Creatine kinase and blood pressure: Clinical and therapeutic implications

Oudman, I.

Citation for published version (APA):

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Hypertension affects more than one billion people worldwide and is one of the most powerful contributors to cardiovascular morbidity and mortality. Black people of African descent are more often affected by the disease. One of the biological factors contributing to this difference is the enzyme creatine kinase, a key player in cellular energy metabolism. High activity of this enzyme, common in black people, has been linked to hypertension. In this thesis, we aimed to provide further insight into the association of creatine kinase with blood pressure. Furthermore, we extended our research to the role of creatine kinase in hypertension associated conditions, as ethnic disparities are also found in these entities. Finally, we focused on the creatine kinase system as a new target for hypertension treatment.
Creatine Kinase and Blood Pressure
Clinical and Therapeutic Implications
Creatine Kinase and Blood Pressure
Clinical and Therapeutic Implications

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties
ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op woensdag 20 maart 2013, te 16.00 uur

door

INGE OUDMAN
geboren te Haarlem
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Prof. dr. K. Stronks

**Faculteit der Geneeskunde**

Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door de steun van de Nederlandse Hartstichting.
Voor mijn ouders
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Chapter 1

Introduction and outline of the thesis
**INTRODUCTION**

Coupling of intracellular ATP-producing and consuming processes that are spatially separated is essential to the bioenergetics of living organisms. The creatine kinase (CK; ATP:creatine N-phosphoryl transferase) system is thought to play a key role in the intracellular energy homeostasis. The CK-system couples cellular ATP-producing with ATP-consuming processes, by catalyzing the reversible transfer of a high-energy phosphate moiety (Pi) between creatine and ADP, via the reaction:

\[
\text{MgADP + CrP + H}^+ \leftrightarrow \text{MgATP + Cr.}
\]

CK is specifically located at subcellular energy producing compartments, including the mitochondrion and near glycolytic enzymes, as well as energy consuming compartments, such as Na\(^+/\)K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase at cellular membranes and myosin light chain kinase and myosin ATPase at the contractile proteins. Due to this specific localization, ATP generated by glycolysis and oxidative phosphorylation, is shuttled as phosphocreatine to subcellular locations of ATP utilization, where ATP is regenerated (Figure).\(^1^\)\(^-^\)\(^3^\)

The CK-system is of particular importance in tissues that display high and variable rates of ATP turnover, including skeletal muscle, the cardiovascular system, brain, and the kidney.\(^4^\)\(^-^\)\(^6^\) In these tissues the enzyme provides ATP for muscle contraction and ion transport. There is a widespread interindividual variability in activity of the enzyme. Relatively high tissue and serum CK activity occurs commonly in the population, but is typically found in men, obese, and black people from African descent. The high CK state is a generalized condition with morphological and functional effects on different organ systems.\(^5^,^7^\)

High CK activity was previously postulated as a genetic factor that could explain the higher blood pressures found in black people, a population subgroup with a greater prevalence of hypertension and its complications. Pressor responses were proposed to be enhanced via increased ATP availability for cardiovascular contractility, renal sodium retention, and capillary rarefaction of skeletal muscle.\(^4^\) In line with this, population studies showed that serum CK activity was associated with blood pressure, independent of age, sex, BMI, and ethnicity.\(^8^,^9^\) In addition, subjects with high CK activity were reported to display increased vascular contractility.\(^10^\) However, it remains to be studied whether high CK activity explains the greater tendency for renal salt retention in black people. Furthermore, high tissue CK activity may be involved in the higher prevalence of other conditions that frequently coexist with hypertension in this population subgroup, such as obesity and uterine fibroids.
Hypertension affects more than a quarter of the adult population worldwide, nearing 1 billion people, and is the leading risk factor for cardiovascular morbidity and mortality.\textsuperscript{11-13} Therefore, research on biological pathways leading to hypertension and its unequal distribution among population subgroups is needed. We will focus on a genetically determined high CK phenotype with effects on skeletal muscle, heart, blood vessels, and the kidney, in relation to hypertension and other clinical conditions that increase hypertension risk.

\begin{center}
\textbf{Figure. The creatine kinase system.}
\end{center}

The creatine kinase (CK) system shuttles ATP, generated by oxidative phosphorylation in the mitochondrion or by glycolysis, as phosphorylcreatine (CrP) to sites of ATP utilization, including Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, Ca\textsuperscript{2+}-ATPase, and myosin-ATPase, where ATP is regenerated. CM, cellular membrane; MEM, mitochondrial outer membrane; MIM, mitochondrial inner membrane; Matrix, mitochondrial matrix; Cr, creatine; CT, creatine transporter; CK\textsubscript{cyt}, the cytosolic isoform of CK; CK\textsubscript{mt}, the mitochondrial isoform of CK; SER, sarcoplasmatic reticulum.
Vascular system and smooth muscle

An elevated arterial blood pressure is achieved either by constriction of arterioles causing diminished volume capacity or by fluid overload exceeding the capacity of the arterial tree, both resulting in increased pressure against the arterial wall. In hypertensive patients, the increased pressure is predominantly the result of increased total peripheral resistance of blood vessels, determined by the amount of vasoconstriction of small arteries and arterioles, or “resistance sized arteries”. These vessels are characterized by the presence of myogenic tone, i.e. their intrinsic ability to contract in response to a sudden increase of transmural pressure. This myogenic tone becomes more vigorous as vessel size decreases. In these arteries, CK is tightly bound near vascular smooth muscle contractile proteins, including myosin ATPase and myosin light chain kinase, where the enzyme provides ATP for smooth muscle contraction. In addition, high activity of the enzyme is thought to keep ADP levels near the contractile proteins low. Smooth muscle contraction consists of a fast, force generating component at high energy costs, and a slow tonic maintenance of tension at low energy costs which is thought to depend on the ability to have attached but dephosphorylated crossbridges. For this maintenance ADP is required. If ADP at the contractile proteins does not achieve the required level, excessive shortening may occur before crossbridge formation, leading to increased vasoconstriction. In accordance, microvascular contractility was shown to decrease with inhibition of intravascular CK.

In chronic hypertension vascular tone is only a short-term modulator, while structural adaptation of resistance vessels is an obligatory requirement for elevated blood pressure to be maintained for a long time. With sustained hypertension vascular smooth muscle hypertrophy leads to an increase in wall thickness and narrower lumen. As CK activity has been reported to be upregulated in trophic responses of vascular tissue to meet the increased energetic demands, high CK activity may enhance smooth muscle proliferation in hypertension. Smooth muscle proliferation and remodeling is known to cause several other clinical conditions, including uterine fibroids, the most common pelvic benign neoplasm. Hypertension and uterine fibroids are more frequently diagnosed in black and obese women. As CK stimulates growth responses as well as vascular contractility, it may be hypothesized that high CK predisposes to the development of both conditions.
Skeletal Muscle

The vascular peripheral resistance is partly dependent on the morphologic characteristics of skeletal muscle. Muscle is a heterogeneous tissue comprising fibers which vary in their metabolic and contractile nature and which occur in varying proportions in individual muscles. Muscle fibers are classified on the basis of these properties into two major 'types'; type I and type II. Highest CK activity of all tissues is found in type II fibers. These fibers are typically fit for burst exercise with a fast time to peak tension, with CK as the main ATP buffer. Cytosolic CK is tightly coupled to anaerobic glycolysis, whereas mitochondrial fatty acid oxidation capacity and glucose uptake are limited, rendering them relatively insulin resistant. In addition, high CK activity in these fibers is associated with capillary rarefaction and relatively high vascular resistance. In contrast, type I or "slow twitch" fibers have a slow time to peak tension, rich in mitochondria, derive ATP mainly from oxidation of fatty acids, and have a high glucose uptake, rendering them relatively fatigue-resistant. These fibers are densely vascularised.

In line with the morphological and metabolic characteristics of skeletal muscle fibers, high CK activity, as in type II fibers, may contribute to increased peripheral resistance and higher blood pressures. Furthermore, the tight coupling of CK with anaerobic glycolysis, may limit the capacity of muscle to oxidize fatty acids and glucose, leading to storage as lipid instead of utilization. Therefore, the high CK phenotype might be hypertension and obesity prone.

