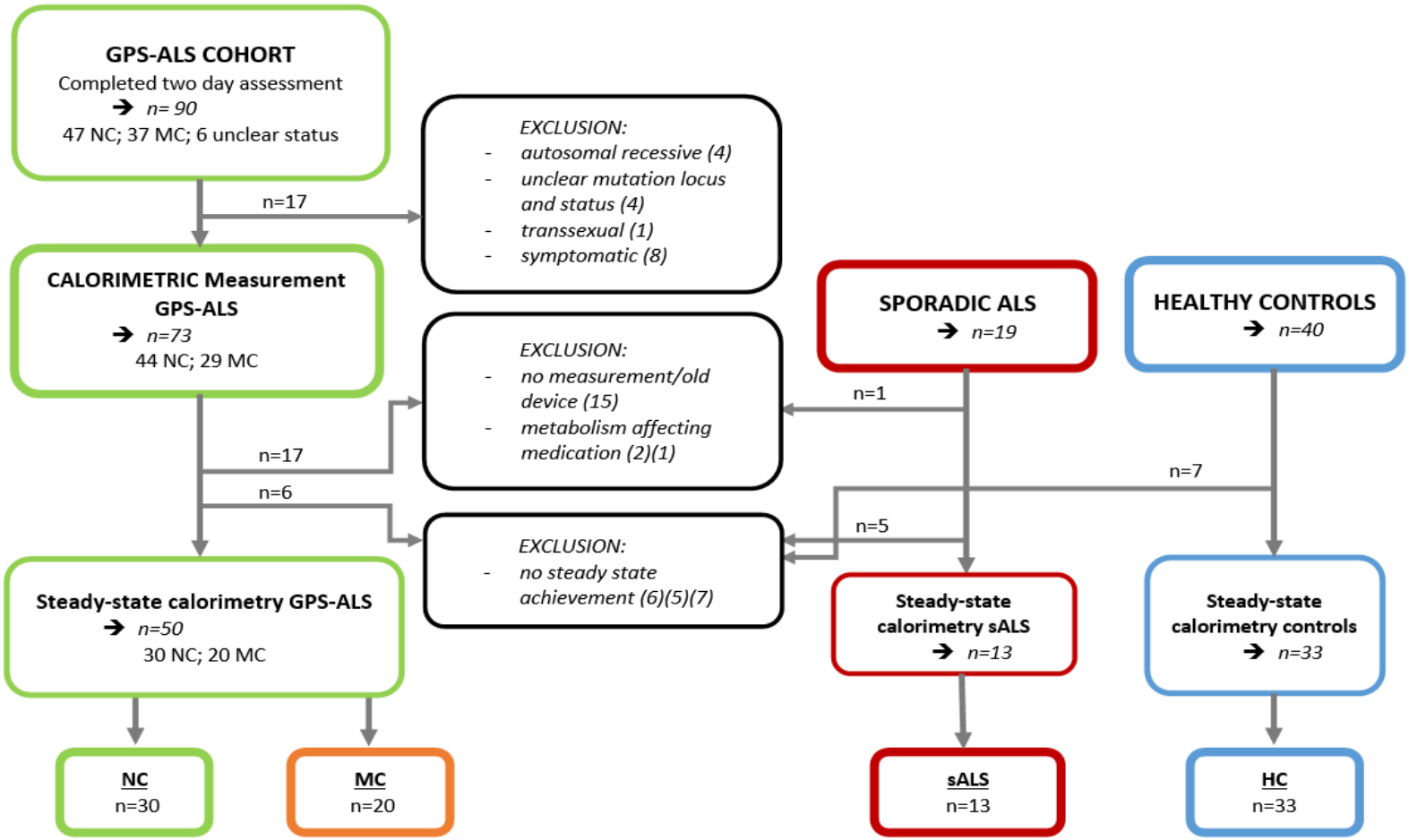


Figure 8: Flowchart of the different study groups concerning calorimetric measurement including GPS-ALS study participants, healthy controls (HC) and sporadic ALS patients (sALS). Abbreviations: ALS: Amyotrophic Lateral Sclerosis; GPS-ALS: German PreSymptomatic amyotrophic lateral sclerosis study; HC: healthy controls; MC: mutation carriers; NC: non-mutation carriers; n: amount of; sALS: sporadic Amyotrophic Lateral Sclerosis.

Table 3: Characteristics of the study groups with a calorimetric measurement fulfilling steady-state criteria: participants of the GPS-ALS study carrying the mutation (MC); those not carrying the mutation (NC), healthy controls (HC) and sporadic ALS (sALS) patients. Abbreviations: HC: healthy controls; MC: mutation carriers; m: mean; n: amount of; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis; SD: standard deviation; mRMR: measured resting metabolic rate; cRMR: calculated resting metabolic rate; WHO: World Health Organization; MR: metabolism ratio.

n = 96	NC n = 30	MC n = 20	HC n = 33	sALS n = 13
Age (years): m ± SD	41.1 ± 10.9	42.6 ± 10.9	34.0 ± 13.6	63.3 ± 9.0
Sex (n): male/female	12/18	9/11	14/19	8/5
BMI (kg/m²): m ± SD	26.8 ± 6.1	24.7 ± 4.5	23.8 ± 3.8	24.0 ± 3.9
WHO BMI classification (n)	Underweight: 1 Normal: 15 Pre-obese: 6 Adipose I: 4 Adipose II: 2 Adipose III: 2	Underweight: 1 Normal: 11 Pre-obese: 5 Adipose I: 2 Adipose II: 1 Adipose III: 0	Underweight: 2 Normal: 22 Pre-obese: 5 Adipose I: 4 Adipose II: 0 Adipose III: 0	Underweight: 2 Normal: 5 Pre-obese: 5 Adipose I: 1 Adipose II: 0 Adipose III: 0
Gene Mutation (n)	C9orf72: 21 SOD1: 4 FUS: 2 TDP43: 3 Senataxin: 0	C9orf72: 11 SOD1: 5 FUS: 3 TDP43: 0 Senataxin: 1	-	-

