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Repetitive Subconcussive Head Impact – Magnetic Resonance Spectroscopy in young athletes

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Summary

While sports around the world and its beneficial effects on body and mind and overall health are commonly acknowledged, many sports have faced an increased focus on player's safety and health over the last few years. Researchers around the world are looking more closely at sports with potential detrimental effects on the body, brain and long-term-trajectories. The United States National Institute of Health (NIH) just recently publicly concluded that repetitive traumatic brain injuries can cause chronic traumatic encephalopathy (CTE) despite the resisting position of many official sport governing bodies of contact sports[1].

Soccer, being one of the most played sports, globally and nationally, has also been more closely looked at. Soccer players are not only at risk of unintentional tackles and collisions but face the challenge of heading the ball as intended part of games and practice. Many soccer players start practicing headers at a young age and continue to perform headers throughout their career. While there is some research hinting at increased risk for soccer players and other athletes of contact sports of developing neurodegeneration of the brain, it is important to understand the changes when they begin to be detectable and possibly reversible. The brain, as a remarkable sensitive, yet robust organ, is the main focus of this thesis. As part of a larger study called RepImpact, its main objective was the study of the effect of headers at a young age (14-16 years old); looking at the brain itself, its structure, pathways and its metabolites, but also at motor and balance skills, cognitive performance as well as blood and saliva markers. In order to detect early effects and minimal changes of repetitive subconcussive head impacts such as headers, Magnetic Resonance Spectroscopy (MRS) as a sequencing part of Magnetic Resonance Imaging (MRI) was used to detect changes in brain metabolites.

This dissertation focused on brain metabolites of young soccer players and controls from Germany and possible changes before, during and after season, as well as in comparison to controls.

Typical developmental changes of the brain – as seen through changes in metabolite levels of total N-acetylaspartate (tNAA) dependent on age and consistent with the limited research available – could be presented. tNAA is shown to be a sensitive marker of age during the age of 14 to 16, indicating ongoing brain development. Further research of the brain metabolite levels during crucial developmental phases is necessary to establish baseline levels of the metabolites during development. This would be an important first step to further investigate possible changes brought on by repetitive subconcussive head impacts (and concussion). Using the data at hand, differences in metabolite levels between soccer players and controls were not detectable.

A larger data set with longer observation period, more sensitive methods to count headers (and possible other collisions and concussions to evaluate “realistic risks”) and more knowledge about baseline metabolite levels at different ages might further help to understand and see the effects of repetitive subconcussive head impact (and biological compensatory and repair mechanisms). This could provide a basis for guiding and protecting children from detrimental outcomes in their future.

Zusammenfassung

Während Sport und seine positive Wirkung auf Körper und Geist sowie auf die allgemeine Gesundheit weltweit allgemein anerkannt sind, sind viele Sportarten in den letzten Jahren mit einem Fokus auf die Sicherheit und Gesundheit der Spieler konfrontiert worden. Forscher auf der ganzen Welt konzentrieren sich auf Sportarten mit potenziell schädlichen Auswirkungen auf Gehirn und Körper. Das United States National Institute of Health (NIH, Behörde des US-amerikanischen Gesundheitsministeriums) schlussfolgerte erst vor kurzem öffentlich, dass repetitive traumatische Gehirnverletzungen zu chronisch traumatischer Enzephalopathie (CTE) führen können, trotz erkennbarer Widerstandshaltung vieler offizieller Verbände von verschiedenster Kontaktsportarten[1].

Fußball, eine der weltweit und national am meisten gespielten Sportarten, wird in diesem Kontext ebenfalls zunehmend genauer betrachtet. Fußballspieler sind nicht nur durch unbeabsichtigte Zweikämpfe und Zusammenstöße gefährdet, sondern stehen auch vor der Herausforderung, den Ball während des Spiels und Trainings viele Male zu köpfen. Viele Fußballspieler fangen schon in jungen Jahren an, Kopfball zu trainieren und praktizieren Kopfbälle während ihrer gesamten Karriere. Da es vermehrt Hinweise gibt, die zeigen, dass Fußballer und Sportler anderer Kontaktsportarten ein erhöhtes Risiko für die Entwicklung von Neurodegeneration des Gehirns haben können, ist es notwendig, Anfänge und Gründe dieser (möglichweise biologisch reversiblen) Veränderungen zu verstehen. Das Gehirn als bemerkenswert empfindliches und dennoch robustes Organ steht im Mittelpunkt dieser Arbeit. Als Teil einer größeren Studie namens RepImpact war das Hauptziel die Untersuchung der Wirkung von Kopfbällen in jungem Alter (14-16 Jahre alt), wobei das Gehirn selbst, seine Struktur, die Verbindungen und seine Metaboliten, aber auch motorische und Gleichgewichts-Fähigkeiten, kognitive Leistungsfähigkeit, Blut- und Speichelmarker untersucht wurden.

Um schon die minimalen und frühen Veränderungen durch die repetitiven subconcussiven Kopfeinwirkungen, in diesem Fall Kopfbälle, möglichst früh im Gehirn zu erkennen, wurde Magnetresonanzespektroskopie (MRS) genutzt. Diese Sequenz der Magnetresonanztomographie (MRT) ermöglicht die Auswertung der Metaboliten und deren Level im Gehirn.

In dieser Arbeit wurden Gehirn Metaboliten von jungen Fußballspielern und Kontrollen aus Deutschland und mögliche Veränderungen vor, während und nach einer Saison – sowie im Vergleich zu Kontrollen – untersucht.

Veränderungen des Gehirns als Teil der typischen Entwicklung konnten durch Veränderungen des gesamt N-Acetyl-Aspartat (tNAA) Levels gezeigt werden, welche sich abhängig vom Alter der Probanden veränderten. Diese Ergebnisse sind in Einklang mit der bisherigen, jedoch stark limitierten Literatur. tNAA konnte als sensitiver Marker von Alter (zwischen 14 und 16 Jahre) gezeigt werden und wird mit weiter ablaufenden Entwicklungen im Gehirn in Verbindung gebracht. Weitere Erforschung der Gehirn-Metabolite in anderen typischen Entwicklungsphasen ist aber notwendig, um für die Vielzahl der Metaboliten und alle Altersklassen die Level der Metaboliten zu kennen. Dies wäre ein wichtiger erster Schritt, um darauf aufbauend Abweichungen der Level durch repetitive subconcussive Kopfeinwirkungen (und Gehirnerschütterungen) zu bestimmen. Signifikante Unterschiede in den Metaboliten-Spiegeln zwischen Fußballspielern und Kontrollpersonen waren nicht nachweisbar, die in der Studie gegebene – begrenzte – Gruppengröße wird dazu diskutiert.

Ein größerer Datensatz, mit längerem Beobachtungszeitraum, empfindlicheren Methoden zum Zählen von Kopfbällen (und möglichen anderen Kollisionen sowie Gehirnerschütterungen, zur Darstellung der „realistischen Risiken“) und mehr Kenntnisse über die Referenzwerte der Meabolitenlevels in verschiedenen Altersklassen könnte dazu beitragen, die Auswirkungen von repetitive subconcussive Kopfeinwirkungen auf die Gehirne von Kindern – einschließlich biologischer Kompensations- und Reparaturmechanismen – zu verstehen und eine Grundlage für Empfehlungen zum Schutz von Kindern vor schädlichen Kurz- und Langzeitfolgen zu erarbeiten.

List of Abbreviations

ACG	Anterior cingulate gyrus
ALS	Amyotrophic Lateral Sclerosis
BOLD	Blood-Oxygen-Level-Dependent
CHO	Choline
CR	Creatine
CRLB	Cramer Rao Lower Bound
CTE	Chronic Traumatic Encephalopathy
DAI	Diffuse Axonal Injury
DLPFC	Left Dorsolateral Prefrontal Cortex
DTI	Diffusion Tensor Imaging
DWI	Diffusion Weighted Imaging
FA	Fractional Anisotropy
FLAIR	Fluid-Attenuated Inversion Recovery
FIFA	Fédération Internationale de Football Association
fMRI	Functional Magnetic Resonance Imaging
Glx	Glutamate/Glutamine
LAC	Lactate
LMEM	Linear Mixed Effects Model
MD	Main Diffusivity
mI	Myo-Inositol
MRA	Magnetic Resonance Angiography
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy
MS	Multiple Sclerosis
M1	Dominant Primary Motor Cortex
NAA	N-acetyl aspartate
NAAG	N-acetylaspartylglutamate
PCG	Posterior Cingulate Gyrus
PWM	Parietal white matter
rs-fMRI	Resting State – Functional Magnetic Resonance Imaging
RSHI	Repetitive Subconcussive Head Impact
SD	Standard Deviation
SWI	Susceptibility Weighted Imaging
TBI	Traumatic Brain Injury
tNAA	Total N-acetyl aspartate
TP 1	Timepoint 1
TP 2	Timepoint 2
TP 3	Timepoint 3
TE	Echo Time
TR	Repetition Time

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1 Introduction

Soccer is one of the most popular games worldwide, with 265 million players being actively involved in the sport around the world, according to FIFA's (Fédération Internationale de Football Association) Big Count survey from 2006. Over 54% of registered male players are under the age of 18 according to FIFA[2].

According to the German Soccer Association (DFB; Deutscher Fußball Bund), over 215.000 male soccer players aged 15 to 18 and over 735.000 boys aged 14 and below are currently actively playing soccer in Germany in 2020/2021, illustrating the unaltered fascination of the sport for children and adolescents[3]. Most children start playing at a young age and remain avid soccer players throughout their teenage years, accumulating a tremendous number of practice hours, soccer games and consequently, headers.

There is no doubt that sports for children and adolescents should be actively promoted, with an array of positive effects, not only physically but also mentally, including better academic performances[4,5]. But the more popular a sport, especially among children and adolescents, the more one needs to continuously evaluate its (long-term) safety for body and mind. Playing soccer combines several risks: the "classical" orthopedic injuries, next to others, and its injuries concerning the head and the brain. Players may either hit the head on the ground during physical contact, have head-to-head contact, or experience an intended (or unintended) head-to-ball contact, called heading.

This thesis will focus on assessing the effect of headers and its biological consequences on the developing brain.

Adults can, in moderation, self-sufficiently decide whether to head the ball and weigh the risks of injury. Children and adolescents are dependent on rules and guidelines, which should protect – first of all – the developing brain.

In a study published by Sandmo and colleagues, they studied an international youth soccer tournament and recorded 2.7 headers per player hour with heading rates increasing with age, reaching elite senior level for players 16 years and older[6]. This only illustrates a small portion of the number of headers that might be performed additionally during practice sessions and designated header practices. While headers mostly lead to no visible type of injury or discomfort, repeatedly heading the ball and its impact on the brain without leading to symptoms (as opposed to the clinical diagnosis of a concussion, which is based on acute symptoms) has been described as repetitive head impact or repetitive subconcussive head impact (RSHI)[7]. A study by Koerte et al in 2012 showed changes of the brain's white matter in soccer players without a history of concussion[7]. Additional cognitive, functional and metabolic changes could be found in athletes without a history of concussion and display the effects of RSHI[8-12]. With accumulation over many years of playing soccer, the impact and effect of those repetitive subconcussive head impacts due to heading the ball should be explored. With the high number of children playing soccer all over the world, the early starting age of many children and the accumulation of headers over a career of one soccer player, it is of increasing relevance and importance to investigate potential effects those headers and repetitive subconcussive head impacts might have for a child's brain.