Heart

The heart contains 20-40% of skeletal muscle CK activity. To maintain an adequate cardiac output, the myocardium consumes more energy than any other organ. Because the amount of ATP is small (10 mM, enough for only a few beats) compared with the demand (10,000 times greater), the myocardial cell must continually re-synthesize ATP to maintain cardiac pump function. In the heart, the CK-system is of particular importance to maintain local ATP levels constant and contribute to myocardial contractile capacity. Myofibrillar CK, functionally coupled to myosin ATPase, maintains high ATP/ADP ratios and limits the rate of ADP release, which prevents a decline in maximum shortening velocity of the myofibrils. The importance of the CK-system in the myocardium is illustrated by the finding that CK activity and other components of the CK-system are reduced in the failing heart, and that intervention in the CK-system is studied as treatment for patients with heart failure.
Kidney

The cardiac output largely dependent on sodium and volume homeostasis, with the kidney as the major regulator. It is long known that sodium plays a major role in the regulation of blood pressure. However, there is a wide interindividual variability in renal sodium handling and the effect on blood pressure. The amount of sodium excreted by the kidneys depends on the balance between filtration by the glomeruli and reabsorption in the tubuli. After filtration more than 99% of the filtered sodium is reabsorped. This process is achieved by tight cooperation of exchangers, transporters, and ion channels in the nephron. Proximal tubule sodium handling accounts for 60-70% of reabsorption of all filtered sodium, 20 to 30% of the filtered load is absorbed in the thick ascending loop of Henle, and 5 to 10% in the distal tubule. Importantly, in all parts of the nephron, Na+/K+-ATPase resides at the basolateral surface, where it provides the force for the vectorial transport of sodium from the tubular lumen to the blood compartment, by coupling hydrolysis of ATP to the active exchange of three intracellular Na+ ions for two K+ ions. In the kidney, CK is functionally coupled to renal Na+/K+-ATPase and the ATP produced by colocalized CK is preferentially used for the high and fluctuating ATP demand of sodium transport across the tubular epithelial cells. Thus, high CK activity in the kidney tubule cells may lead to increased availability of ATP for the active process of sodium reabsorption. This may underlie the reduced ability to excrete sodium and the greater prevalence of sodium-sensitive hypertension in black people.

Evolutionary viewpoint

Humans as a genus appeared in the African savanna about 2 million years ago. The rapid expansion of brain size starting at that time is thought to be associated with upregulation of the CK-system in the brain, in order to adapt to the increasing metabolic demands. In the context of human evolution, the high CK phenotype was likely exceptionally efficient for the hunter-gatherer ancestors: high cardiovascular contractility and the ability to retain sodium in the kidney would help to maintain adequate blood pressures in times when sodium and volume depletion by heat exhaustion was a daily threat (the ancestors survived on a diet with only a fraction of the salt that we consume). Furthermore, the predominance of fast type II fibers in skeletal muscle would enhance the capacity for short bursts of running for survival and facilitate optimal storage of carbohydrates as lipid in times of food shortage. However, evolution has transformed the human environment, including the rapid transformation of human lifestyles from...
small food-foraging societies to large and economically complex states in less than 5000 years with increased access to high-caloric and sodium rich food and lack of physical activity. Some of us may then be unfortunate and inherit a predisposition to conditions that in our modern society have harmful effects.\textsuperscript{33}

**Outline of the Thesis**

This thesis consists of two parts. **PART I** focuses on CK in the kidney and resistance arteries, as a sustained elevation of blood pressure is only achieved by excess renal sodium retention or general vasoconstriction. Furthermore, associations between CK, hypertension, and other clinical conditions that frequently coexist with hypertension are studied. In **Chapter 2** we assessed whether subjects with high CK activity display enhanced sodium retention. In **Chapter 3** it is studied whether transcriptional activity of CK in resistance sized arteries correlates with blood pressure. In **Chapter 4** the association of CK activity with obesity, a risk factor for hypertension and, is addressed. In search for other risk factors we assessed whether the prevalence of hypertension is increased in women with uterine leiomyomata in **Chapter 5**.

In **PART II** we focus on the CK-system as a possible therapeutic target in hypertension and cardiovascular disease and on the effect of CK inhibition in tissues with high energy demands. If high CK activity promotes sodium retention and vascular contractility, hypertension may be more difficult to treat in subjects with high CK. Therefore, we assessed in **Chapter 6** whether serum CK is associated with hypertension treatment failure in the general population. As the CK-system is thought to be involved in hypertension and cardiovascular disease, we questioned what was known regarding therapeutic intervention in the CK-system. Thus, in **Chapter 7** we systematically searched the literature for the existing evidence on interference in the CK-system in hypertension and cardiovascular disease. In Chapter 8 we performed a systematic review on the effect of beta-guanidinopropionic acid, an inhibitor of the flux through the CK reaction, on function and tissues with high energy demands in **Chapter 8**. Finally, in **Chapter 9A** we assessed the effect of inhibition of the CK-system with beta-guanidinopropionic acid on blood pressure and properties of resistance arteries in hypertensive animals, whereas **Chapter 9B** describes a protocol for the first-in-man study with this compound.
REFERENCES

PART 1
Clinical conditions

PART 1
Clinical conditions
Healthy men with high serum CK activity display reduced sodium excretion after a high salt diet

I. Oudman
I. Khoyska
L. M. Brewster

Submitted
ABSTRACT

Background Serum creatine kinase (CK) was reported to be the main predictor of blood pressure in the general population, with an increase in SBP and DBP of resp. 14 and 8 mm Hg per log CK increase. The enzyme is tightly bound near Na^+/K^+-ATPase in the kidney, where it rapidly regenerates ATP for tubular sodium reabsorption. Therefore, we hypothesized that sodium excretion is reduced in subjects with high serum CK activity.

Method Eligible for inclusion were healthy men with normotension or untreated hypertension, younger than 50 years, on 7 days of low sodium (<50 mmol/d) followed by 3 days of high sodium (>200 mmol/d). Examination included sitting resting blood pressure, serum CK after rest, and sodium, potassium, and creatinine in serum and 24-h urine. After correlation analysis, we assessed the difference in sodium excretion after a high salt diet between the lowest and the highest CK tertile.

Results We included 52 subjects, 27 white and 25 black, with a mean age of 37.0 (SE 1.4) y and mean BMI of 24.7 (0.4) kg/m^2. Median serum CK activity was 205.0 IU/L (Range, 63 to 1648 IU/L). After logarithmic transformation, serum CK was negatively correlated with 24-h urinary sodium excretion after a high salt diet; a correlation coefficient of -0.40 (p=0.002). Sodium excretion after a high salt diet was significantly lower in the highest compared to the lowest log CK tertile; respectively 279.6 (34.1) versus 403.9 (25.5) mmol/day (p=0.007).

Discussion Subjects with high CK activity display reduced sodium excretion after a high salt diet. Under the assumption that serum CK reflects tissue CK, this may imply that high CK activity promotes sodium reabsorption in the renal tubules, as the enzyme regenerates ATP near Na^+/K^+-ATPase, the primary force for tubular sodium transport.
BACKGROUND

Salt plays a major role in the regulation of blood pressure and an extensive amount of evidence links higher salt intake with higher blood pressures and increased cardiovascular risk. There is a wide interindividual variability in sensitivity to changes in salt balance. Salt sensitivity is associated with increased cardiovascular risk and mortality in hypertensive and normotensive subjects. However, the pathophysiological mechanism leading to sodium retention in some individuals, whereas others exposed to the same intake are able to excrete sodium, has not yet been defined.

We propose that differences in activity of the enzyme creatine kinase (CK) may be related to differences in sodium handling. By catalyzing the reversible transfer of a phosphate group from phosphocreatine to ADP, the enzyme connects sites of ATP production (glycolysis and mitochondrial oxidative phosphorylation) with subcellular sites of ATP utilization, including myosin ATPase and myosin light chain kinase at the contractile proteins and Ca2+-ATPase and Na+/K+-ATPase at cellular membranes, where it rapidly regenerates ATP in situ from phosphocreatine. In this way, the enzyme is thought to lead to greater ATP buffer capacity for cardiovascular contractility and renal sodium retention. In line with this, serum CK was found to be the main predictor of blood pressure in the population, independent of age, sex, BMI, or ethnicity, with an increase in systolic and diastolic blood pressure of resp. 14 and 8 mm Hg per log CK increase.

