CHAPTER 16

CREATINE AND CREATINE KINASE IN HEALTH AND DISEASE – A BRIGHT FUTURE AHEAD?

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Abstract: Many links are reported or suspected between the functioning of creatine, phosphocreatine, the creatine kinase isoenzymes or the creatine biosynthesis enzymes on one hand, and health or disease on the other hand. The aim of the present book was to outline our current understanding on many of these links. In this chapter, we summarize the main messages and conclusions presented in this book. In addition, we refer to a number of recent publications that highlight the pleiotropy in physiological functions of creatine and creatine kinase, and which suggest that numerous discoveries on new functions of this system are still ahead of us. Finally, we present our views on the most promising future avenues of research to deepen our knowledge on creatine and creatine kinase. In particular, we elaborate on how state-of-the-art high-throughput analytical ("omics") technologies and systems biology approaches may be used successfully to unravel the complex network of interdependent physiological functions related to creatine and creatine kinase

G.S. Salomons and M. Wyss (eds.), Creatine and Creatine Kinase in Health and Disease, 309–334.

1. SHORT SUMMARY OF KNOWLEDGE PRESENTED IN THIS BOOK

For this book, a number of eminent players in the creatine (Cr) and creatine kinase (CK) research arena joined forces to summarize the state-of-the-art in their respective fields of expertise, and to share their views on future scientific developments in these fields. The first six chapters of the book discuss our current knowledge on the origins and physiological functions of the main players of Cr metabolism, namely Cr, phosphocreatine (PCr), L-arginine:glycine amidinotransferase (AGAT), guanidinoacetate methyltransferase (GAMT), the Cr transporter (SLC6A8/CT1) and the different CK isoenzymes. In chapter 2, the evolutionary history of CK isoenzymes, of the Cr transporter and of the Cr biosynthesis enzymes is presented, suggesting that all these processes may have evolved already in the earliest (invertebrate) animals to facilitate high-energy phosphate transport in flagellated cells (Ellington and Suzuki, 2007). Thermodynamic and kinetic aspects relevant for the CK phosphotransfer network are discussed in chapter 3, as are attempts to devise informative mathematical models that appropriately describe the physiological functions of the CK system (Saks *et al.*, 2007). Besides reporting on the modeling achievements already made, this chapter also provides a glimpse at the challenges faced when attempting to find the appropriate balance between (i) a proper description of a highly compartmentalized enzyme system that is functionally coupled to a multitude of dynamic physiological processes and (ii) the level of abstraction required to make the modeling both feasible from a computational perspective and amenable to experimental validation. Chapters 4–7 provide insights into the tissue-specific expression, localization and function of AGAT, GAMT, the Cr transporter and the different CK isoenzymes (Braissant *et al.*, 2007; Christie, 2007; Heerschap *et al.*, 2007; Tachikawa *et al.*, 2007). They report, among others, on new structural insights into the mechanism of action of the Cr transporter based on a comparison with the recently published high-resolution structure of a prokaryotic homologue of the SLC6 transporters; on the distinct cell type-specific expression of AGAT, GAMT and the Cr transporter in the brain; on the functional relevance of transport processes for Cr between different cell types and across the blood-brain and blood-retinal barriers; and on the use of gene knock-out mouse models to elucidate the physiological functions of GAMT and of the different CK isoenzymes.

The second part of the book focuses on clinical aspects of Cr metabolism. As a logical continuation and complementation of chapter 7 on GAMT- and CK-knockout animals (Heerschap *et al.*, 2007), chapters 8 and 9 summarize our current knowledge on inherited disorders of Cr biosynthesis and transport in man – i.e., on (cerebral) creatine deficiency syndromes (CDSs) that are due to pathogenic mutations in the *AGAT*, *GAMT* or Cr transporter genes –, together with therapeutic approaches to treat Cr biosynthesis defects (Schulze and Battini, 2007; Stockler *et al.*, 2007). Key messages from these chapters are that CDSs may be more prevalent than typically presumed; that the severe neurological sequelae due to AGAT or GAMT deficiency may be prevented completely if appropriate

therapeutic intervention is initiated early after birth; and that, thus, at least some CDSs may qualify for inclusion in neonatal screening programs. In chapters 10 and 11, the promise of Cr supplementation as a therapeutic strategy in neurological, neuromuscular and neurometabolic disorders is discussed (Klein and Ferrante, 2007; Tarnopolsky, 2007). While benefits of Cr supplementation were reported in some studies and for several disorders, other trials failed to provide a positive effect. Nevertheless, the authors conclude that Cr supplementation is a potentially powerful therapeutic approach, but that additional, larger-scale clinical trials on homogeneous patient groups are required to determine whether these disorders are responsive to Cr supplementation, and which supplementation regime is optimal for what kind of disorder. Since the benefits of Cr supplementation in these disorders are most likely rather modest, it would seem desirable to pursue combination therapies together with other neuroprotective agents that display different mechanisms of action than Cr. The evidence for ergogenic actions of Cr is presented in chapter 12 (Hespel and Derave, 2007). It is shown that – although Cr's effects are usually limited – consistent improvement in exercise performance can be obtained with Cr supplementation, mostly in explosive sports with repeated contraction series. In addition, Cr supplementation shows promise as an adjuvant therapy in rehabilitation from immobilization-induced muscle atrophy.

In the last part of the book, several aspects relevant for Cr supplementation in man are featured. Chapters 13 and 14 discuss the pharmacokinetics of Cr and the safety of oral Cr supplementation, respectively (McCall and Persky, 2007; Persky and Rawson, 2007). Surprisingly little is as yet known on the pharmacokinetics of Cr, especially for clinically relevant patient populations and for special populations such as the elderly. Although many 'clinical' studies on the safety of oral Cr supplementation have been published, most of them involved only a small number of subjects, thus allowing at best preliminary conclusions. So far, Cr supplementation at the recommended dosages has proven to be safe, although more extended studies on large cohorts are required to allow definitive conclusions on the long-term safety of chronic oral Cr supplementation especially at higher dosages. In chapter 15, an industrial perspective is provided on the commercial production and use of supplementary Cr (Pischel and Gastner, 2007). The different chemical production methods as well as analytical methods used for quality control are presented, as is the regulatory status for Cr as a dietary supplement in the USA and Europe. Emphasis is placed on implementing an appropriate quality assurance policy to maintain high quality standards for oral Cr supplements.