1.1 Repetitive subconcussive head impact (RSHI)

1.1.1. Definition

While concussion nowadays is a defined and much looked-at phenomenon in many sports, repetitive subconcussive head impact (RSHI) has been described as repetitive head impacts below the level of a clinical concussion[13]. Repetitive heading of a soccer ball has been considered a source of RSHI[14].

1.1.2 Etiology

Subconcussive repetitive head impact has been part of studies concerning variable sports, including Lacrosse and American football and will be the focal point of this thesis[15,16]. While those studies have looked at sports with subconcussive repetitive head impacts being a result of direct physical contact while wearing a helmet, heading a ball as part of playing soccer has been found to provide numerous subconcussive head impacts, with every header being another "repetitive" event[17].

Imaging studies have shown that exposure to RSHI leads to multiple neurophysiological changes within the brain such as increased connectivity within the brain as a compensatory mechanism of the brain, microhemorrhaging and changes within the white matter[18-21]. While data concerning changes of metabolites after RSHI is limited (as reviewed below), some research has been done concerning changes in metabolites in children after concussion and traumatic brain injury (TBI). Children (aged 11.2 +/- 5.4) with TBI were found to have reduced levels of N-acetyl aspartate to creatine ratio (NAA/Cr) and increased levels of choline to creatine ratio (Cho/Cr) compare to controls within 1–16 days after injury, indicating diffuse axonal injury[22]. Adolescents with persistent cognitive symptoms have been shown to also have reduced levels of NAA/Cre and NAA to choline ratio (NAA/Cho) 3-12 months post-SRC (sports-related concussion), reaching physiological levels after this period[23].

To look at the effect those neurophysiological and metabolic changes might have, some research has been published showing short- and long-term effects.

MacAllister et al. looked at cognitive performance of college athletes, comparing contact sports and non-contact sports: contact sport athletes (without concussion) had poorer performance scores on new learning tests and furthermore, higher scores on several head impact exposure metrics were associated with poorer performance on cognitive tests[8].

Looking at long term effects, a recent UK study has shown a higher prevalence of

neurodegenerative diseases among former soccer players when compared to the general population, with position and career length being discussed as relevant factors for an increased risk of developing neurodegenerative diseases[24].

1.1.3 Epidemiology

Research has begun to shift the attention from the already affected adults to the children that are currently playing sports with headers, head impact or even head-on collisions. The interest is not only in being able to detect those changes early on but generating tools and safety measures to ensure that children's brains are being protected from permanent and irreparable damage. The question concerning RSHI and its effects for young athletes' brains is the main basis of my thesis. As the cohort examined for this study is based on competitive soccer players aged 14 to 16, it is relevant to know that most soccer teams are starting to train specifically for headers around this age, making it a pivotal age as far as this study is being concerned. It has been reported that professional soccer players perform 6-12 headers per game, totaling thousands of headers over one's career[25]. Furthermore, soccer practice was not included in this above-mentioned study and would add even more headers. Looking at the cohort of my thesis, the participants reported between 228 and 2958 headers over a single season, with most headers occurring during practice. In a recent british study, the average number of headers during a professional career was 52.000 (self-report)[26]. This illustrates the high number of headers that might be performed over a player's career and the importance of investigating its potential effect.

With current research focusing on the short- and long-term effects of heading a ball numerous times during practice sessions and games, England, Scotland and Northern Ireland became one of the first countries (next to the United States of America) to effectively ban training headers in practice for young athletes under the age of 12. The most recent guidelines by the English Football Association are further regulating the number of headers for youth soccer

players and amateur levels. Soccer players under the age of 11 should not practice any headers, with a graduate increase in headers after this age (U13 only 1 session per week and maximum of 5 headers) and amateurs are advised to only have one header session per week with a maximum of 10 headers[27].

In 2022, the German Soccer Association published a set of "training and instruction rules" but avoided the anglo-american "ban of heading" in younger age groups[28].

Those developments highlight the seriousness of possible long-term effects of RSHI and the importance of further research in order to protect athletes. In order to further understand the timeline of those changes to the brain and its effects, it is crucial to examine the most vulnerable time period of a developing brain and the effects of RSHI within that window.

1.1.4 Lack of knowledge:

While short-term effects (such as reduced cognitive performance) and long-term effects such as higher prevalence of neurodegenerative diseases among former soccer players have been discussed in the literature, it is important to further examine the underlying metabolic and structural changes that lead to those observable outcomes. Research concerning the very basis of those observable changes has been limited. Understanding the possible metabolic changes as an immediate detector of change within the brain that might be the basis for short- and long-term cognitive impairments is crucial in order to adequately protect children while not hampering their athletic career.

1.2 RSHI and brain development

This leads to the second focal point of this thesis, as my cohort is based on young soccer players aged 14 to 16. This is an under-investigated age group, as many papers concerning RSHI, sports and the brain have examined either adult ex-professionals or, as many papers are

being based on cohorts in the USA, college-aged young adults (age 17-24). Additionally, it has been shown that in former NFL players, “age of first exposure” is associated with thalamic atrophy (the younger the player was when he started to play, the smaller the right thalamic volume), indicating a younger starting age as a more vulnerable time period for RSHI[29]. Stamm et al. showed that a younger “age of first exposure” is associated with changes within the white matter and long-term cognitive difficutlties[30,31]. The age of the study participants examined, as presented above, is unique and scientifically of interest due to the occurring brain development during those years. While it is known that major parts of the structural and functional motor system development are completed by 12 years of age, the adolescent brain is nonetheless under constant “higher order” development driven hormonally by what is called puberty. Those changes are especially forceful at the prefrontal cortex, the structural basis of a wide range of high-level executive and cognitive performances, a sense of self, decision making, planning, self-control and self-awareness as well as social interactions. With the ongoing changes within the prefrontal cortex including synaptic pruning and increased myelination, it is not surprising that environmental factors might play a role in the development during this sensitive period. Repetitive subconcussive head impacts during those critical years of development concerning predominantly the prefrontal cortex should be looked at carefully, as potentially hazardous effects might affect the individual “later in life” [32].

1.3 Neuroimaging of RSHI

While diagnosing a clinical concussion can already be difficult, showing the effects of exposure to RSHI has proven to be even more complex. Advanced Imaging has been used to detect potential effects of exposure to RSHI over time,

A frequently used method to assess tissue changes within the brain is Diffusion Tensor Imaging (DTI). While several studies have presented changes within the white matter after

RSHI (such as decreased fractional anisotropy (FA), with lower FA being associated with increased RSHI exposure and increases in main diffusivity (MD; increased axial and radial diffusivity). Making predictions concerning the clinical outcome and future possible effects has been difficult when using DTI[14,18-20,33].

Functional MRI (fMRI) and resting state fMRI (rs-fMRI) are another tool used to (indirectly) detect neuronal changes brought on by exposure to RSHI. While DTI is using the characteristics of water, fMRI and rs-fMRI are using the blood-oxygen-level-dependent (BOLD) changes within the brain, based on the neuronal activity (and therefore increased blood flow) to the region of interest within the brain. One possible part of fMRI is the rs-fMRI, which examines the brain activity during rest (no active task is being performed)[34].

Looking at the effect of RSHI, literature suggests an increased connectivity correlating to a stronger BOLD signal, which has been discussed to be due to compensatory mechanisms of the brain[21,35,36].

Susceptibility weighted imaging (SWI), is used to depict neurovascular integrity and possible microhemorrhages. A single study looked at collegiate football players and repetitive subconcussive head impacts over the course of a single season and found significant regional decreases when comparing pre to post-season in SWI signals, indicating damaged blood vessels and blood being outside the vessels as part of microhemorrhaging[37].

DTI, fMRI and SWI have been very useful in demonstrating, in vivo and non-invasively, structural changes within the brain associated with exposure to RSHI.

1.4 Magnetic Resonance Spectroscopy

MRI offers many settings, with the sequence of interest in this dissertation being the Magnetic Resonance Spectroscopy. While the above-mentioned sequences provide tools to visualize structural and functional changes within the brain, brain metabolism as an opportunity to show immediate responses to change is not detectable with those methods. Brain metabolism

is much more sensitive and flexible, and deviations can be swift and even reversible. Changes in metabolite levels can be a first predictor of permanent damage and might be the first warning sign before the level of structural and functional change is being reached.
In the context of most scientific research concerning RSHI and brain injury, MRS has been used as a predictor of individual outcome and for allowing long-term prognosis[38-41].

MRS allows for the quantification of metabolites of the brain, either looking at the entire brain or a small voxel/region of interest. Those qualities permit MRS to be most sensitive to even the smallest changes in the brain, as metabolite levels are an imminent marker of changes in the brain, even before structural changes can be observed using structural MRI Sequences. MRS is the only available technique to non-invasively depict the energy metabolism of the brain and to reveal biochemical disturbances.
MRS has proven to be very sensitive to metabolic changes although the brain's metabolite levels in general have been shown to be remarkably constant[42]. Proton MRS displays brain metabolites while making use of the characteristics of ubiquitous hydrogen groups and their behavior when under magnetic exposure. Each metabolite can be distinguished using the behavior of the hydrogen groups and represents one or multiple peaks on the magnetic resonance spectrum (Figure 1). By calculating the height of the peak(s), a quantitative analysis can be created and by comparing the amount of metabolite present to controls or known average values, a statement can be made concerning the state of the brain at a certain point in time and place [43].
The most commonly quantified brain metabolites are N-acetyl aspartate (NAA), choline (Cho), and creatine (Cr), whereas NAA is believed to be a sufficient marker for neuronal integrity, Cho representing a marker for membrane turnover, whereas Creatine represents cellular energy. It is common to also look at metabolites such as glutamine, which is released after brain injury as well as myo-Inositol (mI), an indicator of astroglial proliferation.

Focusing on the brain, several metabolites as listed below have shown to be of particular interest.

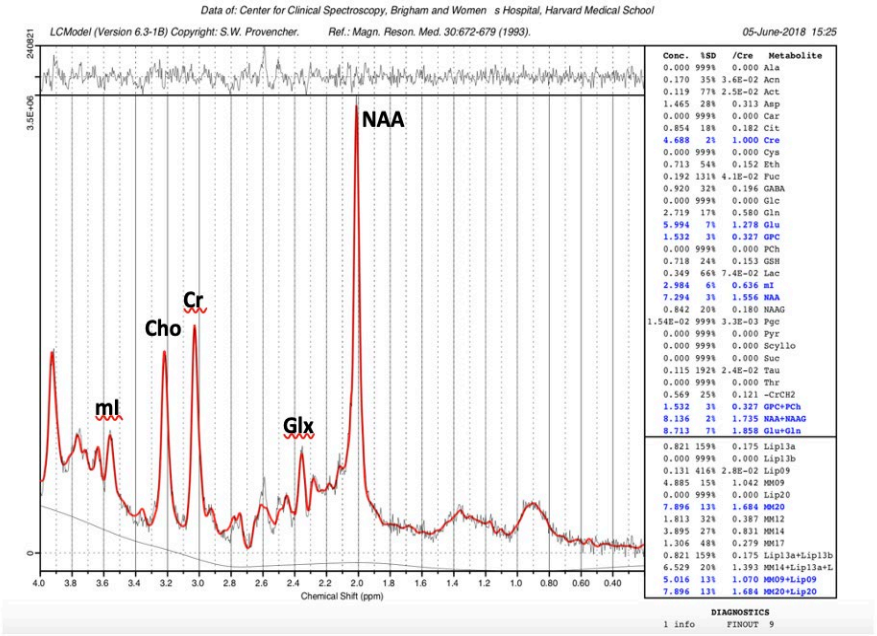


Figure 1: MR spectroscopy result (example)

1.4.1 Water

Using in vivo proton spectroscopy of the brain, water resonance has to be suppressed in order to be able to detect all other metabolite signals, many of which are 10 000 times less concentrated in the brain than water [44].

