*Dissertation title*

# *CREATINE HOMEOSTASIS AND METABOLISM IN RESPONSE TO DIFFERENT STRESS INTERVENTIONS: EFFECTS OF CREATINE FORMULATIONS ON PERFORMANCE OUTCOMES IN HEALTHY YOUNG MEN AND WOMEN*

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# Abstract

Creatine is a semi-essential nutrient that plays a central role in cellular bioenergetics through its function in the phosphocreatine system. Endogenously synthesized by the kidneys, pancreas, and liver in a two-step process, creatine can be obtained in a typical omnivorous diet or as a dietary supplement. In humans, concentrations of creatine and its metabolites–its direct precursor guanidinoacetic acid and the end metabolite creatinine–fluctuate in response to different physiological stimuli and energy demands. Monitoring these changes in biomarker concentrations provides insights into their dynamics and turnover, reflecting unique adaptations of creatine metabolism homeostasis. This dissertation attempts to explore how circulating biomarkers of creatine metabolism respond to different physiological stressors and whether dietary supplementation can improve creatine status and performance outcomes.

In sum, this dissertation is composed of five peer–reviewed studies, fully available in Chapters II and III, investigating physiological fluctuations of biomarkers of creatine metabolism to high-energy demands and their potential upregulation via dietary supplementation strategies. Chapter II outlines a series of three quasi-experimental studies focused on acute metabolic stress in young, healthy men and women. These studies explore how circulating creatine related biomarkers respond to short–term, energy demanding conditions, including 24–hour fasting, esports, and running-to-exhaustion interventions. Our results consistently suggest significant changes in biomarker concentrations in biological fluids, indicating that creatine homeostasis is sensitive to different prolonged physiological stimuli. Building on these findings, Chapter III investigates whether creatine status and related performance outcomes can be positively influenced by supplementing creatine–guanidinoacetate formulation. In two additional studies, the effects of a mixture were assessed: the first one investigating the acute effects of supplementation when consumed promptly before committing to the incremental running to exhaustion;

and the second, evaluating a four-week supplementation protocol in esports players' performance. While the observed effects were modest, both dietary interventions demonstrated significant improvements in biomarker profiles, with a tendency to improve performance metrics.

Chapter IV integrates these findings, discussing their implications for creatine metabolism under stress and the potential role of dietary supplementation in preserving or enhancing tissue bioenergetics. Although the studies are preliminary and not without limitations, they contribute new insights into how creatine status responds to both physiological demands and targeted supplemental strategies. Collectively, this dissertation provides early evidence that creatine metabolism is responsive to short-term physiological stress, with potential relevance for optimizing human performance in physically and mentally exhaustive environments post-supplementation.

Finally, Chapter V comprises the personal experiences and acquired skills resulting from the additional scientific work conducted by the candidate in the field of public health through international collaborations with renowned labs and institutions globally.

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# CHAPTER I: INTRODUCTION

## 1.1 A Brief Historical

*Today, creatine (Cr) is considered a member of the large and important family of guanidines, which comprises more than a hundred different naturally occurring compounds (Ellington, 2001). Despite being considered a rather simple guanidino compound, its relevance among scientists rapidly escalated in the 20th century, nearly a hundred years after its reported discovery in 1832 by the French chemist Michel Eugène Chevreul (Chevreul, 1835). To bridge the gap in such a long timeline of events, we dedicate this relatively short paragraph to its instigators and meaningful past events related to creatine, primarily dissecting pieces originating from the early eighteenth century towards the end of the twentieth century. Creatine was first isolated by basification of skeletal muscle and therefore was named "creatine" after the Greek κρέας, the word for flesh. At the time, Chevrul was already a renowned scientist in the field of lipid chemistry, and creatine was a mere detail in the rich resume among numerous discoveries he made in 102 years of his life, including more than 800 publications and several books authored. Interestingly, being remembered for his groundbreaking work on fatty acids, including the chemistry of dyeing, which is perhaps why extracting a white, tasteless, and odorless crystallized structure from commercial beef-tea ("bouillon de la Compagnie Hollandaise") did not seem as appealing after all (Chevreul, 1889). Of course not. He immediately reported a discovery of a new organic compound to the French Academy of Sciences; however, its chemical structure remained undefined at the time. Fifteen years later, it was the German chemist, Justus von Liebig, who continued the research and confirmed the presence of creatine in the flesh of several wild animals, classifying it as a methylguanidino-acetic acid in 1847 (Strecker, 1861). Shortly after, Liebig himself successfully marketed and sold the Liebig Flesh Extract (Extractum Carnis Liebigii, in Latin), produced from the*

*leftover meat of cattle slain for leather in South America (Gratzer, 2005). Eventually, the product quickly became popular in Europe, primarily as the mixture was used as a remedy for the exhausted and wounded soldiers; however, soon it found its way into every grocery and chemist shop (Lankester, 1870). According to the discussions at the meeting of the Royal Medical and Chirurgical Society in 1865, the substance appeared to be nothing like what supplemental creatine looks like today; instead, it resembled a concentrated, brown-colored syrup with a strong smell of cooked meat, easily administered when mixed with salt, pepper, and boiling water. However, one pound of extract was said to correspond to the soluble matters of thirty pounds of ox-flesh ("Extractum Carnis Liebig," 1865). The growing popularity of Bavarian "Juice of Flesh" became a remedy not only for the wounded soldiers and the poor, but also across healthy households of Europe. As outlined by Lankester and his peers in 1870, physicians initially prescribed the meat juice to the poor and wounded soldiers, eventually realizing the remedy was too valuable to the exhausted nervous powers of the wealthy. Around the same time, dietary recommendations were also made for individuals engaging in prolonged mental tasks to increase their fish intake, due to their flesh containing more creatine than other food sources. Anyhow, little could they explain, but they knew that the consumption of albumen, fibrine, fat, and gelatine alone or combined did not provide similar effects without the meat juice. Yet, one could not entirely rule out the possibility that the extracts' mineral and nutritive values exerted significant positive effects (Lankester, 1870). However, the famous drink was harshly criticized in public for its nutritive value, which, according to the British chemist Vosper was purely made of "excremental substances" (Vosper, 1865). Regardless, the production of meat extract began by the private manufacturers across Europe, Scotland, England, including America, costing approximately 10 to 12 shillings per pound of extract. While its appearance and pricing slightly varied depending on the choice of animal flesh used in the process, the meat juice approximately contained about 8% of creatine (Sulser, 1968).*

*Over time, a significant amount of experimental work centered around understanding the physiological roles of creatine by addressing a series of burning questions that arose – to begin with, how does it work? Until the early 1900s, little beyond its presence in skeletal muscles was known until 1866, when Max Jaffe first discovered that creatinine (Crn) forms a clearly colored complex with alkaline picrate (Jaffe, 1886). However, at the time, the creatinine: creatine relationship was yet imperfectly understood, mainly because strong evidence on their physiological functions and distribution was still missing (Mendel, 1909). In a series of breakthrough investigations to come, Swedish-born scientist Otto Folin, initially a research biochemist at the McLean Hospital in the suburbs of Boston and later Professor at Harvard Medical School, developed and applied a colorimetric method to the Jaffé reaction to in order to estimate the circulating creatinine and creatine levels (Folin & Denis, 1914). This quickly brought another series of inquiries on this matter, such as questions on tissue distribution and relationship between creatine and creatinine, and whether factors like starvation or muscle activity perhaps alters their concentrations (Mendel, 1909; Myers & Fine, 1912, 1915). In 1922, Andrew Hunter, Professor from the University of Toronto devoted an entire monograph to physiology of creatine and creatinine metabolism, providing first estimates of tissue creatine and creatinine concentrations in a wide range of animal and human organs. Indeed, it was estimated that more than 95% of the total creatine pool resides within the muscle tissue, with notable quantities also found within the brain, he assumed creatine is a substance tightly connected with the neuromuscular abilities, including those powerful muscle contractions (Hunter, 1922). Around December of 1926, the British couple Eggleton discovered a labile form of organic phosphate which tended to decrease with the onset of muscle fatigue, roughly around the same time as Fiske and Subbarow, and named it phosphocreatine (Eggleton & Eggleton, 1927; Fiske & Subbarow, 1927). In the next several years, adenosine triphosphate (ATP) was discovered in 1929, and Lohmann and Lehmann showed that the reversible reaction catalyzed by creatine kinase (CK) yields*

*phosphocreatine (PCr) and adenosine diphosphate (ADP) (Lohmann, 1931). However, evidence on the precursors involved in creatine metabolism was relatively limited, although Hunter already touched upon this and hypothesized that creatine may be looked upon as a derivative of guanidinoacetic acid (GAA), based on its guanidino group and distinct connection with arginine. Perhaps contrary to the common wisdom, the first evidence of its direct precursor appeared more than a century after the discovery of creatine. In 1935, Dr. Clarence J. Weber from the University of Kansas School of Medicine first isolated guanidinoacetic acid (also known as glycocyamine) from dogs urine, while Borsook and Dubnoff showed that GAA is methylated to creatine in slices and cell-free extracts of kidney and liver in the presence of methionine (Borsook et al., 1941; Weber, 1935). Its role as an intermediary product in the endogenous synthesis of creatine metabolism was subsequently beautifully elaborated in detail by James B. Walker (Walker, 1979).*

*In the upcoming years, both creatine and its direct precursor were investigated in ergogenic, therapeutic, and nutritional contexts, particularly due to their benefits in cellular bioenergetics. With the pioneering investigation led by Chanutin in 1926, "Fate of creatine when administered to a man", it was demonstrated for the first time in human history that orally administered creatine is absorbed by the intestine (Chanutin & Guy, 1926). However, at the time, guanidinoacetic acid was largely overshadowed by creatine, not living up to its potential, and was instead positioned as an effective nutritive aid in the livestock industry. However, although rudimentary, the pioneering studies testing GAA supplementation back in the 1950s predominantly investigated its efficacy in clinical populations (Ostojic & Jorga, 2023). More interestingly, they highlighted that its physiological potential was superior in restoring creatine stores than administering purely creatine alone.*

*Nonetheless, in 1992, it was Roger Harris who made a turning point in the history of sports science literature, producing the first robust, placebo-controlled data showing that oral creatine*

*supplementation improves intramuscular creatine levels and repeated high-intensity exercise performance (Harris et al., 1992). Concomitantly, these findings were anecdotally supported by reports of creatine use among the athletes and gold medalists during the 1992 Olympics in Barcelona. Soon after, interest in creatine research took off with a momentum that continues today, confirmed by thousands of scientific papers on this matter (Stout et al., 2025).*

*While many questions remain, this dissertation offers just one small thread in the twisted rope connecting decades of scientific research to the present dissertation. Written with curiosity and passion for the scientific process, it hopefully contributes to the ongoing discussions, perhaps by raising new questions or revisiting old ones. Lastly, the true value of this dissertation lies not only in its key findings but in the ideas it explored and its imperfections.*

## 1.2 Creatine: a miserable life without it

Creatine (Cr) is an essential component of cellular bioenergetics, especially in tissues with high and fluctuating energy demands (Wallimann et al., 2011). Although creatine deficiency is not lethal, animals for instance, could not survive for any significant length of time in the wild, whereas human with any of the creatine deficiency syndromes would suffer from lifelong health impairments (Schulze, 2013). Its primary function lies in maintaining the availability of adenosine triphosphate (ATP), the universal energy currency of the cell essential for any biological work. This is accomplished through a reversible reaction catalyzed by the enzyme creatine kinase (CK), in which creatine in its energy–charged form (PCr) donates a phosphate group (P) to adenosine diphosphate (ADP) enabling immediate ATP resynthesis when and where energy is necessary (Walker, 1979; Wallimann et al., 2011). Together with CK, the Cr/PCr system acts as both energy buffer and a shuttle for cellular energy. Cellular stores of PCr can be up to 10 times greater than ATP, securing reserves of energy pool during periods of escalating ATP demands (Wallimann et al., 2011). More specifically, at the onset of intense activity–whether muscular or cognitive, the combustion of a phosphate bond in PCr enables immediate ATP resynthesis, sustaining work capacity before slower systems such as glycolysis or oxidative phosphorylation can engage (Gastin, 2001). This rapid reaction, involving a single enzyme, maintains the ATP/ADP ratio above 100:1, which is critical for ensuring the high free energy release ($\Delta G$) required for cellular processes such as muscle contraction, membrane transport, and signaling (Weiss et al., 2005). Naturally, the direction of the CK reaction depends on the energetic status of the cell and follows the law of mass action. At sites of energy consumption, such as myofibrils, the reaction proceeds towards ATP resynthesis. In contrast, at energy production sites like mitochondria, the reaction proceeds towards PCr synthesis using newly formed ATP in the process. This bidirectional flow creates the basis of the creatine phosphate shuttle, connecting the sites of mitochondrial ATP production to sites of cellular energy

expenditure, while minimizing the need to transport large and unstable molecules of ATP across intracellular distances (Wallimann et al., 1992) This process is facilitated by the spatial organization of several CK isoforms. Briefly, the CK exists in four different isoforms, with MM-CK predominantly expressed in skeletal muscle, while MB-CK and BB-CK are specifically distributed across the heart and brain tissues (Fritz-Wolf et al., 1996; Hemmer & Wallimann, 1993). A mitochondrial isoform, mtCK, is strategically localized at the inner mitochondrial membrane where it catalyzes the phosphorylation of creatine using oxidative ATP (Gastin, 2001; Schlattner et al., 2006). The newly formed PCr then diffuses through the cytosol to locations such as myofibrils, the sarcoplasmic reticulum, or ion pumps on the membrane, where it regenerates ATP via cytosolic CK. The coordinated activity of mtCK and cytosolic CK isoforms supports a continuous energy loop within the cell (Wallimann et al., 2011).

Furthermore, creatine's function is not limited to energy buffering, as rapid resynthesis of ATP via PCr prevents the excessive buildup of ADP and subsequently adenosine monophosphate (AMP). The accumulation of AMP activates an enzyme AMP deaminase, converting it to inosine monophosphate and ammonia, resulting in a net loss of adenine nucleotides from the cell (Dunn & Grider, 2025). With the assistance of creatine, the CK can mitigate this by rapidly phosphorylating ADP, thereby preserving the nucleotide pools, and ensuring contractile and metabolic functions are within the biological variation. Furthermore, the CK reaction assists by binding free hydrogen ions during ATP resynthesis, contributing to intracellular pH buffering during increased energy demands. Emerging evidence also suggests creatine plays a role in redox homeostasis in mitochondria as the primary site of reactive oxygen species (ROS) production, particularly during processes of oxidative phosphorylation (Meyer et al., 2006).

## 1.3 Creatine Metabolism: Biosynthesis, Transport and Regulation

### 1.3.1 Biosynthesis of Creatine

Creatine (methylguanidine–acetic acid) is a simple yet ubiquitous guanidino compound found in skeletal muscles tissue of most vertebrates (Walker, 2006). Naturally, humans can independently synthesize creatine although it may often be considered semi-essential due to our physiological needs often exceeding the endogenous capacity under certain conditions (Ostojic, 2025). A healthy adult male weighing 70 kg contains approximately 125 mmol/kg of dry muscle mass of total creatine (TCr), with 40% stored in its phosphorylated form, while the remaining is available as free creatine (Harris et al., 1992). These values are generally lower for vegetarians, elderly populations, and patients suffering from muscle-wasting diseases (Forsberg et al., 1991; Smith et al., 1998; Wyss & Kaddurah-Daouk, 2000). Interestingly, sex does not appear to significantly affect cellular concentrations, although females tend to exhibit higher resting muscle TCr concentrations despite having lower dietary protein intake (Tarnopolsky, 2000). Furthermore, there appears to be an upper limit for muscle total creatine concentration of approximately 160-180 mmol/kg dry muscle mass (Harris et al., 1992; Hultman et al., 1996).

Each day, approximately 1.7% (about 2 grams per day) of the total creatine pool is being non-enzymatically converted into creatinine and is spontaneously lost via urinary excretion with an approximate half–life of 40 days at physiological pH (Chanutin & Guy, 1926; Myers & Fine, 1912). In healthy individuals, majority of daily creatine turnover is reflected by the continuous excretion of its end-metabolite creatinine, which can be altered during extreme fluctuations of creatine intake (e.g., dietary supplementation, vegetarian diet), energy demands, or due to medical conditions, including inborn genetic errors causing defects in protein or creatine metabolism (Benedict & Osterberg, 1923; Cajori, 1928; Kim et al., 1983). However, the pool of creatine excreted in the urine in the form of

creatinine, but also guanidinoacetic acid and creatine, with the latter two being almost insignificant amounts compared to creatinine (Valongo et al., 2004), is being continuously replenished and maintained in balance by the seesaw with endogenous synthesis at one and dietary food intake on the other side (M. E. Brosnan & Brosnan, 2016). Principally, both sources should contribute equally in resupplying the body with creatine, with roughly one gram being endogenously synthesized and another gram obtained from the regular food intake, to compensate for the estimated daily turnover of ~2 grams per day (Balsom et al., 1994). The greatest dietary source of creatine comes from products of animal origin, for instance beef containing around 0.9 grams of creatine in 200 grams of uncooked beef (Harris et al., 1992). Apart from beef, other foods rich in creatine include pork (5 g/kg), seafood, herring (6.5-10 g/kg), salmon (4.5 g/kg) and tuna (4 g/kg) among other fishes. Albeit at significantly lower concentrations, dairy products such as milk (0.02g/kg), fruits such as berries (0.01g/kg), and vegetables contain only trace amounts of creatine, which leaves vegans and vegetarians dependent almost entirely on endogenous synthesis (Balsom et al., 1994). Consequently, vegetarians/vegans often exhibit lower muscle creatine levels, serum creatinine and urinary creatinine, emphasizing the lack of exogenous creatine and inability of the endogenous system to fully compensate for the daily turnover (Delanghe et al., 1989).

The biosynthesis of creatine occurs at highest rates under anabolic conditions, hormonal balance, optimal food supply, and creatine precursors available in ample supplies. The synthesis mainly occurs in the liver, kidneys, and pancreas, which are the primary organs, but not the only sites of creatine biosynthesis. It occurs as a two-step reaction and involves 3 out of 20 proteinogenic amino acids, arginine, glycine, and methionine (Wyss & Kaddurah-Daouk, 2000). In the first step, the amidino group from arginine is transferred to glycine molecule to form guanidinoacetate and ornithine, a reaction that is catalyzed by the enzyme L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1) (Borsook et al., 1941). The reaction catalyzed by AGAT takes place mainly in the kidney and pancreas, with renal GAA accounting

for approximately ≥ 20% of the GAA synthesized in total (Edison et al., 2007). In the second step, newly synthesized GAA reaches the circulation to be subsequently taken up by the liver, also the brain, myocardium, reproductive organs, where GAA is methylated by S–adenosylmethionine (SAM) to finally yield creatine and S–adenosyl homocysteine (SAH) via the catalytic action of guanidinoacetate *N*-methyltransferase (GAMT; EC 2.1.1.2), see Figure 1 (Brosnan et al., 2004).

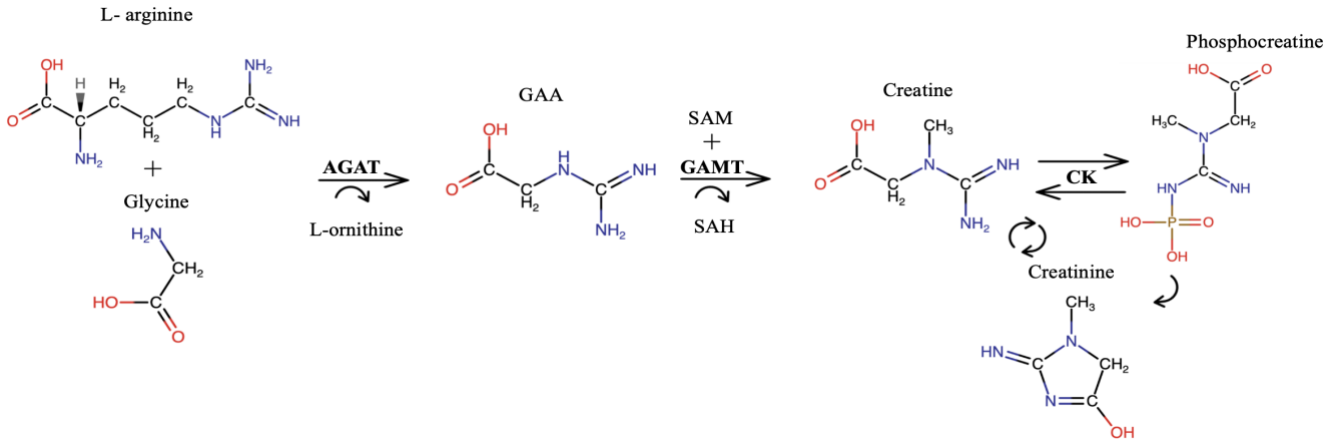



Figure 1. Endogenous synthesis of creatine, chemical structure and enzymes involved.

Naturally, the expression of AGAT and GAMT is often spatially separated, but necessarily not mutually exclusive, with AGAT being predominantly expressed in pancreas and kidney and GAMT activity in the hepatic cells of the liver, brain, and testes. On the other hand, expression of both AGAT and GAMT often coexists in other ATP-demanding tissues outside the liver, including the mammal's pancreas, which was the first organ shown to utilize arginine, glycine, and S-adenosylmethionine (SAM) in the formation of creatine (Walker, 1958). In the adult mammalian brain, primary brain cell cultures possess the enzymatic machinery to synthesize creatine independently, likely due to limited permeability of creatine through the blood-brain barrier (BBB) despite significant CrT expression across specialized endothelial cells forming the barrier. Although the largest stores of creatine are located within the skeletal muscles (up to 95%) the brain is the main organ heavily affected by creatine deficiency, caused by either

inability to fully synthesize or import creatine caused by mutations of CrT, leading to severe neurological impairments (Item et al., 2001; Salomons et al., 2001a; Stöckler et al., 1996). At the basic level of endogenous creatine synthesis, regulatory mechanisms also involve the feedback inhibition, allosteric regulation, or expression of the enzymes involved in Cr metabolism and transport, for a detailed review, see (Walker, 1979). Exogenous creatine *per se* intake directly affects and suppresses the activity of AGAT, which is the key rate-limiting enzyme in the biosynthetic pathway. A rise in plasma creatine concentrations—whether from supplementation, food intake, prolonged fasting induced catabolism, or muscle wasting seem to be directly associated with downregulated AGAT activity. Furthermore, hormonal status has been shown to significantly affect the homeostasis of creatine by modulating the AGAT activity, after administration of estradiol and diethylstilbesterol, caused amidinotransferease levels to increase in the chick liver (Zhu & Evans, 2001). While in rodents like rats, the AGAT activity diminished following hypophysectomy or thyroidectomy, but was restored with success after administration of growth hormone and thyroxine, respectively (Van Pilsum et al., 1970). Similarly, adding growth hormone and anabolic androgens such as methyltestosterone have been shown to enhance AGAT expression and thus overall creatine synthesis (Hoberman et al., 1948; McGuire et al., 1980). Essentially, it is the amidinostransferase step, in which the entire molecule of glycine and the amidino group from arginine are utilized to synthesize GAA, that initiates the biosynthetic pathway but also serves as feedback control loop and the rate–limiting step in the biosynthesis of creatine by end – product repression (Brosnan & Brosnan, 2016). In contrast, the activity of GAMT appears to be primarily driven by the substrate (guanidinoacetate) availability. Conversely, if the AGAT step was bypassed by addition of exogenous GAA, biosynthesis proceeds an excessive rate with consequent depletion of methyl groups. This is beautifully illustrated in several experiments, where feeding GAA has been shown to be far

superior in increasing creatine cause an increase in the biosynthesis of creatine even surpassing the creatine concentration after injecting the equimolar amounts of creatine (Walker & Wang, 1964).