Table 4: Characteristics of the study groups with a calorimetric measurement fulfilling steady-state criteria: participants of the GPS-ALS study carrying the mutation (MC); those not carrying the mutation (NC), healthy controls (HC), and sporadic ALS (sALS) patients. Abbreviations: HC: healthy controls; MC: mutation carriers; m: mean; n: amount of; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis; SD: standard deviation; mRMR: measured resting metabolic rate; cRMR: calculated resting metabolic rate; WHO: World Health Organization; MR: metabolism ratio.

n = 96	NC n = 30	MC n = 20	HC n = 33	sALS n = 13
mRMR (kcal/day): m ± SD	1844 ± 396	1707 ± 373	1601 ± 244	1495 ± 271
cRMR (kcal/day): m ± SD	1670 ± 325	1627 ± 359	1617 ± 258	1473 ± 246
MR: m ± SD	1.106 ± 0.12	1.05 ± 0.10	0.99 ± 0.08	1.01 ± 0.05
median	1.12	1.05	0.99	1.02

3.4.2. Calorimetry – results

For calorimetric analysis, differences in metabolism ratio (MR) were the center of interest. MR was calculated putting the calorimetrically measured RMR in relation to the calculated RMR according to the respective Harris Benedict formula. Thereby, a comparable value concerning age, gender, and body composition was applicable. Mean values and standard deviation for each group are stated in Table 5.

Comparing the MR between the different groups, differences in means were found ($F(3,92) = 7.252$; $p = 0.000$) (Table 5 and Figure 9). Looking closer, there is a significantly higher MR in mutation carrying participants compared to HC ($t(51) = 2.2$; $p = 0.032$). No statistically significant difference was found comparing MC with sALS patients ($t(31) = 1.232$; $p = 0.227$) or GPS-ALS participants not carrying the mutation ($t(48) = -1.646$; $p=0.106$). Furthermore, a significantly higher MR is found in those GPS-ALS participants not carrying a mutation compared to HC ($t(61) = 4.291$; $p = 0.000$) as well as to sALS patients ($t(41) = 2.662$; $p = 0.011$).

MR in MC being higher than in HC was a sex specific effect: in the male cohort of which there were 9 MC, 12 NC, 14 HC and 8 sALS patients, MR was significantly higher in MC ($m = 1.07 \pm 0.12$) compared to HC ($m = 1.01 \pm 0.062$; $t(21) = 2.363$; $p = 0.028$). In the female cohort though, with 11 MC, 19 NC, 19 HC and 5 sALS patients, MR was not significantly elevated in MC ($m = 1.04 \pm 0.09$) compared to HC ($m = 1.01 \pm 0.09$; $t(28) = 0.868$; $p = 0.393$). In Figure 10 the underlying mutations in male MC are shown with the corresponding MR (C9orf72 n = 2; SOD1 n = 4; FUS n = 2; Senataxin n = 1).

Using solely data of participants with a BMI under 30 kg/m^2 no remarkable changes to the results were observed. MC still have a higher MR compared to HC ($t(44) = 2.372$; $p = 0.022$).

Table 5: Statistical analysis of differences in mean metabolism ratio. Student's t-test were used with a significance level of α = 0.05. Abbreviations: GPS-ALS: German PreSymptomatic amyotrophic lateral sclerosis study. HC: healthy controls; n: amount of; ns: not significant; MC: mutation carrier; NC: non-mutation carriers; sALS: sporadic amyotrophic lateral sclerosis.

n = 96	HC	NC	MC	sALS
HC (n = 33)	X	p = 0.000	p = 0.032	ns
NC GPS-ALS (n = 30)	p = 0.000	X	ns	p = 0.011
MC GPS-ALS (n = 20)	p = 0.032	ns	X	ns
sALS (n = 13)	ns	p = 0.011	ns	X

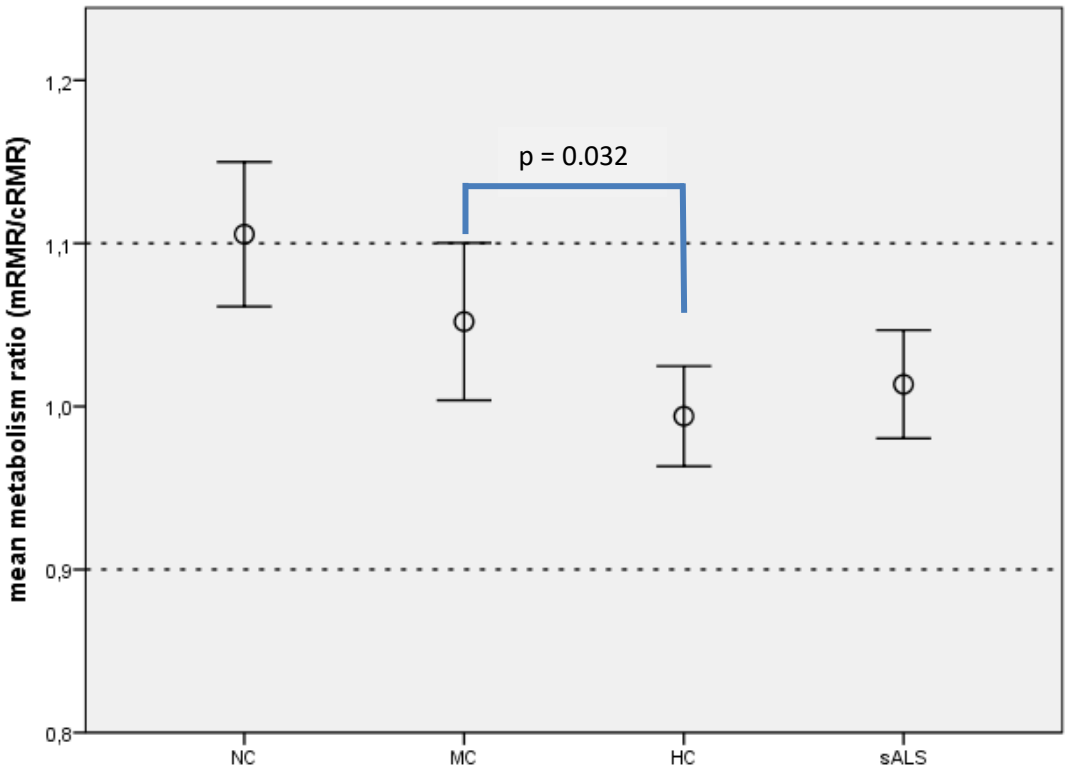


Figure 9: Mean of metabolism ratio for the four different groups: GPS-ALS participants not carrying the respective mutation (NC), mutation carrying GPS-ALS participants (MC), healthy controls (HC), and sporadic ALS patients (sALS). The blue line is marking a significant difference concerning MC with the respective p-value. The circle is marking the mean and the error bars are marking the 95% confidence intervals. The dashed lines are marking the considered range of normal metabolism ratio with 0.9 and 1.1 as thresholds. Abbreviations: cRMR: calculated resting metabolic rate; HC: healthy controls; MC: mutation carriers; mRMR: measured resting metabolic rate; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis.