1.4.2 NAA

The highest and most prominent peak in an MR Spectrum, looking at a healthy adult brain (at 2.01 degree of chemical shift as expressed in part-per-million (ppm)) is due to N-acetylaspertate (NAA). Like most of the distinguishable metabolites in MRS, the exact role of NAA has yet to be conclusively evaluated and determined. It is known that NAA is most abundant in neurons and their projections, therefore resulting in almost equal concentrations in the gray matter as well as the white matter[45]. Its role in the brain however remains open to discussion, with theories of NAA being a signaling molecule of neurons, facilitating communication with oligodendrocytes and astrocytes and presenting an important part of

bidirectional cell-to-cell communication that allows for construction and up-keep of healthy, normal brain development[46]. Several metabolic pathways and functions of NAA are being discussed, with NAA acting as an osmolyte by moving water out of neurons as well as countering anion deficits[47]. NAA is also being looked at for being a part of the process of producing N-acetylaspartylglutamate (NAAG) (a neurotransmitter of the nervous system), supplying acetate for myelination in oligodendrocytes and facilitating the removal of nitrogen from the brain[48]. While the visible peak at 2.01ppm is commonly referred to being due to NAA, this is often considered a simplification. NAA is closely related to and difficult to distinguish (via MRS) from NAAG, a metabolite and neurotransmitter mentioned above. NAA, as used in many papers, is therefore more precisely the combination of NAA + NAAG, which can be noted as tNAA (totalNAA), as NAAG can take up to 20% of the measured peak depending on the imaging parameters[48].

Both NAA and NAAG are used as reservoirs for aspartate and glutamate, with NAAG being synthesized from NAA and glutamate, leading to a release of NAAG to astrocytes, where it is cleaved back into NAA and glutamate. Glutamate can be taken up by astrocytes, with the glutamate-glutamine cycle offering a way of bringing glutamate back to the neurons. Oligodendrocytes hydrolyze NAA back to aspartate and acetate[44].

The intensity and peak height of NAA signal has been used as a general marker of neuronal health. Several studies looking at diseases that are linked to neuronal or axonal cell death (such as brain tumors, infarctions and Multiple Sclerosis (MS)) have confirmed a decrease in NAA, visible by using in vivo MR spectroscopy[49]. Additional studies with a cohort of MS patients have shown a correlation between the NAA value and the physical and mental impairment of those patients, with high NAA values being indicative of less neuronal loss and impairments[50]. It has been shown, however, that reduced levels of NAA can be reversible, indicating NAA to be a marker of not only permanent cell death but also temporary neuronal cell dysfunction. Its increase has been found in cases such as MS, AIDS, mitochondrial

disease, ALS and temporal lobe epilepsy in response to therapy[49,51]. It should be kept in mind that there are a few exceptions where NAA cannot be used as a liable neuronal marker. In patients with Canavan disease (a genetic disorder characterized by a malfunction of NAA metabolism), NAA reaches exceptionally high levels without being indicative of increased neuronal communication while a decrease of NAA can be found in patients with Alexander disease[52,53]. With that in mind, NAA can be seen as a valuable indicator of neuronal health, with decreased levels indicating temporary or permanent cell dysfunction.

1.4.3 Glutamate/Glutamine

Glutamate is one of the most abundant metabolites in the brain and functions as a major neurotransmitter of the brain. Glutamate and Glutamine are often combined and noted as Glx[49]. Glutamate and Glutamine are part of a metabolic cycle between neurons and astrocytes. As an excitatory neurotransmitter, Glutamate is released from neurons and reabsorbed by astrocytes. Within the cell, Glutamate is being converted back to glutamine with via glutamine synthetase. The astrocytes then release glutamine and it is taken up by neurons, which can convert glutamine back to glutamate and can store it as a neurotransmitter [45]. Those steps are energetically costly and may be responsible for 80 - 90% of the glucose consumption of the brain[54]. The Glx peak appears at about 2.1 to 2.4ppm[45]. Due to the nature of being a neurotransmitter, Glx values are significantly higher in the gray matter of the brain compared to white matter, as both glutamate and glutamine should be found in close proximity to the synapses and astrocytes[55].

1.4.4 Choline

The choline peak can be found at 3.21 ppm and consists of signals from glycerophosphocholine (GPC), phosphocholine (PC) and a small amount of choline[56]. A lot of times, this signal is marked as tCho, indicating total Choline consisting of above-mentioned substrates as a distinction is not possible with standard MRS methods used for in vivo

experiments. Both PC and GPC are metabolites of lipid pathways and constitute to the building of myelin sheets as well as other lipid layers and membranes. They are important building blocks of membrane lipids such as phosphatidylcholine and sphingomyelin but both substrates are not visible using in vivo MRS, as PC and GPC are bound in the membrane and cannot move freely[57]. Correlations can be made between the membrane lipids and the substrates of the metabolic pathway (PC and GPC) and a higher signal value of tCho in the whiter matter compared to gray matter can be partially explained. Additionally, the highest values can be obtained when looking at the cerebellum, followed by the thalamus, white matter and cortical gray matter[45]. Increased signals of tCho can be found when looking at processes of membrane degradation but also with increased production of lipid membranes of the brain [58]. Ischemia, as looked at in several studies, also has implications on the tCho level. Despite an initial increase of choline levels, a decrease could be detected 5 months post-ischemic events[59]. Increased tCho levels are generally thought to be an indicator of pathological events, and are often described as a marker of diffuse axonal injury[22]. With an additional decrease in tNAA levels, the ratio tNAA: tCho might be a valid indicator of pathological brain processes[45].

1.4.5 Myo-Inositol

Myo-inositol (mI) is a pentose sugar and an important metabolite and osmolyte of the brain, which can be found primarily in astrocytes [60]. Several studies have shown that extracellular hypertonicity have led to an increased uptake of mI into the cell via a specific sodium myo-inositol transporter (SMIT)[61,62]. This pathway might potentially be a reason for increased brain volume and edema of the brain after traumatic brain injuries and the resulting diffuse axonal injuries (DAI)[61]. Increased levels of mI in the brain have been found in children after TBI, possibly in relation to the process of reactive astrocytosis shortly after injury, which describes glial cell proliferation as an protective mechanism of the brain to create a “barrier” around the possibly irreversibly damaged brain area[63]. Higher levels of mI could be correlated

to worse outcome in children with TBI and DAI[64]. Physiologically increased levels of mI have been documented in children and infants[61], while pathophysiological increases have been showcased in patients with Down Syndrome[65], Alzheimer, hypoxia, hyperosmolarity, bipolar disease and renal failure[61]. Decreased levels are known to be present in patients with stroke, hepatic encephalopathy, tumor and infection[60].

1.4.6 Creatine

Creatine is often mentioned as tCre, being composed of two substances, creatine (Cre or Cr) and phosphocreatine (PCr), with a peak at 3.03 ppm. It is often used as an internal concentration reference for other metabolites, as it has been shown to be at relative constant levels within the healthy brain[45]. While the levels stay relatively stable between 4.5 -5.6 mmol/L in adult aging brains, tCre variability could be shown in infant rats, with levels increasing significantly from day 7 to day 28 postnatal[66]. Creatine is involved in the energy metabolism via the creatine kinase and producing ATP, the storage unit of energy within the brain. While neurons contain much less creatine than glial cells (in vitro), higher levels of tCre have been found in cortical gray matter compared to white matter with the highest levels of Cr in the cerebellum[67,68].

tCre should not be used as a reference metabolite in hypoxia and trauma, as those instances have shown to reduce the levels of tCre within the brain[45]. Furthermore, tumors have also been shown to lead to decreased or even absent tCre signals[69].

1.4.7 Lactate

In a healthy human brain, lactate levels, referred to as lac, are below the threshold of detectability and are only increased in pathological situations. Lack of oxygen, such as an ischemic event or hypoxia, halt the metabolism of glucose and lead to an increase in lactate levels, visible at 1.3ppm[49,70]. Lactate can also be visible in high-grade tumors and abscesses of the brain[70].

1.4.8 Lipids

Lipids, just as lactate, are not usually detectable in a healthy human (adult) brain. Elevated levels have been described in tumors and pediatric brains prior to complete myelination[70]. Furthermore, various hereditary leukodystrophies have been shown to lead to elevated levels of lipids[48]. Its broad peak is between 0.9 ppm and 1.3 ppm.

1.5 Metabolite changes during development

The focus of this thesis, as mentioned earlier, are young soccer players (and controls) aged 14 to 16. While the general description of the metabolites above and its implications are the same for adults and children, a few papers have looked at the differences in MRS spectra of children and adults and during development, which should be mentioned here.

While it has been shown that adults have stable levels of most metabolites, drastic changes in metabolite levels have been found to happen within the first months of life[42].

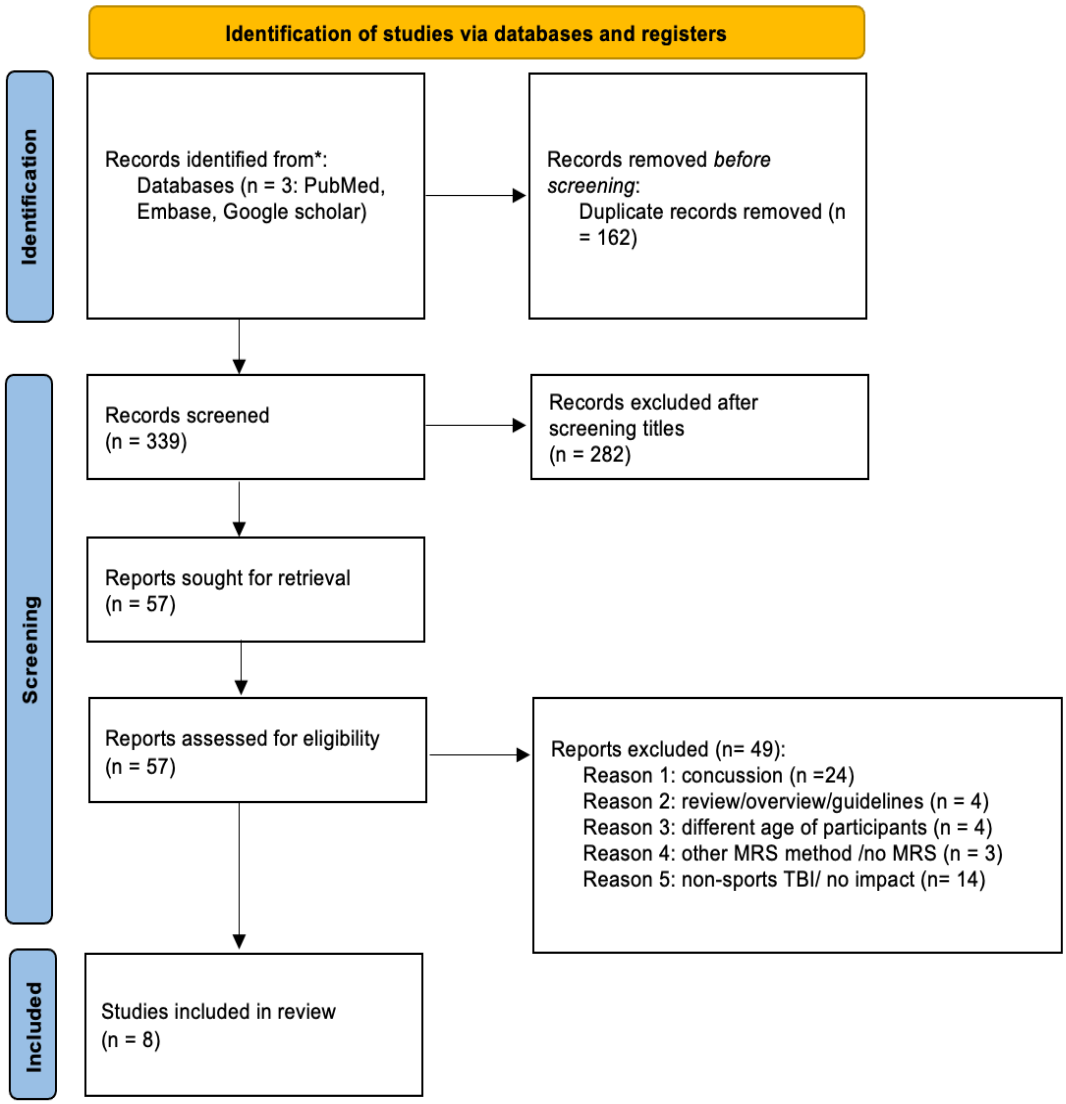
NAA levels, unlike most other metabolites, have been shown to increase within the white matter over the course of childhood up to the age of 18, with drastic increase within the first 3 months, a plateau phase after and another significant increase between the age of 5 and 12. Thereafter, levels of tNAA are steadily continuing to increase up to the age of 20, especially within the frontal and parietal white matter[71-73]. This is probably due to neuronal maturation during childhood as well as an increasing numbers of axons as part of brain development[46,71]. Most other metabolites, including Cre, Cho and mI are reaching relatively constant levels within the first year of age, with mI initially declining and Cho increasing[52,71,73-75]. Young adults (age 18 – 20) have fairly constant metabolite levels, including tNAA levels[76].