### 1.3.2 Transport and Regulation

Once synthesized or taken up from dietary sources, creatine is released into the bloodstream at physiological concentrations ranging from 40–100 μM (Delanghe et al., 1989; Harris et al., 1992), to be delivered to tissues with high energy demands, with highest concentrations found in skeletal muscle, heart, spermatozoa and photoreceptor cells of the retina. Other tissue containing intermediate levels of creatine is the brain, brown adipose tissue, intestine, seminal vesicles, seminal vesicle fluid, endothelial cells, and macrophages, with low levels found in lung, spleen, kidney, liver, white adipose tissue, blood cells, and serum (Wyss & Kaddurah-Daouk, 2000). What's interesting is that sites of creatine synthesis and its utilization are spatially separated, since tissues with highest amounts of creatine such as skeletal muscles, lack enzymatic machinery required to undependably synthesize creatine. As a workaround, these cells naturally rely on the ability to transport creatine and its precursors to allow an entire sequence of reactions to unfold. Given that approximately 95% of the body's creatine pool is located within the skeletal muscle, supplies of creatine are essentially being continuously replenished by importing creatine from circulation (Ostojic, 2021). This is allowed via secondary active transport by the creatine transporter (CrT) located in the plasma membrane.

CrT belongs to the solute carrier family 6 of transporters, a large group of membrane proteins encoded by the SLC6A8 gene, responsible for the import and export of creatine across the plasma membrane (Chen et al., 2004). To overcome the steep concentration gradient between intracellular and extracellular matrix (up to 200:1), the transport of creatine occurs through a saturable, sodium-dependent mechanism mediated by the electrochemical gradient maintained by the $Na^+/K^+$-ATPase pump (Béard & Braissant,

2010; Daly & Seifter, 1980; Loike et al., 1988). In the process, two sodium ions are transported for each creatine molecule transported, making the process energetically favorable. The affinity of the transporter for sodium is characterized by the Michaelis–Menten constant (Km) 1, defined as the substrate concentration at which transport proceeds at half of the maximal velocity (Vmax). For CrT, the Km is approximately 55 mM, implying that relatively high extracellular sodium concentrations are required for the transporter to operate near its maximum rate (Möller & Hamprecht, 1989). Once creatine enters the cell, it becomes irreversibly phosphorylated and trapped within the cytosol, because its polar nature prevents passive efflux back into the system, keeping circulating concentrations of creatine quite stable (Greenhaff, 1997). The SLC6A8 is highly specific for creatine and is unable to transport PCr nor Crn, however it may occasionally contribute to the transport of GAA, although at much lower affinity (Jomura et al., 2022a). For instance, cerebral uptake of GAA from blood via SLC6A8 is limited under physiological conditions due to 2–50 times higher concentrations of creatine, which competes for the same receptor with higher affinity for creatine transport (Braissant, 2012).

Along with the CK isoenzymes, CrTs are highly expressed in tissues with fluctuating energy demands—including but not limited to the skeletal muscle, heart and brain tissue, testes, retina, and kidneys (Wyss & Kaddurah-Daouk, 2000). In addition to the SLC6A8, other members of the SLC family may also contribute to creatine movement across cell membranes. These include the monocarboxylate transporter 12 (MCT12) encoded by the SLC16A12 gene, being responsible for the efflux of creatine and GAA, especially in renal tissues such as the tubular epithelial cells, sinusoidal membrane of the liver, sarcolemma of skeletal muscle, and the choroid plexus (Jomura et al., 2022b; Tachikawa et al., 2009; Verouti et al., 2021). Furthermore, GAT2 (SLC6A13), a member of the gamma-aminobutyric acid

[1] Km — Michaelis–Menten constant; substrate concentration at which the transport rate reaches half of its maximum (Vmax).

(GABA) transporter family, is the primary hepatic transporter responsible for the uptake of GAA, essentially supplying a direct substrate necessary for the methylation within the liver. Other CrTs, like taurine transporter (TauT, SLC6A6) and organic cation transporter 3 (OCT3, SLC22A3) are primarily responsible for exporting the GAA and Crn across the BBB and CSFB, which is essential in order to prevent the neurotoxic accumulation of guanidino compounds in the brain (Tachikawa & Hosoya, 2011). The regulation of creatine homeostasis is complex and multifactorial. Both endogenous and exogenous factors may be affecting the uptake by modulating CrT activity. These include, skeletal muscle contractions (Harris et al., 1992) and types of skeletal muscle fibers (Brault et al., 2003), co-administration of high doses of carbohydrates (Greenhaff, 1997), hormones like catecholamines, thyroxine and triiodothyronine, insulin – like growth factor 1 (IGF-1) and insulin (Odoom et al., 1996), all of which have been reported to enhance creatine uptake into skeletal muscle by upregulating $Na^+/K^+$-ATPase activity (Haugland & Chang, 1975; Steenge et al., 1998). On the other hand, several factors which may effectively be reducing the uptake of creatine include diet, chronic creatine supplementation, extracellular creatine levels, baseline levels of creatine, prolonged periods of fasting and various pathologies accompanied by muscle dysfunction (Guerrero-Ontiveros & Wallimann, 1998; Kreider & Stout, 2021; Loike et al., 1988; Shearer & Weljie, 2014). Collectively, these transporters play important role in the biosynthesis and distribution of creatine and its precursors across the plasma membrane, maintaining the homeostatic balance between efflux and influx of creatine (Jomura et al., 2022a).

### 1.3.3 Circulating Biomarkers of Creatine Metabolism

Biomarkers of creatine metabolism can be sampled from various biological fluids, offering valuable insights into metabolic status, renal function, and dynamics of tissue–specific bioenergetics. Although blood remains the most widely used and validated matrix for assessing systemic changes in creatine metabolism, other fluids such as urine, cerebrospinal fluid, and saliva may provide additional

information to create a picture more complete. In healthy individuals, average serum creatine concentrations are 62–63 μM with minimal to no differences between sexes (Joncquel-Chevalier Curt et al., 2013). Throughout the lifespan, higher concentrations are commonly observed among children, following a negative correlation with years of age. Other factors influencing levels of creatine may include physical activity, dietary food intake, supplementation, and medical conditions underlying metabolic or neuromuscular disorders (Wyss & Kaddurah-Daouk, 2000). In creatine deficiency syndromes (CDS), which include inherited defects in AGAT and GAMT enzymes, or the SLC6A8 transporters, circulating creatine levels are heavily affected, with plasma creatine values between 0 and 7 μM and even getting < 5 μM (Almeida et al., 2004; Carducci et al., 2002). Alternatively, increased levels of creatine in plasma are often detected among individuals diagnosed with neuromuscular conditions, e.g., Duchenne Muscular Dystrophy, due to the muscle cell membrane instability and subsequent leakage into circulation, where even fairly small amounts of creatine cause a significant spike in plasma concentrations (Wyss & Kaddurah-Daouk, 2000). On the other hand, GAA levels are found at much lower levels in healthy adults, approximately ranging between 1.4–1.5 μM, and have a tendency to increase throughout the lifetime (Joncquel-Chevalier Curt et al., 2013). Interstingly, serum GAA levels do not appear to be influenced by sex but tend to increase with age (Joncquel-Chevalier Curt et al., 2013; Valongo et al., 2004). In the case of GAMT deficiency, plasma GAA can vary in a 12–39 μM range, whereas in AGAT deficiency, GAA concentrations are practically very close to zero (<0.05 μM) (Carducci et al., 2002; Verhoeven et al., 2005). Interestingly, CDS does not necessarily affect GAA in the same direction as creatine. In SLC6A8 transporter deficiencies, for instance, patients typically show normal GAA levels despite lowered creatine levels (Verhoeven et al., 2005).

Creatinine, being a product of spontaneous non-enzymatic conversion of creatine and phosphocreatine, circulates in blood at concentrations of approximately 63–107 μM and 46–103 μM for males and

females, respectively (Junge et al., 2004). These values are in striking correlation to skeletal muscle mass and glomerular filtration rate (GFR), establishing creatinine as a powerful clinical biomarker of renal function (Patel et al., 2013). Creatinine is primarily eliminated through the kidneys, with urinary excretion reflecting both muscle mass and renal clearance capacity. In healthy adults, daily urinary creatinine loss typically ranges between 14–26 $mg \cdot kg^{-1}$ body weight in men and 11–20 $mg \cdot kg^{-1}$ body weight in women, corresponding to approximately 1–2 g/day (Heymsfield et al., 1983; W. Wang et al., 2018). Although renal elimination via urine represents the dominant pathway, small amounts of creatinine are also lost through sweat and fecal excretion, particularly under conditions of high ambient temperature or gastrointestinal dysfunction (Fukumoto et al., 1988; Wixom et al., 1979). In chronic kidney disease (CKD), serum creatinine may rise dramatically, often exceeding 1000 μM in the advanced stages of the disease (Temilola et al., 2019). In the case of inborn errors of creatine metabolism, individuals commonly exhibit specific urinary profiles: GAMT–deficient patients typically have elevated GAA in urine; in contrast, patients deficient in the AGAT enzyme have reduced urinary creatine and GAA output (Almeida et al., 2004; Carducci et al., 2002).

Creatine biomarkers are present in fluids such as saliva, human milk, cerebrospinal fluid (CSF), and semen, perhaps better illustrating tissue-specific distribution (Nedeljkovic & Ostojic, 2024). Cr concentrations in unstimulated saliva have been reported at approximately 9.24 ± 2.76 μM in males and 10.67 ± 5.07 μM in females over the age of 10. In young children (0–10 years), levels are slightly lower, 7.65 ± 3.12 μM in males and 8.07 ± 2.98 μM in females (Martínez et al., 2016). In another study, the authors reported even lower values in healthy adults, averaging 6.18 ± 2.59 μM, which are substantially lower than typical plasma concentrations (Suzuki et al., 2016). This discrepancy may reflect restricted transport or local regulation of creatine in saliva, potentially involving the CrT (SLC6A8), which has high mRNA expression in salivary glands. Regarding GAA, higher salivary concentrations are reported

in individuals under the age of 15, averaging 4.02 ± 2.16 μM and 3.94 ± 2.92 μM for males and females, respectively. Conversely, individuals (aged >15 years) typically show lower values around 2.38 ± 1.44 μM for males and 2.69 ± 1.60 μM for females. Although sex differences were insignificant, age–related trends appeared more pronounced among females (Martinez et al., 2016). Salivary Crn levels in healthy subjects have been measured at 4.59 ± 4.33 μM (Suzuki et al., 2016). However, these values tend to rise significantly in patients with chronic kidney disease (CKD), following a clear correlation with serum creatinine and averaging 11 μM (Temilola et al., 2019). In CKD patients undergoing hemodialysis, studies reported salivary creatinine at 64.53 ± 61.88 μM before treatment and 22.10 ± 16.8 μM posttreatment (Suzuki et al., 2016).

Other biofluids, such as human milk, contain creatine in concentrations from 60 to 70 μM during early lactation, while creatinine remains around 45 μM and GAA at only ~0.3 μM, supporting active creatine metabolism and contributing roughly 9% of an infant's daily requirement (Edison et al., 2013). Creatine has also been recognized for its role in sperm energy metabolism; however, its levels do not consistently predict fertility. In seminal plasma, Cr varies from 544 μM in vasectomized men to 1,818 μM in normospermic men (Srivastava et al., 2009). Based on a single study in infertile men, Crn averages around 316 μM, while GAA is present only in negligible amounts (Allahkarami et al., 2017). In CSF, creatine concentrations typically range between 17 and 87 μM, closely reflecting blood levels (Valongo et al., 2004). However, in individuals with GAMT deficiency, CSF creatine drops dramatically, often at values below 2 μM (Schulze et al., 2003). In contrast, SLC6A8 deficiency usually presents with normal or slightly elevated levels of CSF creatine (Salomons et al., 2001b). According to Almeida et al., estimated normal levels of GAA in CSF are very low, ranging between 0.02–0.56 μM (Almeida et al., 2004), while Crn levels are reported to be around 68 μM; however, they drop in the absence of the enzyme GAMT and rise up to as high as 521 μM in renal failure patients (De Deyn et al., 2009).

### 1.3.4 Biochemical Assessments: Sample Preparation and Analysis

The analytical study of creatine metabolism and its biomarkers has evolved considerably over the past century. Early investigations employed classical chemical assays such as the Jaffe, Barritt, and Sakaguchi reactions for creatinine, creatine, and guanidinoacetate assessment. Although foundational, these methods were limited in specificity and sensitivity, being prone to interference from structurally related compounds such as sugars, ketones, and bilirubin. Enzymatic approaches improved selectivity but required costly reagents and were susceptible to interference and instability. Advances in analytical instrumentation have shifted the field toward gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS). While GC–MS offers high sensitivity, it typically requires laborious derivatization of highly polar molecules. LC–MS/MS, in contrast, combines efficient chromatographic separation with molecular specificity and sensitivity, making it ideal for creatine biomarkers. For this dissertation, a modified LC–MS/MS method employing hydrophilic interaction chromatography (HILIC) was developed, offering strong retention, baseline separation, and a rapid six-minute runtime for creatine and guanidinoacetate without derivatization, thus simplifying sample preparation and preserving analyte integrity.

Plasma samples (250 µL) were vortex-mixed for 30 s with 2 mL methanol to precipitate proteins, refrigerated at 4 °C for 20 min, and centrifuged at 4000 rpm (2240 × g) for 15 min. The supernatant was transferred to clean vials, evaporated under nitrogen at 40 °C, and reconstituted in 1 mL mobile phase before analysis. Separation and quantification were performed on an Agilent 1200 HPLC coupled to a Triple Quad 6410 mass spectrometer using a Kinetex HILIC 100A column (Phenomenex, USA). The mobile phase consisted of acetonitrile (A) with 0.1% formic acid and methanol/water (1:1, v/v) with 100 mM ammonium acetate, adjusted to pH 3.7 with formic acid (B). Isocratic elution (80:20, A:B) was performed at 0.5 mL min−1 with a runtime of 6 min and a 2 min post-run. Mass spectrometry was

operated in positive ion mode using multiple reaction monitoring (MRM), with two transitions per analyte (quantification and confirmation) and stable isotope-labeled internal standards ($D_3$-creatinine, $D_3$-creatine, [^13$C_2$]-GAA) to correct for matrix effects. Chromatographic parameters were optimized using response surface methodology with a Box–Behnken design and Derringer's desirability function. Method validation demonstrated recoveries of 75–102%, intra- and inter-day precision with relative standard deviations below 20%, low limits of quantification (0.025 µg/mL for GAA, 0.006 µg/mL for creatine), and linear calibration curves ($R^2 > 0.99$). This robust and sensitive approach provided a reliable platform for monitoring creatine metabolism in response to supplementation and metabolic interventions, see (Jovanov et al., 2018).

## 1.4 Creatine Supplementation: Dosing and Effects

Since the pioneering study by Roger Harris successfully demonstrated that oral Cr supplementation elevates intramuscular Cr and PCr stores, creatine has quickly gained significant attention in the fields of sports nutrition and exercise physiology (Harris et al., 1992). Around the same time, the 1992 Olympic Games in Barcelona were among the earliest instances where athletes publicly endorsed the benefits of creatine supplementation. In the subsequent years, the first commercial creatine supplements were launched onto the market in 1993, and CrM quickly emerged as one of the most effective and extensively studied ergogenic aids, advocated by more than 10,000 peer-reviewed studies to date (Kreider et al., 2017a). On top of that, creatine remains one of the leading ergogenic supplements on the market, generating more than $400 million in annual sales (Momaya et al., 2015).

Creatine supplements may be available in a variety of forms, including suspensions, capsules, powder, and edible gummies (Kreider et al., 2017b). As a comparison, liquid creatine taken may result in faster absorption kinetics due to a shorter digestion process compared to when taken as regular food,

suspension, or lozenge (Harris et al., 2002). However, while the form of creatine supplements may influence factors such as solubility or convenience, it has a minimal impact on the overall bioavailability and performance compared to the monohydrate powder (Kreider et al., 2022). For instance, administering equal dosages in liquid form of creatine heavily underperforms in comparison to standard CrM powder, in terms of promoting muscle tissue ATP, Cr, and PCr levels. (Kreider et al., 2003). Creatine monohydrate was the first source of creatine ever marketed, and is the most common source of creatine found in supplements today. Because of its well-known physiochemical properties, high efficacy, high bioavailability, low pricing, and safety aspects, monohydrate is considered the gold standard (Escalante et al., 2022; Fazio et al., 2022; Kreider et al., 2022). After ingesting 5 g of creatine, the time to reach maximum plasma concentration ($T_{max}$) is approximately 1.9 ± 0.9 hours, with the concentration peak in plasma ($C_{max}$) of 102.1 ± 11.2 $mg \cdot L^{-1}$ (Persky et al., 2003). The elimination half–life of Cr in plasma is approximately 3 hours, with levels typically returning to baseline within 6 to 8 hours after ingestion (Persky et al., 2003). Despite the development of alternative Cr formulations over time, including creatine citrate, pyruvate, hydrochloride, and ethyl ester, none in the list above demonstrated superior efficacy, safety, and cost compared to CrM (Escalante et al., 2022). Although certain forms may be advantageous due to improved solubility leading to faster plasma peaks, there is no substantial evidence to support a significant advantage in healthy humans when it comes to boosting tissue TCr or exercise performance (Kreider et al., 2022). The typical omnivorous diet provides approximately 1 g of creatine daily, which maintains muscle creatine levels at approximately 60–80% of their maximum saturation. Supplementation enables individuals to increase these stores further, particularly among those with lower baseline tissue creatine levels or athletic individuals with greater lean body mass. In such cases, intramuscular Cr and PCr concentrations may increase by 20–40%, whereas individuals with already high baseline levels tend to experience a slightly lower increase of 10–

20% (Harris et al., 1992; Hultman et al., 1996). Unfortunately, CrM supplementation isn't effective due to a certain proportion of individuals (~20–30%) being classified as "non-responders". In addition, it is important to emphasize that a single dose of creatine taken immediately prior to exercise is unlikely to be beneficial for performance. Creatine must be supplemented daily and consistently over several days in order to induce a significant elevation of intramuscular stores (Hultman et al., 1996; Preen et al., 2003). The most widely adopted supplementation strategy initially designed by Roger Harris, is the high dose loading strategy. The protocol involves consuming approximately 20 to 25 grams of creatine monohydrate (CrM) per day, divided into four to five doses of 5 grams each over 5 to 7 days. This equates to roughly 0.3 grams per kilogram of body weight per day, meaning a 70 kg individual would require about 21 grams of creatine daily (Harris et al., 1992). The main idea behind it is to rapidly saturate intramuscular creatine and phosphocreatine stores, typically reaching maximum saturation within 3 to 5 days. In the subsequent days, a maintenance phase of 3 to 5 grams per day (~0.03 g/kg/day) is recommended to sustain elevated levels of muscle creatine (Hultman et al., 1996). Although it is nowadays accepted that a loading phase is not necessary, the high dose loading protocol remains the fastest way to saturate the skeletal muscle tissue with creatine. Alternatively, a low–dose supplementation protocol typically involves 3 to 5 grams per day (0.03–0.05 g/kg/day) administered consistently over 4 weeks to achieve optimal levels of saturation (Hultman et al., 1996). Such regimen may be equally effective in the long term and may be better suited for individuals prioritizing health–related benefits over immediate performance boost, including elderly or clinical populations (Antonio et al., 2021; Candow et al., 2024; Wallimann et al., 2011). On the other hand, the literature shows the use of moderate–dosing strategies, typically consisting of daily intakes ranging from 6–10 grams (~0.1– 0.14 g/kg/day) for 10 and up to 21 days, again without a distinct loading phase. Both low and moderate dosing strategies resulting in a more gradual increase in intramuscular creatine (Candow et al., 2024;

Willoughby & Rosene, 2003). Regardless of how saturation was accomplished, the intracellular creatine stores naturally return to baseline, approximately 4 to 6 weeks following supplementation cessation (Greenhaff et al., 1993; Hultman et al., 1996) without evidence of consistent adverse effects on health and long–term suppression of endogenous creatine biosynthesis, even after the continuous prolonged creatine supplementation (Kreider et al., 2025).

### 1.4.1 Effects on Athletic Performance

Creatine, particularly the monohydrate formula, has been extensively studied for its effects on lean tissue mass and athletic performance, particularly in anaerobic activities characterized by short–duration and maximal effort such as sprinting, powerlifting, and resistance training (Branch, 2003; Chilibeck et al., 2017; Delpino et al., 2022; Forbes et al., 2021). The primary mechanism underpinning these effects is the elevation of intramuscular PCr stores, which facilitates rapid regeneration of ATP supplies during repeated bouts of high-energy demands (Greenhaff et al., 1993; Kreider et al., 2017a). According to Kreider et al. approximately 70% of studies investigating the efficacy of CrM reported significant improvements in maximal strength, anaerobic power, sprinting performance, and training volume, with effect sizes typically ranging from 5% to 15% (Kreider et al., 2017a). The only consistently reported side effect is a modest increase in body mass (~1 to 2 kg), primarily due to intracellular water retention (Buford et al., 2007). Short-term CrM supplementation, typically involving a 5–7 day loading phase, has been shown to quickly increase muscle Cr and PCr levels, therefore resulting in improved performance in high-intensity, anaerobic, and strength dominant activities. In the study conducted by Law and colleagues, significant increases in back squat strength and anaerobic cycling power was shown after 5 days of CrM loading (20g/day) in recreationally trained men following a structured resistance training program (Law et al., 2009). Similarly, Volek found improvements in bench press and squat performance following 10 days of CrM supplementation (25g/day) in untrained men, even in the absence of an

exercise program, suggesting that increased PCr availability may directly enhance muscular performance (Volek et al., 1997). Furthermore, Greenhaff et al. reported that 5 days of CrM loading (20g/day) substantially increased torque production during maximal isokinetic knee extensions (Greenhaff et al., 1993). However, effects of short-term creatine supplementation are somewhat equivocal, especially as the baseline muscle creatine levels, daily physical activity, or methodology used to analyze performance varies across the studies. For instance, Jakobi et al. reported no beneficial effects of a short term CrM supplementation (20g/day for 5 days) on muscle isometric force, muscle activation, time to fatigue and or recovery (Jakobi et al., 2000). In another study on professional cyclists, short-term creatine supplementation (20g/day for 6 days) showed no consistent positive effects on recovery, lean tissue mass or performance metrics (Barranco-Gil et al., 2024).