Overall, the various chapters of this book provide a very good overview on many of the key topics that are currently addressed in Cr and CK research, and bear testimony to the attractiveness of the field. The last ten years have shown that in man, Cr and CK may play their most crucial functions in the brain. Thus, the neuroprotective and neurostimulatory effects of Cr supplementation may be(come) more relevant for mankind than the ergogenic effects that are currently so popular.

2. FURTHER CONSIDERATIONS AND HIGHLIGHTS FROM THE RECENT LITERATURE

It is far beyond the scope of this book to comprehensively cover all aspects of Cr and CK research. This is understandably so when considering that Cr and CK were discovered about 175 and 70 years ago, respectively. For reviews on some other aspects of Cr and CK research, the reader is referred to Wyss and Kaddurah-Daouk (2000), Wyss and Schulze (2002), Schlattner *et al.* (2006), Vial (2006), Saks (2007), and Brosnan and Brosnan (2007). The purpose of the current section is to convey some additional thoughts and to pinpoint at some recently published studies on aspects not covered otherwise in this book that provide further insights into the pleiotropy of functions exerted by Cr and CK.

Given the recent interest in (cerebral) creatine deficiency syndromes (CDSs) and the realization that this group of inherited diseases may be rather frequent, the question arises of the prevalence and functional implications of CK deficiency. In principle, both Cr and CK deficiency might be expected to similarly compromise the function of the CK/PCr/Cr system. Nevertheless, only five CK-deficient patients have been reported so far (Feng *et al.*, 2007; Nagai, 2000; Oita *et al.*, 1988; Shibuya *et al.*, 1992; Yamamichi *et al.*, 2001). Four of the five patients were diagnosed as suffering from acute myocardial infarction, and three patients died of acute myocardial infarction at age 49–56 years. In two patients, a point mutation in the M-CK gene has been identified, causing the amino acid substitution D54G (p.Asp54Gly) (Nagai, 2000; Yamamichi *et al.*, 2001). Compared to wild-type M-CK, the D54G mutant revealed substantially lower enzymatic activity, substrate affinity and stability (Feng *et al.*, 2007).

The primary reason for the low number of CK-deficient patients recognized so far seems to be the multiplicity of CK isoenzymes in higher eukaryotes, comprising two cytosolic CK isoenzymes, M-CK and B-CK, and two mitochondrial CK isoenzymes, ubiquitous and sarcomeric MtCK (Wallimann *et al.*, 2007). In either muscle or brain, at least two CK isoenzymes are co-expressed: M-CK and sMtCK in muscle, B-CK and uMtCK in brain. In transgenic mice with knock-outs in a given CK isoenzyme, the remaining CK isoenzyme(s) can take over the function of the deleted CK isoenzyme, at least in part (de Groof *et al.*, 2001; in 't Zandt *et al.*, 2003; Streijger *et al.*, 2005). Therefore, the phenotype of CK-deficient patients may be milder than that of patients with deficiencies in either AGAT, GAMT or the Cr transporter. The likelihood of a simultaneous deficiency in two or even more CK isoenzymes can be expected to be extremely low. It is somewhat intriguing, though, that while the brain seems to be the most affected tissue in CDSs in man, only M-CK-deficient, but no B-CK- or uMtCK-deficient patients have been identified so far.

The brain is probably the tissue that is currently most intensively investigated for the physiological functions of Cr and CK. This is reflected in several chapters of this book – most prominently in chapters 4, 5, 7–9, and 11 (Braissant *et al.*, 2007; Heerschap *et al.*, 2007; Klein and Ferrante, 2007; Schulze and Battini, 2007; Stockler *et al.*, 2007; Tachikawa *et al.*, 2007) –, but also in a significant number of

further recent publications. One report indicated that Cr concentrations in the brain may play a role in regulating appetite and weight (Galbraith *et al.*, 2006). According to an earlier suggestion, Cr may act as one of the most relevant osmolytes in brain (Bothwell *et al.*, 2001, 2002; Videen *et al.*, 1995). This may explain the stimulation of Cr transporter expression in conditionally immortalized mouse brain capillary endothelial cells exposed to ammonia, an *in vitro* model system for studying the impact of hyperammonemia (due, e.g., to acute liver failure) on the blood-brain barrier (Bélanger *et al.*, 2007). In tissue culture of human fetal spinal cord, chronic exposure to Cr resulted in significantly higher densities of GABA-immunoreactive neurons while total neuronal cell density was unchanged (Ducray *et al.*, 2007). This finding points to an important role of Cr in inducing differentiation towards the GABAergic phenotype, and for normal brain development and function in general. In addition, it suggests that Cr supplementation may become a promising strategy in cell replacement strategies for central nervous system (CNS) tissue repair.

Several studies have provided evidence for a link between Cr and/or high-energy phosphate metabolism and mental performance (Ferrier *et al.*, 2000; Hoyer *et al.*, 2004; McMorris *et al.*, 2006, 2007; Rae *et al.*, 2003; Valenzuela *et al.*, 2003; Watanabe *et al.*, 2002). Among others, oral Cr supplementation was shown to positively affect working memory and intelligence (Rae *et al.*, 2003). On the other hand, CK has been identified as a primary target for a number of potential neurotoxins: for instance, both mitochondrial and cytosolic CK from rat cerebral cortex were found to be inhibited by 3-hydroxykynurenine, a neurotoxin implicated in neurodegenerative disorders such as Huntington's disease and Parkinson's disease (Cornelio *et al.*, 2006). Similarly, human B-CK was inhibited by micromolar concentrations of 4-hydroxy-2-nonenal, a lipid peroxidation product that is known to be increased in the brain of, e.g., Parkinson's disease patients (Markus Wyss, unpublished data). Administration of arginine to rats significantly reduced the cytosolic and total CK activities in cerebellum, while not affecting mitochondrial CK activity (Delwing *et al.*, 2007). Thus, these results suggest that inhibition of CK by arginine may contribute to the neurotoxicity seen in hyperargininemic patients. Similarly, intrastriatal administration of guanidinoacetate, supposed to be responsible for at least part of the neurological symptoms in GAMT-deficient patients (chapter 9; Schulze and Battini, 2007), was shown to decrease mitochondrial and total CK activity in rat striatum, whereas cytosolic CK activity was not affected (Zugno *et al.*, 2006). Finally, 2-methyl-3-hydroxybutyrate, a substance that accumulates in patients with either mitochondrial β-ketothiolase or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency (which are inherited neurometabolic disorders), was demonstrated to inhibit both total and mitochondrial CK activity from cerebral cortex of developing rats, while not affecting cytosolic CK activity (Rosa *et al.*, 2005).