2. Systematic review of existing literature concerning RSHI, MRS and young athletes

To furthermore evaluate the current scientific consensus on the effects of repetitive subconcussive head impact for children, a systematic review was performed. The search was performed on PubMed, Embase and Google Scholar using the following search terms:

- ((MRS) OR (magnetic resonance spectroscopy) OR (MR spectroscopy)) AND (brain) AND (sport) NOT (review);
- ((MRS) OR (magnetic resonance spectroscopy) OR (MR spectroscopy)) AND (brain) AND ((youth) OR (children) OR (adolescent) OR (pediatric)) AND ((sport) OR (athlete)) NOT (review);
- ((MRS) OR (MR spectroscopy) OR (magnetic resonance spectroscopy)) AND ((repetitive subconcussive head impact) OR (head impact) OR (repetitive head impact)) NOT (review)
- ((MRS) OR (MR spectroscopy) OR (magnetic resonance spectroscopy)) AND ((concussion) OR (head impact) OR (repetitive head impact) OR (RHI) OR (mTBI) OR (TBI) OR (mild traumatic brain injury) OR (mild TBI)) NOT (review) NOT (adult) NOT (animal).

Articles were initially screened by title and abstract, and exclusion criteria were defined as following: studies concerning the investigation of sports-related concussion, non-sports TBI, cohort consisting of adults (single exception: Koerte paper), no MRS or different MRS method. After thorough investigation of all articles, 8 papers were included in this systematic review.



From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

Figure 2. Prisma Flow Chart of systematic literature research

Title	Authors, Year	Cohort	Voxel Position	Study type	Time Points	Screenend for concussion	Findings
Dependence on subconcussive impacts of brain metabolism in collision sport athletes: an MR spectroscopic study	Bari et al. 2019	40 male football athletes, 23 female soccer athletes, 27 athletes participating in non-collision sports (13 female, 14 male); age 15-18 years	M1, DLPFC	Longitudinal	5 scans: Preseason and during season	Prior Concussions noted but not excluded	- Decrease in Glx (Glutamate and Glutamine) in DLFPC from pre- to post-season in male football players - Increase in tCho/tCr in DLFPC from pre- to post-season in male football players - Increase in Glx in M1 from pre- to post-season in female soccer players - Head impact measured by xPatch, sensor placed behind the ear;
Neuro-Metabolite Changes in a Single Season of University Ice Hockey Using Magnetic Resonance Spectroscopy	Panchal et al. 2018	33 collegiate ice hockey players (16 female, 17 male); age 20-25 years	Corpus callosum	Longitudinal	Pre- and post-season	Concussed athletes included	- Significant decrease in NAA from pre- to post-season in both male and female players - males an exhibited a trend towards increased Glu
Structural, Functional, and Metabolic Brain Markers Differentiate Collision versus Contact and Non-Contact Athletes	Churchill et al. 2017	65 athletes overall; 20 non-contact sport athletes (10 female, 10 male), 22 contact sport athletes (14 female, 8 male); 23 collision sports athletes (9 female, 14 male), age 20- 23 years	Left and right hand motor knobs	Crosssectional	Single scan before the respective season Start	Concussion included if at least 6 months prior to the study	- Lower NAA/Cre levels in collision sport athletes compared to non-contact sports athletes.
Increased Myo-Inositol in Primary Motor Cortex of Contact Sports Athletes without a History of Concussion	Lefebvre et al. 2018	72 athletes overall; 24 non-athletes (12 female, 12 male; 24 contact sport athletes (12 female, 12 male; 24 non-contact sport athletes (12 female, 12 male); age 20-27 years	M1 left, left prefrontal Cortex	Crosssectional	Off-season	Excluded when any history of concussion	- Higher mI concentration in M1 in contact sport athletes compared to non-contact sport athletes, and non-athletes - Reduced Glx and GABA in PFC in contact sport athletes compared to non-contact sport athletes
MR spectroscopic evidence of brain injury in the non-diagnosed collision sport athlete	Poole et al. 2014	34 athletes (High school football) in total; plus 10 non-collision sport controls (age 15-18 years)	dlPFC, M1	Longitudinal	Multiple scans: Off-season and in season	No concussion during the study, controls without any history of concussion	- Decreased tCr in athletes in the dlPFC over season at 1, 2, and 3 months - Higher mI at baseline in dlPFC and then a significant decrease over season at 1, 2, and 3 months in athletes - Significantly higher Glx in M1 in athletes at baseline compared to controls - Initially higher tCr in M1 in athletes and then a significant decrease over season at 2 and 3 months - Decreased tCho in M1 at 1 month in athletes
Sub-concussive hit characteristics predict deviant brain metabolism in football athletes	Poole et al. 2015	25 male athletes (High school football), age 15-17 years	dlPFC, M1	Longitudinal	Multiple scans: Pre-season, and at least once during the season	No concussion during the study	- Negative correlation between changes in tCr and mI in the dlPFC as well as GLx (in M1) and the number subconcussive hits experienced in the preceding week. - Positive correlation between changes in tCr (in M1) and number of hits experienced in the previous week
Altered Neurochemistry in Former Professional Soccer Players without a History of Concussion	Koerte et al. 2015	11 male former professional soccer players (age 45-58 years), 14 age- and gender-matched, former non-contact sport athletes (age 39-53)	Posterior cingulate gyrus	Crosssectional	Single scan	Excluded when any history of concussion	- Increase in Cho and mI in former soccer players compared to controls - Correlation between mI and GSH and lifetime estimate of subconcussive head impacts within the group of former soccer players
Brain Metabolite Levels in Sedentary Women and Non-contact Athletes Differ From Contact Athletes	Schranz et al. 2020	54 female varsity rugby players, 23 female non-contact sport athletes, 23 sedentary women; age 18-30 years	Prefrontal WM	Longitudinal	Beginning of season and off-season	Concussion included if at least 6 months prior to the study	- Lower mI and higher Gln in rugby players compared to non-contact athletes - Lower Glu, higher Gln, lower Cr, and lower mI in rugby players compared to sedentary controls - Lower mI in non-contact sport athletes compared to sedentary controls

Table 1: Summary of articles included with cohort characteristics, study design, voxel placement, concussion screening and findings. DLPFC = dorsolateral prefrontal cortex; M1= dominant primary motor cortex

2.1 Summary of systematic review

Eight papers were included in the final discussion. Pachal et al and Churchill et al, both looking at male and female athletes in college, found decreased NAA over the span of a season, with Churchill including controls (non-contact sport athletes). Both studies did however include previously (Pachal) and newly concussed (Churchill) athletes.

Studies from Bari et al, Poole et al 2015 and Lefebreve et al demonstrated significant decrease in Glx, Poole in correlation with the number of subconcussive hits experienced the week prior and Bari comparing pre to post season, while Lefebreve compared single time point contact sport athletes and non-contact sport athletes before the start of a new season. While Bari looked at high school aged athletes and separated between male and female athletes, Pooles' cohort was comprised of male high school footballer. The study of Bari et al contained previously concussed athletes while Poole et al specified only the controls as no history of concussion and no further information was provided for the athletes. Lefebreve investigated male and female college athletes without any history of concussion.

Schranz et al looked at young adult female rugby players at two time points (in-season and off-season) and found, contrasting the finding of Bari, Poole and Lefebreve, higher Gln in rugby players compared to non-contact athletes. Previously concussed athletes were included if it occurred six months prior (or more) to the study.

Poole et al 2014/2015 and Schranz et al showed a decrease in mI in both cohorts over the span of a single season and Poole also described a negative correlation between the number of subconcussive hits and mI levels.

Lefebreve, as well as Poole et al, showed an initially higher level of mI during off season/baseline in both cohorts, while Poole additionally described a following decrease during season.

Poole et al showed an initially higher tCre levels in contact sports athletes at baseline with subsequent significant decrease in DLPFC, while tCre levels in M1 were positively correlated to the numbers of hits received.

Koerte et al, looking at former professional soccer players and chronic outcomes reported an increase in Cho and mI compared to former non-contact sports athletes. Exclusion criterium for both groups were former concussions.

While comparing Voxel placement, differences between the studies could be found. Bari and Poole both placed their voxel in DLPFC and M1, while Lefebvre looked at M1 left and left prefrontal cortex. Schranz placed a single Voxel in the prefrontal white matter and Panchal in the corpus callosum. Churchill looked at the left- and right-hand motor knobs and Koerte at the posterior cingulate gyrus (PCG).

While differences in percentage of white matter, gray matter, and cerebral fluid within one voxel were calculated and factored in during post-processing in all papers, different placements might make exact comparison between the studies more difficult, as different regions of the brain might be differently affected by RSHI and have different levels of metabolites.

Another important factor is the inclusion or exclusion of concussed or previously concussed athletes. As shown in several studies even single concussions have been shown to result in metabolic changes up to a year after the event[77-79]. Only Lefebvre and Koerte did not include participants with a history of concussion, while Poole did not specify it for contact-sports athletes, Bari and Churchill did include concussed athletes, and Schranz did only exclude athletes with a concussion in the last six months. Panchal did not specify if the participants were screened for prior concussions.