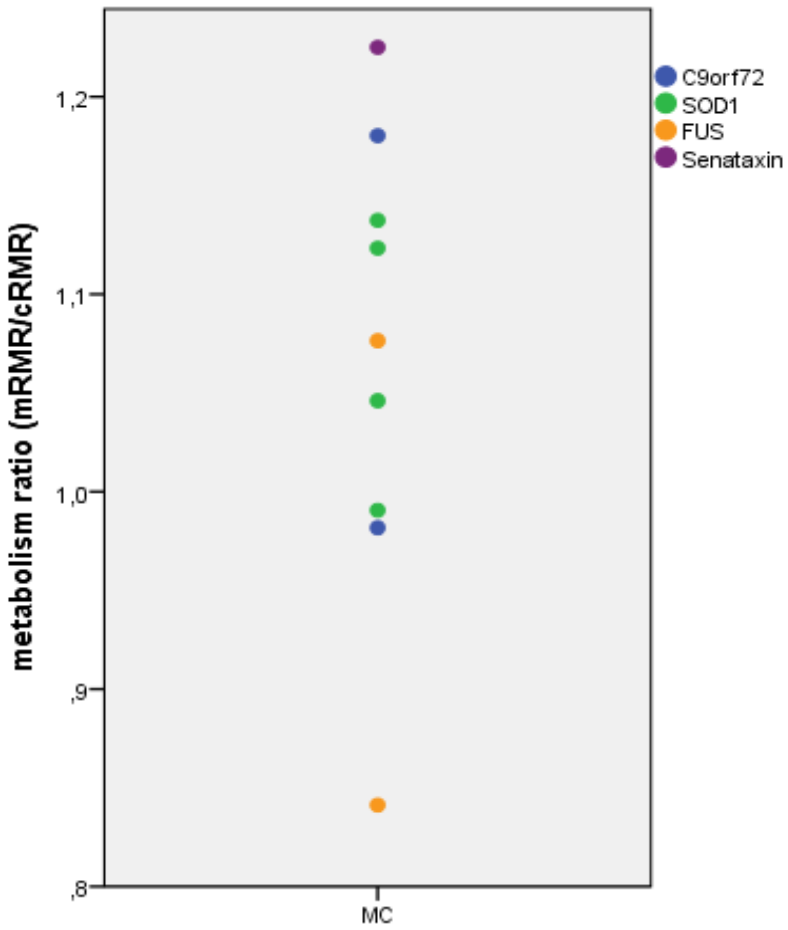


Figure 10: Individual metabolism ratio values of the nine male mutation carriers color-labeled according to the underlying mutation locus. Abbreviations: cRMR: calculated resting metabolic rate; C9orf72: chromosome 9 open reading frame 72; FUS: fused in sarcoma; mRMR: measured resting metabolic rate; SOD1: superoxide dismutase 1.

There was no directional connection between actual age and metabolism ratio in all groups ($r = 0.092$; $p = 0.372$; $n = 96$) (Figure 11).