Together with the neuroprotective effects of Cr seen under a variety of different conditions (see chapter 11; Klein and Ferrante, 2007; Andres *et al.*, 2005a,b; Bender *et al.*, 2007; Braissant *et al.*, 2002; Burklen *et al.*, 2006; Ducray *et al.*, 2006; Lensman *et al.*, 2006; Morton *et al.*, 2005; Pena-Altamira *et al.*, 2005; Sakellaris

et al., 2006; Vasques *et al.*, 2006), the above findings provide a flavour of the potentially tight dependency of optimal brain performance from proper functioning of the CK/Cr system. Although some of the listed studies need independent corroboration, the wealth of data on a critical role of the CK/Cr system in the brain may be sufficient rationale to more thoroughly consider oral Cr supplementation as a means to improve mental performance, mood, and/or general well-being.

Aging and neurodegeneration are thought to share pathophysiological pathways (Bender *et al.*, 2007). Therefore, testing the impact of Cr on the aging process suggested itself. In fact, in aged mice, oral Cr supplementation was found to increase the healthy life span by 9%, to improve neurobehavioral functions, and to reduce accumulation of the "aging pigment" lipofuscin (Bender *et al.*, 2007).

A surprising finding has recently been made on the expression of AGAT in human myocardium, suggesting that this organ is able to synthesize Cr locally (Cullen *et al.*, 2006). AGAT mRNA levels and enzyme activity were found to be increased in end-stage heart failure. Moreover, upon combination therapy consisting of mechanical unloading using a left ventricular assist device and pharmacological intervention, functional recovery of the heart was correlated with a decrease in AGAT expression to baseline values. These findings may suggest a shortage of Cr in the failing heart and are further proof for the relevance of the CK/Cr system in this organ, a conclusion that is supported by other recent findings (chapter 7; Heerschap *et al.*, 2007; Ingwall, 2006; Lygate *et al.*, 2007; Neubauer, 2007; Saks *et al.*, 2006a; Schlattner *et al.*, 2006).

Besides the tissues analyzed extensively for the functions of Cr and CK such as skeletal and cardiac muscle, brain, retina and spermatozoa (Wyss and Kaddurah-Daouk, 2000), new, relevant roles for the CK/Cr system have recently been identified in tissues not studied in detail before. In rat osteoblast-like cells, addition of Cr to the culture medium had a stimulatory effect on metabolic activity, differentiation and mineralization (Gerber *et al.*, 2005). Similarly, supplementation of rats for 8 weeks with Cr increased lumbar bone mineral density as well as the mechanical load at failure of the femur (Antolic *et al.*, 2007). In a study on older men, preliminary evidence was obtained for a positive effect of Cr supplementation on bone mineral content (Chilibeck *et al.*, 2005). Together with previous findings showing that in boys with Duchenne and Becker muscular dystrophy, Cr supplementation increased bone mineral density and reduced urinary excretion of N-telopeptide (Louis *et al.*, 2003; Tarnopolsky *et al.*, 2004), these studies suggest that Cr supplementation might be used as an adjuvant therapy for bone repair *in vivo*. A study on the impact of Cr supplementation, eventually in combination with vitamin D and calcium, on the mineral content and density loss of compact and spongeous bones of post-menopausal women might be most warranted, given their high incidence of osteoporosis.

Aging of the skin is characterized by a decline in cellular energy metabolism, which seems to be caused predominantly by free radicals that are generated by exogenous noxes such as UV radiation. Both the Cr transporter and CK isoenzymes are expressed in human skin (Lenz *et al.*, 2005; Schlattner *et al.*, 2002; see also

Zemtsov, 2007). In cultured human skin fibroblasts and keratinocytes, addition of Cr to the medium increased CK activity and mitochondrial function, and protected against free oxygen radical damage and, in particular, DNA mutations (Berneburg *et al.*, 2005; Lenz *et al.*, 2005). In studies with healthy old volunteers, topical application of Cr prevented the decline in mitochondrial membrane potential due to UV irradiation (Lenz *et al.*, 2005), increased the density of dermal papillae, and reduced the appearance and depth of fine lines and wrinkles (Blatt *et al.*, 2005). Although Cr is already used commercially in skin creams (http://www.beiersdorf.com), more in-depth studies are required to corroborate the relevance of the CK/Cr system for proper function and aging of the skin. It will be particularly interesting to evaluate the beneficial effects of Cr on wound healing, as well as on a variety of pathological skin conditions, such as toxic epidermal necrolysis, psoriasis, chronic ulcers, burns and neurodermitis.

A shotgun proteomics approach identified B-CK as the second most-abundant protein in the sensory hair cells of the inner ear (Shin *et al.*, 2007). Theoretical considerations and experiments in which B-CK was selectively inactivated demonstrated that high-energy phosphate transport in these elongated mechanoreceptor cells is crucially dependent on the CK/Cr system. In another study, detailed analysis of the gene expression profiles in the superior olivary complex, a structure of the mammalian auditory brainstem, as well as in striatum, hippocampus and extraocular muscle tissue revealed increased expression of several genes of Cr metabolism in the superior olivary complex (Nothwang *et al.*, 2006). Expression of the Cr transporter, of AGAT and of B-CK was higher in the superior olivary complex than in the other tissues analyzed, thus also suggesting an involvement of CK and Cr in auditory function. The findings of both studies likely explain the hearing loss and the vestibular dysfunctions observed in transgenic knock-out mice lacking either B-CK alone, or both B-CK and ubiquitous mitochondrial CK (uMtCK) (Shin *et al.*, 2007). In mice lacking both CK isoenzymes, the sensitivity of the auditory system to 8–32 kHz tone bursts was reduced 10- to 30-fold as compared to wild-type mice. Notably, supplementation of guinea pigs with high doses of Cr (3% w/w) has recently been shown to exert remarkable protection from noise-induced hearing loss and degeneration of hair cells (Minami *et al.*, 2007). Therefore, it might be attractive to evaluate Cr supplementation in patients with tinnitus or other degenerative hearing loss conditions, preferably at early time points of the disease. In addition, Cr supplementation might be tested as a preventive measure for people who are chronically exposed to high noise levels.