Age also plays a role in the level of metabolites as described earlier in this paper. While the age of participants in the studies by Bari and Poole are comparable to our age range, other

studies looked at older students and adults, making a comparison once again more difficult. It should be noted that some studies also mixed male and female participants, although it has been shown that male and female MRS spectra vary during development, subconcussive hits and after concussions[41,80].

This investigation of published reports about MRS changes after subconcussive hits shows the limited number of studies that have been published so far. Additionally, when looking at the age range 14-16, and clear separation by sex as well as without any history of concussion, no papers could be found that match all the criteria. While MRS and its expressiveness to detect potentially harmful (long lasting or irreversible) changes after subconcussive hits or concussion is only in its early stages, well designed studies are needed to further grow the understanding of the effects of subconcussive hits on young brains. In order to most reliantly investigate the effect of RSHI on young brains, reducing possible other influences as much as possible, this thesis was looking at male (to avoid any possible sex differences) 14- to 16-year-old soccer players without any history of concussion.

3 Hypothesis

Looking at the current available research, many questions concerning young athletes and the effects of RSHI have yet to be investigated. Additionally, most studies have focused on

concussion and its implications for the brain, and not on repetitive subconcussive head impacts. In order to protect children from potentially harmful long-term effects, a sensitive and non-invasive method such as MRS might be helpful. This thesis is looking at young male soccer players (aged 14-16) and athlete controls over the course of a season (including pre-season and off-season) and focusing on the MRS data that was collected. Looking at three time points (pre-season, in season, off-season), MRS data of the soccer group was compared to controls as well as within the groups.

I hypothesize that

1. Soccer athletes display metabolic changes within the brain during the season due to RSHI (in this case headers). We expect
    - decreased levels of NAA
    - increased levels of choline
    - lower Glu
    - an initially higher tCre level in contact sports athletes with positive correlation to RSHI.
    - decreasing levels of mI in the soccer population, especially in season, as a result of RSHI,

All seen in comparison to the control group.

2. Correlation between the exposure (based on numbers of years played and number of headers over single season) and levels of metabolites with above mentioned tendencies

Lastly, I hypothesize that

3. The control group, containing age-matched athletes (non-contact sports) is not showing significant changes of their MR spectra except age-related changes to tNAA (increasing tNAA levels with increasing age)

4 Methods

4.1 RepImpact

The data for the doctoral thesis presented here stems from a project managed by the REPIMPACT consortium. The scientific research project was launched under the leadership of Prof. Dr. med. Inga Koerte in 2017 and includes scientists, statisticians and physicians from Germany, Belgium, Israel, Norway, Slovakia, and The Netherlands. Its main objective is to investigate the implications of RSHI, looking at elite youth soccer players (plus matched athlete controls from sports without RSHI) using a number of tools to detect brain alterations and possible clinical consequences: MRI (structural MRI (T1 and T2), fMRI, FLAIR, DTI and MRS), saliva, blood, balance testing as well as neurocognitive and neuropsychological tests were used to comprehensively capture any biological, structural and cognitive changes that might occur due to RSHI.

Funding occurred through the framework of ERA-NET Neuron. The separate national funding agencies are the German Ministry for Education and Research (Germany), Slovak Academy of Sciences and Ministry of Education of Slovak Republic, the Research Foundation Flanders and Flemish Government, the Dutch Research Council, the Norwegian Research Council and the Ministry of Health, Israel. All institutional review boards approved the study and prior to participation, written informed consent was provided by all participants and their legal guardians.

4.2 Participants

In Germany, 50 athletes, including 35 soccer players and 15 controls were included in the study. Recruitment was conducted via several Bavarian sports club and interested parties were initially screened via phone call. Soccer players had to match following inclusion criteria: age 14-16 (mean= 14.80, SD= 0,85), male, righthandedness, proficient in the German language, competitive soccer practice for at least three times a week. Control athletes were matched in age (14 to 16 years old, mean= 14.98, SD= 0.59), handedness, language proficiency, and competitive practice in a non-contact sport for at least three times a week and no history of contact sports within the last 12 months.

A history of physician-diagnosed concussion, contradictions to MRI, recent brain surgery, neuroleptic or psychiatric medication, substance abuse or a neurological or developmental physician-diagnosed pathology or disability as well as premature birth (prior to week 37 gestational age) were criteria of exclusion for both soccer players and controls. All participants were invited for three timepoints within 15 months: Timepoint 1 (TP1) before the beginning of the season (pre-season), TP2 within/towards the end of the season and TP3 two months after TP2 (post-season)[81]. At all three time points, MRS spectra were acquired from two different voxel locations: parietal white matter (PWM) and anterior cingulate gyrus (ACG) (Figure 4).

During data acquisition, at TP 1, different MRI sequences could not be acquired due to technical difficulties (missing data for n=2). At timepoint 2, n=2 were missing MRI sequences, while n=10 participants did not participate in the data acquisition that day.

At Timepoint 3, no data could be collected for n=13 due to not participating on that day.

Quality control of MRS data was taken by analyzing the Cramer Rao lower bound (CRLB) (more detailed description in the Methods section) and by manually checking voxel placement in order to estimate the level of measurement error and to estimate the standard deviations.

CRLB with estimated percentage standard deviations (%SD) of less than 20 % was used as recommended by previous studies using the LCModel[82-84] (*Table 2*).

	Glu%SD	GSH%SD	mI%SD	tNAA%SD	tCho%SD (GPC+PCH)	tCre%SD
ACG	≤ 12	≤ 20	≤ 8	≤ 5	≤ 5	≤ 5
PWM	≤ 12	≤ 20	≤ 8	≤ 5	≤ 5	≤ 5

Table 2: Cramer Rao Lower Bound values of the metabolites analyzed. Glu%SD stands for metabolite Glutamate and percentage standard deviation, in the case of Glutamate less or equal than 12% standard deviation in ACG and PWM.

During quality control at TP 1, n=3 were excluded due to artefacts brought on by tooth braces and consequently higher Cramer Rao lower bound values with standard deviations of more than 20%. n=1 was excluded due to motion artefacts of the MRS sequence and consequential CRLB higher than 20%.

At timepoint 2, n=4 were excluded due to artefacts by braces and increased CRLB, n=1 was excluded due to motion artefacts.

At timepoint 3, n=5 were excluded due to artefacts brought on by braces and consequential higher CRLB with standard deviation of more than 20%.

After data acquisition and quality control (QC) for MRS and data, TP1 consisted of 25 soccer players (mean age = 14,80 years; standard deviation (SD)= 0,85) and 7 (mean age = 14.98, SD= 0.59) controls, TP2 consisted of 17 soccer players (mean age = 15,6, SD= 0,68) and 4 controls (mean age 15,52; SD = 0,81) and TP3 consisted of 15 soccer players (mean age= 15,91; SD = 0,77) and 5 (mean age= 15,88; SD= 0,90) controls (Figure 3).

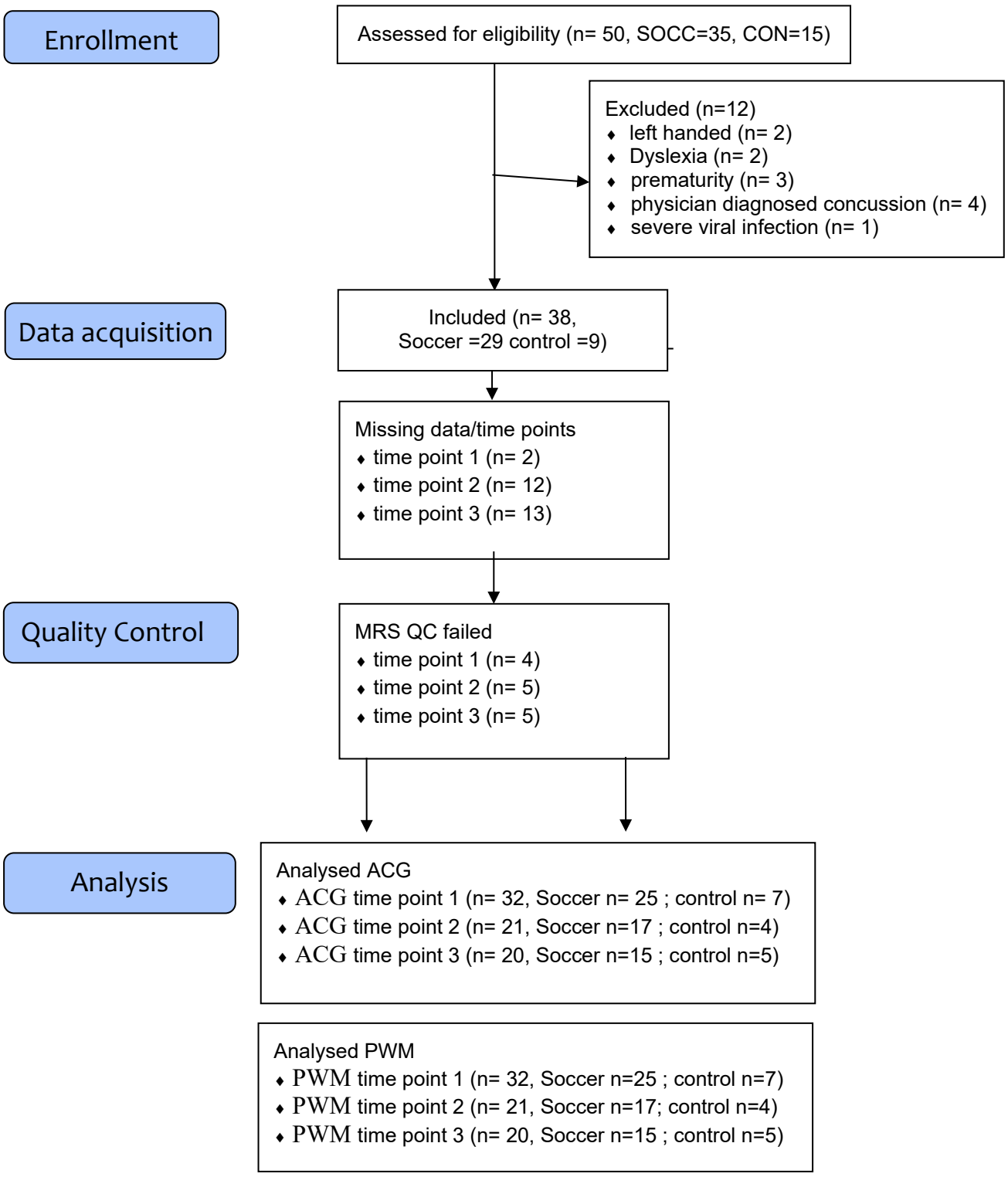


Fig. 3: Flow chart of data acquisition, quality control and number of participants

Soccer Cohort								
age in years	**soccer position**	**school**	**grade/class**	**BMI**	**EHI handedness**	**TP1**	**TP 2**	**TP3**
15,92	wingback	Gymnasium	9	21	right	yes	yes	yes
14,92	midfield defensive	Gymnasium	8	18	right	yes	yes	yes
14,08	wingback	Hauptschule	8	21	right	yes	yes	no
15,33	wingback	Gymnasium	9	19	right	yes	yes	no
14,17	wingback	Gymnasium	8	20	right	yes	no	no
14,42	midfield offensive/wingback	Gymnasium	9	20	right	yes	yes	yes
14,33	midfield offensive/wingback	Realschule	9	19	right	yes	yes	yes
14,17	wingback	Middle school	8	18,75	right	yes	yes	yes
13,00	striker	Realschule	7	19	right	yes	no	no
14,08	wingback	Gymnasium	8	20 *(measured at TP2)*	right	yes	yes	yes
14,92	midfield offensive/wingback	Gymnasium	9	21	right	yes	yes	yes
15,00	center back	Gymnasium	9	21	right	yes	no	yes
15,50	goalkeeper	Gymnasium	9	20	right	yes	no	yes
16,42	midfield defensive	*not recorded*	10	*not recorded*	right	no	yes	yes
15,25	center back	Gymnasium	9	20	right	yes	yes	yes
16,17	midfield offensive/wingback	Gymnasium	9	20	right	yes	yes	yes
14,00	midfield offensive/wingback	*not recorded*	7	18	right	yes	yes	no
13,67	midfield offensive/wingback	Realschule	8	16	right	yes	no	yes
14,58	striker	Realschule	9	19	right	yes	yes	yes
14,58	midfield defensive	Gymnasium	9	19	right	Yes	yes	no
13,83	wingback	Gymnasium	9	16	right	yes	yes	yes
14,42	midfield defensive	Gymnasium	9	20	right	yes	yes	no
15,92	midfield offensive/wingback	Realschule	10	21	right	yes	no	no
16,00	striker	Gymnasium	11	21	right	Yes	no	no
15,83	midfield offensive/wingback	Gymnasium	10	19	right	yes	no	no
15,83	goalkeeper	Gymnasium	11	20	right	yes	no	no

Table 3: Soccer Cohort with position, school and grade as well as BMI (body mass index) and EHI handedness (Edinburgh Handedness Inventory), defines handedness of subject based on daily activities. TP1/TP2/TP3 refer to the Timepoints 1,2, and 3 and indicates if data for this time point exists.