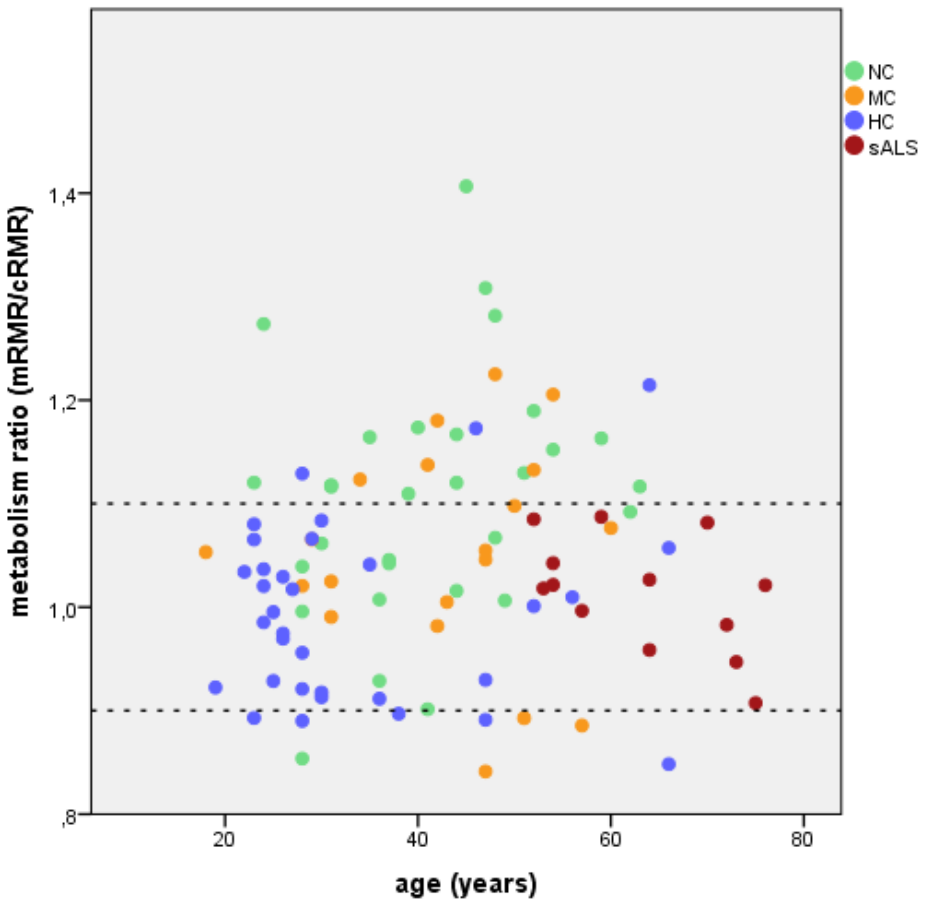


Figure 11: Connection between age and metabolism ratio of all participants (n = 96). Dots are the individual values and the colors apply to the respective group. The dashed lines are marking the considered range of normal metabolism ratio with 0.9 and 1.1 as thresholds. Abbreviations: cRMR: calculated resting metabolic rate; HC: healthy controls; MC; mutation carriers; mRMR: measured resting metabolic rate; NC: non-mutation carriers; sALS: sporadic Amyotrophic Lateral Sclerosis.

3.4.2.1. Mutation-specific analysis

Of participants with families having a C9orf72 history, there were 11 calorimetric measurements from MC and 21 from NC available. No significant difference was to be found between MC and HC ($t(42) = 1.088$; $p = 0.283$) or sALS patients ($t(22) = 0.454$; $p = 0.654$) (Figure 12). Merely the NC GPS-ALS participants are significantly higher in MR compared to all other groups (NC vs. MC: $t(30) = 2.440$; $p = 0.021$; NC vs. HC: $t(52) = 4.718$; $p = 0.000$; NC vs. sALS: $t(32) = 3.261$; $p = 0.003$). Excluding C9orf72 and looking at the other mutations altogether, the means in MR between MC ($m = 1.08 \pm 0.12$) and NC ($m = 1.07 \pm 0.13$) are at the same level. For SOD1 MC an analysis was not sensible due to even smaller group sizes.

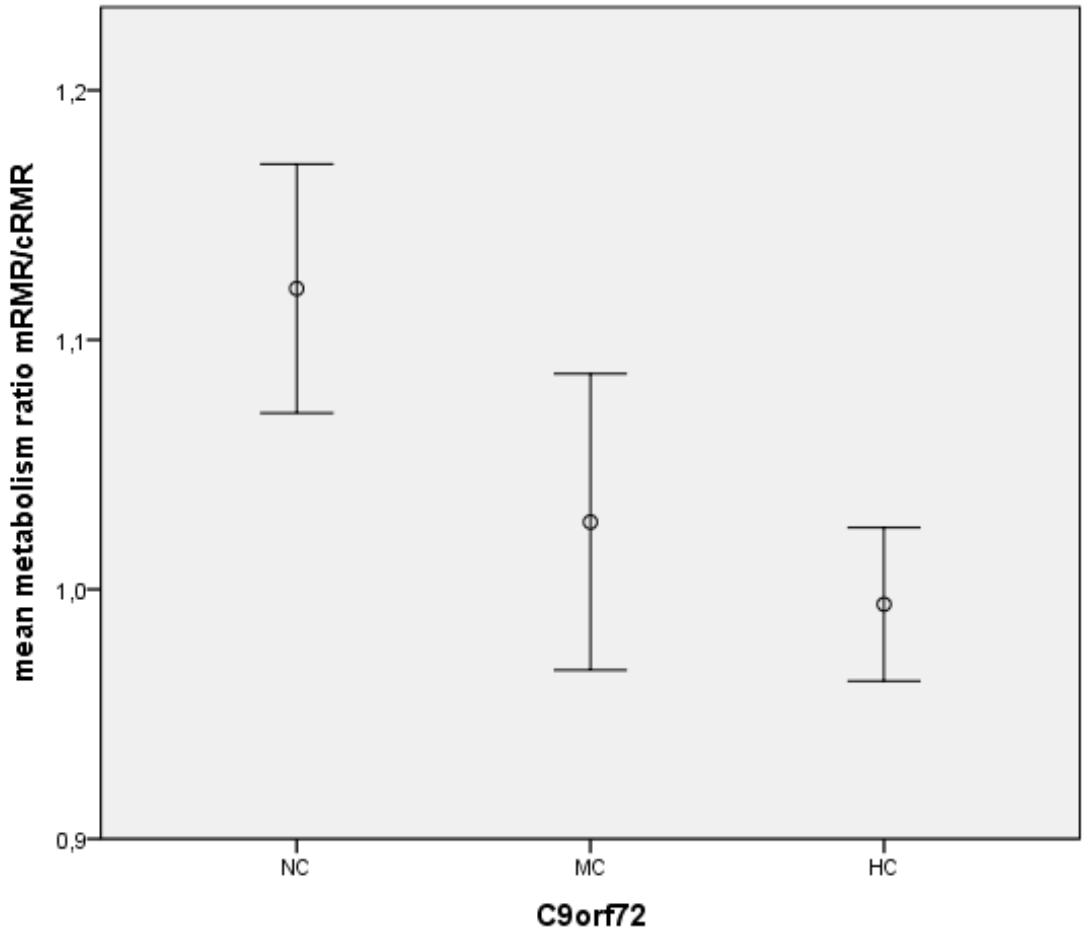
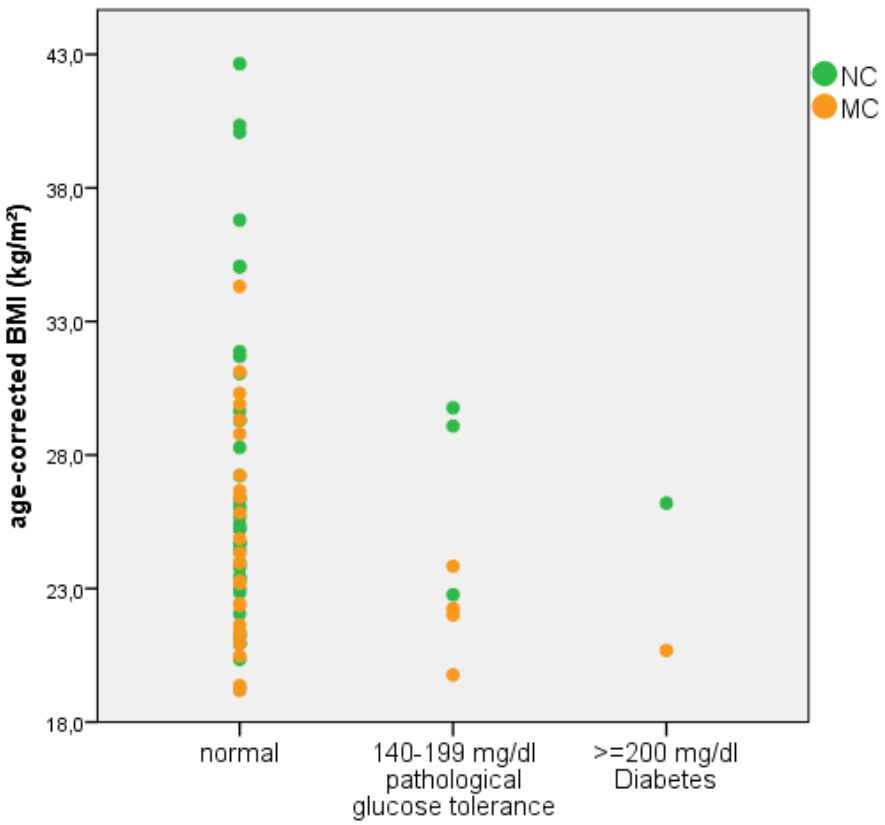