In the kidney, sodium reabsorption is primarily driven by basolateral Na+/K+-ATPase, coupling hydrolysis of ATP to the active exchange of three intracellular Na+ ions for two K+ ions. CK is tightly bound near this enzyme, where it regenerates ATP for tubular sodium reabsorption. High CK activity near this enzyme is may thus enhance ATP availability for this highly energy demanding process (Figure 1). Therefore, we hypothesized that sodium excretion is reduced in subjects with high CK activity.
Figure 1. Mechanism of sodium reabsorption in the kidney.
Basic mechanisms of sodium transport in the proximal tubule, thick ascending limb, distal tubule, and collecting duct, modified from Greger et al. In all parts of the nephron, basolateral Na+/K+-ATPase is the primary force for the vectorial transport of sodium from the tubular lumen to the blood compartment by coupling hydrolysis of ATP to the active exchange of three intracellular Na+ ions for two K+ ions. Evidence indicates that creatine kinase (CK) is functionally coupled to renal Na+/K+-ATPase and that ATP produced by colocalized CK is preferentially used for the high and fluctuating ATP demand of sodium transport across the tubular epithelial cells. Both mitochondrial and the cytosolic B isoform of CK have been found in mammalian kidney. NHE3, Na+/H+ exchanger; CA, carbonic anhydrase; CK, creatine kinase; Cr, creatine; CrP, phosphocreatine; ENaC, epithelial sodium channel; matrix, mitochondrial matrix; IMS, mitochondrial intermembrane space; TAL, thick ascending limb.

METHODS

Participants and protocol
The protocol was approved by our local institutional review board. All of the procedures were in accordance with institutional guidelines. All participants gave written informed consent. We included healthy men, normotensive or with untreated
primary hypertension, of self-reported white or black ethnicity. Subjects with treated or secondary hypertension, glucose, lipid spectrum, thyroid, kidney, or liver abnormalities, CK-increasing drugs including statins, cardiovascular, neuromuscular, or endocrine disorders, vasculitis, HIV infection, or infectious hepatitis were excluded. Participants were instructed to abstain from heavy exercise three days before the baseline visit to our hospital. Physical examination was unremarkable. After the baseline visit, subjects were instructed to adhere to a low sodium diet (50 mmol Na\(^+\) per day, LS) during 7 days followed by a high sodium diet (>200 mmol Na\(^+\) per day, HS) during 3 days. During HS a minimal daily amount of sodium (200 mmol) was provided to the participants by the research physician. A dietician was consulted before start of the protocol. During the study, participants were supported daily by the research physician. At baseline, day 1, and day 4 of LS overnight urine was sampled to assess dietary compliance. At the final day of each level of sodium intake all participants collected 24-h urine, and, after an overnight fast, body weight and blood pressure was measured, and blood were sampled.

**Study measures**

Physical examination included height, weight, and blood pressure levels. Office blood pressure was measured with an Omron M4 oscillometric device (Omron Healthcare Europe BV, Hoofddorp, the Netherlands). In a quit room with the subject seated following a 5-minute rest period. An appropriately adjusted cuff size was used on the non-dominant arm, supported at heart level. Blood pressure was calculated as the mean of the second and third reading, with a maximum of 5 mm Hg difference.\(^{13}\) Body mass index (BMI) was calculated as weight (kg) divided by the height (rounded to the nearest centimeter) squared. Laboratory studies included serum CK activity after 3 days of rest, creatinine, sodium, potassium, urea, fasting glucose, fasting lipids, including total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglycerides. Urine analysis included sodium, potassium, creatinine, and urea. All laboratory analyses were performed on a Modular Cobas 8000 (Roche Diagnostics, Darmstadt, Germany). Plasma levels of CK, glucose, total cholesterol and triglycerides were estimated by enzymatic spectrophotometric; high-density lipoprotein – cholesterol by colorimetric/spectrophotometric; plasma creatinine and urea by kinetic/spectrophotometric; and sodium and potassium by Indirect Ion-Selective Electrode methods.
Data analysis and statistics

The primary outcome was the difference in 24-h urinary sodium excretion after a high salt diet between the lowest and the highest serum CK tertile. Other outcomes included correlations of 24-h sodium excretion after high salt with serum CK, age, and BMI, and ethnic differences in sodium excretion. Finally, we assessed whether correction for 24-h creatinine excretion, as a measure of the accuracy of sampling, influenced the outcomes.

Based on previously reported differences in sodium excretion of 10-50% between salt sensitives and controls, we calculated to need 40 subjects to detect this difference with a one-sided alpha of 0.05 and a 1-beta of 0.80. Because serum CK distribution was expected to be extremely skewed to the right, a logarithmic transformation to base 10 was performed to achieve a more symmetrical distribution. For statistical analysis, unpaired and paired t-tests were used for between group (low vs high CK tertile, white vs black ethnicity) and within-group (HS effect) comparisons respectively. To assess associations, 1-tailed Spearman's rank correlations were calculated for 24-hour urinary sodium excretion versus log serum CK, age, and BMI.

We considered a one-sided probability value of <0.05 to be significant. Data were analyzed with SPSS statistical software package for Windows, version 19.0 (SPSS Inc., Chicaco, IL, USA). Data are presented as mean with the standard error in square brackets unless stated otherwise.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Total* (n=52)</th>
<th>White (n=27)</th>
<th>Black (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)†</td>
<td>37.0 (1.4)</td>
<td>33.7 (1.7)</td>
<td>40.6 (2.0)</td>
</tr>
<tr>
<td>BMI (kg/m2)†</td>
<td>24.7 (0.4)</td>
<td>23.7 (0.5)</td>
<td>25.8 (3.4)</td>
</tr>
<tr>
<td>CK (IU/L)‡§</td>
<td>205.0 (63.0 to 1648.0)</td>
<td>132.0 (63.0 to 680.0)</td>
<td>319.0 (116.0 to 1648.0)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)‡§</td>
<td>96.4 (1.6)</td>
<td>92.8 (2.1)</td>
<td>100.3 (2.4)</td>
</tr>
<tr>
<td>SBP (mm Hg)†</td>
<td>125.6 (1.8)</td>
<td>122.8 (2.0)</td>
<td>128.7 (3.0)</td>
</tr>
<tr>
<td>DBP (mm Hg)†</td>
<td>76.8 (1.4)</td>
<td>74.7 (1.8)</td>
<td>79.0 (2.0)</td>
</tr>
<tr>
<td>Hypertension (N)</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)‡§</td>
<td>4.8 (0.1)</td>
<td>4.9 (0.2)</td>
<td>4.8 (0.1)</td>
</tr>
<tr>
<td>Glucose (mmol/L)‡§</td>
<td>5.5 (0.1)</td>
<td>5.4 (0.1)</td>
<td>5.6 (0.1)</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, DBP, systolic/diastolic blood pressure; Hypertension, systolic/diastolic blood pressure ≥140/90 mm Hg; CK, serum creatine kinase after 3 days of rest; * Includes participants with "other" ethnicity (n=5); † Mean (SE); ‡ Median (Range); § fasting plasma concentration
RESULTS

Fifty-two men, including 27 white and 25 black, were included, with a mean age of 37.0 (1.4) y and mean BMI of 24.7 (0.4) kg/m². Mean systolic/diastolic blood pressure (SBP/DBP) was 125.6 (1.8)/76.8 (1.4) mm Hg. Eight subjects were hypertensive (SBP/DBP ≥ 140/90 mm Hg). The baseline characteristics of the included participants are shown in Table 1. Crude CK activities ranged from 63.0 to 1648.0 IU/L (Median, 205.0 IU/L). We identified one possible outlier (1648 IU/L, black ethnicity), and after applying Dixon’s one-third rule, this subject was excluded from further analysis. As the data still showed significant skewness and kurtosis, a logarithmic transformation was performed to acquire a normal distribution.