However, when long–term supplementation strategies are employed, such as administering 3–5 g/day for periods longer than 4 weeks, CrM tends to consistently produce ergogenic benefits, especially when combined with resistance training (Forbes et al., 2021; Lanhers et al., 2015, 2017; Rawson & Volek, 2003). Based on 22 studies, Rawson et al. reported that CrM supplementation resulted in an average increase of 8% in muscular strength and 14% in muscular endurance compared to group doing resistance training alone (Rawson & Volek, 2003). The meta-analyses conducted by Lanhers et al. corroborates with these findings, reporting significant improvements with effect sizes (ES) of 0.336, 0.297 0.266 for back squat and leg press, and lower-body strength, respectively (Lanhers et al., 2015). In the follow-up study on the upper body strength, significant improvements were observed in bench press (ES = 0.265), chest press (ES = 0.677), and an overall upper-body ES of 0.317 (Lanhers et al., 2017). Furthermore, Branch et al., found CrM supplementation to be effective on high-intensity anaerobic activities not exceeding 30 seconds in duration, while no positive effect of supplementation was found in activities beyond 3 minutes. Similarly, based off 13 eligible studies included in the most recent meta-analysis,

CrM supplementation did not show beneficial effects on aerobic performance in trained healthy populations, regardless of the supplementation protocol used (Branch, 2003; Fernández-Landa et al., 2023). However, Forbes highlights the ability of CrM to support aerobic activities where all out end-spurts are critical for performance and can decide the winner (Forbes et al., 2023).

### 1.4.2 Effects on Cognitive Performance

The role of creatine extends well beyond the skeletal muscle into brain energy metabolism, where it helps maintain the ATP levels, especially under energy–challenging tasks or stressful environments (Dolan et al., 2019). Brain effectively accumulates creatine from both endogenous and exogenous sources in concentrations several fold higher than in circulation, however, due to limited permeability of BBB, supplementation has a limited effect on increasing the brain creatine levels, with short–term regimens using lower doses (< 5 g/day) often reporting insignificant improvements (Candow et al., 2024b; Rawson & Venezia, 2011; Wilkinson et al., 2006). To overcome the limited uptake of creatine across the BBB and the independent ability of the brain to endogenously synthesize creatine, higher dosages of creatine over prolonged periods are often recommended. Accordingly, evidence shows that supplementing 0.03g/kg (≥ 20 g/day) of CrM elevates total cerebral creatine content by 3–10%, depending on several factors including the baseline levels, dosing strategies employed (Dechent et al., 1999; Lyo et al., 2003; Turner et al., 2015). Regardless, the question remains whether the increase translates into a significant boost in cognitive performance. This is especially ambiguous in young, healthy individuals in non-stressed conditions, where effects CrM supplementation shows mixed results, ranging from limited improvements in some aspects of cognition to no benefits at all. According to Rawson et al., supplementing CrM (0.03 g/kg/day; ~ 2.2 g/day) for 6 weeks in 11 healthy young adults (aged 21.0 ± 2.1 years) did not improve components of cognitive processing (code substitution, logical reasoning, math processing, running memory, memory recall) (Rawson et al., 2008). Similarly, no

improvements on executive function and verbal learning were reported after one week of CrM supplementation (0.3 g/kg/day for 7 days) in 67 young adolescents (aged 10-12 years) (Merege-Filho et al., 2017). Nonetheless, creatine levels in several brain regions, including the left dorsolateral prefrontal cortex, hippocampus, and occipital lobe, remained unchanged after supplementation. Alternatively, McMorris et al. showed positive effects on cognition in 32 healthy men and women (aged 68–85 years), reporting significant improvements in memory (recall and long-term memory tasks) after administering 20 grams of CrM per day for 7 days (Rawson et al., 2008). On the other hand, when under stressed conditions, current evidence shows that even a low-dose supplementation (8 g/day for 5 days) reduced cerebral oxygenated hemoglobin desaturation and decreased mental fatigue during repeated calculation tasks, indicating more efficient oxygen utilization (Watanabe et al., 2002). In case of mild and acute hypoxia (10% $O_2$ for 90 min), supplementing healthy adults with 20g of CrM for 7 days resulted in ~ 9% increase in total brain creatine, while also maintaining levels of attention and preventing the hypoxia-induced declines in cognitive task performance compared to placebo (Turner et al., 2015). Furthermore, following a challenging mental task (~ 90 minutes), CrM supplementation (20 g/day) did not attenuate fatigue – induced decline in short sport-specific psychomotor and cognitive task performance in 14 healthy adults. Nonetheless, it did lead to improved executive function assessed by a prolonged version of the Stroop accuracy test (van Cutsem et al., 2020).

Sleep deprivation is another energy–demanding condition where CrM supplementation may be effective. In a recent study by Gordji-Nejad et al., 15 healthy young adults received a single high oral dose of CrM (0.35 g/kg) during sub–total 21-hour sleep deprivation. A single dose of creatine significantly attenuated declines in PCr and pH levels, while enhancing ATP metabolism and cognitive performance and processing speed (Gordji-Nejad et al., 2024). In another study, a total of 20 healthy young adults underwent 24 hours of sleep deprivation while receiving CrM supplementation (20 g/day) for one week.

The creatine group significantly improved performance compared to placebo, including random movement generation, verbal and spatial memory, choice reaction time, static balance, and mood regulation (McMorris et al., 2006). Building upon these findings, the authors then examined the short–term effects of a 7-day CrM supplementation protocol on cognitive functions after 18, 24, and 36 hours of sleep deprivation on the same group of subjects. Unfortunately, no improvements in executive function, short-term memory, choice reaction time, balance, or mood were found at 18, 24, and 36 hours of wakefulness; with only the creatine group demonstrating superior performance on a random number generation task (McMorris et al., 2007). In a cohort of elite male rugby players, the authors investigated the acute effects of CrM administered 1.5 hours at two dosages (50 mg/kg and 100 mg/kg) before performing the passing accuracy test following normal sleep (7–9 hours) versus restricted (3–5 hours) sleep. CrM supplementation attenuated the decline in performance associated with sleep deprivation, causing sleep deprived athletes to maintain significantly better execution of accuracy related skills (Cook et al., 2011).

Since dietary creatine intake differs between omnivores and non-meat eaters, it has been hypothesized that vegetarians may experience greater benefits from supplementation (Avgerinos et al., 2018). To that end, Benton and Donohoe conducted a double-blind, placebo-controlled study on 128 young healthy females (67 vegetarians, 61 omnivores), orally consuming 20 g/day of CrM for 5 consecutive days. The creatine group improved memory performance in vegetarians, while no effect was found in the group of omnivores (Benton & Donohoe, 2011). In addition, they did not report improvements in verbal fluency or vigilance tasks, while reaction times variability decreased in both omnivores and vegetarians. Another large double-blind, placebo-controlled crossover trial (n = 123, ~ 50% vegetarians) investigated effects of 6 weeks of CrM supplementation (5 g/day) on cognitive performance, reporting small, yet significant, improvements in working memory. Additionally, side effects were more frequently reported in the CrM

group, while no differences in performance were observed between the vegetarians and omnivores (Sandkühler et al., 2023).

While an increasing number of studies focuses on cognitive effects of CrM supplementation, the results remain equivocal. Approximately, supplementation regimens administered ~ 2.2 – 20g/day and lasting 5 days up to 24 weeks, reporting only modest improvements in short-term memory, intelligence, and reasoning tasks (Avgerinos et al., 2018; Prokopidis et al., 2023). In the most recent systematic review by Morris et al., the evidence suggests that while CrM elevates the cerebral creatine content, it often fail to show consistent significant cognitive improvements in young non–stressed healthy individuals (McMorris et al., 2024). Nevertheless, CrM supplementation seems to be particularly potent under energy–challenging conditions due to e.g., aging, unbalanced diet, hypoxia, sleep deprivation, or prolonged physical and mental efforts, which essentially served as a rationale for original studies shown in Chapters II and III, respectively.

## 1.5 Guanidinoacetic acid (GAA) Supplementation

Being a direct creatine precursor, GAA (also known as guanidinoacetate, glycocyamine, and betacyamine) is the essential compound in the homeostasis of creatine metabolism (Davenport et al., 1938). Naturally, GAA can be supplied via endogenous synthesis and regular food intake, with the total GAA output directly being synthesized into equimolar amounts of creatine, at the expense of methyl groups and homocysteine as a byproduct (Stead et al., 2001). Mainly produced in the human kidney and pancreas, but also the brain, liver, endocrine tissues, and skin, approximately 1 gram of endogenously synthesized GAA is being methylated to creatine every day (Edison et al., 2007). On average, dietary food intake of GAA in U.S. adults is estimated to be ~10 mg per day, suggesting that diet provides only a small portion of GAA, and that de novo synthesis essentially secures the majority of the daily requirements (Ostojic, Stajer, et al., 2022; Ostojic & Jorga, 2023). Very similar to creatine, meat–based

products contain the highest relative amounts of GAA (~50 mg per kg), followed by dairy products (~0.3 mg per kg), while amounts found in plant–based foods are almost negligible (~1 μg per kg). Other viable sources of GAA include dietary supplements, with several products available on the market providing up to 1000 mg of GAA per serving, predominantly in the form of powder (80.5%), caplets (15.9%), and liquid (3.7%). These products often combine GAA with Cr and/or other amino acids, varying in total amounts from 72.5 mg to 38.0 g per serving, which is a substantially higher source of GAA as compared to traditional foods (Ostojic, Ratgeber, et al., 2022). As a reference, consuming meat products such as pork and poultry, renders to up to 160 mg of GAA per kilogram, with the average intake on a regular diet appears to be ~10 mg per day (Ostojic, Stajer, et al., 2022). Alternatively, dietary supplementation can deliver up to 1000 mg of GAA per serving, essentially providing up to two orders of magnitude more GAA than in a regular omnivore diet (Ostojic, 2022; Ostojic, Ratgeber, et al., 2022).

In comparison to creatine, the GAA is more bioavailable, stable, and cost-effective, which puts it in a position at least equally competent for human nutrition use due to several reasons. The GAA is more soluble in water and a highly stable compound, while Cr quickly converts into creatinine upon contact with water at elevated temperatures and low pH (Wyss & Kaddurah-Daouk, 2000). Additionally, the cost of GAA is approximately 40% cheaper than CrM powder (Ostojic, Ratgeber, et al., 2022). In the pioneering animal studies, it was demonstrated that administering 1 g of GAA to young rats resulted in a 48.5% increase in skeletal muscle creatine, greatly surpassing the increase observed after administering the equivalent dose of creatine (Beard & Barnes, 1931). Due to its demonstrated efficacy to augment creatine levels, GAA was quickly approved for use at recommended dosages of 600–800 mg/kg in animal feed by the European Food Safety Authority (“Safety and Efficacy of Guanidinoacetic Acid for Chickens for Fattening, Breeder Hens and Roosters, and Pigs,” 2016). These findings quickly positioned GAA as an effective feed additive for livestock, contributing to enhanced growth, improved feed

conversion, and elevated muscle creatine concentrations in poultry and swine (Eckhardt et al., 2024; Majdeddin et al., 2023). Unfortunately, despite the earliest evidence of human use of supplemental GAA being first introduced approximately more than sixty years ago, research on its bioavailability, safety, and effects on human performance and health outcomes remained poorly explored compared to creatine supplementation (Ostojic, Ostojic, et al., 2016; Ostojic & Ostojic, 2018; Ostojic & Vojvodic-Ostojic, 2015; Semeredi et al., 2019).

### 1.5.1 Metabolic Effects

Over the years, scientific community has gradually started to investigate the potential metabolic and ergogenic effects of GAA supplementation in young healthy populations. So far, several studies have been able to demonstrate that orally administered GAA (up to 4.8 g/day for 6 weeks) in healthy adults significantly upregulated serum and urinary levels of GAA, Cr and Crn, alongside the serum homocysteine levels (Ostojic et al., 2013b), even after a single oral administration (Ostojic & Vojvodic-Ostojic, 2015). Studies show that after orally administering GAA in three different doses, peak in plasma creatine occurs in < 2 hours following a dose-dependent manner, with doses of 1.2 g, 2.4 g, and 4.8 g of GAA elevating creatine concentrations by approximately 80%, 116%, and 293%, respectively (Ostojic & Vojvodic-Ostojic, 2015). Regarding tissue concentrations, studies showed that 12 weeks of GAA supplementation (2.4g/day) caused GAA to undergo rapid conversion to creatine, thus boosting muscle tissue creatine concentrations by 36% (Ostojic, Niess, et al., 2014). Furthermore, administering 36 mg/kg of GAA (~3g/day) to young healthy adults over a period of 4 weeks yielded significantly superior improvements on muscle and brain bioenergetics than equimolar doses of creatine (~3.4g/day) alone. The addition of GAA to creatine has been shown to elevate creatine levels in both gray matter and skeletal muscle, increasing creatine levels by 14.9% in muscle and 4.4% brain tissue compared to administering creatine alone (Ostojic, Drid, et al., 2016). These effects may be partly attributed to its

versatile transportability, as GAA seems to take advantage of multiple membrane transporters in order to reach target tissues and undergo methylation (Stead et al., 2001; Tachikawa et al., 2012). Furthermore, GAA's ability to engage with the multiple transporters may have additional beneficial effects indirectly related to creatine synthesis. Activation of the SLC6A6 transporter can induce metabolic effects of taurine, such as increasing buffering capacity and maintaining redox balance, while activation of the SLC6A13 GABA transporter inhibits the Na++K+-ATPase, thereby conserving the ATP reserves within the cell (Ostojic et al., 2017). Additional evidence shows that GAA has other physiological roles, acting as potent modulator of insulin, including the stimulation of hormonal release and neuromodulation, an alteration of metabolic utilization of arginine, for a review, see (Ostojic, 2015).

### 1.5.2 Effects on Health and Performance Outcomes

Going back to human trials, the first studies investigating the effects of supplemental GAA on performance and health outcomes date back before the expansion of research on creatine supplementation. The pioneering study on the effects of supplementary GAA in humans emerged in the early 1950s when a group from Caltech and the US Naval School of Aviation Medicine supplemented GAA (~5 g/day) in combination with betaine to patients with cardiovascular and neuromuscular disorders with the interventions lasting one to up to twelve months (Borsook & Borsook, 1951; Graybiel & Patterson, 1951; van Zandt & Borsook, 1951). The majority of patients had improved health outcomes, including a subjective sense of well-being, experienced less fatigue, and had positive clinician-reported outcomes. Over the subsequent years, the clinical effectiveness of GAA supplementation was studied across different populations, including patients diagnosed with arthritis, acute and chronic poliomyelitis, anxiety and depression and motor-neuron disease (Aldes, 1957; Basom & Leonard, 1955; M. E. Borsook et al., 1962; Borsook & Borsook, 1951; Dixon et al., 1954; Fallis, 1952; Graybiel & Patterson, 1951; Higgins et al., 1952; Liveksedge, 1956; van Zandt & Borsook, 1951; Watkins, 1953). Since then, the

research from early 20th century continued exploring other applications of supplemental GAA, including neuromuscular disease and genetic disorders of Cr biosynthesis (Stöckler et al., 1996), chronic renal failure (Tsubakihara et al., 1999) and chronic fatigue syndrome (Ostojic, Stojanovic, et al., 2016). Interestingly, Borsook and colleagues were among the first to hypothesize that GAA could offer greater efficacy in restoring creatine levels than creatine alone. However, most of the studies from the 1950s had several limitations to look for, including small sample size, lack of blinding, absence of a control group, and no objective performance metrics. For a detailed summary of all studies, please refer to work by Ostojic (Ostojic, 2015).

More interestingly, in a series of studies over the last two decades Ostojic and colleagues attempted to address the potential novel applications and safety of dietary interventions using either GAA alone or in combination with CrM. In a cohort of 48 healthy men and women (aged 20–25 years), GAA (1.2g, 2.4 or 4.8 g/day) was orally administered over a period of 6 weeks. The results showed that even at low doses (1.2g/day), GAA supplementation significantly improved upper body strength performance up to ~25% compared to placebo. However, no significant effect on body composition, aerobic or anaerobic performance, and dose-response differences were found (Ostojic et al., 2015). In another human trial, Ostojic et al. compared the efficacy of CrM (3g/day) and GAA (3g/day) supplementation over 4 weeks and found that 3 g/day of GAA led to significantly higher boost (up to 16.2%) in tissue creatine levels in vastus medialis muscle, middle-cerebellar peduncle, and paracentral grey matter; whereas CrM failed to achieve similar effects (Ostojic, Ostojic, et al., 2016). Co–administration of creatine and GAA has recently emerged as a promising strategy to target tissue bioenergetics, as their complementary properties may facilitate the mixture to target the energy–demanding tissues more effectively than conventional supplementation strategies. In another superiority trial on 14 healthy young adults, supplementing with GAA and CrM in a 1:3 ratio for 4 weeks was shown superior to 4 g/day CrM in boosting brain and

muscle creatine levels. Specifically, the creatine-GAA mixture was significantly superior compared to CrM, increasing creatine levels in skeletal muscle (16.9% vs. 2.0%) and grey matter of the brain (5.8% vs. 1.5%), in addition to improved bench press performance (6.0% vs. 5.1%) (Semeredi et al., 2019). In a randomized crossover trial, 19 healthy young adults orally administered 4 grams of the CrM–GAA mixture (2g/day of CrM + 2 g of GAA/day) or a placebo for 7 days. Oxygen saturation and level of total hemoglobin in the prefrontal cortex were measured at rest, during meditation, a cognitive task, and recovery. Compared to placebo, the CrM–GAA combination significantly improved oxygen saturation during rest, meditation, and recovery phases, suggesting upregulated cerebral oxygenation (Zanini et al., 2025). In another randomized crossover trial conducted on 21 healthy individuals ≥ 65 years of age, orally administered the GAA-creatine mixture (2g/day of CrM and 2g/day of GAA) for 8 weeks. The mixture significantly increased brain and muscle creatine levels from baseline, measures of functional performance, however, no improvements on measures of cognitive function and aspects of quality of life (Seper et al., 2021). This approach may especially be of interest when targeting the brain and skeletal muscle tissue, since supplementing GAA has superior creatine restoring effect versus CrM ingestion alone. However, one should be exogenous GAA has raised several safety concerns due to elevated fasting homocysteine levels, an independent risk factor for neurodegenerative and cardiovascular diseases (Ostojic, Niess, et al., 2014).

### 1.5.3 Dosing and Safety

Early evidence suggested that chronic GAA administration might negatively affect the availability of key methyl donors such as methionine, folate, choline, and B–complex vitamins (Borsook & Borsook, 1951; Obeid, 2013). However, available evidence does not entirely support this notion. Ostojic et al. found that daily GAA doses up to 4.8 g for six weeks did not significantly alter serum concentrations of folate, vitamin B6, or B12, similarly, in a 12-week trial using 3 g/day (Ostojic, Stojanovic, et al., 2014).

Furthermore, no adverse changes in global DNA methylation were reported, suggesting that the endogenous methylation capacity remains intact under moderate GAA dosing (Ostojic, Mojsin, et al., 2018). On the other hand, several studies have shown that supplemental GAA may increase circulating homocysteine formation. However, the observed increase in Hcy was clinically insignificant, and could be partially corrected by coadministration of methyl donors (Ostojic et al., 2013a). A single orally administered dose of 2.4 g GAA is followed by an increase in fasting serum total homocysteine by ~ 40%. Fortunately, the effects seem to be acute, with the peak of concentrations at the 12-hour mark and returning to baseline values 24 hours later. However, even at its peak ~ 13.1 ± 2.1 μmol/L, the levels observed were not exceeding 15 μmol/L, an upper limit of clinically established hyperhomocysteinemia (Ostojic & Vojvodic-Ostojic, 2015). In a dose-response study by Ostojic et al., daily GAA intakes of 1.2 g, 2.4 g, and 4.8 g over six weeks produced mean Hcy increases of 1.4, 2.6, and 6.6 μmol/L, respectively, with 25% of participants in the group ingesting 4.8g of GAA exceeding the clinical threshold (>15.0 μmol/L) for hyperhomocysteinemia (Ostojic, Stojanovic, et al., 2014). In another study, 3 g of GAA per day for 10 weeks led to a mean increase in total Hcy of 73.5% (5.0 μmol/L), with 20% of subjects showing clinically elevated concentrations (Ostojic, Trivic, et al., 2018). However, elevation of Hcy was not accompanied by cardiometabolic or inflammatory biomarkers and quickly returned to baseline values (Ostojic et al., 2018b; Ostojic et al., 2021). To address this, concurrent intake of methyl donors has been shown as an effective mitigation strategy. In a series of studies, Ostojic and colleagues demonstrated that combining 2.4 g/day of GAA with betaine, folic acid, and vitamins B6 and B12 fully prevented the development of hyperhomocysteinemia in healthy adults in contrast, when an equal dose of GAA was administered without a methyl donor, more than 55% of the participants had Hcy levels in the range of clinical hyperhomocysteinemia (Ostojic et al., 2013a). Furthermore, besides the addition of methyl donors, CrM was also shown to exert a protective effect against elevated Hcy levels, suggesting

that the homocysteine response is both predictable and modifiable by either adding creatine or methyl donors to the mixture (Farkas et al., 2024; Ostojic et al., 2021; Semeredi et al., 2019). In the most recent open label study, 0.5g/day of GAA supplementation (increased by 0.1g/day per week up to 1 g/day) has shown a favorable safety profile in a cohort of twelve healthy young men without significant changes in Hcy throughout 17 weeks of supplementation. Furthermore, individual variations in THcy were falling within the range of biological variability (~25%) in 83.3% of the subjects (Todorovic et al., 2025). In another study, Ranisavljev et al. conducted a post-marketing surveillance study and found no adverse effects on health after 6 months of creatine-GAA supplementation (1.5g of GAA and 1.5g/day of CrM) in physically active young men and women. Out of 38 participants (aged 21–40 years) included in the study, only 5% reportedly experienced transient gastrointestinal disturbances, while levels of homocysteine significantly lowered, even getting below the study baseline levels (Ranisavljev et al., 2024). In another 12-week open-label study, eight healthy adults administered escalating daily doses of CrM and GAA in the mixture (1g, 2g and 3g each). Plasma total Hcy levels remained stable throughout the study, with no cases of hyperhomocysteinemia (>15 μmol/L) or any adverse effects reported. These findings are consistent with earlier studies indicating that creatine–GAA supplementation may not result in hyperhomocysteinemia, potentially through mechanisms involving modulation of endogenous creatine biosynthesis or the provision of methyl group donors. Collectively, GAA supplementation at doses up to 3 g/day may be considered safe and well tolerated in healthy individuals, particularly when supported by coadministration of methyl donors or creatine (Farkas et al., 2024).