Figure 12: Mean of metabolism ratio in C9orf72 GPS-ALS participants (MC n = 11; NC n = 21) compared to healthy subjects (n = 33). The circle is marking the mean and the error bars are marking the 95% confidence intervals. Abbreviations: cRMR: calculated resting metabolic rate; C9orf72: chromosome 9 open reading frame 72; HC: healthy controls; MC: mutation carriers; mRMR: measured resting metabolic rate; NC: non-mutation carriers.

3.5. Glucose metabolism

73 subjects of the GPS-ALS study went through an OGTT. One test was excluded because of a missing two-hour value. A normal reaction to glucose feeding occurred in 63 subjects, whereas in seven participants a pathological glucose tolerance was observed (NC: n = 3; MC: n = 4). Two subjects had blood sugar levels considered as manifest diabetes (MC: n = 1; NC: n = 1). Looking at the relationship to BMI mainly a graphical analysis is possible due to small group sizes. In this cohort, NC with a pathological OGTT had on average a higher BMI compared to the respective MC (NC: m = 26.9 ± 3.2; MC: m = 21.7 ± 1.6), which is a significant difference according to Mann-Whitney-U testing (p = 0.032). OGTT and calorimetric data were available for analysis in 49 GPS-ALS study participants (NC: n = 29; MC: n = 20) of which six subjects had a pathological glucose tolerance and two diabetes. Mean MR in those subjects with pathological values was 0.97 ± 0.6 in NC and 1.05 ± 0.1 in MC (Mann-Whitney-U-test: p = 0.25).



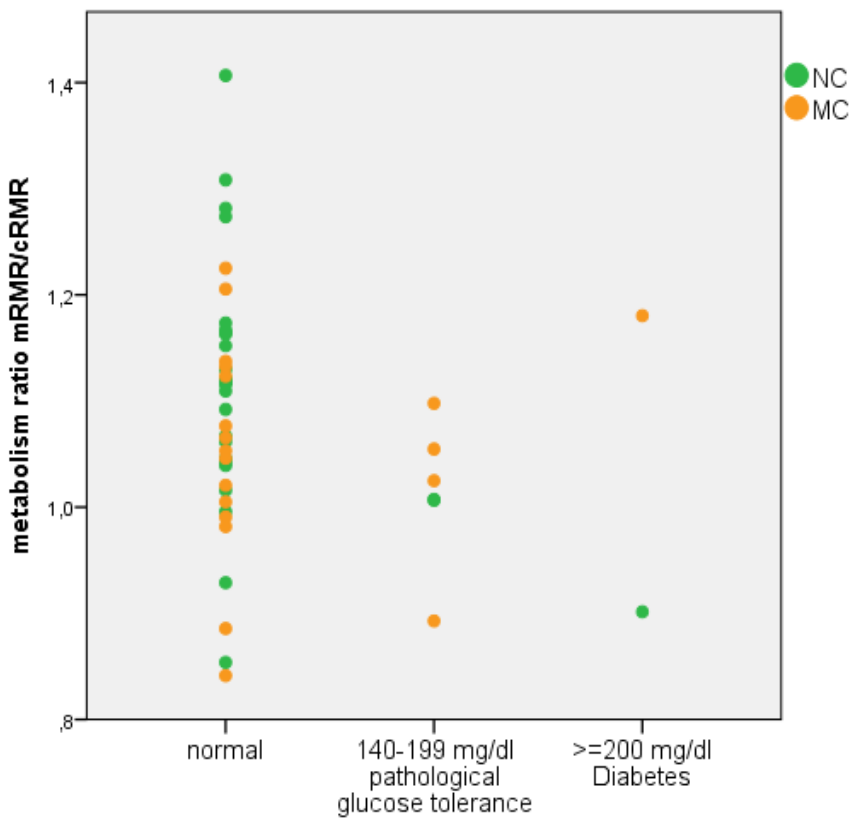


Figure 13: Results of the oral glucose tolerance test in non-mutation carriers (NC; n = 43) and mutation carriers (MC; n = 29) in relation to BMI (kg/m²) and to metabolism ratio (NC: n = 29; MC: n = 20). Abbreviations: BMI: body mass index; cRMR: calculated resting metabolic rate; MC: mutation carriers; mRMR: measured resting metabolic rate; NC: non-mutation carriers.

3.6. Estimated time to disease onset

In average mutation carrying GPS-ALS participants had nine years to estimated disease onset ([-32 years; 29 years]; SD = 14.4; median = -13 years).

3.6.1. Body composition

There was no robust connection to be found between BMI and ETTO ($r = 0.02$; $p = 0.869$; $n = 73$).