Investigations on the regulation of methyl balance in humans have shown that approximately 40% of methyl groups from *S*-adenosylmethionine (SAM) are used for Cr biosynthesis (see Brosnan *et al.*, 2007a,b). In addition, about 10% of dietary glycine and as much as 20% of dietary arginine are used for Cr synthesis. Since elevated plasma homocysteine levels have been implicated as a risk factor for a number of chronic diseases, Cr supplementation may prove useful in situations of compromised SAM homeostasis and /or increased demand for methyl groups from SAM.

Recent studies also provide further evidence for a critical role of the CK/Cr system in diseases other than those discussed elsewhere in this book. *S*-adenosylhomocysteine hydrolase deficiency is a rare disease with primarily neuromuscular symptomatology (among others, hypotonia, psychomotor delay, and delayed myelination). In the three patients described so far (Baric *et al.*, 2004, 2005; Buist *et al.*, 2006), plasma concentrations of SAM and *S*-adenosylhomocysteine (AdoHcy) were largely increased. A slight increase in guanidinoacetate and a slight reduction in phosphatidylcholine and free choline concentrations were also observed occasionally. Dietary methionine restriction and supplementation with Cr and phosphatidylcholine markedly reduced plasma SAM and AdoHcy concentrations, and improved myelination and psychomotor development (Baric *et al.*, 2004, 2005). In patients with argininosuccinate synthetase or argininosuccinate lyase deficiency treated with dietary arginine supplementation, cerebral guanidinoacetate concentrations were found to be increased (Sijens *et al.*, 2006; van Spronsen *et al.*, 2006). Considering the neurotoxicity of guanidinoacetate (see chapters 8 and 9; Schulze and Battini, 2007; Stockler *et al.*, 2007), these findings – if confirmed – may advocate a reduction in arginine supplementation as well as complementary Cr supplementation in these urea cycle defects (van Spronsen *et al.*, 2006). In a rat model of endotoxin-induced sepsis, MtCK content, MtCK activity and Cr-stimulated mitochondrial respiration were all largely reduced in diaphragm and heart (Callahan and Supinski, 2007). MtCK inactivation may be linked to the increase in mitochondrial free radical generation observed in sepsis, and may contribute to the functional abnormalities occurring in this syndrome. Again, it might be worthwhile to test whether Cr is able to protect against the sequelae of sepsis. Cystinosis is an autosomal recessive disorder associated with lysosomal cystine accumulation and involves, among others, visual deficits. In cytosolic and mitochondrial fractions isolated from pig retina, CK activity was inhibited in a dose- and time-dependent manner by cystine (Pereira Oliveira *et al.*, 2007). Cysteamine prevented and reversed the inactivation caused by cystine, suggesting oxidative modification of the reactive sulfhydryl groups of CK by cystine as the underlying mechanism for the observed effects. Given the likely relevance of the CK/Cr system for high-energy phosphate transport in photoreceptor cells of the retina (Hemmer *et al.*, 1993; Wegmann *et al.*, 1991), inhibition of CK by cystine may contribute to visual impairment in cystinosis patients. Cachexia is prevalent in cancer and in certain infectious diseases such as AIDS or tuberculosis. In patients with colorectal cancer undergoing milder chemotherapy, preliminary experiments suggested oral Cr supplementation to be a potentially promising adjuvant therapy for preventing cachexia (Norman *et al.*, 2006). Patients on aggressive chemotherapy, however, did not seem to benefit from Cr supplementation. Further experiments are required to evaluate the potential benefits of Cr for increasing body weight and muscle mass in cachexia patients and whether it would seem desirable to add Cr to high-caloric clinical nutrition. Finally, as an extension to previous studies, Ghosh *et al.* (2006) demonstrated that in tumor-bearing mice, a combination of methylglyoxal, ascorbic acid (both injected intraperitoneally) and Cr (intraperitoneally or supplied in the

drinking water) completely inhibited proliferation of tumor cells and significantly increased the life span of the animals. Nearly 80% of the tumor-bearing mice were completely cured. Therefore, such a combination therapy might be considered as a low-cost alternative to the exquisite cancer drugs marketed by the leading pharmaceutical companies.

A number of recent publications also provide new insights into the mechanisms of action of Cr and CK. Epand *et al.* (2007) presented striking experimental evidence for octameric MtCK to mediate lipid transfer between two membranes. Through their particular symmetry and membrane-binding properties, MtCK octamers can simultaneously bind to two nearby membrane bilayers, e.g. in mitochondrial contact sites. In the *in vitro* experiments performed, the lipid transfer process was suggested not to involve vesicle fusion or loss of the internal contents of the liposomes used. These findings are remarkable when considering that interbilayer transfer of lipids between the inner and outer mitochondrial membranes may play an important role in the regulation and initiation of apoptosis (Epand *et al.*, 2007).

Exposure of C2C12 murine myoblasts or porcine endothelial cells to hypertonic stress induced an increase in the expression of the Cr transporter as well as in Cr transport activity (Alfieri *et al.*, 2006). When exposed to hypertonic conditions, survival of C2C12 cells was enhanced by Cr in a manner similar to that of betaine, taurine, or *myo*-inositol. Thus, in muscle as in brain (see above), Cr may act as a compatible osmolyte.

In rats selectively bred for high vs. low aerobic running capacity, muscle mitochondria displayed higher Cr-stimulated respiration despite similar mitochondrial density (Walsh *et al.*, 2006). This suggests increased functional coupling between MtCK and adenine nucleotide translocase of the inner mitochondrial membrane. Remarkably, rats bred for high aerobic capacity also revealed a lower susceptibility to ischemia-reperfusion-mediated ventricular tachyarrhythmias (Lujan *et al.*, 2006) which is reminiscent of the anti-arrhythmic effects of PCr reported previously (Cisowski *et al.*, 1996; Fagbemi *et al.*, 1982; Hearse *et al.*, 1986; Ruda *et al.*, 1988). In a further series of experiments on isolated rat brain mitochondria and cultured embryonic rat cortical neurons, MtCK was demonstrated to favour internal ADP cycling in mitochondria, resulting in tight coupling of the respiratory chain to ATP synthesis, and thereby to diminish production of reactive oxygen species, particularly under hyperglycemic conditions (Meyer *et al.*, 2006). The preventive anti-oxidant role depended on both the PCr/Cr ratio and MtCK activity, as evidenced by a lack of anti-oxidant action of Cr in rat liver mitochondria which are devoid of MtCK.