## 1.6 Research Framework

This dissertation aims to advance our understanding of creatine metabolism by synthesizing existing evidence with original experimental data collected from healthy young adults. This dissertation is essentially structured around two series of original investigations designed to address different aspects that affect the homeostasis of creatine metabolism, particularly under physiologically stressful or energy–challenging conditions. The core experimental work is presented in Chapters II and III, which together represent the central axis of this dissertation. Briefly, Chapter II comprises a series of studies investigating creatine homeostasis in response to acute physiological stress. This was accomplished by examining differences in changes in circulating biomarkers before and after exposure to multiple physiological demands, providing insight into systemic responses and regulatory mechanisms. Building on these findings, Chapter III presents three intervention trials evaluating the efficacy of alternative creatine formulations. These studies focused on exploring their capacity to enhance tissue creatine levels and potential effects on physical or cognitive performance outcomes, offering practical insights into alternative supplementation strategies. Additional study articles published by the candidate are included in Chapter V. While these studies are not directly related to the dissertation topic, they reflect the broader scientific engagement and independent research activity undertaken throughout the course of doctoral training.

# CHAPTER II: ADAPTATIONS OF CREATINE HOMEOSTASIS AND METABOLISM IN RESPONSE TO DIFFERENT STRESS INTERVENTIONS

## 2.0 Chapter II Overview

This chapter presents the first of two series of investigations exploring the adaptations of creatine homeostasis in response to physiological stimuli by employing three quasi–experimental studies in a sample of healthy young men and women. By synthesizing evidence in this chapter, this dissertation aims to contribute to our understanding of creatine homeostasis by analyzing changes in biomarkers of creatine metabolism in both salivary and blood samples after three different interventions of varying physiological stimuli. Accordingly, we analyzed the changes in guanidinoacetic acid, creatine, and creatinine indices at baseline and following three energy–demanding tasks: (1) short-term fasting, (2) competitive video gaming, and (3) running to exhaustion interventions. We hypothesized that immediately after the subjects complete the designated intervention, a significant change in the concentration of circulating biomarkers of creatine metabolism from baseline would be detected.

Chapter II is in full based on the previously peer-reviewed published articles:

2.1 Ranisavljev, M., Todorovic, N., Panic, J., Andjelic, B., Vranes, M., & Ostojic, S. M. (2023). Short-term fasting affects biomarkers of creatine metabolism in healthy men and women. Human Nutrition & Metabolism, 34, 200217.

2.2 Andjelic, B., Todorovic, N., Panic, J., Vranes, M., & Ostojic, S. (2024). Video Gaming Reduces Circulating Creatine Levels in Young Male E-Gamers. Journal Of Endocrinology And Metabolism, 14(1), 59-62.

2.3 Andjelic, B., Lainovic, T., Todorovic, N., Panic, J., Vranes, M., Stajer, V., & Ostojic, S. M. (2025). Exercise affects salivary biomarkers of creatine metabolism in healthy adults. Sports Medicine and Health Science.

## 2.1 “Short-term fasting affects biomarkers of creatine metabolism in healthy men and women”

### Introduction

Creatine is considered conditionally essential nutrient, having vital role in cellular energy utilization and transfer across the human body (Ostojic & Forbes, 2022). It can be synthesized endogenously by humans and obtained through an omnivorous diet. The breakdown of creatine involves a non-enzymatic conversion to creatinine, which is excreted by the kidneys as a waste product (Wyss & Kaddurah-Daouk, 2000). However, routine measurement of creatine turnover is not commonly performed in healthy populations. In healthy adults, plasma levels of creatine range from 12.3 to 99.1 μmol/L, while guanidinoacetic acid (GAA), a direct biosynthetic precursor of creatine, and creatinine levels range from 1.2 to 3.6 μmol/L and 53.0–114.9 μmol/L, respectively (Joncquel-Chevalier Curt et al., 2013). It has been observed that meat-free diets result in lower concentrations of creatine indices (Delanghe et al., 1989), while chronic food deprivation (*e.g.*, anorexia nervosa) could diminish creatinine clearance (Boag et al., 1985; Delanaye et al., 2008; Downey et al., 2022). Interestingly, there is limited information available on whether creatine status (serum creatine in particular) is influenced by other dietary patterns in humans, including acute fasting. Previous animal studies have presented conflicting findings, with some indicating that creatine levels remain relatively stable during fasting (Chanutin & Shearer, 1931), while others suggest a significant increase in creatine levels during fasting periods (Walker, 1979), making this a topic of debate. To date, no human studies have investigated a potential cause-and-effect relationship between fasting and creatine biomarkers. In this preliminary study, we aimed to assess the effects of a 24-h fasting period on serum levels of GAA, creatine, and creatinine in non-vegetarian healthy men and women.

## Methods

A total of twenty-four (n = 24) healthy young adults, with a mean age of 23.5 ± 3.3 years and a body mass index of 25.1 ± 2.5 kg/m2, including 12 females, volunteered to participate in this non-randomized quasi-experimental before-after study. The appropriate sample size was calculated using power analysis (effect size 0.5, alpha error probability 0.05, power 0.80) for the primary outcome measure, which was the fasting-induced increase in serum creatine (G-Power 3, Heinrich Heine University Düsseldorf, Germany), with the sample size in accordance with previous short-term interventional study (Stajer et al., 2016). All participants were non-vegetarian, without any acute or chronic disorders, and had abstained from consuming any dietary supplements containing creatine for at least four weeks prior to the start of the study. Written informed consent was obtained from all participants, and the study was approved by the local Institutional Review Board at the University of Novi Sad, in accordance with the principles of the Declaration of Helsinki.

During the study, participants underwent a single 24-h fasting session, during which they were restricted from consuming any calorie-containing foods and drinks. To minimize the risk of dehydration, participants were instructed to consume at least 1.5 L of water during the fasting session. They were also instructed not to engage in any planned and structured physical activity (including exhaustive exercise) 24 h prior to and during the monitoring period. Body weight and blood biomarkers were assessed at baseline (after an overnight fast) to ensure accurate pre-intervention readings, and following the 24-h fasting period. Laboratory tests were conducted between 07:30 and 10:00. Body weight was measured using a digital scale (Omron BF508, Tokyo, Japan). Venous blood samples were analyzed for hemoglobin (Hb) and hematocrit (HCT) using an automated analyzer (Hitachi, Tokyo, Japan), and serum samples were analyzed for GAA, creatine, and creatinine using modified liquid chromatography–tandem mass spectrometry (Agilent 1200 Series LC System, Agilent Technologies Inc., Santa Clara, CA, USA)

(Jovanov et al., 2018). Baseline and follow-up values for Hb and HCT were used to correct creatine biomarkers for changes in plasma volume after fasting, as previously described (Matomäki et al., 2018).

## Results

The baseline values for the study outcomes are presented in Table 2.1 Most participants had serum GAA, creatine, and creatinine levels within the reference ranges specific to their gender. However, one female participant (age 25 years, weight 62.5 kg) had low serum creatine levels of 7.3 μmol/L, and two females had elevated serum creatinine levels (147.3 μmol/L in a 23-year-old woman weighing 68.6 kg, and 106.6 μmol/L in a 22-year-old woman weighing 64.4 kg).

*Table 1. Baseline characteristics of the study sample. Values are mean ± SD.*

| | Women ($n$ = 12) | Men ($n$ = 12) | Total ($n$ = 24) |
|---|---|---|---|
| Age, years | 22.4 ± 2.2 | 24.5 ± 3.9 | 23.5 ± 3.3 |
| Weight, kg | 70.2 ± 6.5 23.9 ± 2.2 | 88.8 ± 11.4 | 79.5 ± 13.1 |
| Body mass index, kg/m$^2$ | 23.9 ± 2.2 | 26.3 ± 2.4 | 25.1 ± 2.5 |
| Hemoglobin, g/L | 130.4 ± 9.0 | 150.5 ± 11.9 | 140.5 ± 14.6 |
| Hematocrit, % | 40.1 ± 2.2 | 46.1 ± 3.2 | 43.1 ± 4.1 |
| GAA, μmol/L | 2.0 ± 0.5 | 2.4 ± 0.6 | 2.2 ± 0.6 |
| Creatine, μmol/L | 26.6 ± 8.4 | 23.4 ± 3.5 | 25.1 ± 6.6 |
| Creatinine, μmol/L | 90.2 ± 21.1 | 76.8 ± 11.9 | 83.8 ± 18.2 |

Fig. 2.1 illustrates the percentage changes observed in the study outcomes within the sample. The average change in plasma volume used for adjusting GAA, creatine, and creatinine follow-up concentrations was − 0.57% (95% confidence interval, − 1.29 to 0.15). A 24-h fasting session led to a significant decrease in serum GAA levels (from 2.2 ± 0.6 μmol/L at baseline to 1.3 ± 0.4 μmol/L at follow-up; $p < 0.001$), with a large effect size (d = 1.77). Serum creatinine showed a significant increase after fasting across the entire sample (from 83.8 ± 18.2 μmol/L to 94.9 ± 22.0 μmol/L; $p < 0.01$), with a moderate effect size (d = 0.54). In the follow-up, two males and five females had creatinine levels above

the reference values (>114.9 μmol/L for men and >97.2 μmol/L for women). Serum creatine levels remained unaffected by the fasting session (25.1 ± 6.6 μmol/L at baseline vs. 23.4 ± 6.7 μmol/L at follow-up; $p = 0.29$). Gender-specific analysis largely confirmed the aforementioned findings regarding changes in creatine biomarkers across the entire sample, except for serum creatinine, which remained stable after fasting in the female subgroup (90.2 ± 21.1 μmol/L at baseline vs. 93.5 ± 25.4 μmol/L at follow-up; $p = 0.10$). Moreover, all participants (100%) exhibited a reduction in serum GAA levels at follow-up, while serum creatine was lower in approximately three out of five participants (57.9%), and creatinine increased in about nine out of ten participants (89.5%).

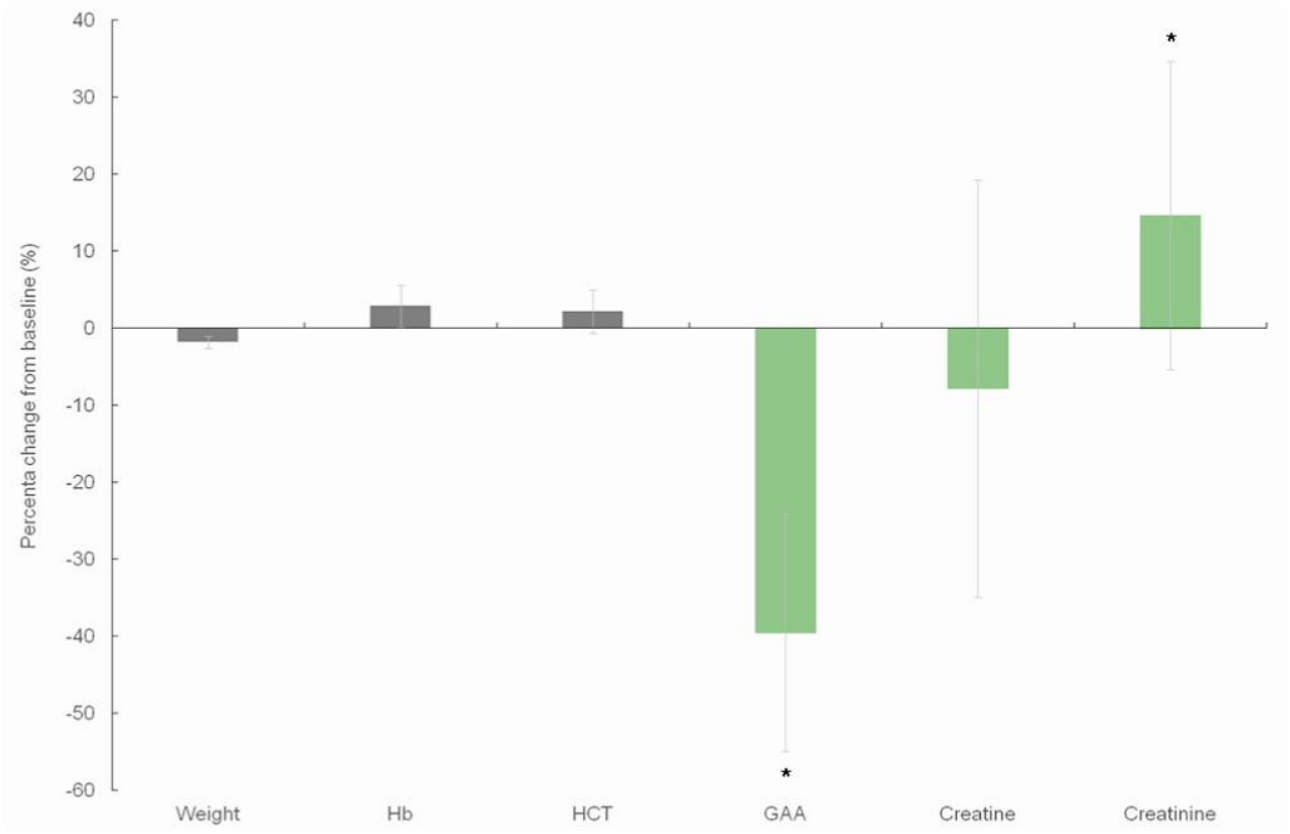



*Figure 2. The percent change of study outcomes following a 24-h fasting session; error bars represent SD. Asterisk (*) indicates significant change from baseline values at P < 0.05.*

## Discussion

Our study revealed significant effects of short-term fasting on specific biomarkers related to creatine metabolism in healthy men and women. It was observed that the balance between creatine precursor (GAA) and breakdown product (creatinine) is responsive to food abstention, while the concentration of creatine itself remains unchanged. Notably, even after accounting for changes in plasma volume induced by fasting, the levels of GAA and creatinine remained significantly altered. This suggests a distinct role of these compounds in adapting to a 24-h fasting period.

Only a limited number of studies have examined the effects of fasting and starvation on creatine status, and the findings have been somewhat contradictory. In a seminal study by Myers & Fine, it was observed that the creatine content in rabbit muscle increased during the early stages of starvation but decreased towards the end of the 6 to 27-day period of starvation (Myers & Fine, 1913). Similarly, both animal and human studies have shown that short-term fasting or an inadequate diet can lead to increased blood and urine levels of creatine, resulting in creatinemia and creatinuria (Walker, 1979). Another experiment conducted on mice confirmed that 24-h fasting increased plasma creatine specific activity, indicating that fasting can influence the entry of creatine into target tissues (Kim et al., 1983). However, a 4-day period of starvation in adult male Sprague-Dawley rats led to an increase in total myocellular creatine levels, while other indicators of creatine metabolism, such as GAA, creatine kinase, creatine transporter expression, and guanidinoacetate *N*-methyltransferase, remained unaffected (Zhao et al., 2002). Furthermore, a clinical study showed elevated creatine levels in decompensated heart patients undergoing prolonged fasting treatment (Feruglio & Toso, 1958). On the contrary, Chanutin & Shearer reported that the percentage of creatine concentration in the whole organic white rat remained remarkably stable throughout a period of food restriction lasting up to 19 days (Chanutin & Shearer, 1931). Our study findings differ from previous research as we observed a trend of lower serum creatine

levels after a 24-h fast in healthy adults, although the reduction was not statistically significant. Fasting, being an energy-demanding condition (Wang & Wu, 2022), could potentially strain high-energy phosphate pathways, leading to a decrease in circulating creatine levels. However, creatine levels remained relatively stable, possibly due to an increased internal utilization of GAA to produce creatine. This is supported by a notable drop (39.5%) in serum GAA levels observed after fasting. It is possible that circulating GAA plays a role in maintaining creatine levels during fasting by serving as an immediately available precursor for creatine synthesis, which supports its buffering function in cellular energy metabolism. Discrepancies among studies regarding creatine dynamics after fasting may be attributed to various factors, including differences in fasting protocols, species-specific variations in creatine metabolism, and the lack of plasma volume correction for creatine concentrations observed in most studies. The changes in serum GAA and creatinine observed in our study may also be explained by concurrent changes in renal function induced by fasting. The kidneys, which are key organs involved in creatine metabolism (Wyss & Kaddurah-Daouk, 2000), undergo metabolic and vascular adaptations during fasting, including impaired function of the renal cortex and decreased glomerular filtration rate (Amlal et al., 2001; Rapoport et al., 1965). Since GAA is primarily synthesized by the renal tubules located in the cortex (Takeda et al., 1992), a reduction in cortical function during fasting is likely to decrease GAA production, as evidenced by the significant decrease in serum GAA levels observed in our study. Similarly, a mild-to-moderate increase in serum creatinine (14.7%) observed may indicate a decrease in glomerular filtration rate after fasting, as previously described (Mascioli et al., 1984). Interestingly, GAA dynamics appear to be particularly responsive to fasting, as all participants experienced a distinct decrease in serum GAA levels, ranging from 6.7% to 66.8%, perhaps advancing GAA as an innovative indicator of kidney stress in food restriction.

While gender has been found to impact creatine status through various mechanisms (Muccini et al., 2021), no studies have investigated the effect of fasting on creatine homeostasis while considering gender-specific responses. Interestingly, we observed a difference between men and women in their serum creatinine response, as the levels remained relatively stable in the female subgroup despite a 24-h fasting session. The underlying mechanism is not yet understood, but it could potentially involve a lesser degradation of creatine to creatinine in fasted women, possibly due to a more favorable acid-base balance (Worcester et al., 2018). Creatine tends to be favored at higher pH levels, while creatinine is favored in acidic environments. Further investigation is warranted to explore fasting-induced changes in creatinine status in women. It is noteworthy that the baseline serum creatinine levels in the female subgroup of our study were already at the upper end of the normal range (90.2 ± 21.1 μmol/L), which may mitigate any changes induced by food abstinence in serum creatinine for other quartiles of the reference range.

This study has certain limitations that should be acknowledged. Firstly, our sample size was small, consisting of young and healthy participants, and the fasting period was of short duration. Therefore, investigating the effects of longer periods of food deprivation, including distinguishing between fasting and starvation, in a larger and more diverse participant pool across various health domains may reveal different adaptations in creatine balance. Secondly, we did not assess changes in the excretion of GAA, creatine, and creatinine through urine and feces, nor did we investigate tissue-specific kinetics, particularly in the kidney, liver, and muscle, using isotopic tracers. Such assessments would provide valuable insights into the turnover of these compounds during fasting. In addition, we were unable to ensure participants were fed to energy balance in the days prior to fasting intervention to reduce the risk of premature energy deficit during fasting; this should be addressed in future studies assessing the link between fasting and creatine status. Lastly, incorporating more sensitive and specific measures

of kidney function under acute stress, such as cystatin C, kidney injury molecule-1, and uromodulin, could help differentiate kidney tubular health based on anatomical localization and potentially validate the use of serum GAA as a potential marker of kidney viability.

In conclusion, our findings demonstrate that a short-term fasting period led to a decrease in serum GAA levels and an increase in serum creatinine levels in healthy adults. The reduction in GAA during fasting may indicate an increased demand for creatine production and/or potential kidney dysfunction. Further research is needed to explore the underlying causes, severity, progression, and potential implications of this GAA decrease, including the need for replenishment strategies. Finally, serum GAA should be further scrutinize as a new fasting-specific biomarker of possible kidney damage.

## 2.2 "Video Gaming Reduces Circulating Creatine Levels in Young Male E-Gamers"

### Introduction

Video gaming has grown rapidly across the globe during the past few years. According to the latest data, there are approximately 3.09 billion active video gamers worldwide in 2023 (Exploding Topics, 2023), and the global average for a single gaming session duration was up to 5.1 h (Statista, 2021). The health consequences of excessive video gaming drive much attention (Griffiths, 2005; Prot et al., 2012), with the World Health Organization adding gaming disorder to the 11th Revision of the International Classification of Diseases based on reviews of available evidence on repetitive patterns of gaming behavior (World Health Organization, 2022). However, the impact of a single session of video gaming attracted much less research interest, giving grounds for quantifying a possible acute metabolic burden of this ever-popular form of leisure activity. Recent studies demonstrated a number of biochemical correlates of single-session video gaming (Carpita et al., 2021), including an increase in glucose

metabolism and energy expenditure (Chaput et al., 2011), cortisol/catecholamines alterations (Phan-Hug et al., 2011), and oxidant-antioxidant perturbations (Podrigalo et al., 2020). Interestingly, no studies so far assessed whether acute video gaming affects creatine metabolism, a metabolic pathway critical in replenishing immediate energy for cells and tissues with high and intermittent energy fluctuations (Wallimann et al., 2011). The aim of this study was to explore whether a single session of prolonged video gaming alters circulating biomarkers of creatine metabolism in young male e-gamers. We hypothesized that an extended video gaming session would reduce blood biomarkers related to creatine metabolism.

## Materials and methods

A total of 12 young men (age 25.6 ± 3.8 years, body mass index 25.9 ± 2.5 kg/m$^2$) signed an informed consent to volunteer in this quasi-experimental before-after pilot trial. All participants had six or more years of experience in video gaming, spending 3–9 h a day playing games from first-person shooting (FPS) and multiplayer online battle-arena (MOBA) genres. The participants were apparently healthy non-vegetarians, free from using any creatine-containing supplements in the past 3 months. Additionally, all participants were asked to refrain from extensive physical activity and video gaming at least 24 h prior to the study kick-off. The gaming session was performed in a private professional e-sports club (Matrix Gaming, Novi Sad, Serbia) between 08:00 and 15:00. Each participant took part in a single 6-h session of competitive online ranked matches in CounterStrike: Global Offensive™ (Valve, Bellevue, WA, USA), a popular tactical FPS. Throughout the 6-h gaming period, each participant was provided with 2 L of water to prevent dehydration, and a vegetarian sandwich (250 kcal) to break fasting but minimize interference on creatine turnover via food sources. Blood samples were collected after an overnight fast immediately upon arrival at the club (baseline) and after the end of gaming session

(follow-up). All samples were immediately taken to the lab, centrifuged within 15 min at 3,000 × g, with serum stored at - 80 °C for further analyses. The serum was analyzed for creatine, guanidinoacetic acid (GAA, a direct precursor of creatine), and creatinine (an endproduct of non-enzymatic conversion of creatine) using modified liquid chromatography-tandem mass spectrometry (1200 Series LC System, Agilent Technologies Inc., Santa Clara, CA, USA), as described previously (Jovanov et al., 2018). The ethical approval to conduct the study was granted by the local IRB at the University of Novi Sad (# 23-1462). The study was conducted according to the guidelines of the Declaration of Helsinki.