Control Cohort								
age in years	Sport	school	Grade/class	BMI	EHI handedness	TP1	TP2	TP3
15,75	rowing	Gymnasium	9	20	right	yes	no	yes
15,08	track&field	Gymnasium	9	22	*not recorded*	yes	yes	yes
14,17	swimming	Gymnasium	9	20	right	yes	yes	yes
14,67	track&field	Gymnasium	10	*not recorded*	right	no	yes	yes
15,58	rowing	Gymnasium	9	19	right	yes	yes	yes
15,08	*not recorded*	*not recorded*	9	*not recorded*	right	yes	no	no
14,33	tabletennis	Realschule	8	*not recorded*	right	yes	no	no
14,83	tabletennis	Gymnasium	8	19	right	yes	no	no

Table 4: Control Cohort with the respective sport, the school and grade as well as BMI and handedness. TP1/2/3 refers to the timepoints of data collection and scans and indicates if data for this time point exists.

The initial visit (TP1) to the MRI site at the Ludwig-Maximilians-University (LMU) Munich was conducted prior to soccer season and included an MRI, blood and saliva samples, balance testing, neurocognitive testing, a neurological exam, an intelligence test and a self-report. Those tests (excluding the intelligence test) were repeated within the season and post-season.

4.3 Magnetic Resonance Imaging

The MRI was done using a Phillips Scanner (3 Tesla, Version 5.3) Two regions of interest were determined: parietal white matter and anterior cingulate gyrus (gray matter) (Figure 4). Voxel of 2x2x2 cm^3 were placed manually in each region of interest. PRESS MRI sequence was used with echo time (TE) = 30 ms, repetition time (TR) = 2 s and bandwidth = 2 kHz. Prior to acquisition, automated shimming was performed over the voxel volume followed by manual first-order shimming to ensure that line widths are less than 14 Hz. This ensured clearly separated individual resonances of the metabolites.

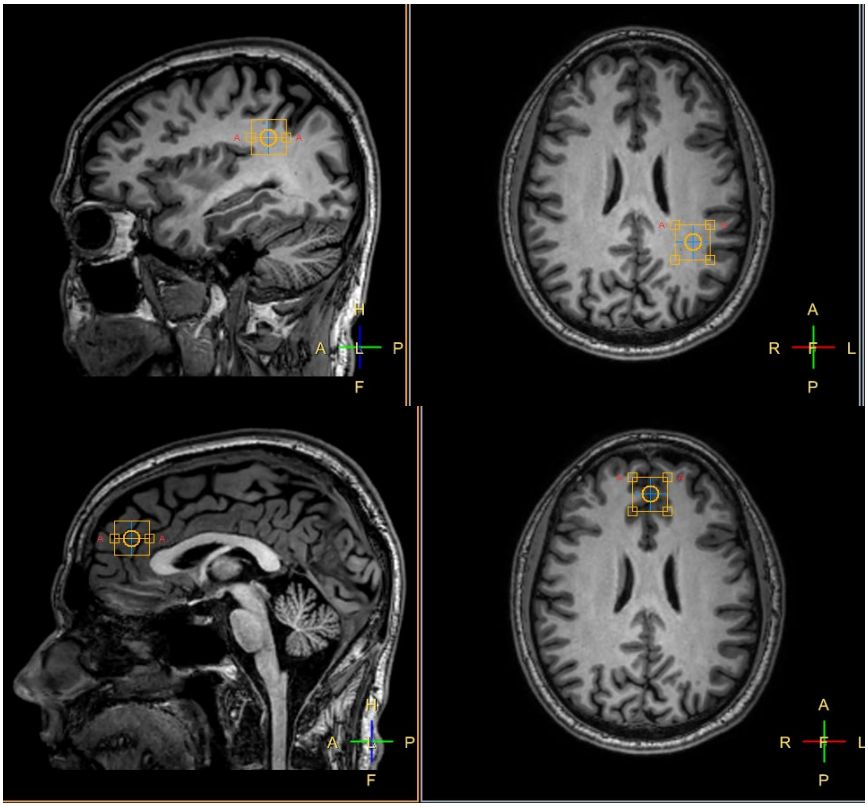


Figure 4: Voxel placement in parietal white matter (PWM, upper row) and anterior cingulate gyrus, gray matter (ACG, second row). Voxel size: 2x2x2cm^3

4.4 Magnetic Resonance Spectroscopy pipeline

Quality control was initially started by manually screening all MRS Voxel placements for proper position and possible motion artifacts or technical issues. Most data in this step were lost due to imaging artefacts brought on by dental braces.

PRESS spectra were analyzed by LCModel (Version 6.3– 1B), a fully automated software that adjusts the experimental spectrum with a linear combination of model spectra at the Center for Clinical Spectroscopy, Brigham and Women's Hospital, Harvard Medical School, using water as an internal reference.

In a next step, a general visual quality control of the T1 weighted imaging was performed using 3D Slicer (version 4.5). Skull-stripping was performed using FSL bet[85]. In order to segment the T1 weighted data (the entire brain) in grey matter (GM), white matter (WM), and cerebrospinal fluid (CSF), FSL fast was used[86]. In a final step, the content in the MRS voxels of interest was determined by performing voxel segmentation using an in-house script of the Psychiatry Neuroimaging Laboratory, Brigham and Women's Hospital, Harvard Medical School. This step ensured the absolute quantification of metabolites, as CSF, being mostly water and containing next to no metabolites, could be subtracted. GM and WM differentiation was furthermore achieved by picking two voxel locations: Anterior cingulate gyrus (ACG) with predominantly gray matter and parietal white matter (PWM).

Analyzed were the following six metabolites: Glu, GSH, mI, tNAA, tCr and tCho.

Another quality control step was taken by analyzing the Cramer Rao lower bound (CRLB) in order to estimate the level of measurement error and to estimate the standard deviations.

4.5 Statistics

Preliminary statistics were done using SASUniversityEdition and T-Test. Final statistics were done using IBM© SPSS© Statistics, Version 27.0.1.0.

Initially, all data was screened for normal distribution using Shapiro-Wilk at all three timepoints before any statistical analysis was performed. Bonferroni Correction was used in order to correct for multiple comparison and to reduce type I error. In order to correct for multiple comparison, initial p values were multiplied with 12 (number of analyzed metabolites; 6 metabolites in PWM and 6 metabolites in ACG).

To address the hypothesis 1, initially a single time point ANCOVA (+ Bonferroni) was used to evaluate baseline metabolic levels at a single timepoint and compare it to the control group, performed using “age in years” as a covariate, “group” as the fixed effects and dependent variable being metabolites. All results were corrected for multiple comparison using Bonferroni correction.

To further address the longitudinal aspect of the hypothesis 1 and 3, Linear Mixed Effects Model was used (additionally useful due to missing data points, making LMEM most useful in this case). The dependent variable was the metabolites, fixed effect was the group (either soccer or control) and the covariates were “timepoint”, “age in years” and “Subject_ID”.

To evaluate hypothesis 2, and therefore in order to correlate the exposure (based on number of years playing soccer in one analysis as well as the number of headers over a single season in another analysis), Bivariate Correlation was used.

5. Results

The aim of this study was to evaluate the effects and consequences of headers within a single season for adolescent male soccer players. MRS was chosen as a sensitive method to detect small changes within the brains' metabolite levels as a first indicator of disturbance. After careful selection of participants, both soccer and control athletes, to allow for best possible comparability, data from MRI imaging sequences and MRS data were obtained and screened for quality (final cohort see Table 3 and 4). In a final step, statistical analysis of the data was performed.

5.1 Hypothesis 1: Metabolic differences at different timepoints using Single Timepoint ANCOVA

Looking at hypothesis 1 and possible metabolic differences concerning tNAA (hypothesis: decreased levels in soccer population) between soccer and control, single timepoint ANCOVA was used.

tNAA levels were not significally different at timepoints 1, 2 and 3 betweeen soccer and control looking at both voxel places (*Table 5*). Furthermore, as postulated in the first hypothesis, increased levels of choline compared to controls could not be found (*Table 6*) nor lower Glx in soccer compared to control (*Table 7*) or differences in mI levels (*Table 8*)

tNAA

Timepoint	Voxel placement	p-value after Bonferroni
Timepoint 1	ACG	10,32
	PWM	1,82
Timepoint 2	ACG	4,17
	PWM	8,63
Timepoint 3	ACG	7,82
	PWM	7,79

*Table 5: **tNAA** level p-values at Timepoint 1, 2 and 3 in both Voxel locations comparing soccer and control group. No significant differences could be found, as seen in the corrected p-values after Bonferroni*

Cho

Timepoint	Voxel placement	p-value after Bonferroni
Timepoint 1	ACG	4,416
	PWM	11,28
Timepoint 2	ACG	10,75
	PWM	9,71
Timepoint 3	ACG	7,20
	PWM	2,63

*Table 6: **Cho** level p-values at Timepoint 1, 2 and 3 in both Voxel locations comparing soccer and control group. No significant differences could be found, as seen in the corrected p-values after Bonferroni*

Glx

Timepoint	Voxel placement	p-value after Bonferroni
Timepoint 1	ACG	7,44
	PWM	3,30
Timepoint 2	ACG	4,12
	PWM	7,87
Timepoint 3	ACG	9,40
	PWM	10,91

*Table 7: **Glx** level p-values at Timepoint 1, 2 and 3 in both Voxel locations comparing soccer and control group. No significant differences could be found, as seen in the corrected p-values after Bonferroni*

mI

Timepoint	Voxel placement	p-value after Bonferroni
Timepoint 1	ACG	11,63
	PWM	6,56
Timepoint 2	ACG	4,85
	PWM	9,40
Timepoint 3	ACG	8,87
	PWM	8,22

*Table 8: **mI** level p-values at Timepoint 1, 2 and 3 in both Voxel locations comparing soccer and control group. No significant differences could be found, as seen in the corrected p-values after Bonferroni*

5.2 Hypothesis 1: Examining longitudinal data using Linear Mixed Effects Model

In order to analyse our longitudinal data, LMEM was used in order to evaluate the fixed and random effects. There were no significant differences in metabolite levels between the soccer and the control group (Table 9).