In cultured MIN-6 β-cells, addition of Cr in the presence of glucose elicited a significant increase in insulin secretion (Rocic *et al.*, 2007). On the other hand, Cr had no effect in the absence of glucose, suggesting that Cr acts as a potentiator rather than an initiator of insulin secretion. In addition, Cr increased cellular ATP levels, independently of the presence of glucose. These mechanisms of action are likely an underlying basis for the observed hypoglycemic effects of Cr.

Cr supplementation has been shown in rat skeletal muscle cells to increase the phosphorylation of AMP-activated protein kinase (AMPK) (Ceddia and Sweeney, 2004), a key sensor and fuel gauge of cellular energetics (Hardie *et al.*, 2006), as well as to stimulate expression of the glucose transporter GLUT4 in rat muscle (Ju *et al.*, 2005). These findings are in accord with the fact that AMPK is a well-known regulator of glucose transport (Fujii *et al.*, 2004). According to preliminary data, AMPK also seems to regulate the Cr transporter (Darrabie *et al.*, 2007). Elucidation of the detailed interplay between AMPK and regulation of the Cr transporter on one hand, and between Cr supplementation and AMPK signalling on the other hand (Neumann *et al.*, 2007) deserves further attention in the future.

Let us conclude this section on recent Cr- and CK-related reports with some rather unrelated and preliminary findings. Given the ergogenic effects of oral Cr supplementation (see chapter 12; Hespel and Derave, 2007) and the popular use of Cr as a strength- and muscle mass-enhancing aid in explosive sports and bodybuilding, it seemed logical to test the impact of dietary Cr on growth performance and meat quality in livestock production. Disappointingly, however, current evidence does not seem to provide sufficient rationale for regular, economical use of Cr in livestock feed (Lindahl *et al.*, 2006; Stahl *et al.*, 2007). One reason for the inconclusive data so far might be – as in man – the occurrence of Cr "responders" and "non-responders" among different breeds of, e.g., pigs (Young *et al.*, 2007).

An intriguing hypothesis was raised by Zhou *et al.* (2006) who linked a genetic polymorphism in the 3'-region of the M-CK gene to differences in the response of running economy to endurance training in man. Finally, in a study on a multiethnic population involving 1444 Dutch citizens, a significant correlation was seen between serum CK activity and both systolic and diastolic blood pressure (Brewster *et al.*, 2006). Higher serum CK activity may be a reflection of higher tissue CK content which, in turn, may trigger higher pressor responses in heart and vasculature. Clearly, many of the findings reported in this section and, in particular, the last-mentioned hypotheses require further experimental corroboration.

The collection of articles presented in this section is by no means meant to be exhaustive. Rather, it should provide a flavour and further proof for the pleiotropy of effects exerted by Cr and CK. Despite 175 years since the discovery of Cr, new disclosures are still being made on a regular basis on the localization and function of CK and Cr in many different organs, tissues and cell types of living organisms across large evolutionary distances.

3. FUTURE AVENUES OF RESEARCH

Considering the recent emphasis on (cerebral) Cr deficiency syndromes (CDSs) and the multitude of cellular processes with which the CK/Cr system interacts, what may be the future avenues of research that promise the most significant gains in our knowledge on the relevance of the CK/Cr system in both health and disease? What obstacles may be encountered, and how may they be circumvented? The

following facets of the picture will be discussed in this section: (i) the relevance of suitable animal models; (ii) the need for better understanding of compartmentalization, transmembrane transport processes and cell-to-cell trafficking involved in Cr metabolism; (iii) the expected contributions of "omics" and "systems biology" to knowledge gain; (iv) neuroprotection and CDSs – what is next?; (v) suggestions for optimized Cr dosages and supplementation regimes; and (vi) why we cannot expect the large pharmaceutical companies to drive future developments in this field.

3.1. Relevance of Suitable Animal Models

Given the functional redundancy among different CK isoenzymes and among different high-energy phosphate transport systems, standardization of experimental conditions is a key prerequisite for the detection of subtle functional differences. There are limits to such standardization in human studies (different ethnicities, genotypes, living styles or diets), so that suitable animal models will remain a crucial instrument in furthering our knowledge on the functions of the CK/Cr system in health and disease. In knock-out studies done so far, certain CK isoenzymes or GAMT were deleted globally by disruption of the corresponding gene in the genome. Thus, the phenotypic expression of the genetic deficit in these studies may have been a composite effect of compromised cellular functions in many different cell types at once. This makes proper dissection of cause-and-effect relationships very difficult if not impossible. Studies on the liver of transgenic mice expressing either cytosolic or mitochondrial CK isoenzymes have been one example to address the functions of CK and Cr in one particular tissue only (see chapter 7; Heerschap *et al.*, 2007). Similarly, CK genes have been introduced into distant model organisms that do not normally express these enzymes, i.e. *Escherichia coli* and *Saccharomyces cerevisiae* (Canonaco *et al.*, 2002, 2003).

In the future, with further improvements in genetic tools, more targeted and better controlled changes in expression of genes involved in Cr metabolism should be attempted, for instance specific expression of such enzymes in cell types or tissues in which they are not normally expressed, or tissue-specific gene knock-outs. In addition, conditional knock-out and knock-in strategies are desirable to study the time-course of functional responses to such modifications at different developmental stages. Furthermore, rather than studying just all-or-none effects, more graded changes in gene expression should be targeted to establish dose-response relationships, and to provide better resolution for establishing the logical sequence of events in response to the given genetic derangement. RNA interference strategies may be a suitable instrument in many of these endeavors.

Studies on cell cultures, either with a single cell type or with, e.g., brain cell primary 3D cultures composed of mixed oligodendrocytes, astrocytes and neurons (chapter 4; Braissant *et al.*, 2007), may be a means of attaining even better experimental standardization. However, extrapolation of results obtained in cell culture

to the *in vivo* situation may not be trivial. Finally, we still await the first (transgenic) animal models with targeted alterations in expression of AGAT or the Cr transporter.

3.2. Relevance of Transport Processes in Cr Metabolism

It is broadly believed that in the human body, sites of Cr biosynthesis are typically separated from tissues and cell types that most rely on the CK/Cr system for high-energy phosphate recycling and transport such as striated muscle. The reason for this may be the need for independent regulation of Cr biosynthesis and CK function. The direct implication is that transport processes between different tissues and cell types and across cellular membranes play a crucial role in overall Cr metabolism. Possibly due to the fact that membrane proteins and processes are more difficult to study than soluble, cytosolic enzymes, our knowledge on transport processes important for Cr metabolism is still limited. For instance, it is still largely unknown how guanidinoacetate enters hepatocytes in the liver and how Cr is exported from the liver into the blood to be directed to the target organs.