## Results

Eleven participants (n = 11) completed the trial, with one participant (age 23) deciding to discontinue participation and thus being removed from the further analyses. All participants had baseline serum creatine, GAA, and creatinine levels within the reference ranges, except for one participant (age 23, weight 81.6 kg) who had mildly elevated serum GAA (5.1 μmol/L) and creatinine levels (116.2 μmol/L). A 6-h video gaming session resulted in a statistically significant drop in serum creatine levels (from 27.6 ± 7.5 μmol/L at baseline to 22.9 ± 8.3 μmol/L at follow-up; P = 0.029). The mean reduction in serum creatine was 4.70 μmol/L (95% confidence interval (CI): - 2.3 to 11.7), with a moderate-to-large effect size (d = 0.59). The highest reduction in serum creatine (57.8%) was found in a 29-year old participant. Serum creatinine concentrations tended to drop after the gaming session (from 88.1 ± 15.5 to 78.2 ± 19.8 μmol/L; P = 0.077), and the effect size was moderate-to-large (d = 0.56). Circulating GAA non-significantly diminished after the session (2.3 ± 1.0 μmol/L at baseline vs. 2.2 ± 0.4 μmol/L at follow-up; P = 0.359). In the follow-up, most participants had creatinine levels within the reference values (61.9 to 114.9 μmol/L), except for two men, aged 29 and 22, who had creatinine concentrations below a lower cut-off value (58.8 and 36.2 μmol/L, respectively). Individual changes for creatine biomarkers after a single session of e-gaming are shown in Figure 3 below.

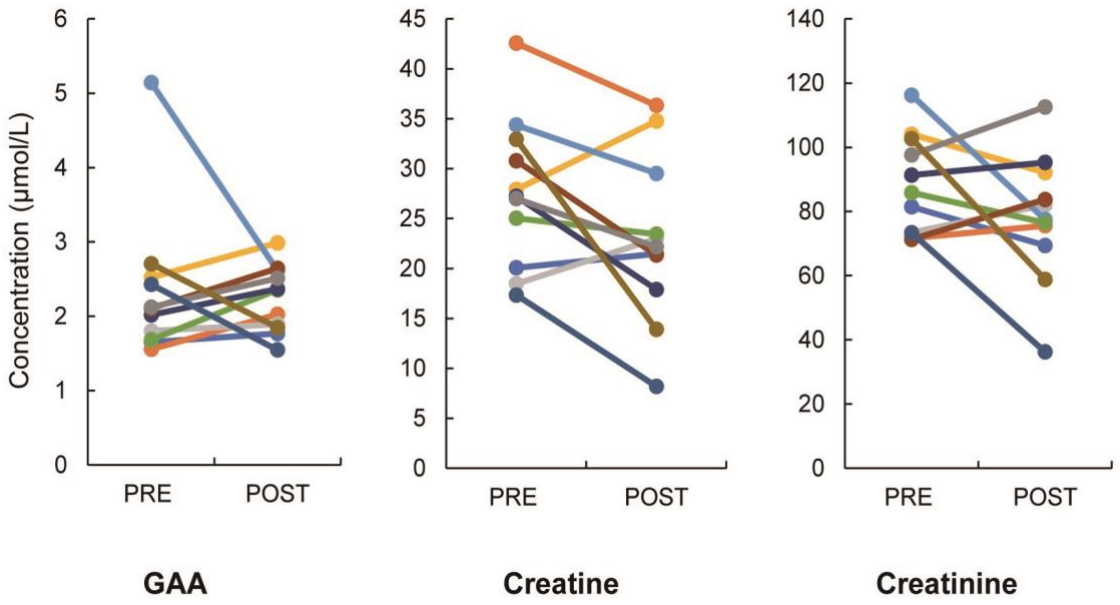



Figure 3. Individual changes in serum guanidinoacetic acid (GAA), creatine and creatinine after a single session of e-gaming in young men (n = 11).

## Discussion

Our preliminary trial demonstrated a significant effect of single-session video gaming on circulating creatine levels in male e-gamers. Six hours of competitive online game reduced circulating creatine for up to 19.1 μmol/L, with other biomarkers of creatine turnover (GAA and creatinine) tending to drop after the gaming session. This indicates that creatine homeostasis is sensitive to video gaming perhaps owing to more creatine from the circulation utilized as an energy source for active tissues, including the brain. Previous studies showed that creatine levels can decrease acutely upon brain activation (Rango et al., 1997). Since video gaming increases neural activity across several brain regions (Brilliant T et al., 2019), a 6-h session of FPS game might deplete brain creatine reservoir, leading to cerebral uptake of creatine from the circulation as an alternative source of rapidly mobilizable energy. Creatine fall-off might also be due to enhanced utilization via other energy-demanding tissues active during video gaming (Park et al., 2017), and/or impaired production due to a video gaming-induced circulation restriction in organs involved in creatine biosynthesis (Gwinup et al., 1983). It remains currently unknown whether

creatine deficit is associated with compromised game performance or any health consequences in e-gamers.

Our study is not without limitations. These involve the absence of a control group showing typical daily variations in creatine levels, which would enable a comparison with the experimental group. In this study, our focus was on a relatively small sample of young male e-gamers. Whether single (or repeated) gaming sessions produce comparable effects in other gaming demographics, such as women, children, or middle aged adults, is currently unknown and necessitates further investigation. In addition, we have not evaluated video game performance during the study, and a possible correlation between game performance (also subjective feeling of fatigue and concentration difficulties) with respect to the highest and lowest creatine values. Further studies are warranted to expand our findings by recruiting more diverse and larger samples, and accounting for different games and play time spans, encompassing shorter gaming intervals along with morning versus evening plays. In addition, addressing creatine biodynamics during video gaming across the human body via isotopic tracer studies and magnetic resonance spectroscopic imaging would help to understand organ-specific creatine turnover. A separate interventional trial should also evaluate a possibility to acutely correct creatine shortage driven by video gaming via dietary supplementation or providing foods rich in creatine in this population.

## 2.3 “Exercise affects salivary biomarkers of creatine metabolism in healthy adults”

### Introduction

During physical exertion, particularly during high-intensity exercise, the body's energy requirements escalate. Creatine, predominantly stored in skeletal muscles, plays a crucial role in supporting cellular energy production during heavy exercise by maintaining adenosine triphosphate (ATP) levels. This process can lead to the well-documented temporary depletion of muscle creatine stores; (Sahlin et al.,

1979) however, there is limited information available regarding exercise induced changes in creatine metabolism in other tissues. High-intensity exercise could result in fluctuations in creatine levels in bodily fluids such as serum and saliva. For example, an acute session of exhaustive exercise induces transient changes in circulating biomarkers of creatine metabolism, suggesting a significant exercise-induced disruption in bioenergetics (Al Fazazi et al., 2019; Stajer et al., 2016, 2020). Using blood as a sample for monitoring creatine biodynamics is not always feasible due to concerns regarding invasiveness, hygiene, and the potential infection risks associated with its collection and handling (Martínez et al., 2016). In contrast, saliva appears relatively clean and the samples can be quickly and noninvasively collected and easily stored (Suzuki et al., 2016).Still, no studies so far evaluated whether exercise affects salivary biomarkers of creatine metabolism, and do creatine levels in saliva mirror those found in serum before and after exercise. The primary objective of this study was to observe the variations in salivary creatine levels before and after a single session of high-intensity exercise among young adults. Additionally, the study aimed to assess the potential correlation between salivary creatine levels and those present in the serum of the study participants. We hypothesized that high-intensity exercise induces transient changes in salivary creatine levels, with fluctuations that correspond to alterations observed in serum creatine levels before and after exercise. These variations in salivary creatine could potentially serve as a non-invasive biomarker for monitoring exercise-induced shifts in creatine metabolism within the human body.

## Materials and methods

Ethical clearance for conducting the study was obtained from the local Institutional Review Board at the University of Novi Sad (Approval # 49-03-14/2023-1), with the research adhered to the principles outlined in the Declaration of Helsinki. Fifteen young adults (mean age 23.9 ± 2.9 years, eight females), provided informed written consent to participate voluntarily in this study. Participants were required to

meet specific inclusion criteria, including an age range of 18.0–29.9 years, a body mass index (BMI) between 18.5 and 24.9 kg/m², a non-vegetarian diet, and a minimum of three years of training experience as collegiate athletes, engaging in at least five hours of structured exercise per week. Exclusion criteria encompassed the presence of major chronic diseases or acute medical conditions, recent use of dietary supplements (within four weeks prior to study enrollment), unwillingness to comply with follow-up assessments, and concurrent participation in other clinical trials. During the laboratory visit, participants were subjected to an incremental running-to-exhaustion treadmill test. The test protocol began with a 3-minute warm-up walk at 5 km/h, followed by running at 8 km/h. The workload was progressively increased by 1.5 km/h every 60 seconds until the point of exhaustion was reached. A specimen of unstimulated whole saliva (approximately 0.5 mL) was collected from beneath the tongue using a disposable Pasteur pipette. Saliva collection took place ~ 30 minutes before exercise after mouth cleansing, and immediately after exercise. After protein precipitation with methanol, the saliva sample was centrifuged at 6000 rpm. The supernatant was dissolved in the mobile phase (80% acetonitrile and 20% 25 mM ammonium formate, pH = 3.3) and analyzed at a flow rate of 0.3 mL/min. Blood samples were collected from the antecubital vein using a gel vacutainer both before and after exercise. The samples were then centrifuged within 10 minutes at 3,000 *g* to isolate the serum. The serum and salivary samples were stored at –80ºC and subsequently analyzed for biochemical markers following the completion of the trial. Both samples underwent analysis for guanidinoacetic acid (GAA, a precursor of creatine), creatine, and creatinine (an end-product of creatine) utilizing a modified liquid chromatography–tandem mass spectrometry method (Agilent 1200 Series LC System, Agilent Technologies Inc., Santa Clara, CA), as previously described (Jovanov et al., 2018). The coefficients of variation for the salivary and serum creatine assays were 14.0% and 3.6%, respectively. All measurements were performed between 07:00 and 10:00 following an overnight fasting period, and

participants refrained from vigorous exercise within the preceding 24 hours. All participants completed a familiarization session with the testing procedures prior to assessments, adhering to a standardized sequence of tests. Differences in salivary and serum creatine and creatinine levels at baseline and post-exercise, as well as the slopes of the two regression lines (saliva *vs.* serum), were compared using paired two-tailed *t*-tests. Pearson's correlation test was utilized to evaluate the linear relationship between serum and salivary biomarkers at both time points of assessment. A significance level of $P < 0.05$ was applied. All statistical analyses were performed using SPSS version 24.0 for Mac (IBM SPSS Statistics, Chicago, IL).

## Results

The average duration of running until exhaustion was 668 ± 64 seconds. A bout of high intensity exercise resulted in a notable rise in salivary biomarkers related to creatine metabolism. Specifically, creatine levels demonstrated a significant elevation from 17.5 ± 14.2 μmol/L at baseline to 43.6 ± 30.4 μmol/L post-exercise ($P < 0.001$). This coincided with a notable alteration in salivary creatinine, which rose from 11.3 ± 5.8 μmol/L to 17.0 ± 9.3 μmol/L ($P = 0.04$). The levels of salivary GAA remained undetectable (below the limit of detection, 0.1 μmol/L) both before and after the exercise session. In contrast, serum creatine levels were unaffected by exercise ($P = 0.80$), while creatinine levels exhibited a strong tendency to decrease post-exercise (from 81.8 ± 17.5 μmol/L to 73.1 ± 11.6 μmol/L; $P = 0.06$). Furthermore, the levels of creatine and creatinine in saliva were significantly lower compared to the corresponding serum levels, both at baseline and after exercise ($P < 0.05$). Figure 4 illustrates the individual variations in salivary and serum levels of creatine and creatinine.

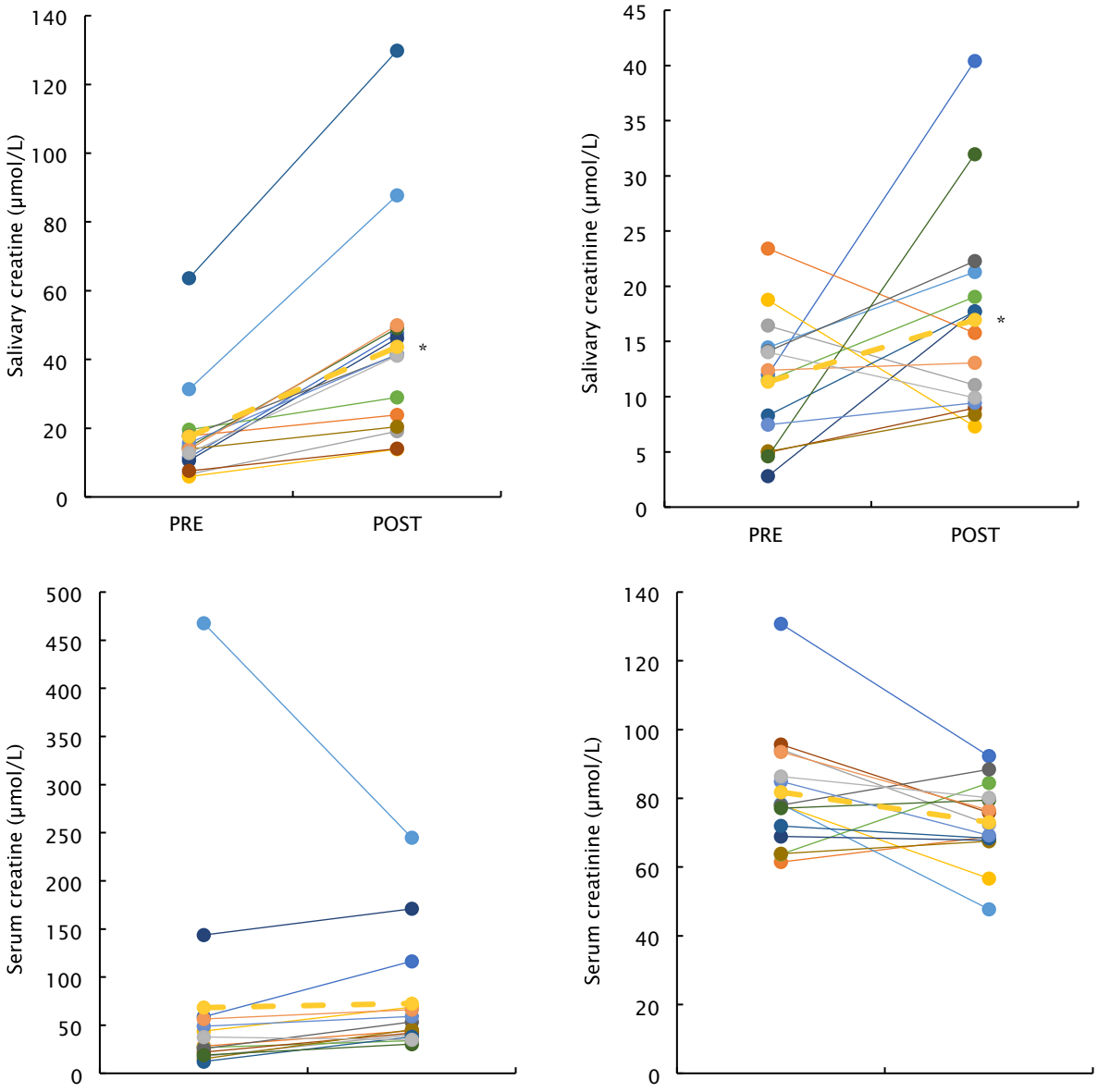



*Figure 4. Variations in individual levels of salivary and serum creatine and creatinine before (PRE) and after exercise (POST). The dashed lines represent the mean values and The asterisk (*) denotes statistical significance at $P < 0.05$.*

The salivary indicators of creatine metabolism showed a non-significant correlation with the corresponding serum indicators. The Pearson two-tailed correlation coefficients for creatine concentrations at baseline and post-exercise were $r = 0.18$ ($P = 0.51$) and $r = 0.33$ ($P = 0.21$), respectively. The corresponding correlation coefficients for creatinine were $r < 0.01$ ($P = 0.99$) for baseline values, and $r = 0.40$ ($P = 0.13$) at post-exercise. However, a comparison of the slopes of the two

regression lines (saliva *vs.* serum) revealed significant differences for both creatine ($P$ = 0.01) and creatinine ($P$ = 0.03).

## Discussion

The present pilot study potentially represents the pioneering demonstration of exercise- induced alterations in salivary biomarkers related to creatine metabolism. We observed that high-intensity exercise led to an escalation in both salivary creatine and creatinine levels among young healthy adults. A rise in salivary creatine was consistently observed in all fifteen participants (100%), whereas an increase in salivary creatinine was evident in eleven out of fifteen participants (73.3%). The elevated concentrations of creatine and creatinine in saliva might be attributed to an exercise-induced concentration gradient, potentially facilitating greater diffusion of these compounds from the serum into saliva. The specific impact likely depends on factors such as the intensity and duration of exercise, individual metabolism, hydration status, and other physiological factors (Al Fazazi et al., 2019; Snow & Murphy, 2003), which requires further investigation. Interestingly, the correlation between salivary biomarkers and concentrations measured in serum was weak, indicating a potentially distinct biodynamics of creatine metabolites in these two bodily fluids, both before and after exercise. Given that serum creatine levels reportedly remain largely unchanged following acute exercise (Andjelic et al., 2024), the utilization of salivary creatine might be suggested as a more responsive biomarker to exercise, and perhaps illustrate changes in creatine metabolism outside of the muscle tissue. This may be attributed to the bioenergetics specific to salivary glands during exercise and/or inflammation induced by exercise. Still, factors like individual differences in metabolism, the route of creatine excretion, and the influence of other components in saliva may affect the reliability of using saliva as a proxy for creatine turnover. Our results partially corroborate findings from a study demonstrating a difference between GAA and creatine concentrations in saliva and plasma of healthy subjects (Martínez et al., 2016). Still, the authors

provided no correlation indices between creatine levels in two fluids, and concluded that salivary biomarkers could be used as a noninvasive, safe, inexpensive tool for diagnosing creatine disturbances across various conditions. Based on our findings, there is no observed association between salivary and serum biomarkers, indicating that they should not be utilized interchangeably.

Despite its interesting findings, our study has several limitations that impact its conclusions. The small sample size (15 participants) reduces statistical power and limits generalizability, especially given the homogeneous population of young, physically active adults. A broader study including individuals of varying fitness levels, ages, and health statuses would be necessary to validate these results. Additionally, the study does not account for potential confounding factors such as hydration status, diet, and individual differences in creatine metabolism, all of which could influence biomarker fluctuations. Methodologically, while saliva offers a non-invasive alternative to blood sampling, its reliability as a biomarker source remains uncertain. The study does not clarify whether salivary creatine increases due to diffusion from the blood or whether it is influenced by exercise-induced changes in salivary gland metabolism. Furthermore, the storage conditions, sample processing, and potential degradation of creatine in saliva over time could introduce variability in the results. Given these concerns, more research with larger sample sizes, improved methodological rigor, and varied exercise protocols is necessary before saliva can be considered a reliable biomarker for creatine metabolism in response to physical exertion.

# CHAPTER III: EFFECTS OF CREATINE FORMULATIONS ON PERFORMANCE OUTCOMES IN HEALTHY YOUNG MALES AND FEMALES

## 3.0 Chapter III Overview

Building upon the findings presented in Chapter II, we conducted a second series of experiments focusing on investigating effects of dietary supplementation as a strategy to improve measures of human performance creatine status induced by physiological stressors—specifically, exhaustive running and prolonged competitive video gaming, as previously described. In Chapter III, our focus shifted toward evaluating the effects of alternative creatine formulation on creatine status, endurance capacity, and cognitive performance in a population of healthy young men and women. In the first study, we hypothesized that a single orally administered dose of creatine – GAA mixture would elicit significant improvements in endurance running performance and creatine status relative to placebo. In the second experiment, our team investigated the effects of the same supplemental regimen on improving esports and cognitive performance by offsetting the decline in performance caused by prolonged mental efforts. This chapter examined whether co-administration of creatine and GAA can be considered as an advanced dietary strategy to optimize tissue bioenergetics, running to exhaustion, and cognitive performance outcomes.

Chapter III is in full based on the previously peer-reviewed published original articles:

3.1 Andjelic, B., Todorovic, N., Vranes, M., & Ostojic, S. M. (2024). Dietary Creatine-Guanidinoacetate Acutely Improves Circulating Creatine Levels and Running Performance in Healthy Men and Women. *Progress in Nutrition*, *26*(1), e2024003.

3.2 Andjelic, B., Todorovic, N., Vranes, M., & Ostojic, S. M. (2025). Creatine-Guanidinoacetic Acid Supplementation Improves Esports Performance in Young Men. *Current Nutrition & Food Science*, *21 (3).*

## 3.1 “Dietary Creatine-Guanidinoacetate Acutely Improves Circulating Creatine Levels and Running Performance in Healthy Men and Women”

### Introduction

Creatine is considered a conditionally essential nutrient that plays a critical role in sustaining high-energy phosphate bioenergetics across the human body (Ostojic & Forbes, 2022b). Several recent studies demonstrated an exercise-induced impairment in creatine status, with circulating levels of guanidinoacetate (GAA), a direct biosynthetic precursor of creatine) particularly sensitive to heavy exercise (Stajer et al., 2016, 2020). Interestingly, females appear more prone to creatine and GAA depletion due to lower endogenous creatine stores compared to males, and higher excretion of those compounds by the kidneys (Joncquel-Chevalier Curt et al., 2013), making this subpopulation especially eligible for a replenishment. Supplying dietary GAA and creatine before exercise has been anecdotally suggested as a viable strategy to prevent creatine reduction. However, no studies to date have evaluated the potency and gender-specific response of this combination in humans. In this pilot trial, we assessed the effects of a single-dose creatine-GAA intake on creatine biomarkers during a running-to-exhaustion test in young men and women.

### Methods

A total of sixteen ($n$ = 16) young men and women (age 22.8 ± 1.9 years, body mass index 23.8 ± 2.8 kg/m$^2$; eight females) signed an informed consent to voluntarily participate in this open-label interventional trial. All participants were non-vegetarian, free from acute and chronic disorders, and had not consumed any dietary supplements for at least four weeks prior to the study. During the trial, all participants were assessed on two occasions separated by a 7-day interval. At the first laboratory visit, participants were subjected to an incremental running-to-exhaustion treadmill test, with breath-to-breath metabolic assessment conducted during the test (Quark CPET, COSMED, Rome, Italy). The test

commenced with a 3-minute warmup walk at 5 km/h, followed by running at 8 km/h with a workload increment rate of 1.5 km/h every 60 seconds until reaching the point of exhaustion. For a second visit, a mixture containing 1 gram of creatine monohydrate and 1 gram of creatine (CreGAAtine$^{TM}$, Carnomed, Novi Sad, Serbia) was consumed 30 minutes prior to the exercise test. All measurements were conducted between 07:00 and 10:00 after an overnight fast, and participants refrained from engaging in heavy exercise within the previous 24 hours. Blood samples were collected from an antecubital vein into a gel vacutainer before and immediately after exercise at each lab session. The blood samples were analyzed for GAA, creatine, and creatinine using a modified liquid chromatography–tandem mass spectrometry method (Agilent 1200 Series LC System, Agilent Technologies Inc., Santa Clara, CA, USA), as described previously (Jovanov et al., 2018).