Looking at the entire cohort (soccer player and controls together) at timepoint 1 + 2 + 3 "age in years" and "timepoint" was shown to have a significant effect on tNAA levels of the PWM (p= 0,04 (age in years) and p= 0,01 (timepoint) after Bonferroni Correction).

All other metabolites were not significant after Bonferroni Correction (see Table 9).

Looking only at soccer players at timepoints 1+2+3, the effect could once again be shown (p= 0,024 after Bonferroni Correction).

Only looking at controls for all three timepoints (n= 3 Controls for all three timepoints), no significant results could be seen (p = 6,67).

	ACG Glu	ACG GSH	ACG mI	ACG tNAA	ACG tCre	ACG tCho	PWM Glu	PWM GSH	PWM mI	PWM tNAA	PWM tCre	PWM tCho
group	p=4,8	p=3,7	p=9,0	p=11	p=1,8	p=7,2	p=6,6	p=6,3	p=10	p=8,0	p=6,7	p=4,5
age_years	p= 10	p=9,9	p=3,7	p=0,9	p=3,6	p=2,3	p=1,8	p=0,2	p=4,4	**p=0,04**	p=2,9	p=4,1
TP	p= 0,6	p=4,1	p=6,9	p=3,3	p=5,9	p=9,4	p=2,5	p=7,5	p=7,9	**p=0,01**	p=0,07	p=0,1

Table 9: LMEM results of all soccer players and controls together, looking at all metabolites (and its voxel location) with p-Values for fixed effects "Group", "age_years" and "TP" (timepoint). All p-values are listed after being corrected using Bonferroni.

5.3 Hypothesis 2: Effect of headers and exposure, using Bivariate Correlation

Looking at hypothesis 2 to assess the effect of headers as well as years of soccer, Bivariate Correlation was used.

A significant positive correlation was found between exposure (years of soccer) and PWM tNAA levels (p=0.024 after Bonferroni Correction) (Figure 5). Due to the limited number of data points, Bonferroni correction was used to limit the accumulation of type I errors when making multiple comparisons.

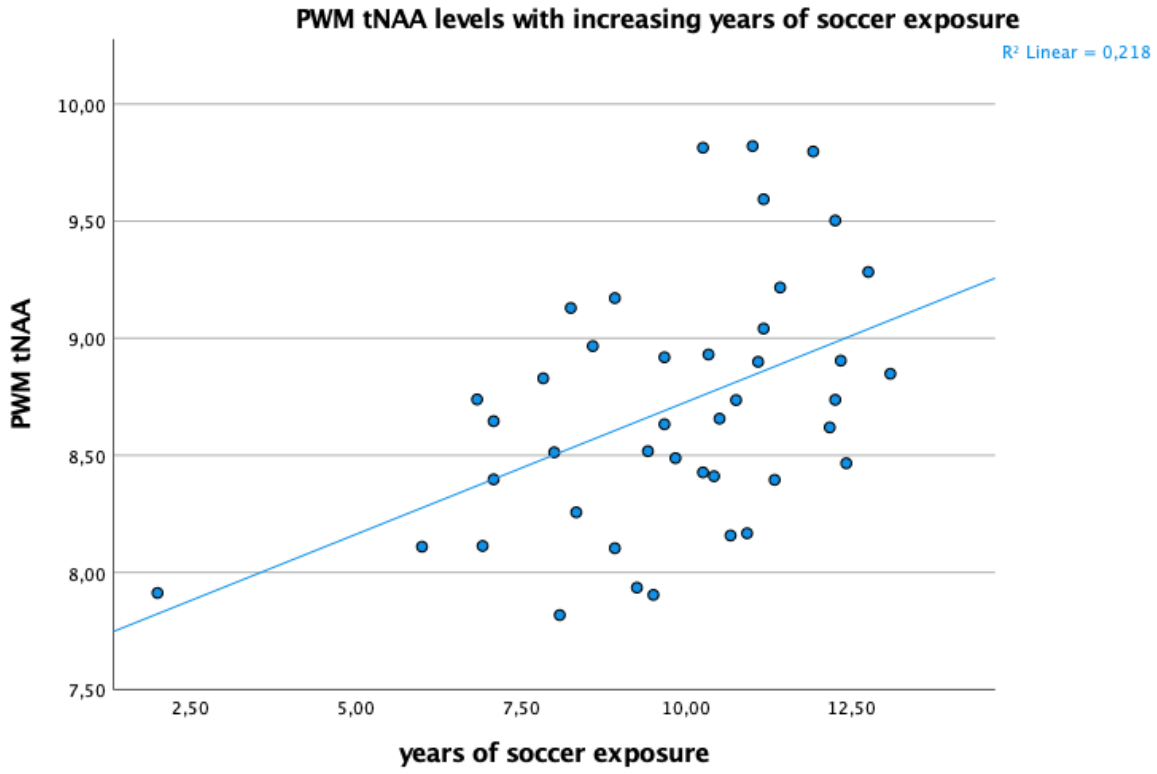


Figure 5: PWM tNAA levels with increasing years of soccer exposure.

Looking at exposure and all other metabolites, no significant results after Bonferroni correction could be reported (Table 10). No significant bivariate correlation between the number of headers over a single season (calculated from memory from each player, asking the players about the number of games, practices, and headers during the season) and metabolite levels could be found (Table 10)

Metabolit	Years_soccer_exposure	headers_overseason
ACG Glu	p= 11,02	p= 2,80
ACG GSH	p= 11,65	p= 3,23
ACG mI	p= 8,23	p= 6,98
ACG tNAA	p= 11,94	p= 2,50
ACG tCre	p= 6,72	p= 2,03
ACG tCho	p= 4,56	p= 8,04
PWM Glu	p= 1,07	p= 0,59
PWM GSH	p= 3,21	p= 4,67
PWM mI	p= 7,55	p= 8,30
PWM tNAA	**p= 0,02**	p= 1,12
PWM tCre	p= 0,36	p= 0,80
PWM tCho	p= 8,94	p= 1,02

Table 10: p-Values after Bonferroni Correction for all metabolites in correlation to years of soccer exposure and headers over a single season. Only significance can be seen looking at the correlation between PWM tNAA and years of soccer exposure. Headers_overseason was calculated by asking players to estimate the number of headers performed during a single season. Years of exposure was calculated by looking at the starting age and the time passed since then.

5.4. Hypothesis 3: Metabolite levels depending on age in controls (and soccer athletes), using Single Timepoint ANCOVA and LMEM

At timepoint 1, tNAA levels of the PWM were significantly correlated to the age in years of all subjects (p=0,036) using Single Timepoint ANCOVA (Table 11; Figure 6); looking only at soccer players or only controls, no significant results could be found (soccer p= 0,12; control p=2,09 after Bonferroni). No significant results for all participants could be found at timepoint 2 and 3 (p-value at TP 2: p = 1,98; p-value at TP 3: p = 2,72). All results after Bonferroni Correction.

All other metabolites did not show any significant results at any timepoint in correlation to age using Single Timepoint ANCOVA (Table 11)

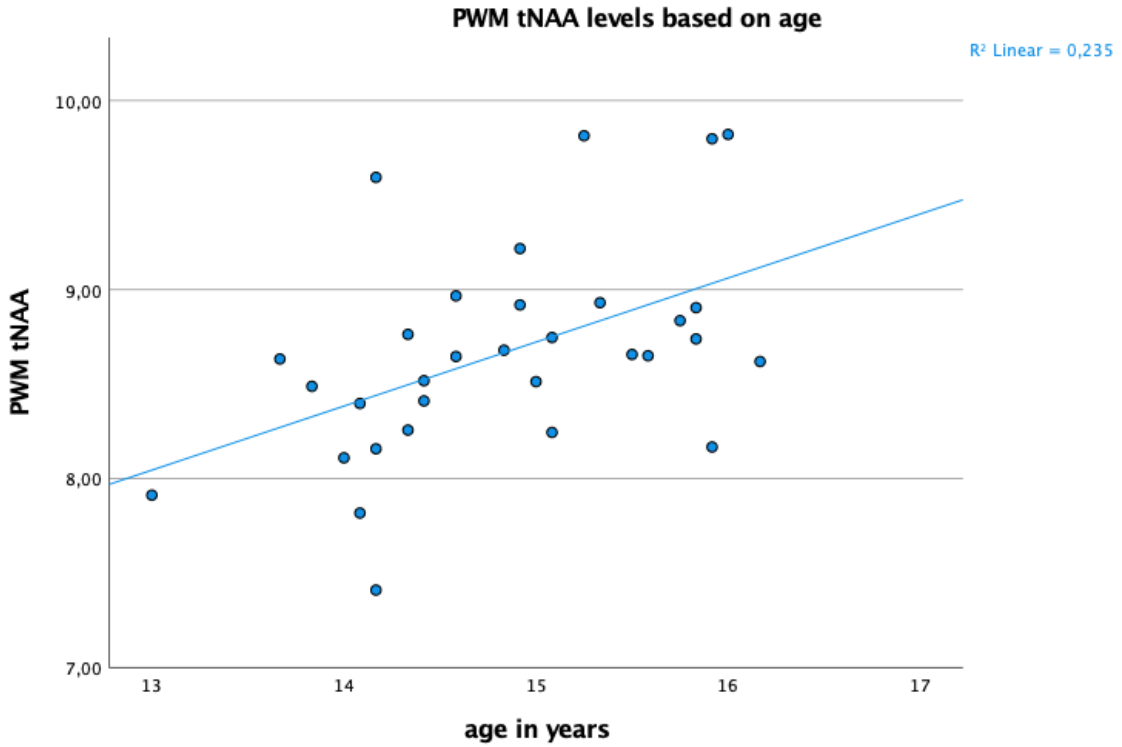


Figure 6: PWM tNAA levels of all participants (soccer and controls) at Timepoint 1 based on age in years

TP 1

Metabolites	Age_in years
ACG Glu	p= 7,02
ACG GSH	p= 6,86
ACG mI	p= 3,02
ACG tNAA	p= 0,88
ACG tCre	p= 2,18
ACG tCHo	p= 2,96
PWM Glu	p= 0,37
PWM GSH	p= 2,12
PWM mI	p= 4,09
PWM tNAA	p= 0,04
PWM tCRe	p= 2,23
PWM tCho	p= 6,94

TP 2

Metabolites	Age_in years
ACG Glu	p= 3,73
ACG GSH	p= 10,28
ACG mI	p= 10,82
ACG tNAA	p= 9,37
ACG tCre	p= 11,03
ACG tCHo	p= 6,70
PWM Glu	p= 7,51
PWM GSH	p= 1,98
PWM mI	p= 2,1
PWM tNAA	p= 1,81
PWM tCRe	p= 4,42
PWM tCho	p= 11,62

TP 3

Metabolites	Age_in years
ACG Glu	p= 8,74
ACG GSH	p= 7,02
ACG mI	p= 7,61
ACG tNAA	p= 3,88
ACG tCre	p= 6,77
ACG tCHo	p= 1,07
PWM Glu	p= 4,34
PWM GSH	p= 2,00
PWM mI	p= 4,96
PWM tNAA	p= 4,39
PWM tCRe	p= 5,24
PWM tCho	p= 8,12

Table 11: Depiction of soccer and control group combined, p-values (after Bonferroni) of all metabolites at all three timepoints (TP1/2/3) using Single Timepoint ANCOVA to correlate age in years to metabolite levels are displayed. Only PWM tNAA was significantly correlated to age in years at TP1.