General parameters during LS and HS are shown in Table 2. Mean 24-h urinary sodium excretion during LS and HS was 28.1 (3.1) and 328.0 (23.0) mmol/day respectively (p<0.001), indicating an adequate dietary compliance. Mean body weight and SBP increased significantly during HS, as expected, whereas DBP was not significantly changed. The 24-h urinary creatinine, potassium, and urea excretion were higher during HS.

Table 2. General parameters during low and high sodium diet (N=52).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Low sodium</th>
<th>High sodium</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>79.1 (1.6)</td>
<td>81.0 (1.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>120.9 (1.4)</td>
<td>124.6 (1.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>73.7 (1.0)</td>
<td>74.4 (1.1)</td>
<td>0.353</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>66.3 (1.5)</td>
<td>60.7 (1.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary sodium excretion (mmol/day)</td>
<td>28.1 (3.1)</td>
<td>328.0 (23.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary creatinine excretion (mmol/day)</td>
<td>14.1 (0.6)</td>
<td>15.8 (0.9)</td>
<td>0.036</td>
</tr>
<tr>
<td>Urinary potassium excretion (mmol/day)</td>
<td>68.9 (4.5)</td>
<td>77.7 (5.3)</td>
<td>0.079</td>
</tr>
<tr>
<td>Urinary urea excretion (mmol/day)</td>
<td>308.9 (19.0)</td>
<td>387.3 (20.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma sodium (mmol/L)</td>
<td>139.9 (0.2)</td>
<td>142.1 (0.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Plasma potassium (mmol/L)</td>
<td>4.12 (0.04)</td>
<td>4.08 (0.03)</td>
<td>0.208</td>
</tr>
<tr>
<td>Plasma creatinine (μmol/L)</td>
<td>97.5 (1.8)</td>
<td>92.4 (1.5)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

SBP, DBP, systolic/diastolic blood pressure; All values are mean (SE).
After logarithmic transformation, we found a negative correlation between serum CK and 24-h urinary sodium excretion after a high salt diet, with a correlation coefficient of -0.40 ($p=0.002$). Subsequently, we assessed differences between the lowest and highest CK tertile. Parameters during LS and HS for the first and the third CK tertile are shown in Table 3. The highest tertile included more black men, as expected. Sodium excretion after a high salt diet was significantly lower in the highest compared to the lowest log CK tertile; respectively 279.6 (34.1) versus 403.9 (25.5) mmol/day ($p=0.007$) (Figure 2). In addition, the urinary 24-h potassium excretion was significantly lower in the highest CK tertile.

Table 3. Parameters during low and high sodium in the lowest versus the highest log CK tertile.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tertile I (N=17)</th>
<th>Tertile III (N=16)</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>35.7 (2.5)</td>
<td>35.5 (2.4)</td>
<td>0.973</td>
</tr>
<tr>
<td>BMI</td>
<td>23.6 (0.6)</td>
<td>25.8 (0.9)</td>
<td>0.053</td>
</tr>
<tr>
<td>Black ethnicity (%)</td>
<td>17.6</td>
<td>76.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Sodium excretion (mmol/day) LS</td>
<td>27.9 (4.9)</td>
<td>25.5 (4.4)</td>
<td>0.0543</td>
</tr>
<tr>
<td></td>
<td>403.9 (105.3)</td>
<td>279.6 (34.1)</td>
<td>0.007</td>
</tr>
<tr>
<td>Creatinine excretion (mmol/day) LS</td>
<td>15.4 (1.0)</td>
<td>13.1 (0.8)</td>
<td>0.089</td>
</tr>
<tr>
<td></td>
<td>18.3 (2.1)</td>
<td>14.9 (0.84)</td>
<td>0.145</td>
</tr>
<tr>
<td>Potassium excretion (mmol/day) LS</td>
<td>90.4 (7.9)</td>
<td>60.1 (6.3)</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>100.5 (10.7)</td>
<td>63.9 (6.8)</td>
<td>0.008</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg) LS</td>
<td>119.3 (2.7)</td>
<td>121.8 (2.6)</td>
<td>0.778</td>
</tr>
<tr>
<td></td>
<td>124.8 (3.1)</td>
<td>124.1 (2.6)</td>
<td>0.630</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg) LS</td>
<td>75.0 (1.8)</td>
<td>73.4 (1.5)</td>
<td>0.464</td>
</tr>
<tr>
<td></td>
<td>74.7 (1.7)</td>
<td>73.7 (1.7)</td>
<td>0.448</td>
</tr>
</tbody>
</table>

LS, low sodium diet; HS, high sodium diet; All values are mean (SE)
Healthy men with high serum CK activity display reduced sodium excretion after a high salt diet. In line with previous reports on ethnic differences in sodium handling, the 24-h urinary sodium excretion during HS was lower in blacks compared to whites, the 24-h urinary sodium excretion during HS was lower in black compared to white men, 213.4 (24.3) and 434.2 (24.2) mmol/day respectively ($p<0.001$). The 24-h potassium excretion was 58.8 (6.8) and 95.0 (6.5) mmol/day for black and white men respectively. Correlation coefficients for age and BMI with 24-h sodium excretion were -0.30 ($p=0.014$) and -0.12 ($p=0.203$). Correction for 24-h creatinine excretion, as a measure for urine sampling accuracy, had no major influence on the direction and magnitudes of the outcomes.

**Figure 2. Urinary sodium excretion after a high salt diet in the lowest and the highest creatine kinase tertile.**

The 24 h urinary sodium excretion after a high salt diet is shown for the lowest (N=17) and the highest (N=17) log creatine kinase (CK) tertile. Sodium excretion was significantly higher in the lowest compared to the highest tertile ($p<0.001$).

**DISCUSSION**

In this study we report for the first time that serum CK is associated with urinary sodium excretion after a high salt diet. This may imply that CK has a contribution to sodium reabsorption in the renal tubules (Figure 1). In the kidney, Na+/K+-ATPase is the primary force for regulating sodium handling and plays a key role in both ion homeostasis and
blood pressure regulation. Evidence indicates that CK is functionally coupled to renal Na+/K+ ATPase and that ATP produced by colocalized CK is preferentially used for the high and fluctuating ATP demand of sodium transport across the tubular epithelial cells. Thus, high CK activity in the kidney tubule cells may lead to increased availability of ATP for the active process of sodium reabsorption. This process is achieved by tight cooperation of exchangers, transporters, and ion channels in the nephron. Proximal tubule sodium handling accounts for 60-70% of reabsorption of all filtered sodium, 20 to 30% of the filtered load is absorbed in the thick ascending loop of Henle, and 5 to 10% in the distal tubule. Importantly, in all parts of the nephron, Na+/K+ ATPase resides at the basolateral surface, where it provides the force for the vectorial transport of sodium from the tubular lumen to the blood compartment, by coupling hydrolysis of ATP to the active exchange of three intracellular Na+ ions for two K+ ions.

In the absence of tissue damage, serum CK at rest is thought to reflect tissue CK activity. In the mammalian kidney, both the cytosolic B isoform and a mitochondrial form of CK have been found. Healthy tissue looses a small fraction of intracellular CK into the interstitial space, which is transported to the bloodstream via the lymphatic system. This release is proportional to tissue CK activity. Therefore, as the high CK state is a generalized condition, high serum CK activity may partly reflect high renal CK activity.