## Results

Exercise itself induced no statistically significant drop in creatine biomarkers across the sample ($P > 0.05$), yet a strong trend was reported for reduced serum GAA (8.1%) and creatine levels (30.3%) after exercise in a female and male subsample, respectively. Dietary intake of creatine-GAA mixture instigated a significant elevation in serum GAA by 62.4% (95% confidence interval [CI], from 37.0 to 87.8) ($P < 0.0001$). This was accompanied by a remarkable rise (660.3%) in serum creatine levels at post-exercise follow-up (95% CI, from 383.9 to 936.7) ($P < 0.0001$). Serum creatinine remained unaffected by the intervention ($P > 0.05$). The creatine-GAA mixture non-significantly affected the running performance across the whole sample by extending the time to exhaustion by 4 sec (95% CI, from -7 to 15), raising anaerobic threshold for 1.2 units (95% CI, from -0.5 to 2.9), and improving end-test oxygen uptake by 0.8 ml/kg/min (95% CI, from -0.5 to 2.1) ($P > 0.05$). Still, the magnitude of improvement after creatine-GAA intake was more pronounced in a female subsample ($P < 0.05$) for time to exhaustion (7 sec, 95% CI, from -7 to 15), anaerobic threshold (2.7 units, 95% CI, from 0.9 to 4.5),

and oxygen uptake by 1.6 ml/kg/min (95% CI, from 0.4 to 2.8). In a subsample of men, no significant changes for running performance indices were demonstrated after creatine-GAA intake ($P > 0.05$), with time to exhaustion increased by 1 sec (95% CI, from - 66 to 68), and anaerobic threshold and oxygen uptake decreased for 0.3 units (95% CI, from - 4.1 to 3.6) and 0.1 ml/kg/min (95% CI, from - 5.8 to 5.6), respectively. No participants reported any side effects of the intervention.

## Discussion

The present trial demonstrated a significant impact of supplemental creatine-GAA consumed immediately before exercise on circulating GAA and creatine levels in young adults, with women benefiting more from the mixture to acutely improve running to-exhaustion indices. A single-dose supplementation with GAA and creatine could be suggested as a safe pre-exercise ergogenic formulation for upholding creatine status. This trial corroborates previous research where medium-term co-administration of GAA and creatine positively affected creatine metabolism and exercise performance in young men (Semeredi et al., 2019). Our findings extend previous research by using a more brief protocol of supplementation while addressing gender–specific responses in creatine biodynamics. Interestingly, a comparatively low dosage of creatine and GAA used here (a single gram of each component) appears capable to acutely augment creatine status perhaps due to favorable bioavailability and transport kinetics of this nutritional combination (Ostojic, 2017).

Further randomized controlled trials are required to validate our findings by assessing short-term uptake and transfer of exogenous GAA and creatine across target organs (*e.g.*, the kidneys, liver, skeletal muscle, brain) while accounting for gender-specific impact(s) of sex hormones on creatine metabolism. Whether the mixture triggers creatine independent mechanisms to improve performance, such as insulin stimulation, arginine sparing and antioxidant support (Ostojic & Jorga, 2023), also necessitates scientific inquiry. In addition, investigating the effects of medium- to long-term supplementation is warranted to

assess the pharmacovigilance of GAA-creatine combination, and ergogenic capacity for other components of physical fitness and exercise performance (*e.g.*, metabolic fitness, body composition, muscular strength and endurance, flexibility, skill-related fitness components).

## 3.2 "Creatine-guanidinoacetic acid supplementation improves esports performance in young men"

### Introduction

Creatine stands as one of the most widely used ergogenic supplements, with extensive evidence supporting its efficacy in enhancing performance across various tasks, for a detailed review, see Wax et al., 2021). However, its potential to enhance cognitive performance in young healthy individuals remains a subject of debate. While several studies have reported positive effects of creatine supplementation on cognitive performance in semi-professional athletes (Borchio et al., 2020), healthy young adults (Benton & Donohoe, 2011; Hammett et al., 2010), and sleep-deprived individuals (Cook et al., 2011; McMorris et al., 2006), others have found no significant effects in preadolescents (Merege-Filho et al., 2017) or young adults (Moriarty et al., 2023; Rawson et al., 2008). A systematic review of randomized controlled trials revealed no notable improvements in cognitive task performance following creatine supplementation in young individuals (Avgerinos et al., 2018), likely due to the limited transport capacity of creatine to the brain (Roschel et al., 2021), necessitating the use of high doses (*e.g.*, ≥ 20 g per day). One potential solution to overcome the transport limitations of creatine is the administration of guanidinoacetic acid (GAA), a direct precursor of creatine believed to be more effective in increasing cerebral creatine levels than creatine itself (Ostojic, Ostojic, et al., 2016). Interestingly, co-administration of creatine and GAA has been shown to outperform creatine alone in elevating brain creatine levels when administered in lower doses in young healthy individuals (Semeredi et al., 2019). However, whether this combined supplementation affects cognitive performance remains unclear. This presents a particular

interest in the realm of esports, where cognitive performance plays a crucial role in competitive success (Szot et al., 2022). Therefore, the primary objective of this preliminary study was to investigate the effects of co-administered creatine and GAA on esports performance, neuropsychological outcomes, and creatine status in young male esports athletes.

## Methods

This pilot study employed an open-label quasi-experimental design. The eligibility criteria for participant inclusion in the trial encompassed several aspects: age 18-35 years, at least ten years of experience in multiplayer (player *vs.* player) esports, spending a minimum of three hours per day gaming, being apparently healthy non-vegetarians, and not using any creatine-containing supplements in the past three months. Eligible participants provided voluntary informed written consent, and ethical clearance was obtained from the local Institutional Review Board (IRB) at the University of Novi Sad (#23-1462-2). The study adhered to the Declaration of Helsinki (7$^{th}$ revision). During the study, all participants were administered a daily dosage of a GAA-creatine mixture (CreGAAtine™, Carnomed, Novi Sad, Serbia), containing two grams of creatine and two grams of GAA. Participants were instructed to take the intervention twice daily, prior to breakfast and dinner, by dissolving the GAA-creatine powder in 250 mL of lukewarm water and consuming it immediately. The intervention spanned four weeks, during which participants were asked to refrain from using any other nutritional supplements. All participants were also asked to refrain from making major changes in their diet and physical activity during the study. All assessments were conducted at two time points: baseline (pre-administration) and the 4-week follow-up (post-administration). Preliminary measurements included demographics and fasting blood sampling for creatine metabolism biomarkers (see below).

After these preliminary measurements, a session of esports multiplayer online game (Dota 2™, Valve, Bellevue, WA, USA) was conducted in a professional esports club (LevelUp, Novi Sad, Serbia). Dota 2™ is a tactical multiplayer online battle arena esports game in which players engage in battle to destroy the enemy base on a virtual map. More details about the game can be found at https://www.dota2.com/home. During a game, players collect experience points and items for their heroes to engage in player versus player combat against the opposing team's avatars (heroes). A session of Dota 2™ was organized in a best-of-three-games format. For the purpose of game-specific performance analysis, we extracted data on Kills Death Assists (KDA, number of kills and assists divided by deaths), Gold Per Minute (GPM, currency used to buy items or instantly revive a hero), Experience Per Minute (XPM, experience points accumulated during the game), and Last Hits (LH, a number of final blows to enemy entities to be awarded with gold and experience) for each of three parts during a gaming session, with total performance scores (KDA + GPM + XPM + LH) for each game calculated; higher values of those outcomes indicate better Dota 2™ performance. Throughout the gaming session that lasted approximately three hours, each participant was provided with 2 L of water to prevent dehydration, and a vegetarian sandwich (250 kcal) to break fasting but minimize interference with creatine turnover via food sources. No caffeinated foods or beverages were consumed before the assessment. Immediately after finishing the esports session, participants underwent two computerized neuropsychological tests, the Stroop Color and Word Test (SCWT) and the Attention Network Test (ANT), using online testing platform (Inquisit 6, Milliseconds Software, Seattle, WA). The SCWT is a widely-used neuropsychological test employed to evaluate the capacity to inhibit cognitive interference; stimuli types were classified as control, congruent or incongruent (Scarpina & Tagini, 2017). The ANT is an individually administered computer-based test that offers assessments of the alerting, orienting, and executive attention networks within a unified task (MacLeod et al., 2010). After the tests, blood

collection was repeated. The blood samples were analyzed for GAA, creatine, and creatinine using a modified liquid chromatography–tandem mass spectrometry method (Agilent 1200 Series LC System, Agilent Technologies Inc., Santa Clara, CA, USA) (Jovanov et al., 2018). Data collection took place at the Applied Bioenergetics Lab at the University of Novi Sad between September 2023 and March 2024. Baseline and follow-up values were compared using the paired *t* test for normally distributed data and the Wilcoxon test for non-normally distributed data. Effect sizes after the intervention were assessed by Cohen statistics, with $d \geq 0.8$ indicating a large effect. Values exceeding three standard deviations from the mean, or falling below three standard deviations, are identified as outliers in the output results, and excluded from the analysis.

## Results

Ten young male esports athletes ($n = 10$) were enrolled in the study, received the designated treatment, and were analyzed for the study outcomes. The baseline characteristics of the participants are summarized in Table 3.1.

*Table 2. Baseline characteristics of the study participants (n = 10).*

| *Variables* | |
|---|---|
| Age, years, mean ± SD | 28.4 ± 4.4 |
| Weight, kg, mean ± SD | 85.5 ± 20.3 |
| Height, cm, mean ± SD | 184.6 ± 7.5 |
| Body mass index, kg/m$^2$, mean ± SD | 24.9 ± 4.4 |
| Fat mass, %, mean ± SD | 24.4 ± 3.5 |
| Gaming experience, years, mean ± SD | 16.1 ± 5.6 |
| Weekly frequency of gaming, days, mean ± SD | 5.5 ± 1.8 |
| Daily exposure to gaming, hours, mean ± SD | 5.8 ± 3.5 |
| Total number of Dota 2™ games played, mean ± SD | 7269 ± 2861 |
| Players who competed at a professional level in Dota 2™, % | 50.0 |

| | |
|---|---|
| Players with highest level of expertise in Dota 2™, % | 80.0 |
| Highest ranking points achieved in Dota 2™, range | 4000 – 11000 |
| Percentile rank in Dota 2™, mean ± SD | 97.6 ± 1.8 |

Table 3.2 illustrates the changes in game-specific scores and post-game neuropsychological outcomes following creatine-GAA supplementation. The total Dota 2™ performance scores for the first two games showed a non-significant increase after the intervention ($P < 0.25$). However, the mean total scores obtained after the final game significantly improved by 30.1% (95% CI, -17.3 to 77.5; $P = 0.05$). A significantly lower control reaction time was observed after the intervention ($P = 0.02$), with an average reduction of 19.2% (95% CI, 2.2 to -40.6), while incongruent and control reaction times tended to decrease post-administration ($P < 0.15$). Additionally, both incongruent and overall accuracy showed significant improvements following the intervention ($P \leq 0.05$). On average, incongruent accuracy increased by 10.2% (95% CI, -1.2 to 21.7), and overall accuracy improved by 4.4% (95% CI, -0.9 to 9.7). Furthermore, the attentional network for executive control improved after the intervention ($P = 0.01$), with levels reduced by 16.2% on average (95% CI, from -3.4 to -29.0). In addition, large effect sizes of the intervention ($d \geq 0.8$) were demonstrated for game 2 Dota 2™ performance (1.28), control reaction time (1.11), incongruent accuracy (0.91), and overall accuracy (0.81).

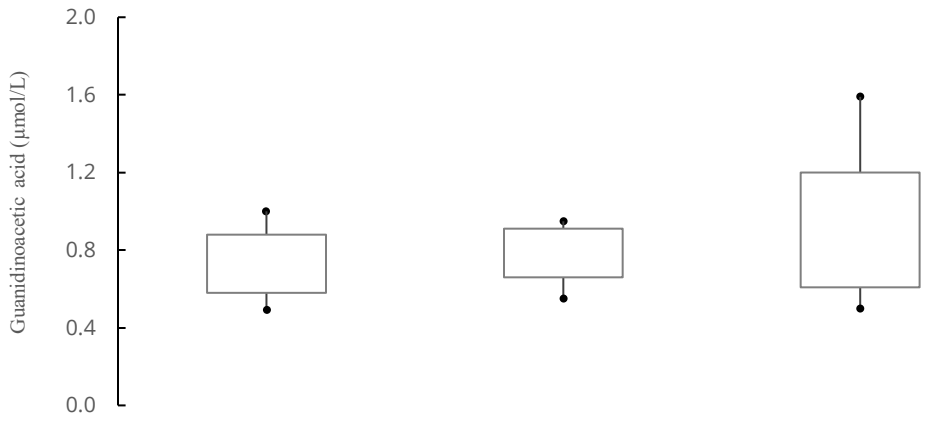
*Table 3. Game-specific outcomes and neuropsychological test results at baseline and following a 4-week supplementation with creatine and guanidinoacetic acid. Values are mean ± SD.*

| | *Baseline* | *Follow-up* | *P ** | *Cohen d* |
|---|---|---|---|---|
| *Dota 2™ performance* | | | | |
| Game 1 (total score) | 9.0 ± 4.0 | 9.9 ± 3.4 | 0.22 | 0.24 |
| Game 2 (total score) | 5.8 ± 3.1 | 9.3 ± 2.3 | 0.06 | 1.28 |
| Game 3 (total score) | 8.0 ± 3.9 | 9.6 ± 2.7 | 0.05 | 0.48 |
| *Stroop Color and Word Test* | | | | |
| Congruent reaction time (ms) | 811 ± 128 | 800 ± 268 | 0.46 | 0.05 |
| Incongruent reaction time (ms) | 955 ± 201 | 837 ± 235 | 0.08 | 0.54 |
| Control reaction time (ms) | 931 ± 145 | 731 ± 210 | 0.02 | 1.11 |

| | | | | |
|---|---|---|---|---|
| Overall reaction time (ms) | 897 ± 99 | 789 ± 226 | 0.11 | 0.62 |
| Congruent accuracy (%) | 93.2 ± 5.4 | 94.6 ± 5.1 | 0.31 | 0.27 |
| Incongruent accuracy (%) | 86.1 ± 10.0 | 93.6 ± 6.0 | 0.04 | 0.91 |
| Control accuracy (%) | 93.2 ± 5.9 | 95.4 ± 4.1 | 0.16 | 0.43 |
| Overall accuracy (%) | 90.8 ± 5.2 | 94.5 ± 3.8 | 0.05 | 0.81 |
| | | | | |
| *Attention Network Test* | | | | |
| Alerting (ms) | 37.5 ± 19.0 | 42.6 ± 12.1 | 0.14 | 0.32 |
| Orienting (ms) | 16.6 ± 11.6 | 16.7 ± 11.5 | 0.50 | 0.01 |
| Executive control (ms) | 75.4 ± 18.0 | 62.9 ± 17.6 | 0.01 | 0.70 |
| Reaction time (ms) | 398 ± 34 | 400 ± 39 | 0.43 | 0.06 |
| Test accuracy (%) | 96.5 ± 2.4 | 96.6 ± 2.7 | 0.43 | 0.04 |

*Notes*: Total scores for Dota 2™ performance are computed for each game in a session by summing KDA (total number of kills and assists divided by deaths), GPM (gold earned per minute), XPM (experience gained per minute), and (LH, number of final blows to enemy entities). An asterisk (*) denotes statistical significance levels for comparisons between baseline and follow-up values, utilizing the paired *t* test for normally distributed data and the Wilcoxon test when the normality assumption is not met.

The mean fasting plasma levels of GAA, creatine, and creatinine were 0.7 ± 0.2 μmol/L, 32.5 ± 12.5 μmol/L, and 87.9 ± 20.5 μmol/L, respectively, with all individuals falling within the normal ranges. Figure 5 illustrates the changes in creatine metabolism indices following a single session of esports gaming at baseline (post-game) and at the 4-week follow-up (post-game post-supplementation).



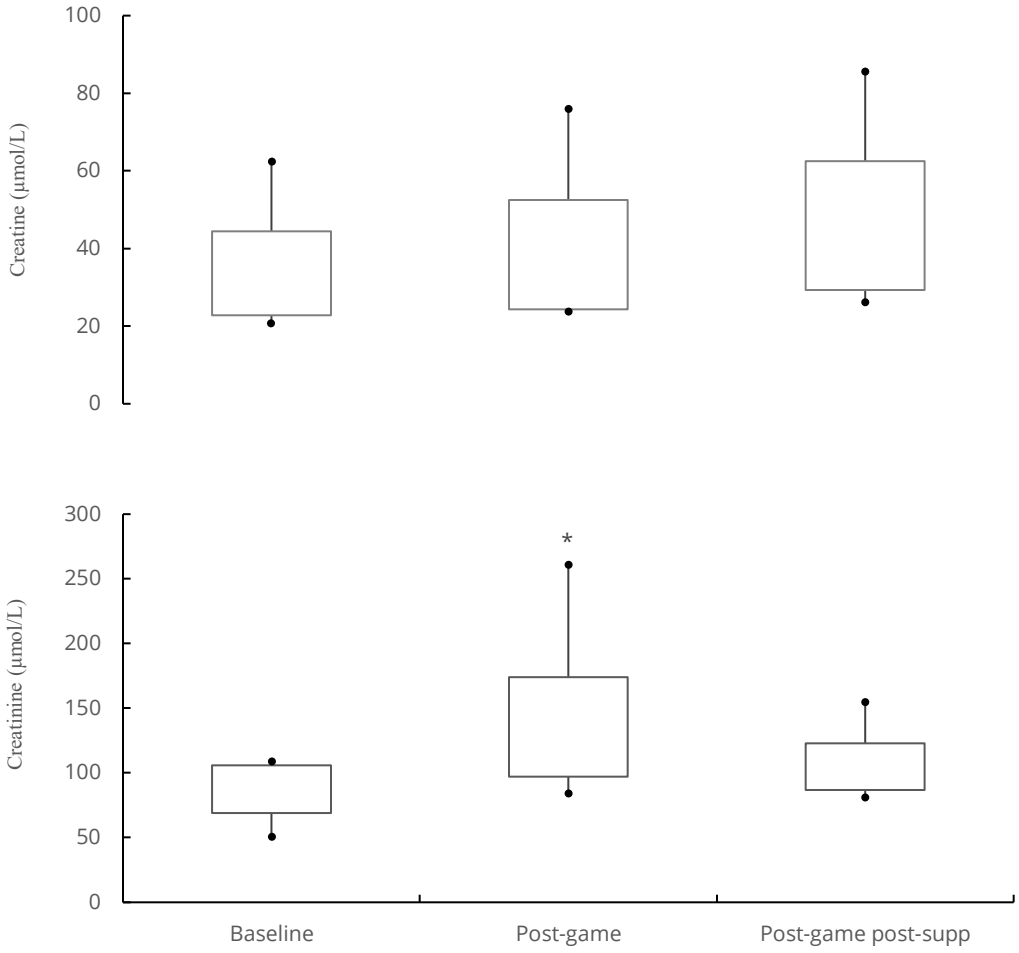



*Figure 5. The alterations in plasma indices of creatine metabolism subsequent to a gaming session of gaming before the intervention (post-game), and after 4-week supplementation with creatine and guanidinoacetic acid (post-game post-supp). The box illustrates the 95% confidence intervals, with lines extending to the minimum and maximum values. An asterisk (*) denotes significantly different mean values compared to baseline at $P \leq 0.05$.*

Circulating GAA levels remained unaffected by esports gaming both at baseline (mean change 10.8%; 95% CI, -10.3 to 31.9%; $P = 0.37$) and after supplementation (mean change 24.2%; 95% CI, -6.0 to 54.4%; $P = 0.21$). Plasma creatine levels showed no significant change following esports session at baseline (mean change 20.6%; 95% CI, -16.5 to 57.7%; $P = 0.31$) but tended to increase post-game after the supplementation (mean change 51.2%; 95% CI, -3.8 to 106.2%; $P = 0.10$). On the other hand, plasma creatinine levels increased significantly following an esports gaming session (mean change 48.3%; 95% CI, 15.0 to 81.6%; $P = 0.008$); the post-game increase was nonsignificant after the supplementation (mean change 12.2%; 95% CI, -28.7 to 53.1%; $P = 0.09$). Interestingly, five participants (50%) exhibited

an increase in creatinine levels surpassing the reference values of 114.9 μmol/L after an esports session at baseline, whereas only two participants (20%) showed a similar elevation in plasma creatinine after four weeks of creatine-GAA supplementation ($P = 0.08$). None of the participants reported experiencing any adverse events from the supplementation.

## Discussion

Our open label pilot study represents a pioneering investigation into the effects of creatine supplementation in esports. We observed that co-administering creatine with GAA for four weeks led to significant improvements in esports performance during a session of competitive Dota 2™ playing, and neuropsychological outcomes evaluated immediately after the game. These enhancements included notable increase in total scores for a final game in a Dota 2™ session, along with improved reaction time, accuracy and executive control. Concurrently, we observed alterations in circulating markers of creatine metabolism, with the intervention demonstrating a tendency to elevate creatine levels during post-game assessment, and reduce the occurrence of game-induced rise in creatinine levels. These preliminary findings suggest that creatine-GAA supplementation may serve as an effective performance-enhancing intervention in esports. Interestingly, the dosage of creatine and GAA utilized in our study was comparatively lower than in other studies investigating creatine for enhanced cognition (Benton & Donohoe, 2011; Borchio et al., 2020; Hammett et al., 2010). The effectiveness of these lower dosages demonstrated in our study might be attributed to the superior capacity of GAA to be transported into the brain (Ostojic, Ostojic, et al., 2016), thereby sustaining cerebral creatine synthesis and supporting the high-energy demands of esports performance. However, further randomized controlled studies are necessary to validate our results across various esports cohorts and games while employing different supplementation protocols.

Our preliminary study has several limitations that must be considered when interpreting the results. Firstly, the absence of a control or placebo group diminishes the robustness of our findings due to potential confounding factors. For instance, observed improvements in performance may be attributable to a learning effect—where results from the second experiment may be influenced by the first—or to increased effort by the participants, rather than the intervention itself. Secondly, our trial was conducted exclusively with male esports athletes and involved a relatively small sample size. This raises concerns about the generalizability of our results to other demographic groups, such as young recreational video gamers or individuals of different genders. Additionally, the study did not assess cerebral creatine and GAA levels, limiting our understanding of whether the intervention affects brain bioenergetics. Given the limited ability of creatine to cross the blood-brain barrier, future research should evaluate the effects of pure GAA—potentially a critical compound—on brain bioenergetics and esports performance. Finally, the short duration of the intervention restricts our ability to draw conclusions regarding the prolonged effects of creatine-GAA intake on esports performance. Long-term studies are needed to evaluate the sustainability of the observed benefits and to assess any potential safety concerns.