Tissues most relying on the CK/Cr system take up Cr from the bloodstream against a large concentration gradient, a process that is mediated by a Na^+- and Cl^--dependent Cr transporter (chapter 6; Christie, 2007). Increasing the extracellular Cr concentration – either by addition of Cr to the culture medium *in vitro* or by oral Cr supplementation *in vivo* – is known to downregulate the Cr transporter in striated muscle cells (Guerrero-Ontiveros and Wallimann, 1998; Loike *et al.*, 1988). This is one of the reasons why in the sports and bodybuilding arena, the daily dose of oral Cr supplementation is reduced from 15–20 g/day in the initial loading phase lasting 3–5 days to 2–5 g/day in the maintenance phase. In addition, it is often recommended to regularly pause Cr supplementation for several weeks, so as to allow the body to restore baseline expression of the Cr transporter and, thus, to prepare the cells and tissues for a next cycle of Cr supplementation. However, the dynamics of downregulation and recovery of Cr transporter expression have been studied only poorly so far, and almost nothing is known in this respect about tissues other than striated muscle. Furthermore, Cr transporter expression *per se* as well as its regulation may differ between health and disease, an area that is also explored to only a very limited extent so far (chapter 6; Christie, 2007).

Recent evidence summarized in chapters 4 and 5 of this book (Braissant *et al.*, 2007; Tachikawa *et al.*, 2007) suggests that Cr can be synthesized locally in the brain, but that also there, Cr biosynthesis is physically separated from cells most relying on the CK/Cr system. How Cr biosynthesis is regulated in the brain, how the different cell types in the brain communicate to balance Cr biosynthesis to demand, and which role transport processes play in the overall scheme requires further in-depth studies. The Cr transporter is expressed in the brain and is very likely responsible for uptake of Cr into cells depending on the CK/Cr system. However, neither in the brain nor elsewhere in the body, it is known how guanidinoacetic acid and Cr are exported from cells and tissues involved in Cr biosynthesis.

It will be interesting to explore whether this is just an example of passive diffusion through non-specific membrane channels, whether a specific Cr exporter exists, and/or whether the Cr transporter also contributes to Cr export in these cells and tissues.

The Cr transporter is also supposed to be responsible for uptake of Cr across the blood-brain and blood-retina barriers (chapters 4 and 5; Braissant *et al.*, 2007; Tachikawa *et al.*, 2007). However, uptake of Cr from the bloodstream into the brain is very slow, requiring several weeks or even months for seeing significant benefits of oral Cr supplementation on the Cr content in brain. Considering (i) that the Cr transporter plays such a crucial role in delivering Cr to its sites of action, (ii) that increasing extracellular Cr concentrations reduce rather than promote cellular uptake of Cr, (iii) that Cr transport across the blood-brain barrier is severely restricted, and (iv) that, on the other hand, Cr supplementation offers a plethora of potential health-beneficial effects as well as clinical benefits in patients with CDSs (see this book and Wyss and Schulze, 2002), well-designed comprehensive studies addressing the regulation of Cr transport are clearly warranted. In addition, the large body of evidence for a neuroprotective role of Cr (chapter 11, Klein and Ferrante, 2007) offers great opportunities for Cr analogues with better blood-brain barrier transport properties. Either these analogues might be neuroprotective themselves, or they should be readily converted to Cr selectively in the brain. On a more hypothetical level, Cr analogues that are potent substrates of CK but cannot undergo cyclization and/or the phosphorylated forms of which have a higher phosphorylation potential than PCr, might perceivably offer even better health benefits than Cr itself. Finally, Cr analogues that would allow more rapid uptake into cells and tissues might allow applications not only in prophylaxis, but also in cure. Towards these goals, first Cr analogues have been designed (Lunardi *et al.*, 2006); however, their properties do not yet warrant testing in clinical studies.

3.3. The Promises of "Omics" and "Systems Biology"

Functional genomics technologies aim at the global quantification of all mRNA transcripts (transcriptomics), all proteins (proteomics), or all metabolites (metabolomics) in a given biological sample. The target of "systems biology" is to understand a living cell at the system level, based on mathematical models of whole-cellular metabolic and regulatory networks, and using functional genomics and other experimental data to constrain the solution space of these models. It is tempting to believe that if you just measure 'everything', you should also be able to fully comprehend the physiological processes in a living cell. The reality is quite different, though. Therefore, as with any other technology, asking the right questions and taking care of the most appropriate experimental design is key to leverage most value from functional genomics technologies and systems biology concepts. How, then, can those up-to-date scientific approaches be used for studying the functions of the CK/Cr system?

We know the reaction catalyzed by CK, but we have serious problems comprehending Cr's overall role in a living cell. This is not entirely surprising, since Cr probably has only few direct effects. On the other hand, through regeneration of ATP from ADP – two molecules acting as substrates, products or effectors of a multitude of cellular reactions –, the CK/Cr system most certainly has pleiotropic effects which, in addition, may influence each other in complex interplays. Therefore, an in-depth understanding of Cr's role will depend on taking all those pleiotropic effects into consideration, in 3D-space and time. This will require complex models, which can only be validated with comprehensive sets of high-throughput functional genomics data. This will not be an easy win. Modeling at this level of complexity is not yet feasible for even a simple prokaryotic cell. It must be even less feasible then for a complex higher eukaryotic cell, and very difficult to forecast when we may reach this stage. However, the prospects of such models are tempting; once these models are able to meaningfully describe a biological system under a wide range of environmental conditions, they may potentially be used not only to describe the system, but also to predict its behavior under situations not tested experimentally before. This may, in turn, guide further experimental design and/or reduce the need for experimentation altogether. And eventually, it may become an ideal aid for predicting optimal therapeutic interventions in CDSs. For the current status of mathematical modeling and systems biology related to the CK/Cr system, the reader is referred to chapter 3 (Saks *et al.*, 2007) as well as to some recent articles (Beard *et al.*, 2006; Saks *et al.*, 2006a,b; Weiss *et al.*, 2006). Fast progress in this field will depend on further improvements in the collaboration between experimentalists and theoreticists (bioinformaticians, mathematicians, statisticians, etc.).