Our findings are in accord with previous studies showing that serum CK was the main predictor of systolic and diastolic blood pressure in the population, independent of age, BMI, sex, and ethnicity. Regarding the effect of sodium retention on blood pressure, sodium and commensurate water induce an expansion of intravascular fluid volume, leading to an increase in cardiac output, that alone was primarily thought by some to initiate the pressor effect of salt. However, it was shown in salt sensitive but not salt resistant subjects that the increase in cardiac output with dietary salt loading was accompanied by inhibition of normal vasodilatation. Thus, vascular dysfunction with increased peripheral resistance is thought to be critical to the initiation of pressor responses to dietary salt loading. We previously found that patients with relatively high serum CK had greater vascular contractility in vitro, and that CK inhibition reduced contractility. Therefore, the high CK phenotype, including high renal CK and high vascular CK may augment the vasodilatory response to volume expansion with dietary salt loading.
Healthy men with high serum CK activity display reduced sodium excretion after a high salt diet. Relatively high tissue and serum CK activity is found in black people. Black people are known to have a reduced ability to excrete sodium. This is accompanied by lower potassium excretion and higher urine concentration, whereas levels of renin are, on average, lower in blacks than whites. The reduced capacity of the kidney to excrete sodium is thought to play a major role in the greater prevalence of (salt-sensitive) hypertension this population subgroup. The findings of this study are in line, as we reported reduced sodium and potassium excretion after a high salt diet in blacks compared to whites. Besides the relatively reduced ability to excrete sodium, evidence indicates that blacks have enhanced vascular reactivity to sympathetic stimulation, attenuated responses to vasodilators, in particular due to reduced nitric oxide availability, and a relatively narrow vascular lumen diameter. However, despite extensive research, the pathophysiological mechanism underlying enhanced sodium retention and hypercontractility of the vasculature in black people is not completely understood. The results from this study combined with previous reports may suggest that a genetically determined high CK phenotype, including high renal and cardiovascular CK, underlies the greater tendency of black people for sodium retention and attenuated vasodilatory responses, leading to a greater burden of hypertension and its associated cardiovascular morbidity and mortality in this population subgroup.

This study has several strengths and limitations. The main strength is that we were able to show for the first time that healthy men with high serum CK activity excrete less sodium on a high salt diet. Furthermore, we standardised for exercise. Serum CK activity is elevated up to 3 days with regular exercise, and up to a week after strenuous eccentric exercise, where the muscle lengthens and contracts at the same time against an external load. This leads to disruption of muscle fibers, and highly elevated serum CK, up to 10,000 IU/L during a week or longer. However, none of the participants stated to be involved in such eccentric exercise. Still, we cannot exclude an exercise induced component in the CK values. Third, during the diet the participants were closely monitored and supported by the research physician and during HS the minimal daily amount of sodium (200 mmoles) was provided to the participants. However, as the monitoring of sodium intake in a real life setting is difficult, we cannot exclude that the dietary intake of sodium during HS was below the target of minimally 200 mmoles sodium per day.

A limitation is that we were not able stratify for ethnicity, as most subjects with high CK activity were black, in line with the distribution of serum and tissue CK in the population. However, we provide a biologically plausible mechanism for the reduced
ability of black people to excrete sodium, as skin colour per se does not explain the observed ethnic differences. Another limitation is the use of serum CK as an indirect measure of the renal CKB isoform. Although serum CK is known to reflect tissue CK, it is not clear whether this accurately reflects CK activity in the kidney. Therefore, further studies should assess renal CK activity in relation to sodium handling.

In summary, we showed that subjects with high CK activity display reduced urinary sodium excretion after a high salt diet. Under the assumption that serum CK reflects tissue CK, this may imply that high CK activity promotes sodium reabsorption in the renal tubules, as CK regenerates ATP near Na⁺/K⁺-ATPase, which provides the primary force for tubular sodium transport. Our findings support the hypothesis that the high CK phenotype is hypertension prone, through greater ATP buffer capacity for cardiovascular contractility as well as sodium retention in the kidney.
Healthy men with high serum CK activity display reduced sodium excretion after a high salt diet.

REFERENCES


Healthy men with high serum CK activity display reduced sodium excretion after a high salt diet


Overexpression of microvascular CK mRNA in human hypertension

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Submitted
**ABSTRACT**

**Rationale** Hypertension remains the main risk factor for cardiovascular death. Environmental and biological factors are known to contribute to the condition, and recently serum creatine kinase was reported to be the main predictor of blood pressure in the general population. This was proposed to be related to high resistance artery creatine kinase rapidly regenerating ATP for microvascular contractility, but hitherto there were no data to substantiate this.

**Objective** To assess whether creatine kinase mRNA levels in human resistance arteries are associated with blood pressure across the clinical spectrum of normotension, and stage 1 and 2 hypertension.

**Methods and Results** We isolated resistance-sized arteries from omental fat donated by consecutive women during uterine fibroid surgery. Blood pressure was measured in the sitting position. Vessels of 13 women were included, 7 white and 6 African-Dutch, mean age 42.9 (SE 1.6) y; systolic/diastolic blood pressure 144.8 (8.0)/86.5 (4.3) mm Hg; heart rate 79.9 (3.3)/min; and body mass index 26.0 (1.5) kg/m². Microvascular creatine kinase mRNA was assessed using quantitative realtime PCR. Normalized creatine kinase B mRNA copy numbers, ranging between 5.18 and 24.43 (mean 15.0, SE 1.9), were strongly correlated with blood pressure, with correlation coefficients of 0.64 (95% CI, 0.13 to 0.88) for systolic, and 0.88 (0.64 to 0.96) for diastolic blood pressure.

**Conclusion** To our knowledge, this is the first direct evidence suggesting that creatine kinase gene mRNA expression levels in microvasculature progressively increase with blood pressure. This finding adds to the evidence that creatine kinase is involved in the vasculature’s pressor responses.
INTRODUCTION

Hypertension is an important worldwide public-health challenge. It is a common disease, affecting over 25% of the adult population, nearly a billion people worldwide. Hypertension is identified as the leading risk factor for cardiovascular mortality, and is ranked third as a cause of disability-adjusted reduction in life-years. The pathogenesis of hypertension is multifactorial, and environmental and biological circumstances contribute to the occurrence of the disease. We proposed that creatine kinase (CK), the central regulatory enzyme of energy metabolism, is the final common pathway leading to pressor responses. The enzyme regenerates and distributes ATP to subcellular locations of energy demands, catalyzing the reaction:

\[ \text{MgADP} + \text{Pcreatine} + H^+ \leftrightarrow \text{MgATP} + \text{creatine}. \]

CK is tightly bound in the immediate proximity of ATP utilizing enzymes such as Na\(^{+/}\)/K\(^{+}\)-ATPase and Ca\(^{2+}\)-ATPase at membranes, and myosin light chain kinase and myosin ATPase at the contractile proteins, where it rapidly provides ATP to these enzymes. CK is thus thought to fuel highly energy demanding processes such as sodium retention, cardiovascular contractility, and remodeling of arteries. In accord with this, serum CK was found to be the main predictor of blood pressure in the general population. This was proposed to be due to high tissue CK, primarily resistance artery CK-BB isoenzyme rapidly regenerating ATP for microvascular contractility. However, hitherto, there were no microvascular data to substantiate this proposal. The main objective of this study was to assess whether resistance artery CK mRNA levels are associated with blood pressure.

METHODS

Participants
Protocols were in accord with institutional guidelines and approved by the local institutional review board. All participants gave written informed consent. Consecutive self-defined white and African-Dutch women, undergoing an abdominal procedure for fibroid enucleation or hysterectomy for fibroids were eligible for inclusion. Patients with pre-existent vascular abnormalities, such as vasculitis and diabetes mellitus; HIV infection; infectious hepatitis; and bleeding disorders were excluded. Sitting blood pressure was measured at the outpatient clinic with the Datascope Accutorr Plus (Tascope Corp., Paramus, New Jersey, USA). High blood pressure was defined as systolic...
blood pressure (SBP) ≥140 or diastolic blood pressure (DBP) ≥90 mm Hg, or the use of antihypertensive drugs.