6 Discussion

After careful and systematic review of existing literature concerning RSHI, MRS and young athletes, the following hypotheses were postulated:

1) Decreased levels of tNAA in the soccer population as a result of RSHI in comparison to control group. Furthermore, increased levels of choline, lower Glx and decreasing levels of mI in soccer players in comparison to the control group.

2) Additionally, I expected a correlation between exposure (based on the number of years played and the number of headers over a single season) and the levels of metabolites with the tendencies mentioned above.

3) Lastly, I hypothesized increasing tNAA levels within the control group with increasing age as a result of age-related changes of brain metabolites.

6.1.1 Hypothesis 1: results

Looking at Hypothesis 1, differences in metabolite levels between soccer players and the control athletes at all three timepoints using Single Timepoint ANCOVA could not be found. When looking at the data longitudinally, using LMEM, no significant differences in metabolite levels between the soccer and the control group could be presented.
However, looking at the entire cohort (soccer player and controls together, n= 34) at timepoint 1 + 2 +3 and using LMEM, “age in years” and “timepoint” was shown to have a significant effect on tNAA levels of the PWM (p= 0,04 (age in years) and p= 0,01 (timepoint) after Bonferroni Correction). With later timepoints, the age also increased, making age as the basis for the changes seen in later time points a likely candidate.
Looking only at soccer players at timepoints 1+2+3 (n= 29), the effect could once again be shown (p= 0,024 after Bonferroni Correction).

Only looking at controls for all three timepoints (n= 3; controls for all three timepoints), no significant results could be seen, but the limited number of controls certainly had its effect. All other metabolites were not significant after Bonferroni Correction.

6.1.2 Hypothesis 2: results

A significant positive correlation was found between exposure (years of soccer) and PWM tNAA levels (p= 0,024 after Bonferroni).

However, positive correlation was also found when looking at "age in years" and exposure (p= 0,012 after Bonferroni). As the results earlier might indicate, age might have had more impact on the results rather than the header associated with exposure.

All other metabolites were not significant after Bonferroni correction. No significant bivariate correlation between the number of headers (based on players account from memory over a single season) and metabolite levels could be found.

While significant changes could only be seen when either looking at the entire cohort or only soccer players (while lacking enough controls), the results can be, albeit cautiously, compared to existing literature. As mentioned earlier, increased levels of tNAA within white matter have been described in several studies and suggest a healthy development[71-73,75].

6.1.3 Hypothesis 3 results

At timepoint 1 (using single timepoint ANCOVA), tNAA levels of the PWM were significantly correlated to the age in years of the subjects (p=0.036). While significant correlation between the age of our participants and tNAA levels of the PWM can be seen as a sign of developmental changes (as mentioned above in metabolite changes during development as reported by prior research[71,73]), one issue for the missing significance at timepoint 2 and 3 could be the higher number of subjects with sufficient data at timepoint 1 (n=32) vs timepoint 2 (n=21) or timepoint 3 (n=20), which would skew the results and make

significant changes more visible at timepoint 1. This also applies to separately looking at soccer players or only controls. Dividing the participants led to significantly lesser numbers (soccer n= 25 at TP1, controls n=7) and no significant results could be found (soccer p= 0,12; control p=2,09 after Bonferroni). No significant results for all participants could be found at timepoint 2 and 3 (p-value at TP 2: p = 1,98; p-value at TP 3: p = 2,72. All results after Bonferroni Correction).

Additionally, changes of tNAA levels have shown to decrease with higher age, and later timepoints with higher age might have made it more difficult to see significant differences[73]. Nevertheless, the significant increase in PWM tNAA dependent on the age was astounding, independent of being either a soccer player or part of the control group.

6.1.4 Results Summary

While it is known that most metabolic changes happen right after birth and in the upcoming months, this study shows the prolonged period of change within the brain and its metabolites even in adolescents between 14 and 16 years old.

tNAA is a metabolite known to increase in the white matter with the most prominent increase in infants, but it has been shown to further increase up to adulthood[73]. While research concerning changes of metabolite levels during development has been relatively scarce, some research has been done looking at the predictive value of metabolites, as a guidance and possible predictor of timely development in premature births[87,88]. This underscores the possible chances and opportunities that lie within the exact knowledge of metabolite changes during development, as it might further help to detect developmental delay in early stages.

Changes of tNAA have shown to be related to an increasing number of dendrites and synaptic connections and an increasing amount of white matter as part of development[75,89,90]. Our findings therefore describe developments that indicate, based on the MRS findings, no irregular patterns. It is possible that harmful changes based on headers and head impact might

show after a more prolonged exposure to headers. As mentioned later again, a further evaluation at a later age, with those results as a baseline, might therefore be of great value. Nevertheless, research concerning the age group we investigated has been limited (and has looked at both girls and boys combined) and more studies will need to be executed to further underscore our findings and produce and verify set metabolite values as predicators for timely development and in order to differentiate irregular developmental changes.

One should acknowledge that those results, despite the great number of headers that the participants have recorded (between 228 and 2958 headers over a single season, average 809 header; SD 612) with starting age of practicing headers ranging from 4 years to 13 years of age (mean start age of header 8,69 years, SD 2,04), have shown no pathological MRS results. A single soccer player in this cohort, with an average age of 14.98 years, starting headers at a mean age of 8,69 and averaging 809 headers over a single season would have already headed 5.089 times in his life.

6.2 Shortcomings and future direction

6.2.1 Small sample size

One recurrent issue of this study was the limited number of participants

Initial recruitment of young professional soccer players was not simple, neither was recruitment of control athletes with no history of contact sports. Despite financial compensation, not many young boys were willing to spend half day of a weekend as part of this study. Coming in for TP 2 and 3 was additionally made more difficult by the COVID19 pandemic and recruitment of controls, that should have happened in the time during the pandemic, was halted. More data and more subjects for all timepoints, and especially more equal numbers of soccer players and controls would have underscored any result.

Additionally, greater number of players would not just help with sheer numbers but also allow for statistical analysis based on positions, as it is known that certain positions, such as defenders, accumulate the highest numbers of headers while goalkeepers have the lowest numbers. This should be factored in when looking at metabolite changes[24,91,92].
While positions of our cohort were known, due to the limited number of cases no statistically relevant results could be presented.

6.6.2 Exact count of headers

The number of headers for each player was calculated from memory from each player, asking the players about the number of games, practices, and headers during the season (during practice as well as games). Players in this study reported between 228 and 2150 headers (median of 809 headers) during the last season (practice headers and game headers combined). While advances with sensors (e.g., headband-mounted, skin-mounted, mouthguard-mounted or helmet-mounted sensors) have been made, reliable count of headers and sensoring of each headers' force for individual players is not yet readily attainable[93,94].
Making those advances in the future would give the research a more precise and powerful tool to investigate the effects of headers and the implications of the force that is created when heading. As seen in the statistical analysis using LMEM, exposure was closely associated with age. To differentiate the effects of age and head impact more precisely, exact numbers of headers and the timeline concerning the impacts would underscore possible findings related to subconsussive head impact.

6.2.3 Longer observation period

With only one season (and off-season) to look at, the time to observe changes of the metabolites was limited. The young and developing brain is known for its plasticity and disturbance of metabolites might be quick and initially reversible.

As shown in the systematic literature review earlier in this paper, most authors have limited the scope to a single season. While feasibility might play a big role, a longer period of observation might be interesting in order to evaluate the cumulative effect of headers for the developing brain. It might be of great interest to use this data as a baseline and continue to scan this cohort at a later point in life, when changes of metabolites are no longer part of development (roughly ages 18 to 20), aiming for participants aged 20 and above (with continued soccer and header exposure for the soccer players and a control group)[73].

6.2.4 Stricter time protocol

Due to scheduling conflicts, scanner availability and the Covid19 pandemic, the time between the time points as well as the time since the last practice/game often varied. T1-T2 varied between 217 and 420 days, T2-T3 between 14 and 181 days and T1-T3 varied between 335 and 483 days. Athletes therefore were scanned and seen during different times (some closer to season than others) and the time of last headers or impact were different and might have changed the level of metabolites. For better comparison, a stricter time protocol might be useful.

6.2.5 Standardized locations of voxel and 7 Tesla MRI

As seen during the systematic literature search for reference papers, the placement and location of single voxels is different for almost all papers. While voxel segmentation can reduce possible errors due to different composition of the voxel, exact and comparable

placements would facilitate a more comparable and more exact research. Different brain regions, as mentioned earlier, have different patterns of development and different times, making comparison, especially when looking and young and developing brains and its metabolite levels, difficult.

A standardized brain map with set voxel locations and instructions for placement would make comparisons between different publications and results much more efficient and comparable. In our case however, voxel placement was always done by the same person and at the same scanner, making comparisons between our subjects very reliable.

Additionally, to further enhance the sensitivity of the MRS method and detect the smallest changes, further studies could use a 7 Tesla MRI scanner or even compare 3 Tesla and 7 Tesla scanners. With exact knowledge of metabolite levels during development being crucial to further investigate effects (such as headers, concussion, head-to-head injuries) on the brain, 7 Tesla is shown to further increase sensitivity and might show changes that are not detectable with 3 Tesla[95,96].

6.2.6 Selection bias

While it has been incredibly important to carefully select only participants with no history of concussion and no possible brain anomalies or events such as premature birth in order to confidently find changes due to header, it might further be valuable to extend the research to all soccer players, including those with a history of concussion and premature birth as well as a learning or developmental disorder (those were excluded as well). Those children, while also playing soccer like anyone else, might react differently to repeated headers and might experience different effects.

While short and long-term effects of a single concussion have been looked at, for many children, the reality of playing soccer includes both concussions (not just due to headers but also head to body or head to ground impact) as well as frequent headers. The addition of those

events, especially when accumulated over time, might have a greater impact than looking at those instances separately.

Additionally, looking at male and female soccer players would certainly add diagnostic value to any results. Sex differences can be seen in the development of the brain, with different pace when looking at development and known influence of hormones, especially during puberty[97]. Within the white matter, growth during puberty and its development is earlier in females than in males, which may lead to decreased WM density in male adolescents and therefore different levels of metabolites when compared to girls[97,98].

As more and more girls are playing sports and soccer, the impact of headers not just for boys but also girls should be looked at and considered.

6.3 Conclusion

Magnetic resonance spectroscopy is a non-invasive and sensitive method to assess brain metabolite levels. It was used in this thesis to study the effects of repetitive subconcussive head impact in adolescent soccer athletes and controls. Hypothesized changes of brain metabolite levels due to headers in a soccer population over one season and compared to controls could not be found. Developmental changes, as seen by increased tNAA based on age, could be seen and confirmed in soccer players as well as controls.

This study has shown that subconcussive head impacts based on typical seasonal headers in adolescent boys up to the age of 16 by playing soccer has no apparent pathological effect when using the sensitive method of MRS.

Meanwhile MRS is demonstrating its sensitivity by showing known developmental changes within the brain based on metabolites. Further research into the age-related changes of brain metabolites might be useful as a basis for further evaluation of the effect of repetitive subconcussive head impacts.

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Affidavit

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