3.6.2. Metabolism Ratio

In MC there is a linear connection between MR and ETTO ($r = 0.507$, $p = 0.023$, $n = 20$), which is a strong effect according to Cohen. The closer estimated time of disease onset becomes or the more it is overreached, the higher is the metabolism ratio in MC. 26% of variation in metabolism ratio is explained by the estimated time to onset. Looking more closely at this observation, no sex-specific differences were found. In the NC no relationship was found between MR and theoretical ETTO ($r = -0.033$, $p = 0.862$, $n = 30$).

In positively tested C9orf72 MC ($n = 11$) no connection was found between ETTO and MR ($r = -0.026$; $p = 0.939$; $n = 11$). However, in the five positively tested SOD1 participants a strong linear connection was found ($r = 0.940$; $p = 0.017$; $n = 5$).

4. Discussion

4.1. Interpretation of the results and placement within the concept of ALS

The GPS-ALS study was realized to characterize and follow up on a cohort of participants with a family history of fALS. All participants had a first-degree relative suffering or having suffered from fALS with an underlying autosomal dominantly inherited mutation. Hence, participants had a 50 % chance themselves to carry the mutation and eventually suffer from ALS. They are ideal participants to study the presymptomatic phase of ALS in humans in a longitudinal setting. This analysis concentrated on the baseline data of metabolic parameters, namely BMI and resting metabolic rate.

4.1.1. Body composition

In this cross-sectional analysis, a significantly lower age-adjusted mean BMI was found in mutation carrying GPS-ALS participants compared to those not carrying the respective mutation. Patients with sALS had a significantly lower BMI compared to all the other groups, which strengthens existing data concerning weight loss in ALS patients [108]. MC did not differ from the HC regarding mean BMI, but the NC unexpectedly differed significantly from the HC group. One would assume though, that NC are in the same range as a HC group. However, in this case, the HC was not age- and gender-matched whereas NC and MC were similar in age and gender. Since BMI increases in the respective decades, the age difference could partly explain the higher BMI in NC compared to HC, although we did an age-adjustion to limit this effect [137]. Another possible factor contributing to the higher BMI in NC compared to HC might be the stressful situation with fALS running in the family and possibly affecting oneself, which could trigger increased food intake [162]. In MC this would have no effect on body weight because of an increased metabolism [47, 97], whereas NC would gain weight. Since GPS-ALS subjects were only tested on the mutation running in their family, it is still possible that NC carry another mutation having in mind evidence for an oligogenic basis of ALS [165].

Looking at mutation-specific effects, the difference in mean BMI between C9orf72 MC and NC was even stronger than in the analysis of all subjects. Additionally, BMI was also significantly lower compared to HC but remarkably already on the same level as in sALS patients. In participants with an SOD1 history MC, NC and HC had similar BMI levels. Group numbers are small though, so it has to be waited whether this mutation specific effect concerning BMI is a robust finding.

In a recent study, Peter and colleagues [126] compared self-reported changes in BMI over the past decades and found a consistently higher mean BMI in ALS cases than in the controls 20-70 years before the study took place. This changed about ten years before disease onset in which there was a decrease in mean BMI in future ALS patients; eventually, at the time of disease onset mean BMI was lower in ALS patients than in HC. A similar preclinical decrease of BMI one to two decades before estimated time of disease onset has also been reported in studying MC of autosomal dominantly inherited Alzheimer's disease [116]. In these available data it is currently impossible to tell at which point the MC are to be located in matters of their upcoming disease, not knowing when - and if - symptoms will appear. Retrospectively a similar change in body composition might be observed in MC vs. NC as had been published based on self-reports. For now, with the GPS-ALS study group being in average about ten years prior to the epidemiologically most frequent years of disease onset [100], it can just be assumed that lower mean BMI in MC is already reflecting a presymptomatic weight loss. However, this remains to be proven with further observations. It becomes more certain that typical changes in BMI over time, not the state at a specific point in time, seem to be characteristic not only in ALS, but also in other neurodegenerative disorders [116, 126]. Thus, further research should concentrate on longitudinal observations how BMI actually develops in MC and if there are differences between the distinct mutations. Greater group sizes are essential for robust results especially looking at mutation-specific effects.

4.1.2. Glucose metabolism

Evidence for a disturbed glucose metabolism in ALS patients has been found decades ago, although the nature of this relationship remains unknown (chapter 1.2.3). In these data, 11 % of the GPS-ALS study participants had a pathological OGTT, likewise NC and MC.

Interestingly, BMI in those MC with a pathological OGTT was in a normal range, whereas mean BMI of the respective NC is considered within a pre-obese range. It is known that high BMI is associated with the risk of being diagnosed with diabetes [63]. The MC with pathological results on the OGTT are rather slim though, indicating a potentially different pathology behind the disturbed glucose tolerance.

4.1.3. Energy expenditure

As stated above, some studies have observed a hypermetabolic state in ALS patients [15, 41], especially in fALS [62]. Is this hypermetabolism already present in the asymptomatic phase? Our data revealed a higher mRMR in MC compared to the HC. However, this was not an outstanding result of MC since mean MR of NC was also significantly elevated compared to HC. Remarkably, this effect in MC was significant in men, but not in female participants. Putting a longitudinal aspect into this analysis, the connection between ETTO and MR was analyzed. A trend towards hypermetabolism was seen as estimated time of disease onset was getting closer or overreached in both genders. In SOD1 MC a hypermetabolic state was present a decade before estimated time of disease onset. However, these analyses are limited due to small group sizes and ETTO is merely an assumption, so that these results should be handled carefully.