# CHAPTER IV: CONCLUSIONS

In summary, this dissertation explored opportunities in an attempt to improve our understanding of a) homeostasis of creatine metabolism in response to different types of physiological stress, and subsequently, b) the effects of short-term creatine-GAA supplementation, by synthesizing two series of original studies shown in detail across Chapters II and III, respectively. Supported by our preliminary findings from a series of experiments on population of young healthy men and women, this dissertation demonstrates that concentrations of biomarkers of creatine metabolism, its direct precursor GAA and an end–product metabolite Crn, are responsive to different bouts of physiologically demanding tasks, including a single acute session of fasting, maximal effort exercise and prolonged cognitive tasks to exhaustion. As evidence in our first series suggests, short–term fasting, being an energy-demanding process alone, induced significant changes in serum GAA levels, reducing it by an average of 0.9 μmoles, and causing an increase of creatinine by 11.1 μmoles per liter 24 hours from the studyd baseline. Furthermore, significant perturbations were observed in response to a six-hour video gaming session, with a significant decrement of serum creatine by 4.70 μmol/L, whereas its precursor and end-metabolite creatinine both non-significantly diminished by 9.9 μmol/L and 1.2 μmol/L, respectively. In addition to blood analysis, in Study 3, our group also sampled non-stimulated whole saliva to analyze biomarkers of creatine metabolism. This dissertation introduces the pioneering evidence on salivary biomarkers of creatine metabolism before and immediately upon reaching exhaustion, reporting a reduction of salivary (but not serum) creatine and creatinine by 23.1 and 5.7 μmol/L. Subsequently, in our second series of experiments, this dissertation studied the effects of supplementation, aiming to upregulate cellular bioenergetics by increasing available circulating creatine and offsetting the decline previously reported. Accordingly, the outcomes showed remarkably significant positive effects of short-term oral creatine–GAA supplementation in Studies 4 and 5, respectively. Namely, our findings suggest that a single dose

of creatine–GAA enriched circulating creatine levels by 660.3% and improvements in running performance outcomes, especially among the subsample of females. In Study 5, after administering the same formulation twice a day over a period of four weeks, esports players significantly improved their in-game performance in the last game and neuropsychological test outcomes. This coincided with significantly upregulated circulating levels of creatine biomarkers.

## 4.2 Key Findings

In Study 1, 24 young healthy adults (12 females) underwent a full day of water-only fasting and biomarkers of creatine metabolism were analyzed at the study baseline and follow-up. After adjusting for a decrease in plasma volume (~ 0.57%), 24-hour fasting has caused a significant decrease in serum GAA levels (from 2.2 ± 0.6 μmol/L to 1.3 ± 0.4 μmol/L; $P < 0.001$, d = 1.77). Furthermore, our results also showed serum creatinine were significantly elevated (from 83.8 ± 18.2 μmol/L to 94.9 ± 22.0 μmol/L; $P < 0.01$, d = 0.54), which was not the case for creatine levels which remained largely unaffected by the fasting intervention (25.1 ± 6.6 μmol/L at baseline vs. 23.4 ± 6.7 μmol/L at follow-up; p = 0.29). Accordingly, gender-specific analysis confirmed these findings, except for creatinine being relatively steady in the female subsample (n = 12, 90.2 ± 21.1 μmol/L at baseline vs. 93.5 ± 25.4 μmol/L at follow-up; p = 0.10). As current evidence suggests, creatine biomarkers require further investigation on their associations with kidney markers in response to fasting–induced energy demands.

In Study 2, a total of 12 young male esports players volunteered in our before-after quasi-experimental trial in order to investigate the effects of a single gaming session of a popular tactical FPS game on circulating biomarkers of creatine metabolism. Eleven subjects completed the trial and were analyzed for study outcomes, attesting that six hours of competitive CounterStrike: Global Offensive™ online playtime resulted in a significant decrease of serum creatine levels (from 27.6 ± 7.5 μmol/L at baseline to 22.9 ± 8.3 μmol/L at follow-up; P = 0.029), while creatinine (from 88.1 ± 15.5 to 78.2 ± 19.8 μmol/L;

P = 0.077) and GAA non-significantly diminished (2.3 ± 1.0 μmol/L at baseline vs. 2.2 ± 0.4 μmol/L at follow-up; P = 0.359). Our preliminary study supports the initial hypothesis, confirmed by the significant reduction in creatine levels captured after a 6-h session of popular FPS online gameplay.

In Study 3, 15 young healthy adults underwent an incremental running-to-exhaustion treadmill protocol with salivary and blood samples assessed at baseline and post-intervention. In the analysis of two biofluids, we found that running to exhaustion led to a significant rise in salivary creatine levels from 17.5 ± 14.2 μmol/L at baseline to 43.6 ± 30.4 μmol/L post-exercise ($P < 0.001$), and salivary creatinine levels increased from 11.3 ± 5.8 μmol/L to 17.0 ± 9.3 μmol/L (P = 0.04), however, GAA concentrations in saliva were below the detection limit. On the other hand, serum creatine and creatinine levels were unaffected by exercise (P = 0.80, P=0.06), despite creatinine showing a tendency to decrease post-exercise (from 81.8 ± 17.5 μmol/L to 73.1 ± 11.6 μmol/L). Salivary creatine and creatinine were positively correlated to the serum indicators, r = 0.18 (P = 0.51) and r = 0.33 (P = 0.21), respectively. By comparing the slopes of the two regression lines (saliva vs. serum), there was a significant difference for both creatine (P = 0.01) and creatinine (P = 0.03), suggesting that creatine biomarkers perhaps follow their dynamics unique to the biofluid assessed (Figure 3).

In Study 4, a total of 15 young adults from Study 3 were retested using an identical running-to-exhaustion treadmill protocol to investigate the effects of acute creatine-GAA supplementation on performance outcomes and circulating creatine biomarkers. Interestingly, maximal exercise induced no significant change on creatine biomarkers across the entire sample (P > 0.05). However, a single oral dose of creatine-GAA mixture led to a significant elevation in serum GAA by 62.4% and 660.3% in serum creatine levels at post-exercise follow-up (both $P < 0.0001$). At the same time, creatinine remained unaffected by the intervention (P > 0.05). On average, the mixture caused non-significant improvements of the time to exhaustion by 4 seconds, increased the anaerobic threshold by 1.2 units, and end-test

oxygen uptake by 0.8 ml/kg/min (all, P > 0.05). Nevertheless, the acute beneficial effects of supplementation were distinguishably superior in the subsample of females ($P < 0.05$). Our results support the idea of the mixture being a potent supplement even when administered shortly before bouts of endurance running.

In Study 5, we recruited 10 healthy young male esports players to examine the effects of 4-week creatine-GAA supplementation on specific Dota 2™ performance, neuropsychological outcomes, and creatine metabolism biomarkers. Our study shows game-specific scores from the first two Dota 2™ matches showed a non-significant positive trend ($P < 0.25$), with final game performance being improved significantly by 30.1% ($P = 0.05$). For the neuropsychological outcomes, the control reaction times improved by 19.2% ($P = 0.02$), with smaller, non-significant reductions in incongruent and control reaction times ($P < 0.15$). Executive control improved significantly by 16.2% ($P = 0.01$) while incongruent and overall response accuracy improved by 10.2% and 4.4% (both $P \leq 0.05$). Biomarkers of creatine metabolism were not influenced by the gaming session(s) *per se*; however, supplementation affected post-gaming levels of creatine to elevate, while creatinine remained creatinine similar to the baseline values (Figure 5).

## 4.3 Practical Implications

In Study 1, our findings suggest that 24–hour food restriction in healthy young adults can significantly affect circulating GAA across the entire sample of young healthy adults. Being aware of our study limitations, our work here emphasizes the ability of short-term fasting to alter circulating biomarkers of creatine metabolism, especially since five females had creatinine above the reference range values at follow-up. In support of our findings, we highlight the potential of creatinine and GAA as promising biomarkers of kidney stress, specifically in response to extended periods of fasting, perhaps caused by

the limited biosynthesis of GAA by the renal tubules or clearance of creatinine after a full day of food restriction while accounting for sex–specific differences.

Correspondingly, 50% of the sample in Study 5 managed to surpass the reference range of serum creatinine upon completing the best of three consecutive esports matches at study baseline. Along with increased creatinine in Study 5, our findings in Study 2 confirmed that prolonged mental efforts may affect homeostasis of creatine metabolism. Globally, competitive online video gaming has surged in popularity, with over 3 billion players worldwide dedicating up to several hours per day to playtime. Consequently, our novel findings for the first time show that demanding tasks embedded in a virtual world can lead to a significant decrease in circulating creatine. Despite its limitations, our pioneering work here suggests that populations engaging in esports may potentially benefit from the additional supplies of available creatine in circulation. Naturally, endogenous synthesis and regular (non-vegetarian/vegan) diet essentially secure plenty of nutrients, including both creatine and its direct precursor GAA; however, it seems that the supplemental formulation of creatine and GAA administered to esports players has beneficial effects indeed. In Study 5, our preliminary findings comply with the idea that 4–week supplementation, even with lower daily dosages, should be considered effective intervention for current (and perhaps future) esports players. We especially emphasize the potential benefits of supplementation towards the end of prolonged sessions/matches or when final games of the series are being played, as accumulation of creatinine in the blood after three consecutive matches was countered post–supplementation. When it comes to running endurance performance, after administering a single dose of the creatine-GAA mixture, despite being insignificant, the improvements in running performance were especially pronounced in the subsample of females.

Furthermore, another novelty comes from our findings in Study 3, suggesting that salivary concentrations of (not serum levels) creatine and creatinine were significantly altered after exercise–

induced exhaustion. However, one caveat is that our results showed no significant correlations between the two fluids; in fact, we found none. This suggests that saliva should be assessed separately and independently from venous blood. Despite being compelling, our preliminary data warrants larger scale confirmation before applying such assessments to practical settings. Regardless, yet another study using creatine-GAA supplementation concluded that it is a safe and effective tool to boost the creatine availability. Despite the limitations in Study 4, a single dose of creatine-GAA taken ~30 minutes before running led to a substantial rise in blood creatine after completing the running-to-exhaustion protocol lasting about 11 minutes on average. However, while the performance gains were not significant, the overall improvements showed a positive trend, which was especially notable in the subsample of females.

## 4.4 Concluding Remarks

This dissertation aimed to answer a series of questions regarding the homeostasis of creatine metabolism in response to different physiological stimuli, and second, to investigate the effects of alternative creatine formulations on creatine status and performance under energy–challenging environments in healthy young men and women. Besides its strengths, the original studies presented in this dissertation are subject to several limitations. Considering that our trials employed quasi–experimental or open–label designs, with the exception of the running-to-exhaustion study, which included a crossover placebo trial, the absence of consistent randomization and a control group reduces the robustness of our findings and causal inference. This introduces the possibility of several confounding factors, such as the effects of familiarization and learning, the placebo effect, or increased effort by participants. Furthermore, a relatively small sample size and homogeneous cohorts of apparently healthy young males and females further limit statistical power and generalizability to broader populations. Other factors that may potentially limit our findings include uncontrolled dietary patterns, energy balance, or interindividual

variability of creatine metabolism. Across studies, no direct tissue-specific assessments (e.g., isotopic tracer approaches, magnetic resonance spectroscopy) were undertaken, limiting mechanistic insight into creatine kinetics across the tissue of interest and thus leaving the organ-specific bioenergetics unresolved at present. The use of saliva as a biomarker matrix, while certainly novel, remains uncertain due to unclear mechanisms of creatine fluctuation and potential analytical variability in handling and storage. After acknowledging these limitations, our current findings highlight the versatile nature of creatine homeostasis in response to various physiological demands, as confirmed by studies involving prolonged fasting, competitive video gaming, and exhaustive running interventions. Furthermore, while the precise mechanisms underlying the beneficial effects of creatine–GAA supplementation on performance remain incompletely understood, these observations should encourage further investigation into this matter to elucidate the underlying mechanisms and translate these insights into practical applications beyond populations of healthy young healthy adults.

# CHAPTER V: PERSONAL SKILLS AND EXPERIENCE

Throughout the doctoral training, I acquired numerous skills from multiple domains related to scientific research. Briefly, the 3 years could be separated in three distinct phases, starting at University of Palermo, at the Department of Psychology, Educational Science and Human Movement, where I've spent my first months, then joining the Applied Bioenergetics Lab for the next 12 months, upon which my oversea period abroad took me to Harvard University for two semesters as a visiting researcher and member of the Kales Lab. Together, these opportunities allowed me to connect with the global scientific community, enabling me to gain the skills necessary for conducting original research, culminating in numerous achievements, including the submission of this doctoral thesis.

## 5.1 Experiences and Skills

Throughout my doctoral training, my academic path started at the Sports and Exercise Science Research Unit. Some of the essential research skills that would later enable me to identify and pursue my goals throughout my career were cemented there. I had the opportunity to work with a rich milieu of equipment related to human performance, including but not limited to devices from the Microgate system (Microgate, Bolzano, Italy) for athletic performance assessment, Virtual Reality (Meta Quest 2, Menlo Park, USA) for the cognitive aspects, and body composition analysis using body impedance analysis (BIA-AKERN 101-AKERN SRL, RJL Systems, Detroit, USA). After joining the Applied Bioenergetics Lab, this collaboration further advanced my experiences and skills in the context of the experimental design. The majority of my training and skills were closely related to applied physiology and dietary supplementation. I had extensive experience with using multiple assessment systems, anthropometrics (Stadiometer, Seca 202, USA) and body composition (Harpenden Caliper, British Indicators Ltd., St. Albans, UK), cardio pulmonary exercise test (Quark CPET, COSMED, Rome, Italy), 1RM protocols, isometric strength (Jamar J00105; Lafayette Instrument Company, Lafayette, IN, USA), muscle power

(Just Jump System; Probotics, Huntsville, AL, USA) including battery tests for cognitive assessments (Inquisit 6, Millisecond Software LLC, Seattle, WA).

On top of that, I acquired hands-on experience in several human trials, including subject recruitment and selection, defining inclusion and exclusion criteria, allocation concealment, supplement and tool preparation, sample size estimation, and statistical power (G*Power, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). In addition, I gained hands-on experience with different diagnostic tools/equipment, including the collection of physiological signals using the Polar H10 (Polar Electro Oy, Kempele, Finland), a functional near-infrared spectroscopy sensor (Fortiori Design LLC, Hutchinson, Minnesota), and the ActiGraph wGT3X-BT (version 6.13.0, ActiGraph Corporation, http://www.ActiGraphcorp.com). Lastly, I was involved in collecting, managing and preparing the biological fluids for the final analysis by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) (1,200 Series LC System; Agilent Technologies Inc.) All the above experiences contributed in the learning process, to seek and identify the research question, generate a hypothesis, design a sufficient experiment to test the theory behind it, manage, analyze, and interpret the data acquired.

Furthermore, during my two-semester tenure at the Department of Environmental Health, Harvard T.H. Chan School of Public Health, I had the opportunity to collaborate with the Kales Lab, which introduced me to the population of emergency responders, specifically USA firefighters. This included the assessment of several critical health domains, including cardiorespiratory fitness testing, well-being and mental health, lifestyle measures such as, dietary and sleep quality, sedentary behavior and levels of physical activity, collected by the Surviving and Thriving Healthy Lifestyle Mobile App (Harvard T.H. Chan Public School of Health, Boston, MA, USA), Research Electronic Data Capture (RedCap, Nashville, TN, USA). In addition, I was able to develop my research skills further and greatly improve my base knowledge in environmental and occupational medicine, human nutrition, and data science

through lectures and courses I've taken. Specifically, I attended the NUT202: The Biological Basis of Human Nutrition at Harvard Public School of Health and STAT100: Introduction to Statistics and Data Science at the Faculty of Arts and Sciences at Harvard University during the 2023-2024 Spring Term. The NUT202 offered me a great amount of detail regarding the metabolic and nutritive aspects of human nutrition, both through lectures and practical weekly tests and quizzes. The STAT100 course enabled me to deepen my understanding of the R programming language, statistical techniques, and associated R packages, such as {dplyr} and {ggplot2} (RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA).

Lastly, I had honor to present our work at several conferences worldwide, including the oral presentations at the Italian Society of Exercise and Sport Science (SISMES) at the University of Milano, Italy; Third International Video Game Studies Conference (SVI) at the Academy of Arts in Novi Sad, Serbia; and the 2025 Creatine for Health and Performance, held in Münich, Germany. With the addition of poster presentations at 2023 Cretan Lifestyle: Mediterranean Tradition & Modern Applications at Rethymno, Greece; and the 2024 Annual Meeting of the American College of Sports Medicine (ACSM) in Boston, MA, USA. The complete list of abstracts authored and co-authored is available in the Appendix section.

## 5.2 Other published studies

All peer-reviewed publications previously not reported in Chapters II and III are listed below in forms of abstracts, (if available):

1. Lidoriki, I., Anđelic, B., Lan, F.-Y., Hershey, M. S., Georgakopoulos, S., Hadkhale, K., Speros, E., Sotos-Prieto, M., Christophi, C. A., & Kales, S. N. (2025). Beta and Pilot Testing of the Surviving & Thriving Healthy Lifestyle App: A Countermeasure to Firefighters' Occupational Health Risks. *Toxics*, *13*(3), 159. https://doi.org/10.3390/toxics13030159

"Beta and Pilot Testing of the Surviving & Thriving Healthy Lifestyle App: A Countermeasure to Firefighters' Occupational Health Risks"

Background: Firefighters face elevated chronic disease risks, and interventions promoting healthier lifestyles are essential for improving their well-being. This study aimed to beta test and further evaluate a healthy lifestyle app (HLS app) for firefighters. Methods: Beta usability testing was conducted with new firefighters after using the app. Pilot testing was conducted in two cohorts, (1) the Connecticut Fire Academy Class A-CCA after graduation and (2) the Connecticut Class B-CCB and Miami-Dade Fire Rescue Academy, during academy training to evaluate the potential efficacy of the HLS app in improving healthy lifestyle behaviors, mental health, and physical fitness over three months of use. Results: Beta testing (n = 93) revealed positive usability feedback, with 62% finding it useful for their health. Pilot testing after graduation (n = 28) was associated with increased push-up capacity (35.6 ± 11.7 vs. 42.9 ± 16.1, $p = 0.006$) and improved mental health scores. Pilot testing during academy training (n = 90) was associated with improvements in push-up capacity (33.8 ± 10.8 vs. 41 ± 10.6, $p < 0.001$), pull-ups (7 [4–11] vs. 10.5 [6–14], $p < 0.001$), 1.5-mile run time (11.96 ± 1.43 vs. 11.26 ± 1.1, $p < 0.001$), BMI (26.7 [24.3–29.7] vs. 25.95 [24.0–28.8], $p < 0.001$), and mental health scores. Conclusions: The app was well received and showed potential for improving firefighter health. A randomized controlled trial is needed to rigorously evaluate the effectiveness of the HLS app.

2. Milovančev A, Petrović M, Miljković T, Ilić A, Mudrinić TR, Miljković A, Ivanov O, Tripunović J, Anđelic B, Bianco A and Drid P (2022) The elite judo female athlete's heart. *Front. Physiol.* 13:990142. doi: 10.3389/fphys.2022.990142

"The elite judo female athlete's heart"

Purpose: There is a paucity of data on physiological heart adaptation in elite-level judo female athletes. This study aimed to assess left ventricular morphology and function in highly trained elite female judokas. Methods: The study prospectively included 18 females aged 23.5 ± 2.25 years, nine elite level judokas, and nine healthy non-athlete volunteers. All participants underwent a medical examination, electrocardiogram, and transthoracic 2D echocardiogram. Left ventricular diastolic and systolic diameters and volumes were determined, and parameters of left heart geometry and function (systolic and diastolic) were measured, calculated, and compared between groups. Results: When groups were compared, judokas had significantly increased left ventricular cavity dimensions $p < 0.01$, left ventricular wall thickness $p < 0.01$, and volumes $p < 0.01$. Elite female judokas exhibited left ventricular dilatation demonstrated as high prevalence increased end-diastolic volume/index, and increased end-systolic volume/index in 88.9% of judokas vs. 0% in controls, $p < 0.01$. Left ventricle mass/index was significantly increased in judokas, $p < 0.01$), with a 43.3% difference between groups. The majority (77.7%) of judokas had normal left ventricular geometry, although eccentric hypertrophy was revealed in 2 (22.2%) of judokas. Conclusion: Elite, highly trained female judokas exhibit significant changes in left heart morphology as a result of vigorous training compared to non-athletes. These findings suggest that female judokas athletes' heart follows a pattern toward chamber dilatation rather than left ventricular wall hypertrophy.

3. Santibañez-Gutierrez A, Fernández-Landa J, Calleja-González J, Todorović N, Ranisavljev M, Štajer V, Anđelić B, Zenić N, Bianco A and Drid P (2022) Epidemiology of children's swimming competence and water safety. *Front. Public Health* 10:961342. doi: 10.3389/fpubh.2022.961342.

"Epidemiology of children's swimming competence and water safety"

Introduction: The main purpose of this study was to investigate children's swimming competence in primary schools of districts in Vojvodina, Serbia. Methods: Included subjects were primary school students from first to eighth grade ($N$ = 2,778; male = 1,454, female = 1,324; age = 10.73 ± 2.1 years). We used Swimming Competence Questionnaire to acquire and analyze their swimming experience, non-fatal aquatic events, and demographics. For the statistical analysis, logistic regression and hierarchical multiple regression were used to evaluate if the factors and SC and NFAE were associated. The analyses were carried out by using SPSS® software version 24.0 (SPSS, Inc., Chicago, Illinois, USA). Results: Families with more income and education generally have children with more swimming competence, experience, knowledge, and skills related to water safety. First step in analysis revealed that gender ($\beta = 0.05$, $p < 0.01$), education level ($\beta = 0.06$, $p < 0.01$) age ($\beta = 0.171$, $p < 0.01$), and family income ($\beta = 0.04$, $p < 0.01$) were significant swimming competence (SC) predictors (R2 = 0.04). Age (OR = 1.15, $p < 0.01$) was the only significant predictor in Step 1 predicting non-fatal aquatic events (NFAE). In Step 2, variables associated with SC were swimming location ($\Delta R2 = 0.06$, $p < 0.01$), swimming experience ($\Delta R2 = 0.16$, $p < 0.01$), swimming accessibility ($\Delta R2 = 0.05$, $p < 0.01$), and learning experience ($\Delta R2 = 0.03$, $p < 0.01$) (total R2 = 0.26 to 0.47, $p < 0.01$). Only a minority of participants reported that they could not swim further than 5 meters using general stroke (37.15%) Conclusion: National education trainers programs must be prioritized with the primary strategy of transferring knowledge to swimming and water safety. Families with lower income must be included without exceptions. This is perhaps a key factor in preventing NFAE, increasing SC, and increasing water safety.