If whole-cellular modeling is not yet feasible, should we fully restrain from further studying the functions of the CK/Cr system, until the technologies have sufficiently advanced? Clearly not; we simply need to limit our aspirations to what is already feasible now. For instance, increasingly powerful genome (re-) sequencing technologies will allow to identify further genetic variants in the *AGAT*, *GAMT* and Cr transporter genes and to study their impact on the expressed phenotype. In addition, they may enable identification of further CK variants with compromised function.

Use of functional genomics technologies in combination with good (new) animal models with targeted alterations in CK, AGAT, GAMT and/or Cr transporter expression (see above) also offers great promise. It may allow to understand specific features of each of the Cr-deficiency syndromes (particularly in CNS), such as analysis of cell-type-specific alterations or identification of specific groups of genes or metabolites that are altered (neurotransmitter systems, metabolic pathways, signal transduction pathways, etc.). Comparative studies (e.g., between a knock-out mouse model and its reference strain) should be done not only under resting conditions, but also under stimulated or stressed conditions, so as to allow identification of functional dependencies over a broader range of physiological and pathological states. Eventually, such studies may help to pinpoint new ways for treating Cr-deficient patients, or to uncover new roles and functions of Cr in

a high-throughput manner. Studies of this type have already been reported. For instance, a proteomics approach revealed protein-protein interactions *in vitro* and *in vivo* between ubiquitous MtCK preprotein and amyloid precursor protein (APP) family proteins (Li *et al.*, 2006). Likewise, proteomics studies resulted in the identification of CK as one of the primary targets of oxidative modification in the aging brain, and that prevention of aging-related learning and memory deficits by behavioral enrichment and antioxidant-fortified food is correlated with an increased CK content in the brain (Opii *et al.*, 2006; Poon *et al.*, 2004, 2005). These findings may, thus, suggest an involvement of CK in learning and memory (see also above).

What are the limitations of such studies, and what may need to be considered in their design? None of the currently existing or perceivable experimental models (either through genetic changes, Cr analogues or chemical inhibitors) allows an abrupt and specific perturbation of the CK/Cr system. Establishment of the perturbed state requires time intervals that leave sufficient room for compensatory, confounding metabolic adaptation processes. Given the redundancy of high-energy phosphate transport systems in higher eukaryotes, this is a significant complication. On the other hand, the functional adaptations may provide a signature of molecular changes that enables extrapolation back to the start of the perturbation. Use of chemical inhibitors, however specific, always bears the risk of unrecognized side effects and is, thus, recommended only in selected cases.

Carefully designed time-series experiments in which samples for functional genomics studies are taken at multiple time-points after application of a perturbation may be instrumental in discriminating between primary and secondary effects, in dissecting the logical sequence of events, and in unravelling potential compensatory effects that might preclude development of a more severe phenotype in response to the perturbation applied. In terms of maturity of the different "omics" technologies, DNA microarray technology and metabolomics are most advanced and comprehensive, whereas successful use of proteomics in studying higher eukaryotes requires considerably higher technical sophistication due to the high complexity of the proteome (due to posttranslational modification) and because of limited throughput and proteome coverage of the currently available technologies.

3.4. Neuroprotection and Creatine Deficiency Syndromes – What is Next?

Despite a significant number of studies on CDSs (including the development of models such as the GAMT knock-out mouse) and on the expression of AGAT, GAMT and the Cr transporter, important questions remain unsolved on the specific roles of these genes in different tissues and cell types, in particular in CNS. In addition, Cr deficiency characteristics remain to be elucidated, particularly at the cellular level. Advances in our understanding of the links between the CK/Cr system and brain function are expected to come from improved diagnostics, inclusion of CDSs in neonatal screening programs, establishment of CDS disease registries and multi-centre studies, and innovative new concepts for understanding, preventing

and curing neurological diseases that involve derangements in Cr metabolism or CK function.

Screening for (cerebral) Cr deficiency syndromes (CDSs) is currently performed either by proton magnetic resonance spectroscopy (MRS), metabolite screening and/or molecular investigations. Nowadays, in most institutes, urinary analysis is the first line of screening for CDSs. However, it is expected that several cases with AGAT deficiency as well as the majority of females – and likely some males – with SLC6A8 deficiency will be missed by urinary metabolite screening. Despite this limitation, the relatively high prevalence of CDSs and the availability of potential treatment options will likely result, in due time, in a situation where every mentally retarded patient in the Western world, male or female, is screened for CDSs. The question remains how this will be achieved. One option is proton MRS that will certainly become more widely available and has the advantage of disclosing additional diseases. A marked reduction or absence of the Cr signal in MRS is diagnostic for primary Cr deficiency. A disadvantage is the fact that, usually, the patients need to be sedated and that specific training is needed for the analytical laboratories involved. Currently, molecular analysis for the AGAT, GAMT and SLC6A8 genes is mainly performed by direct DNA sequencing and, as yet, may be included only in well-equipped DNA diagnostic laboratories as the primary diagnostic approach; however, combination of molecular analysis with metabolic testing seems to be more preferable for the time being. The development of (re-) sequencing microarrays by companies such as Affymetrix or NimbleGen offers a new perspective in the diagnosis and screening of CDSs, although their diagnostic sensitivity is currently not yet acceptable for routine clinical use.

So far, CDSs are not included in neonatal screening programs. However, tandem mass spectrometry has the potential for simultaneous multi-disease screening and has been applied recently in some neonatal screening programs. Provided that tandem-MS measurement of guanidinoacetic acid in dried blood spots proves to be specific and sensitive enough for detection or exclusion of GAMT deficiency, this disorder should be included in neonatal screening (Bodamer *et al.*, 2001; Carducci *et al.*, 2002; Schulze *et al.*, 2006). On the other hand, AGAT and SLC6A8 deficiency are not yet eligible for neonatal screening since Cr and creatinine do not seem to be informative in the neonatal period (Schulze *et al.*, 2006).

Cr deficiency syndromes are relatively rare diseases. As with many other rare diseases, progress in understanding of the natural history and phenotype of the disease as well as in the efficacy of treatment has been delayed or is still being hampered by the lack of multi-centre studies. Only after half a century of history of inborn errors of metabolism, we begin to understand that worldwide networks and orphan disease registries are needed to facilitate rapid progress in the development of strategies for treatment and prevention. The group of Cr deficiency syndromes should be investigated using these tools in the future.