The fact that an elevated metabolic rate was male-specific is interesting because it is also men that are known to be more frequently affected by ALS than women and have an earlier disease onset [100]. A transcriptional co-activator named PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) has been found to modulate age of disease onset in male ALS patients and in male SOD1 transgenic mice [52]. This is remarkable since PGC-1α is known as a master regulator of metabolism, adjusting cellular function to metabolic demands by modulating the activity of multiple transcriptions [57]. It is expressed in nearly all cell types with a range of tissue-specific isoforms and effects [110]. In SOD1 and FUS/TLS mouse models of ALS it has been shown that PGC-1α expression is differently affected in the brain and the periphery with levels being increased in the periphery, whereas they are decreased in the brain [11]. Peripheral effects of PGC-1α expression include increasing mitochondrial biogenesis and detoxifying reactive oxygen species, being induced by physiological cues such as exercise, cold, and fasting, or by cell-

specific signals [9, 66, 132, 156, 181]. Furthermore, peripheral glucose tolerance, lipid metabolism, and energy expenditure are regulated by PGC-1α [57]. PGC-1α has already been studied in the brain in connection with several neurodegenerative disorders [144]. Neuronal inactivation of PGC-1α results in striatal degenerative lesions and protects these mice against diet-induced obesity [33, 106]. In ALS mice models it has been shown that PGC-1α overexpression increases survival of motoneurons as well as improves motor function [96, 182]. Correspondingly, in SOD1 transgenic mice, PGC-1α activity was blunted leading to mitochondrial dysfunction and increased levels of reactive oxygen species [135]. PGC-1α silencing promoted these developments.

Putting this information together: PGC-1α activity is disturbed in the brain and periphery in ALS. Again, the nature of this relationships remains unknown to date. An interconnection between metabolic alterations in ALS and PGC-1α signaling seems likely. In these data an increased energy expenditure has been shown for mutation carrying men, which matches the findings of a sex-specific effect of PGC-1α. Hence the idea of PGC-1α as a disease modifier and elevated energy expenditure as part of the metabolic phenotype is supported [52].

The hypothalamus is an important regulator of energy homeostasis [78]. Ma and colleagues demonstrated that the PGC-1α activity in specific neurons (expressing a calcium/calmodulin-dependent protein kinase type II (CaMKIIα)) is essential for maintaining energy homeostasis and neuronal health [106]. These neurons are also present in the hypothalamus [172]. Besides transcriptional factors determining the expression level of PGC-1α [156], posttranslational modifications as by the metabolic sensors AMPK (5' adenosine monophosphate-activated protein kinase) or SIRT1 (Sirtuin-1) play a major role in affecting PGC-1α activity [23, 66, 142, 143]. The function of AMPK is to sense energy demands and activate the corresponding short- and long-term mechanisms for maintaining intracellular energy homeostasis. AMPK and PGC-1α not only affect the gene expression of similar genes, but they also affect and need each other mutually in fulfilling their function of regulating mitochondrial and glucose metabolism [77, 161]. AMPK has been identified as a key regulator of balancing hypothalamic functions, namely feeding behavior and energy expenditure in order to maintain energy homeostasis [75]. Summing up, metabolic

sensors such as AMPK and SIRT1 interact with PGC-1α in exchanging information, reviewing the demands, setting goals and conducting the executing hypothalamic cascades to maintain energy homeostasis. Disturbing these well-tuned processes results in metabolic alterations which can be observed not only in ALS but also in other neurodegenerative disorders [116, 131, 148]. Implementing this into the model of Eisen and colleagues [50], ALS mutations increase the susceptibility of suffering from ALS by causing mitochondrial dysfunction, neuroinflammation, excitotoxity and excessive oxidative stress. Throughout lifetime compensatory mechanisms try to conserve these well-tuned networks for the maintenance of neuronal health and cellular energy homeostasis. The overexpression of PGC-1α could be one amongst other possible compensatory mechanisms [96, 182]. Neuronal health and motoneuron survival would be improved [96, 182], as well as several metabolic alterations might occur as side effects such as a facilitation of reaching high fitness levels by the increased PGC-1α activity. This could end up reinforcing itself, raising the question of the "right" amount of exercise and potentially giving an answer to the controversial results which have been found concerning exercise as a risk factor [73, 93, 99]. A progredient "burning out" or difficulties of overexpressing PGC-1α in peripheral and brain cells due to hyperactivity or (epi)genetic processes could explain the diverging observations of metabolic alterations seen in ALS patients. This goes along with the data reported by Peter and colleagues [126] in which body composition had been stable in ALS patients over decades and started to change about ten years before disease onset in ALS. A similar trend is observed in these data with an increasing metabolic rate towards the estimated time of disease onset. Hypothetically this metabolic shift prior to symptom onset is reflecting a decompensation of cells responsible for energy homeostasis of the body of which especially hypothalamic neurons are known to be involved [78, 88]. The hypothalamic atrophy seen in ALS patients and in presymptomatic MC which was reported by Gorges and colleagues [64] could be a morphological counterpart of decompensation and ensuing loss of hypothalamic neurons. Supporting this hypothesis, the hypothalamic volume was positively correlated with BMI and age of onset especially in fALS patients suggesting a failure in maintaining energy homeostasis. Adding the results from Eschbach and colleagues [52] reporting the deficiency of full length PGC-1α being associated with an earlier age of onset and shortened survival in male ALS SOD1 mice, it seems likely that decompensation, and

thereby disease onset, occurs earlier in those with distinctive features in the PGC-1α genome because of an impaired compensatory mechanism.

In summary, alterations in PGC-1α activity could be one compensatory mechanism against damages caused by ALS mutations in the cellular environment over the lifetime. Initially, the overexpression of PGC-1α maintains neuronal health and stable body weight as well as facilitating endurance performances of the individual as a side effect; later when the compensation is breaking down and there is a deficit in PGC-1α expression, metabolic alterations become obvious such as changes in body composition and energy expenditure similar to the observed resistance to diet-induced obesity and the along going increased energy expenditure in PGC-1α deficient mice [97]. Our data imply that the metabolic phenotype might be differently influenced by gender and mutation loci.

It has to be noted though, that both groups from the GPS-ALS study had elevated MR compared to HC and to sALS patients no matter whether they had the respective mutation or not. One possible explanation is that the study participants had more difficulties reaching resting state as they were lying under the canopy and had time to think about why they were taking part in the study, about their relatives' and their own fate with ALS as well as what else to expect from that day. Hence, psychological arousal could have increased MR in both GPS-ALS study groups. Another explanatory point is associated with the way how MR is calculated: the value of mRMR is obtained by indirect calorimetry whereas cRMR is an estimation based on a formula by Harris and Benedict [68]. It takes body weight and height into account as well as age and sex. However, it has been found to be difficult to estimate RMR in obese persons [60] where metabolically active mass is not proportionately increased to body mass. The NC group has a significantly elevated mean BMI compared to the three other groups which could affect cRMR and therefore MR. Yet results did not change in a significant manner excluding all measurements of persons with a BMI > 30 kg/m² or using the Mifflin formula for calculating RMR and subsequently MR, which has been recommended for estimating RMR in obese patients [61, 115].