**CK isoenzyme cDNA**

The two major cytosolic CK protein subunits are CK-brain (B) and CK-muscle (M) that are encoded by the CKB gene on human chromosome 14q32 and the CKM gene on 19q13.32 respectively. The enzymatic functional form can be either a homodimer (BB or MM) or a MB heterodimer, thus creating 3 cytosolic isoenzymes.\(^9,^{11,12}\) CK is also present in the mitochondrion where it facilitates the formation of creatine phosphate, which is transported by CK to subcellular locations of high-energy demands.\(^12,^{13}\) Two mitochondrial CK isoenzymes, an ubiquitous and a sarcomeric form, are encoded by respectively the CKMT1 gene on chromosome 15q15 and the CKMT2 gene on chromosome 5q13.3.\(^9,^{11,12}\) All CK isoenzymes contain a highly conserved catalytic cysteine domain. However the triplet encoding for this catalytic cysteine domain is GCC for cytoplasmic CK and GTC for mitochondrial CK.\(^12\) Cytosolic CK-B and CK-M cDNA share a 78% nucleotide sequence identity and 79% predicted amino acid sequence identities. The human CKMT1 and CKMT2 cDNA share a 73% nucleotide and 80% predicted amino acid sequence identities but have less than 66% identity with the cytosolic CK.\(^12\)

**Microvessel tissue preparation and RT-qPCR**

After omental biopsy, the omental fat pad sample was immediately placed into cold (4 degrees Celsius), oxygenated, physiologic salt solution (PSS) consisting of (mmol/L) NaCl 118.2, NaHCO\(_3\) 24.8, KCl 4.6, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 2, EDTA 0.26, and HEPES 50. Resistance-sized arteries (200-400 μm in diameter) were dissected under a microscope, cleaned of adherent adipose and connective tissue, and stored in Trizol Reagent at -80 degrees Celsius. Total RNA was isolated using the TRIzol protocol, and purified using the QIAGEN RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) with subsequent DNase treatment. RNA clean up was done using the RNeasy Minute cleanup kit (Qiagen). To determine tissue-specific transcription, the Clontech total RNA human tissue panel was used to assess isoenzyme distribution in brain, striated and smooth muscle. First strand cDNA synthesis was performed on 97.5 ng/μl RNA using the Avian Myeloblastosis Virus (AMV) transcriptase kit 0.8 μl (20 units) and random hexamers (Roche Applied Science, Indianapolis, IN, USA), which are short oligodeoxyribonucleotides of random sequence that anneal to random complementary sites on target RNA, to serve as primers for DNA synthesis by the reverse transcriptase.
Specific PCR primers were designed for the CKB, CKM, CKMT1 and CKMT2 transcripts that amplify all alternatively spliced transcript variants that contain the highly conserved cysteine catalytic domain of creatine kinase. The transcription of CK genes was normalized to the reference gene PSMD4. Primers and corresponding probes were identified using the Roche Universal Probe Library (UPL) Assay Design Center (Table 1). Amplicons were cloned in pGEM-T easy (Promega Corp., Madison, WI, USA), sequenced to validate amplification of the intended transcript, and used to prepare amplicon specific calibration curves.

**Table 1. Primer sequence and probe number.**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward primer (5'→ 3')</th>
<th>Reverse primer (5'→ 3')</th>
<th>UPL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKB</td>
<td>TTCTCAGAGGTGGAGCTGGT</td>
<td>AGGCATGAGGTCCGAT</td>
<td>77</td>
</tr>
<tr>
<td>CKM</td>
<td>CCCACAACAGTTCAAGCTG</td>
<td>GGCCATGTGTTTATGTTT</td>
<td>63</td>
</tr>
<tr>
<td>CKMT1</td>
<td>GGTAACTGAAGAGATGTTTGAAAG</td>
<td>CAGCCAGTTCTGGATAAGT</td>
<td>39</td>
</tr>
<tr>
<td>CKMT2</td>
<td>TGAACCGGCAAAAGTGTG</td>
<td>CGCAGGGTCTGGTAGTCG</td>
<td>32</td>
</tr>
<tr>
<td>PSMD4</td>
<td>GGCAAGATCACCTTCTGCA</td>
<td>CTTCCCACAAAGGGAATGAT</td>
<td>21</td>
</tr>
</tbody>
</table>

*UPL indicates the number of the Universal Probe Library Probe (Roche).

**RT-qPCR**

Quantitative real time PCR (qPCR) was performed on a LightCycler 480 system (Roche), according to the manufacturer’s protocol. Reaction mixtures contained 2.5 μl cDNA, 0.4 μmol/L of each primer (Invitrogen, Carlsbad, CA, USA), 100 nmol/L UPL probe (Roche), 2.5 μl water and 10 μl Absolute qPCR mix (Thermo Fisher Scientific, Asheville, NC, USA), in a total volume of 20 μl. Reactions were run in duplicate. Data were analyzed and quantified, using the second derivative maximum for Cp determination, with the LightCycler 480 software 1.5.0 (Roche).

**Statistical analysis**

The main outcome was the strength of the association between blood pressure and CKB mRNA as measured with the Pearson product-moment correlation coefficient. Based on animal studies showing a 1.5 to 4.0-fold increase in cardiac CK or CK-mRNA with SBP rising from 120 to 150-180 mm Hg,10,13,14 we estimated to need 8 patients to assess a similar association with an alpha of 0.05 and a 1-beta of 0.8. Other outcomes were correlations of blood pressure with non-CKB isoenzymes, and with total CK; and the
difference in CK mRNA expression between different stages of hypertension. Because of the expected small sample size, the distribution of the data could not be formally tested. Parametric analysis may not be accurate with small sample sizes, and non-parametric analysis may lack power to detect a significant difference. Therefore, we used parametric statistics as our primary analysis (i.e. arrhythmic mean with SEM; Pearson product-moment correlation coefficient \( r \), the unpaired \( t \) test, and 1-way ANOVA with the appropriate post-test with Bonferroni correction); and reanalysed the data as a sensitivity analysis with non-parametric methods (i.e. median with interquartile range; Spearman rank order correlation coefficient (rho); Mann-Whitney test, or Kruskal-Wallis test with a Dunn's post-test). We considered a one-sided probability value of <0.05 to be statistically significant. Data in brackets are 95% confidence intervals, unless stated otherwise. Data were analysed with IBM SPSS statistical software package for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

Table 2. Clinical characteristics of the participants.

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
<th>Normotensive (n=6)</th>
<th>Stage 1 HT (n=3)</th>
<th>Stage 2 HT (n=4)*</th>
<th>Total group (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>41.2 (2.7)</td>
<td>42.7 (2.8)</td>
<td>45.5 (2.3)</td>
<td>42.9 (1.6)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>124.2 (4.5)</td>
<td>142.3 (5.5)</td>
<td>177.8 (13.7)</td>
<td>144.8 (8.0)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>72.7 (3.3)</td>
<td>91.7 (3.3)</td>
<td>103.5 (2.7)</td>
<td>86.5 (4.3)</td>
</tr>
<tr>
<td>Heart rate / min</td>
<td>70.8 (3.6)</td>
<td>81.3 (4.8)</td>
<td>90.3 (3.8)</td>
<td>79.9 (3.3)</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24.8 (1.0)</td>
<td>24.0 (2.6)</td>
<td>32.5 (6.5)</td>
<td>26.0 (1.5)</td>
</tr>
</tbody>
</table>

Values are expressed as mean (SE). Normotension, Stage 1, and 2 hypertension (HT) indicate SBP <140, 140 to 159, and ≥160; DBP <90, 90-99, and ≥100 mm Hg respectively. Stage 1, only 1 patient treated, stage 2, 3 patients treated. None of the patients reached control. *In the Dutch setting, according to national guidelines treatment of uncomplicated hypertension is only imperative at systolic pressure levels ≥180 mm Hg.\(^{15}\)

**RESULTS**

Vessels of 13 women, 7 white and 6 African Dutch, mean age 42.9 (SE 1.6) y; SBP 144.8 (8.0); DBP 86.5 (4.3) mm Hg; heart rate 79.9 (3.3)/min; body mass index (BMI) 26.0 (1.5) kg/m², were included. The clinical characteristics of these normotensive, and stage 1 and 2 hypertensive participants are depicted in Table 2, indicating the poor treatment status of the included subjects.
Overexpression of Microvascular CK mRNA in Human Hypertension

Figure 1. CK isoenzyme mRNA in different human tissues.
Real-time quantitative polymerase chain reaction was performed on RNA isolated from different human tissues as indicated on the left. Values represent the average of duplicate quantitative polymerase chain reaction experiments measuring copy number of the four creatine kinase (CK) transcripts (CKB, CKM, CKMT1, and CKMT2) normalized to the PSMD4 copy number. The total transcript level per tissue is set to 100% total normalized CK copy numbers respectively 331.3 for skeletal muscle; 85.9 for heart; 10.1 for renal artery; 4.8 for small intestine; 14.8 for colon; and 12.5 for brain tissue, in accord with tissue differences in CK protein levels as previously reported. Individual transcript fractions were calculated and marked as indicated. The results show CKM/CKMT2 transcription mainly in striated muscle, and CKB/CKMT1 transcription mainly in smooth muscle and other tissue as expected.7-10,12

Table 3. Correlation coefficient of microvascular CKmRNA copy numbers and blood pressure.