In many instances, our understanding of pathobiochemistry and, thus, of possible therapeutic interventions is still superficial. The pathobiochemical actions and pathophysiological consequences of the accumulation of guanidinoacetate,

guanidinoacetate's interaction with brain function, and pharmacological inhibition of guanidinoacetate's action in the brain need to be understood fundamentally to allow development of more effective treatment strategies for GAMT deficiency. New concepts are also needed for understanding the pathogenesis of brain dysfunction in Cr transporter deficiency. What is the pathogenic impact of Cr deficiency, and are there other functions of the Cr transporter which we do not yet know but contribute to pathogenesis? Deeper knowledge about the regulation of Cr transporter activity and its interaction with other genes might provide promising targets for alternative treatment strategies such as pharmacological gene therapy.

Treatment of SLC6A8 deficiency is one of the big challenges. Clinical improvement has been observed in patients with Cr biosynthesis defects (i.e., AGAT and GAMT deficiency) upon treatment comprising Cr supplementation, with almost complete restoration of Cr in brain. This proves that restoration of cerebral Cr levels is essential. Once a vehicle for Cr uptake into brain will be found (see above), treatment should also be successful in SLC6A8 deficiency. Further elucidation of Cr's biosynthesis and function in the brain may also increase the success rate of treatment. For example, "omics" studies may reveal increased levels of compensating genes and their products, which may lead to clever design of specific drugs for the restoration of Cr or its function(s).

AGAT knock-out mice would represent an ideal model system of Cr depletion and might be fundamental for understanding Cr's effects beyond its high-energy phosphate buffering function. This animal model may also allow to investigate the possible neuroprotective role of Cr which still has not been demonstrated convincingly in humans. The role of Cr as a neuroprotective substance has to be critically reconsidered in non-Cr-deficient conditions. It has been shown in numerous studies that under physiological conditions, Cr is present in high concentrations in brain and muscle, the sites where we most expect a neuroprotective or ergogenic effect, respectively. It has also been shown that in these organs with high baseline Cr concentrations, oral Cr supplementation only leads to a minor further increase in cellular Cr content. Therefore, the hypotheses that a further minor quantitative increase in cellular Cr content can be achieved and that it truly provides health benefits have to be revisited.

3.5. Suggestions for Optimized Cr Dosages and Supplementation Regimes

A considerable number of health-beneficial effects have been ascribed to oral Cr supplementation (chapters 9–12; Hespel and Derave, 2007; Klein and Ferrante, 2007; Schulze and Battini, 2007; Tarnopolsky, 2007; see also Wyss and Schulze, 2002). So far, the most clear-cut effects have been observed in animal models of disease, mostly in mice or rats. On the other hand, studies in humans often resulted in marginal to weak benefits only, e.g., in amyotrophic lateral sclerosis (Groeneveld *et al.*, 2003) or Parkinson's disease (Bender *et al.*, 2006). A reason for this discrepancy may be the largely different dosages of Cr used in experimental

animals vs. man. In animal studies, Cr is typically administered at 2–3% (w/w) in the feed. Considering that a 40 g adult mouse consumes approximately 4.0 g feed per day, this corresponds to an estimated Cr intake of 2.0–3.0 g·(kg body weight)$^{-1}$·d^{-1}. On the other hand, daily dosages of 4–10 g Cr have mostly been employed in clinical studies which, for a 70-kg person, translates into an estimated intake of only 0.06–0.14 g·(kg body weight)$^{-1}$·d^{-1}, i.e. 14- to 50-fold less than in animal studies! Remarkably, in one of the few successful – but still preliminary – studies in humans (Sakellaris *et al.*, 2006), protection from complications of traumatic brain injury in children and adolescents was achieved by oral supplementation of Cr at 0.4 g·(kg body weight)$^{-1}$·d^{-1} for six months. Therefore, it would seem desirable to carefully consider significantly higher daily dosages of Cr for clinical studies. However, the potential additional benefits of such higher dosages would need to be balanced properly against the higher risk for potential side effects. As a matter of fact, no side effects of Cr supplementation have been noted in the study of Sakellaris *et al.* (2006), one important prerequisite being that enough liquid is consumed during Cr supplementation.

As pointed out in chapter 12 (Hespel and Derave, 2007), the favourable effects of Cr supplementation on athletic performance may decrease during prolonged supplementation, thus warranting alternating supplementation (2–3 months) and washout periods (4–5 weeks). It will be of great importance to test whether this also holds true for clinical studies involving patients with chronic diseases such as Parkinson's disease, amyotrophic lateral sclerosis or multiple sclerosis, where Cr supplementation would be indicated to last for years.

3.6. Whom can We Expect (or not) to Drive Knowledge Gain

Despite the multiple potential benefits reported for oral Cr supplementation, we cannot expect the large pharmaceutical or nutrition companies to make major investments in the further exploration of the true health-beneficial effects of Cr, the best supplementation regimes, and/or the most appropriate forms of delivery of this compound. The pleiotropic effects of Cr go against the general 'one chemical – one effect' policy of pharmaceutical enterprises. A successful drug should be highly specific at low dosage for a single, clearly defined target, with very limited or, preferably, no side effects. In addition, because Cr is known for about 175 years, product patents on the substance itself are no longer feasible. Last but not least, Cr can be produced easily and cheaply, is consumed orally, and is available over-the-counter worldwide. Therefore, even if application patents existed, an end-consumer would have easy means to circumvent a premium-tagged product with an attached health claim. An attractive business opportunity for pharmaceutical or nutrition companies would only emerge with new, patent-protectable Cr analogues with improved uptake into the brain or if, for distinct health benefits, sophisticated and protectable delivery strategies were required that would allow, for instance, selective uptake of Cr into one particular tissue. For these reasons, generous funding by

public authorities will be essential for fully exploiting Cr's potential for human health and general well-being.

4. SOME FINAL WORDS

In conclusion, we hope that this volume of *Subcellular Biochemistry* and its individual chapters have managed to convey our admiration for nature's complexity and beauty as well as our firm conviction that the CK/Cr system plays an exquisite role in safeguarding proper functioning and an optimally balanced interplay between diverse physiological processes. We also hope that this volume manages to challenge some existing concepts and to stimulate new hypotheses. And last but not least, we are excited about the prospects of emerging scientific concepts and technologies for unraveling the true scope of functions exerted by the CK/Cr system, as well as for harnessing the full potential of dietary supplementation with Cr or its analogues for disease prevention or cure, and for improving general well-being. Although we have identified funding as a potential bottleneck, we are confident that a growing body of evidence for a tight link between the CK/Cr system and health will convince public authorities (and even private institutions?) to generously support the field and to let creative creatures create creatine-based nutritional strategies!

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