4.2. Limitations

This report as a cross-sectional analysis of the baseline data of a study mainly relying on its longitudinal character in following-up on persons at risk of suffering from fALS, obviously has a limited informative value compared to a longitudinal perspective. Interindividual differences in MR are caused by several unswayable factors influencing metabolism [178], which makes it so important to observe intraindividual alterations over time. Crucial basic information concerning the study group such as incomplete penetrance [117] will not be revealed for a long time and for now, complete penetrance in autosomal dominantly inherited mutations has to be assumed for analyzing these data.

Several aspects which are known to affect BMI and metabolism ratio were not included in the assessments. Smoking alters BMI and MR [8, 71, 105], is discussed as a gender-specific risk factor of ALS [6, 176] and therefore would have been good to be recorded. Control groups were not matched in age and gender. Hence age-adjusted BMI values were used and for calorimetric analysis MR was calculated, which took age, gender and BMI into account so that values were comparable. Psychological stress during the measurements was already discussed above as a potential disturbing factor of resting state (4.1), and for objectifying and limiting this effect it would have been helpful to measure heart rate during calorimetric testing [159].

Since BMI and RMR are partly influenced by genetics [154], it is important to know that in our study family members were taking part and share more genetic material than just the respective mutation we were looking for. A family bias is most likely with 90 participants coming from just 48 different nuclear families and being even more interrelated with merging pedigrees.

The number of GPS-ALS participants in general and those willing to participate in this time-consuming and psychologically stressful study is limited. Furthermore, approximately one half is not carrying the respective mutation which reduces the group size of MC even more. Study participant acquisition is not closed yet and stacking up group sizes will reveal more robust results.

4.3. Conclusion

We were looking for distinctive features in asymptomatic mutation carriers of fALS in concern of body composition and resting metabolic rate. MC were indeed slimmer as the non-mutation carrying group of similar age and gender, especially in those carrying a mutation of C9orf72. Yet, longitudinal observations will be essential to observe the development over time, since several studies based on self-reports found a characteristic change of body weight within the presymptomatic phase [118, 126]. MC were not found to be in a hypermetabolic state, although MR was elevated compared to the HC. More data and heart-rate monitoring throughout the calorimetric measurements is needed to see why both, MC and NC had elevated MR compared to HC and sALS patients. MC and HC were alike concerning BMI, so the elevated MR in MC compared to HC seems valid. This difference in MR was even greater in men than in women, fitting into existing data concerning sex-specific differences such as a higher incidence and an earlier disease onset of ALS in men. PGC-1α has been reported to be a male-specific disease modifier in ALS [52] and hypothetically could be involved in causing metabolic alterations as part of a compensatory mechanism maintaining neuronal health. A gender- and mutation-specific effect on metabolic alterations is likely as our data show.

Characterizing the presymptomatic phase in MC is an essential and necessary task for the following years to get further insight and understanding of fALS, potentially opening a new therapeutic window. Longitudinal data are needed to show the development over the years, as well as for limiting interindividual effects and matters of incomplete penetrance.

5. Summary

Metabolic alterations such as weight loss, hypermetabolism, and disturbed glucose metabolism are common findings in patients with Amyotrophic Lateral Sclerosis (ALS). In the framework of a longitudinal study with the objective to characterize the presymptomatic phase in familial ALS (fALS) patients-to-be with inherited mutations in their family history, this cross-sectional, explorative analysis exploits metabolic parameters obtained by anthropometric data, indirect calorimetry, and an oral glucose tolerance test (OGTT).

So far 73 participants (44 non-carriers (NC), 29 mutation carriers (MC) had eligible data for body composition analysis and 30 NC/20 MC for calorimetric analysis. As control groups, 40 healthy participants (HC) and 19 sporadic ALS (sALS) patients were additionally examined.

Age-adjusted body mass index (BMI) was significantly lower in MC compared to NC ($t(71) = 2.21$; $p = 0.031$). This effect was even stronger in those participants with a family history of a chromosome 9 open reading frame 72 (C9orf72) mutation, where mean BMI was also lower compared to HC ($t(53) = 2.243$; $p = 0.029$), while no difference in mean BMI was found compared to sALS patients.

Glucose metabolism was tested with an OGTT, revealing pathological results in four NC and five MC. Of note, these MC had a surprisingly low mean BMI of 21 kg/m² for developing pathological glucose tolerance, whereas the respective NC averaged on a mean of 26.9 kg/m².

Indirect calorimetry was performed to measure resting metabolic rate (mRMR) which was put into relation to calculated resting metabolic rate (cRMR) defining the metabolism ratio (MR = mRMR/cRMR). MC ($n = 20$) had a significantly higher MR compared to HC ($n = 33$; ($t(51) = 2.2$; $p = 0.032$). This effect was stable in the male cohort $t(21) = 2.363$; $p = 0.028$, but not in females ($t(28) = 0.868$; $p = 0.393$). However, NC also had elevated MR compared to HC ($t(61) = 4.291$; $p = 0.000$).

In summary, these findings indicate that metabolic alterations occur already in the presymptomatic phase. Longitudinal data have to show the development of these alterations throughout the lifespan of those eventually going to develop (f)ALS. These cross-sectional data imply that sex and the kind of underlying mutation affect the presence and character of metabolic alterations. It is possible that compensatory mechanisms, such as an upregulation of PGC-1α, try to maintain neuronal health with the side effect of metabolic changes. The hypothalamus as a central regulator of metabolic balance in the brain is a good candidate for a venue of ongoing compensatory and pathophysiological mechanisms in ALS.

Overall, these cross-sectional data have to be supported and enhanced by longitudinal observations and larger group sizes in the future to gain more insight into metabolic alterations during the presymptomatic phase of fALS.

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8. Curriculum Vitae

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