<table>
<thead>
<tr>
<th>CK isoenzyme</th>
<th>Normalized copy number</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>CKB</td>
<td>15.00</td>
<td>1.91</td>
</tr>
<tr>
<td>CKM</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>CKMT1</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>CKMT2</td>
<td>1.53</td>
<td>0.22</td>
</tr>
</tbody>
</table>

CKB, cytoplasmic brain type creatine kinase; CKM, cytoplasmic muscle type creatine kinase CKMT1 and CKMT2 are respectively ubiquitous and sarcomeric mitochondrial creatine kinase. SBP, systolic blood pressure; DBP, diastolic blood pressure. Pearson product-moment correlation coefficient (r) with 95% CI in brackets. *p<0.05; †(0.00 to 0.84), signifies (0.001 to 0.844).
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Figure 2. Correlation between microvascular CK mRNA and blood pressure.

Scatter plot and least squares linear regression lines with Pearson product-moment correlation coefficients ($r$) for the association between systolic (SBP; panel A) and diastolic blood pressure (DBP; panel B) in normotensives and treated and untreated hypertensives ($n=13$) expressed in mm Hg (systolic blood pressure, SBP; open circles and dashed line, scale on left hand side; diastolic blood pressure DBP, closed circles and solid line, scale on right hand side), and resistance artery creatine kinase (CK) B mRNA expressed in normalized copy numbers. CKB is the dominant isoenzyme in vascular smooth muscle. Analysis of the correlation coefficient between total CK isoenzymes (CKB, CKM, CKMT1, and CKMT2) and blood pressure showed identical correlation coefficients and statistical significance (data not shown).

With the CK transcripts as described in the method section, we first assessed CK isoenzyme mRNA in different human tissues as shown in Figure 1. The results indicate that CKM mRNA is predominant in striated muscle and CKB mRNA in other tissue as expected. This confirmed the specificity of our CK isoenzyme transcripts.

Subsequently, we assessed the human microvessels with the validated transcripts (Table 3). Normalized CK mRNA copy numbers of the vascular tissue and the correlation with systolic and diastolic blood pressure are depicted in Figure 2, showing the strong correlation between CKB mRNA and blood pressure. The differences in CK mRNA in different blood pressure strata is shown in Figure 3, indicating a highly significant increment in microvascular CK mRNA with increasing systolic or diastolic blood pressure levels, from normotension, to stage 1, up to stage 2 hypertension. Non-parametric statistical methods did not significantly change the direction or the magnitude of the outcomes, with a Spearman rank order correlation coefficient for the association between CKB mRNA and respectively SBP and DBP of 0.70 ($p=0.002$) and 0.83 ($p<0.001$).
Overexpression of Microvascular CK mRNA in Human Hypertension

**Figure 3. Microvascular CKB mRNA in different blood pressure strata.**
NT, normotension, systolic/diastolic blood pressure up to 139 or 89 mm Hg respectively (n=6); Stage 1 Hypertension (HT), systolic/diastolic blood pressure between 140 to 159 or 90 to 99 mm Hg respectively (n=3); Stage 2 HT, systolic/diastolic blood pressure ≥160 or 100 mm Hg respectively (n=4); $p<0.01$ for differences in CK mRNA copy number between groups assessed with 1-way ANOVA.

**DISCUSSION**

We found a strong association between human resistance arteries CK mRNA and systemic systolic and diastolic blood pressure, including normotension, stage 1 and stage 2 hypertension. We had shown previously that CK is a main predictor of blood pressure, with an adjusted blood pressure increase in a random sample of a multi-ethnic population of 7.98 (3.27 to 12.68) systolic and 4.69 (1.88 to 7.5) mmHg diastolic per log CK increase. This was replicated in a large Norwegian population study.

Furthermore, we had shown that resistance artery contractility highly depends on CK, and that specific creatine kinase inhibitors greatly attenuate human vascular contractility ex vivo. The explanation proposed for these findings, was that in the absence of organ damage, high serum CK activity reflected high tissue CK activity. High CKB activity in resistance arteries was thought to lead to greater vascular contractility and higher blood pressures.

In this study, we have provided the first direct evidence that microvascular CK mRNA expression levels are strongly associated with blood pressure. The correlation coefficient between resistance artery CK mRNA and blood pressure is considerably
Chapter 3

higher than previously reported for serum CK and blood pressure (0.19 for serum CK and SBP vs. 0.64 for CKB mRNA and SBP). The stronger association may indicate that the association with CK mRNA is less likely due to an unmeasured confounder than serum CK, and that microvascular CK mRNA is a more direct estimate of hypertension risk than serum CK.

Even though mRNA expression levels are commonly used as a proxy for estimating functional differences that occur at the protein level, the relation between mRNA and protein expression is not well established. However, in this study, we found an association with a potential functional characteristic of the protein, blood pressure. Therefore, it is likely that the CKmRNA is translated into functional protein. Because of the small size of the microvessels, CK activity could not directly be assessed with the gold standard of spectrophotometric enzyme assays, but using a bioassay with enzyme inhibition, we have previously found evidence that higher microvascular CK activity is associated with enhanced contractility in isolated human resistance arteries. In addition, the tissue isoenzyme mRNA data we present, including renal artery (Figure 1), correspond with the previously reported distribution of CK isoenzyme activity. Finally, in the myocardium and aorta of hypertensive animals or acute pressure overload, CK mRNA was increased with concomitant increase in CK protein levels, as compared to controls. High myocardial CK activity was also reported to precede the development of hypertension in animal models, and to further increase with the development of hypertension, to reduce after successful treatment. Finally, we found evidence in our population study, that otherwise healthy subjects with controlled hypertension have lower CK than those with uncontrolled hypertension. Thus, the existing data indicate that CK mRNA, both constitutive and induced, are likely to be translated into CK protein to meet the increased energy requirements of high blood pressure. Further studies are needed to confirm this, and to assess the relative contribution of constitutive versus induced CK in human hypertensive disease.

As previously reported by us and others, on a protein level, microvascular CK acts as an energy transducer at the smooth muscle contractile proteins, supplying ATP for the contractile process. Calcium dependent, RhoA/Rho kinase and nitric oxide (NO)–guanosine 3', 5'-cyclic monophosphate pathways, the main intracellular effectors of blood pressure-regulating systems in vascular smooth muscle, are thought to converge on contractility responses fueled by CK. ATP is required for each actin-myosin complex formed. Vascular smooth muscle contraction is thought to consist of a fast, force-generating component at relatively high energy costs, and a
slow, tonic maintenance of tension, for which ADP is required. If, because of greater creatine kinase activity, ADP levels at the contractile proteins do not achieve the level required for tonic maintenance of tension, then the smooth muscle tension response could be altered, leading to excessive contractility. As expressed in the Poiseuille-Hagen formula, even a small increase in contractility and reduction in vascular diameter could have profound effect on resistance to flow and hence arterial pressure. Thus, even a small increase in CK activity might have a potentially large impact on blood pressure levels.

Although the resistance artery is central to the generation of blood pressure, to our knowledge, microvascular gene transcription and hypertension in humans have not been widely studied. Schiffrin et al., then using in-situ hybridization, found that small arteries from untreated hypertensive patients with moderate-to-severe hypertension, but not with normotension, or mild hypertension, showed evidence of the presence of endothelin-1 messenger RNA. However, no correlation with blood pressure was reported. We retrieved no further papers that assessed the transcription of genes involved in the intracellular pathways of pressor responses in peripheral, non-coronary resistance arteries in humans, in relation to systemic blood pressure.

The main strength of this study is that we found, for the first time, that mRNA expression levels of the central regulatory enzyme of energy metabolism creatine kinase, shows an almost perfect correlation with diastolic blood pressure, and also with systolic blood pressure. This is in line with previous findings of CK as a main denominator of blood pressure, and reports of significant vasodilation of isolated resistance arteries after CK inhibition. Furthermore, our data were collected in subjects of African and white self-defined ethnicities, and among the wide clinical spectrum of normotension and stage 1 and 2 hypertension. A limitation of the study is the small sample size, related to the nature of isolated vessels studies, which require an invasive harvest procedure. However, the sample size was calculated to be sufficient for the primary outcome.