4. Anđelić, B., Bianco, A., Maksimović, N., Todorović, N., & Drid, P. (2022). A milestone in the era of esports: The Olympics through the lens of virtual reality. *Frontiers in Psychology*, *13*, 990189. https://doi.org/10.3389/fpsyg.2022.990189

"A milestone in the era of esports: The Olympics through the lens of virtual reality"

*No abstract available*

5. Korovljev, D., Todorovic, N., Ranisavljev, M., Andjelic, B., Kladar, N., Stajer, V., & Ostojic, S. M. (2023). Hydrogen-rich water upregulates fecal propionic acid levels in overweight adults. *Nutrition, 116*, 112200. https://doi.org/10.1016/j.nut.2023.112200

"Hydrogen-rich water upregulates fecal propionic acid levels in overweight adults "

*No abstract available*

6. Lan, F. Y., Huang, C. Y., Kao, Y. C., Lidoriki, I., Hadkhale, K., Andjelic, B., Huang, Y. C., Lin, C. H., & Kales, S. N. (2025). Development and validation of a firefighters' organizational health culture score. *Archives of Environmental & Occupational Health*, 1–11. Advance online publication. https://doi.org/10.1080/19338244.2025.2513363

"Development and validation of a firefighters' organizational health culture score"

Firefighters rely on teamwork, but their organizational health culture (OHC) is understudied. We aimed to develop and validate a 34-item OHC measurement tool. Data were collected from 543 Taiwanese and 28 US firefighters between May 2023 and March 2024. Exploratory and confirmatory factor analyses (i.e., EFA and CFA) were conducted, and six factors of OHC-leadership support, leadership commitment, sleep and mental health policy, physical activity and nutrition policy, organizational approach, and sense of belonging-were identified. EFA was conducted using data from 271 Taiwanese firefighters, and CFA was performed on the rest, with the latter showing a good fit. We found that a better OHC was correlated with a healthier lifestyle and fewer posttraumatic stress disorder and depressive symptoms. We offer both English and traditional Chinese versions. The OHC's causal effects require further studies.

7. Lakicevic, N., Andjelic, B., Manojlovic, M., Gentile, A., Bianco, A., Paoli, A., Leonov, S., Pashchenko, A., & Drid, P. (2025). Virtual reality and cognitive function rehabilitation after traumatic brain injury: a systematic review. *European Journal of Translational Myology*, *35*(2), 13275. https://doi.org/10.4081/ejtm.2025.13275

"Virtual reality and cognitive function rehabilitation after traumatic brain injury: a systematic review"

Traumatic Brain Injury (TBI) is the leading cause of injury-related death worldwide. In recent years, Virtual Reality (VR) has emerged as a promising diagnostic and treatment tool capable of improving Cognitive Function (CF) after TBI. We sought to review the literature on this issue systematically. Web of Science, PubMed and PsycINFO were screened for relevant literature. Only randomized control trials whereby TBI-affected individuals underwent VR training and control groups received standard rehabilitative care were included. Screening, quality appraisal and data extraction were conducted by independent reviewers using a standardized protocol. Six studies of ~300 participants met the inclusion criteria and showed that both groups improved their overall CF post-intervention. However, non-immersive and semi-immersive VR groups had markedly better scores in all of the cognitive domains measured when compared to non-VR groups. VR is a potent post-TBI rehabilitative tool that can improve CF in this population and facilitate the return-to-work process. Future studies should adopt a similar design yet use fully immersive VR to enhance CF potentially to a greater degree.

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“Effects of Short-Term Creatine Nitrate Plus Creatinine Intake on Creatine Pharmacokinetics and Safety Biomarkers in Healthy Adults “

Background: A blend of creatine nitrate and creatinine has demonstrated promising bioavailability; however, prior studies have not thoroughly examined its pharmacokinetics and safety profiles, particularly its impact on kidney stress indicators, such as serum cystatin C. Objective: This study aimed to assess the effects of varying doses of creatine nitrate-creatinine intervention on pharmacokinetics and safety in healthy humans. Methods: Ten young adults (mean age 26.1 ± 5.0 years; 5 females) volunteered for this double- blind, crossover, randomized controlled trial. The participants were randomly assigned to receive either a low-dose creatine nitrate-creatinine mixture (CN-CRN-Low; 1.5 g of creatine nitrate and 1.5 g of creatinine), a high-dose creatine nitrate-creatinine mixture (CN-CRN-High; 3 g of creatine nitrate and 3 g of creatinine), or 1.5 g of creatine nitrate (CONTROL) in both a single-dose pharmacokinetics experiment, and a 14-day safety trial. Results: Both CN-CRN-Low and CN-CRN-High interventions displayed increased volume of distribution and total clearance compared to the CONTROL intervention ($P < 0.05$) in a single-dose pharmacokinetics experiment. Additionally, the CN-CRN-High intervention showed significantly higher creatine maximum serum concentrations compared to the other interventions ($P < 0.05$). Serum cystatin C levels remained unchanged across all interventions ($P = 0.65$), with no participants experiencing abnormal cystatin C concentrations or major changes in other safety biomarkers. Conclusion: The present study demonstrates dose-specific utilization of creatine nitrate-creatinine intervention, with the mixture induced no kidney damage. Further studies are needed to explore the potential functional and performance benefits of creatine nitrate-creatinine supplementation in diverse clinical and athletic cohorts.

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# APPENDIX

Conference abstracts authored:

## Gaming consumables: creatine-guanidinoacetic acid improves esports performance in young men

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Abstract: Creatine is among leading ergogenic aids proven effective in enhancing various performance tasks including cognitive aspects. Administration of creatine and guanidinoacetic acid (GAA)— its direct precursor—ought to be superior in increasing cerebral creatine levels than creatine itself, presenting a particular interest in the realm of esports, where cognitive performance is an essential component of success.

A total of ten healthy experienced esports players (age 28.4 ± 4 years, BMI 24.9 ± 4.4 kg/m2, 16.1 ± 5.6 years of playing, all males) consented to this open quasi-experimental pilot study. A primary objective was to investigate the effects of a 4 week supplementation with creatine-GAA blend (3g/day, CreGAAtine™, Carnomed, Novi Sad, Serbia) on in-game performance (KDA ratio) in a best-of-three Dota 2™ games format followed by neuropsychological outcomes (Attention Network Test, Stroop Color and Word Test). Baseline and follow-up values were compared using the paired t-test for normally distributed data, otherwise the Wilcoxon test was applied.

The total Dota 2™ scores for the first two games showed a non-significant increase after the intervention ($P < 0.25$). However, the mean scores obtained after the final game significantly improved by 30.1% (95% CI, -17.3 to 77.5; $P = 0.048$). A significantly lower control reaction time was observed post-supplementation ($P = 0.02$), with a mean reduction of 19.2% (95% CI, 2.2 to -40.6), incongruent and control reaction times tended to decrease ($P < 0.15$). Further, incongruent and overall accuracy significantly improved post-intervention ($P \leq 0.05$). On average, incongruent accuracy increased by 10.2% (95% CI, -1.2 to 21.7), as overall accuracy improved by 4.4% (95% CI, -0.9 to 9.7). Lastly, executive control improved after the intervention ($P = 0.01$), with reaction times 16.2% faster on average (95% CI, from -3.4 to -29.0). These preliminary findings suggest that the blend may serve as an effective performanceenhancing intervention in esports.

# Dietary supplement for esports: creatine loading protocol on cognitive performance in experienced video game players

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Purpose: This is the first study to explore the effects of creatine supplementation on cognitive performance in eSport players. From a bilateral perspective, gamers require improved cognitive function, and secondly, cellular energy homeostasis in the brain highly depends on the ATP/CK/PCr system. Using the Attention Network Test (ANT) our objective was to inspect the influence of oral Cr administration on cognitive performance—as it may be relevant in providing energy to the central nervous system (CNS).

Methods: Six healthy eSport players (25.6 ± 3.7 years and 26.1 ± 4.1 BMI) took part in a 2-week crossover pilot study. All participants were blinded to the supplement content, pre-packed in small plastic bags. Subjects consumed one dose of 12.5 g of glucose (Gl) powder in 150 ml of water every 4 h for one week—placebo week (Gl). They repeated the same protocol in the second week with additional 5 g of creatine monohydrate (CrM) to the package. Players performed ANT on three occasions: (1) at baseline, (2) after the first (Gl) week, (3) after the second (CrM) week. An experienced psy- chologist instructed subjects on how to perform the test and supervised every trial. Each of the primary attention constructs—alerting, orienting, and executive were used for the analysis of the study results. The authors checked the normality of the distribution using Shapiro Wilk test and non-parametric tests to analyze the data using the Statistical Package for Social Sciences (SPSS). Friedman's test was used to establish significant differences and the post-hoc Wilcoxson Signed Ranks test to inspect differences between the individual sample pairs.

Results: Analysis indicated that 7-days of CrM supplementation positively affected reaction time compared to baseline values ($p = 0.046$), where no changes were observed in the placebo group. Other attention composites (alertness and orienting) were insignifi- cant, except for executive attention where improvements in executive function increased after both loading phases—Gl and CrM ($p = 0.043$).

Conclusions: Results from this pilot trial indicate possible implications of CrM supplementation among eSport athletes since competitive video gaming implies higher brain cognitive function and fast response from the players. However, in order to give more credible results, further research with a larger sample size is required to confirm the positive influence of high Gl and CrM loading protocols on reaction time among eSport athletes.

## Effects of 8-Week Creatine Supplementation With and Without Ubiquinol on Sperm Creatine Metabolism Biomarkers

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Background: Rising interest in creatine's role in sperm energy metabolism highlights its potential to enhance male fertility, with semen biomarkers providing insights into its impact on sperm function and fertility.

Methods: Fifteen volunteers (mean age 25.0 ± 6.1 years; BMI 25.1 ± 2.0 kg/m²) were randomly assigned to one of three groups: Group 1 received received 5 g of creatine monohydrate plus 200 mg of ubiquinol, Group 2 received 5 g of creatine monohydrate, and the control group received 5 g of inulin daily for 8 weeks. Semen samples were collected after 3–5 days of abstinence and analyzed for creatine and CK levels.

Results: The sperm CK levels showed a non-significant increase in the creatine-ubiquinol group, from 257.5 ± 140.6 IU/L at baseline to 455.2 ± 249.2 IU/L at 8-week follow up ($P = 0.07$). Sperm creatine concentration also increased non-significantly, from 455.2 ± 188.9 µmol/L to 570.7 ± 228.1 µmol/L ($P = 0.06$). In the creatine-only group, sperm CK decreased from 454.3 ± 106.5 IU/L to 422.9 ± 193.7 IU/L ($P = 0.58$), while sperm creatine increased slightly from 499.4 ± 180.7 µmol/L to 564.3 ± 284.4 µmol/L ($P = 0.71$). In the control group, both sperm CK and creatine concentrations decreased. CK dropped from 462.1 ± 229.1 IU/L to 419.9 ± 248.5 IU/L ($P = 0.76$), and creatine declined from 462.1 ± 229.1 µmol/L to 459.5 ± 236.2 µmol/L ($P = 0.90$). Friedman's two-way ANOVA by ranks indicated a significant interaction effect for both sperm creatine and CK concentrations between the intervention groups ($P < 0.05$).

Conclusion: Creatine, both alone and in combination with ubiquinol, led to increases in sperm creatine and CK concentrations, while these biomarkers remained unchanged in the control group. These results highlight the potential metabolic effects of creatine supplementation, warranting further investigation.

## A Single Bout of High-Intensity Exercise Session Affects Salivary Biomarkers of Creatine Metabolism in Healthy Young Men and Women

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Background: Energy requirements during high intensity exercise rapidly escalate. Phosphocreatine, being the major compound utilized to rapidly replenish the ATP levels, essentially relies on creatine stores which may experience fluctuations in its utilization, transporter release across various bodily fluids. So far, no studies have assessed whether exercise affects salivary biomarkers of creatine metabolism, or whether these metabolites in saliva mirror those found in the serum.

Method: Sixteen healthy adults volunteered in this open-label crossover pilot study. All participants were subjected to the running-to-exhaustion treadmill protocol, starting with a 3-minute warm-up at 5 km/h, progressively increasing the workload by 1.5 km/h every minute. Fasted blood samples were collected from the antecubital vein and unstimulated whole saliva was collected from beneath the tongue ~ 30 minutes before and immediately after intervention. All samples underwent analysis for creatine and creatinine using a modified liquid chromatography–tandem mass spectrometry. Differences were compared using paired two-tailed t-test and Pearson's correlation was used to evaluate the linear relationship.

Results: Fifteen participants (23.9 ± 2.9 years, eight females) were analyzed for the study outcomes. In contrast to serum, salivary creatine and creatinine levels significantly elevated pre- to post-exercise (17.5 ± 14.2 μmol/L to 43.6 ± 30.4 μmol/L and 11.3 ± 5.8 μmol/L to 17.0 ± 9.3 μmol/L, $P < 0.001$, $P = 0.04$). Salivary creatine and creatinine metabolism showed a non-significant correlation with the corresponding serum biomarkers at baseline ($r = 0.18$, $P = 0.51$, and $r < 0.01$, $P = 0.99$) and after the exercise ($r = 0.33$, $P = 0.21$ and $r = 0.40$, $P = 0.13$).

Conclusion: Our preliminary findings suggest a potential difference in the exercise-induced dynamics of creatine metabolism across the two bodily fluids, warranting further research into using saliva as simple and non-invasive proxy for creatine turnover and biodynamics.

## Adherence to the Mediterranean diet in New England and Florida Fire Recruits

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Background: The Mediterranean diet has been proven to provide significant physical health benefits, particularly in the prevention of cardiovascular disease. However, adherence to this nutritional pattern among younger groups in the United States is understudied.

Aims: The aim of this survey study was to utilize the validated Mediterranean Diet Adherence Screener (MEDAS) tool to assess adherence to the Mediterranean diet among New England and Florida fire recruits undergoing fire academy training.

Methods: Surveys containing the 14-item MEDAS as well as general demographic questions were administered to 354 firefighters. Raw MEDAS scores were sorted into tertiles. Variables were then analyzed within each tertile to determine if there were any statistically significant differences.

Results: Age was found to be statistically associated with MEDAS tertiles, with a p-value of 0.0045 (Table). Firefighters with a higher MEDAS (greater adherence to the Mediterranean diet) were older than those with lower scores. Additionally, there was a higher proportion of women in the middle tertile. No regional differences in adherence were found. Unadjusted analyses of body composition did not show significant associations with MEDAS tertiles.

Conclusions: No regional differences in Mediterranean diet adherence were found, while women and older fire recruits were more likely to have higher adherence. Our next steps will be to analyze associations with body composition after adjusting for age and gender, and whether lifestyle education interventions can increase adherence.

## Competitive Video Gaming Reduces Circulating Creatine Levels In Experienced Video Game Players

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Previous studies have highlighted various biochemical changes associated with a single video gaming (VG) session. These involve elevated glucose metabolism and energy expenditure, alterations in cortisol and catecholamines, as well as in oxidant-antioxidant balance. Surprisingly, no studies have yet explored whether acute VG has impact on creatine metabolism, an essential pathway involved in replenishing immediate energy for cells and tissues taxed by rapid and intense energy fluctuations.

Purpose: To observe whether a single prolonged session of competitive VG has impact on circulating biomarkers of creatine metabolism in serum using modified liquid chromatography-tandem mass spectrometry.

Methods: Twelve (n 12) healthy experienced VG players (age 25.6 ± 3.8 years, all male) signed an informed consent to volunteer in this quasi-experimental before-after pilot study. Each subject took part in a single six-hour VG session, playing competitive matches in a popular online multiplayer tactical first-person shooting game.

Results: Eleven (n 11) subjects completed the trial and were included in the analysis. A six-hour VG session resulted in a statistically significant drop in serum creatine levels (from 27.6 ± 7.5 μmol/L at baseline to 22.9 ± 8.3 μmol/L at follow-up; P = 0.029). The mean reduction in serum creatine was 4.70

μmol/L (95% confidence interval [CI], from - 2.3 to 11.7), with a moderate-to-large effect size (d = 0.59). Serum creatinine concentrations tended to drop after the gaming session from 88.1 ± 15.5 μmol/L to 78.2 ± 19.8 μmol/L (P = 0.077).

Conclusion: Our findings indicate that creatine homeostasis is sensitive to VG, perhaps owing to the more creatine from the circulation utilized as an energy source for active tissues, including the brain.

## Association Between Use of the Surviving & Thriving App and Healthy Lifestyle Scores and Physical Fitness Among New U.S. Firefighters

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Introduction: Firefighter training academy programs usually improve the physical fitness and body composition of recruits. However, after graduation, physical fitness and health behaviors often deteriorate rapidly. This study investigates the relationship between using a new healthy lifestyle (HLS) smartphone application (app) and changes in healthy lifestyle scores and physical fitness among new U.S. firefighters.

Methods: The HLS app offers interactive educational content on nutrition, sleep, physical activity, and resilience. Pilot testing was conducted in two academies (Connecticut and Miami-Dade) to evaluate its potential efficacy for improving healthy lifestyle behaviors, mental health, and physical fitness after three months of use. Lifestyle scores were derived from validated, online questionnaires administered before and after app use.

Results: Ninety-seven firefighters participated in the study, with 58 completing the follow-up assessment (Connecticut n = 28, Miami n = 30; male 87.9%, female 12.1%, mean age 27.4 ± 5.7 years). Follow-up results demonstrated significant improvements in push-up capacity (+6.6, $p < 0.001$) and MEDAS score (+1.66, $p = 0.001$). Healthy lifestyle scores also increased (mean difference +0.35, $p = 0.046$), while mental health metrics showed notable improvements in PCL (-2.42, $p = 0.001$) and PHQ (-0.95, $p = 0.005$). Other variables, such as BMI and physical activity levels, remained unchanged (Table 1).

Discussion: These preliminary results support the potential benefits of this user-friendly app for improving young firefighters' health and fitness. The results further support a cluster randomized trial among consecutive classes from different fire academies (classes randomized to HLS app use vs written instructions).

| Variables | Pre-app | Post-app | | | |
|---|---|---|---|---|---|
| | Mean ± SD | Mean ± SD | Mean Difference | p-value | 95% CI |
| Push-up capacity (n=56) | 38.2±10.5 | 44.8±13.7 | +6.6 | <0.001 | 3.8-9.5 |
| HLS score (n=55)<br>*Wilcoxon Signed Rank Test* | 4.73±1.13 (median 5.0) IQR=2.0 | 5.07±1.0 (median 5.0) IQR=2.0 | + 0.35 | 0.046 | |
| BMI (kg/m$^2$) (n=55)<br>*Wilcoxon Signed Rank Test* | 27.4±4.0 (median 26.7) IQR=5.2 | 27.3±4.3 (median 25.9) IQR=5.0 | +0.37 | 0.552 | |
| BMI (kg/m$^2$)<br>≥30<br><30 | <br>12 (21.8%)<br>43 (78.2%) | <br>13 (23.6%)<br>42 (76.4%) | | 1.000 | |
| MEDAS | 5.61±2.23 | 10.1±2.4 | +4.5 | 0.001 | 3.37-5.63 |
| Smoking<br>No (Never/Former>6 months)<br>Yes | N (%)<br>57 (98.3%)<br>1 (1.7%) | N (%)<br>57 (98.3%)<br>1 (1.7%) | | - | |
| Tv watching<br>< 2 h/d<br>≥ 2 h/d | N (%)<br>35 (60.3%)<br>23 (39.7%) | N (%)<br>38 (65.5%)<br>20 (34.5%) | | 0.648 | |
| Sleep<br>7-8 h/d<br>Less/more than 7-8 h/d | N (%)<br>31 (53.4%)<br>27 (46.6%) | N (%)<br>27 (46.6%)<br>31 (53.4%) | | 0.454 | |
| Nap<br>Yes<br>No | N (%)<br>47 (81%)<br>11 (19%) | N (%)<br>58 (100%)<br>0 (0) | | - | |
| Physical Activity<br>≥16 MET-h/wk<br><16 MET-h/wk | N (%)<br>44 (75.9%)<br>14 (24.1%) | N (%)<br>42 (72.4%)<br>16 (27.6%) | | 0.804 | |
| Mental health scores<br>Beck<br>PCL<br>PHQ<br>*Wilcoxon Signed Rank Test* | <br>4.05±3.90 (median 6.0) IQR=1.00<br>16.39±13.10 (median 19.0) IQR=20.25<br>6.26±5.24 (median 4.5) IQR=8.25 | <br>3.47±3.22 (median 3.5) IQR=6.00<br>13.96±11.85 (median 10.) IQR=19.50<br>5.31±4.25 (median 6.0) IQR=9.00 | <br>-0.59<br>-2.42<br>-0.95 | <br>0.071<br>0.001<br>0.005 | |

*Table 1. Pre-and post-app data for Connecticut and Miami cohorts*

# Anti-Mediterranean Blue: the alarming Video Gamer lifestyle

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Purpose: Recently, video gaming (VG) has captivated a global audience, with over 3 billion active players worldwide. As VG surged in popularity, an alarming number of adolescents are engaged in VG, a potentially unhealthy lifestyle with global public health implications.

Methods: Excessive VG is a stressful, high-stress virtual activity that taxes multiple aspects of human health. Research suggests that VG affects various biochemical factors, including glucose metabolism, metabolic rate, stress hormones, and antioxidant levels. Within this scope, our quasi-experimental before-after trial explored the effects of a 6-hour VG session on markers of creatine metabolism using modified liquid chromatography–tandem mass spectrometry.

Results: Preliminary subsample analysis (5 males; age 22.8±1.5 years; BMI 26±1.6) revealed altered guanidinoacetic acid (GAA, a direct precursor in creatine metabolism) levels (pre 2.06 *vs*. post 2.50 μmol/L, $p < 0.05$). Thus, as acute VG session significantly affected serum GAA, perchance high-energy demands during VG led to upregulated GAA levels in order to match the acute ATP demands through phosphagen system.

Discussion: This demographic often exhibits insufficient sleep and physical activity. Additionally, they frequently consume excessive caffeine and adhere to a diet rich in sugars, diverging from the principles of the Mediterranean diet (MD), characterized by a high consumption of whole grains, legumes, vegetables, fruits, olive oil, and moderate fish and dairy intake. Due to the direct relationship between better health and the Mediterranean diet, providing adequate nutrition and lifestyle guidelines (e.g., MD) choices to this demographic may be a promising mitigation strategy. Engaging with this large, worldwide, adolescent community and collecting data on biochemical and physiologic correlates of VG deserve more attention, considering the potential implications for public health.