In summary, we now found evidence that human microvascular CK mRNA levels progressively increase with blood pressure. The association found precludes causal inferences, but together with previous findings that human resistance artery contractility is highly CK-dependent, these new data strengthen the evidence that CK is involved in pressor responses. Hyperexpression of this ATP regenerating enzyme may serve to meet the increased metabolic demands of enhanced microvascular contractility as implicated in hypertension.
References


Creatine kinase predicts obesity in the general population

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Under revision, PLOS One
ABSTRACT

Background There are ample data on environmental factors that affect obesity, but biological factors are understudied. We focus on creatine kinase, the central regulatory enzyme of cellular energy metabolism. Creatine kinase activity is typically high in skeletal muscle type II fibers. There is evidence that predominance of type II, insulin resistant fibers is associated with obesity and weight gain after overfeeding. Therefore, we assessed whether serum creatine kinase after rest is independently associated with obesity in the general population.

Method We analyzed a stratified sample of the multi-ethnic population of Amsterdam, the Netherlands, consisting of 1444 citizens aged 34 to 60 years. We used linear regression analysis to assess the independent association between serum creatine kinase and body mass index.

Results Mean body mass index increased from the first through the third creatine kinase tertile, with values of respectively 26.4 (5.1), 27.2 (5.2), and 27.9 (5.0) kg/m². Creatine kinase was independently associated with body mass index in multivariable regression analysis, with an increase in body mass index of 3.1 (95% CI: 1.8 to 4.3) kg/m²/log creatine kinase increase, after adjustment for age, sex, ethnicity, educational level, and serum creatinine, as a surrogate measure of muscle mass.

Discussion Creatine kinase is strongly and independently associated with body mass index. As creatine kinase has been previously linked to hypertension, the high creatine kinase phenotype might be hypertension and obesity prone. Further studies are needed to assess whether high creatine kinase in obesity is an epiphenomenon, or part of a causal pathway leading to obesity.
BACKGROUND

Obesity has reached epidemic proportions globally. The age-adjusted prevalence is reported to range from 6 to 36% in Western countries. Obesity is part of the metabolic syndrome and a risk factor for hypertension, diabetes mellitus, and cardiovascular disease.\(^1\)\(^-\)\(^3\)

Individuals differ in their susceptibility to gain weight.\(^4\) Although there are ample reports on environmental factors leading to obesity, there is relative lack of research on biological factors related to this condition.\(^5\)\(^-\)\(^7\) In this study we focus on the association of obesity with the enzyme creatine kinase (CK).

CK plays a key role in cellular energy metabolism. By catalyzing the reversible transfer of high energy phosphates from phosphocreatine to ADP generating ATP, via the reaction: \(\text{MgADP} + \text{CrP} + \text{H}^+ \leftrightarrow \text{MgATP} + \text{Cr}\), the enzyme rapidly provides ATP for cellular ATPases.\(^8\)\(^-\)\(^10\) In skeletal muscle, high activity of the enzyme is typically found in type II fibers. Type II fibers are primarily fit for burst exercise, with cytosolic creatine kinase as the main ATP buffer.\(^11\) In these fibers, cytosolic CK is tightly coupled to glycolysis, whereas mitochondrial fatty acid oxidation capacity and glucose uptake are limited, rendering these fibers relatively insulin resistant.\(^8\)\(^,\)\(^12\)\(^-\)\(^14\) In line with this, previous studies have shown an association between predominance of fast, type II skeletal muscle fibers and weight gain.\(^15\)\(^-\)\(^19\) Overfeeding of healthy young men with relatively higher type II fiber proportions induced a greater gain of fat mass compared to men with higher type I fiber proportions.\(^17\)

In the absence of overt tissue damage, serum CK activity after three days of rest is thought to mainly reflect skeletal muscle cytosolic CK activity, which is predominantly derived from type II fibers.\(^20\)\(^-\)\(^23\) Therefore, we assessed whether serum CK is associated with obesity in the general population.

METHOD

Study population

For this study, we used the dataset as described previously of 1444 non-institutionalized persons (including 503 white European and 672 Surinamese-Dutch persons, and 269 with "other ethnicity") aged 34 to 60 years, living in Amsterdam.\(^21\) The Medical Ethnics committee of the Academic Medical Center, Amsterdam, the Netherlands, approved the study and this study was conducted in accord with the ethical principles...
of the Declaration of Helsinki. Cardiovascular risk factors, the use of antihypertensive drugs, socioeconomic status, and self-defined ethnicity were assessed through a questionnaire. We instructed participants to abstain from heavy exercise for three days before visiting our hospital for a physical examination. Walking, driving a car, and normal daily activities were allowed. Physical examination included height, weight, and blood pressure levels. Blood pressure was measured with an Omron M4 oscillometric device (Omron Healthcare Europe BV, Hoofddorp, the Netherlands). Body mass index (BMI) was calculated as weight (kg) divided by the height (rounded to the nearest centimeter) squared. Laboratory studies included serum CK activity after 3 days of rest and serum creatinine, estimated with automated analyzers (Roche/Hitachi Systems, Roche Diagnostics, Indianapolis, Ind), according to procedures recommended by the International Federation of Clinical Chemistry.21,24

**Primary outcome measure**

The primary outcome of this study was the association between serum CK activity after 3 days of rest and BMI after adjustment for age, sex, ethnicity, educational level and serum creatinine, after the exclusion of subjects with renal failure (creatinine >110 μmol/L) as this is proportional to muscle mass.25,26

**Statistical analyses**

To calculate the sample size for the association between serum CK and BMI, we used previously reported data on the correlation of the type II fiber area of the human musculus vastus lateralis and BMI (R=0.18).19 As these fibers are the main source of serum CK, we assumed a similar correlation coefficient between CK and BMI, and calculated that 414 persons needed to enter the study to detect this association with 2-tailed α=0.05 and 1-β=0.80.

**Data analyses**

Because the CK distribution was extremely skewed to the right, 24 outliers were excluded, using the empirical 97.5 percentile point of CK. Furthermore, logarithmic transformation to base 10 resulted in a more symmetrical distribution, as described previously.21 We first estimated differences in BMI across CK tertiles with One-way ANOVA. Thereafter, we calculated 1-tailed Spearman correlations for BMI versus CK activity, and age, sex, ethnicity, educational level, and creatinine. We used univariable and multivariable regression models to assess whether serum CK had predictive value on
Creatine kinase predicts obesity in the general population

BMI, independent of other established predictors. We verified whether the assumptions of the linear regression model, including normality, linearity, and homoscedasticity were met. Data in parentheses are 95% confidence intervals unless otherwise specified. Statistical analyses were performed with the SPSS statistical software package for Windows, version 19.0 (SPSS Inc, Chicago).

Table 1. Baseline characteristics of the participants.

<table>
<thead>
<tr>
<th>Participants (n)</th>
<th>Total (1342)*</th>
<th>White (487)</th>
<th>South Asian (267)</th>
<th>Black (522)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men (%)</td>
<td>40.9</td>
<td>49.5</td>
<td>46.4</td>
<td>31.0</td>
</tr>
<tr>
<td>Age (y)†</td>
<td>45.4 (6.6)</td>
<td>47.8 (6.8)</td>
<td>44.6 (6.6)</td>
<td>43.7 (5.8)</td>
</tr>
<tr>
<td>BMI (kg/m²)†</td>
<td>27.2 (5.1)</td>
<td>26.1 (4.7)</td>
<td>26.7 (4.9)</td>
<td>28.3 (5.3)</td>
</tr>
<tr>
<td>≥ 25.0 (%)</td>
<td>61.9</td>
<td>52.4</td>
<td>61.8</td>
<td>69.9</td>
</tr>
<tr>
<td>≥ 30.0 (%)</td>
<td>24.0</td>
<td>14.8</td>
<td>19.1</td>
<td>34.5</td>
</tr>
<tr>
<td>≥ 35.0 (%)</td>
<td>7.8</td>
<td>4.9</td>
<td>4.5</td>
<td>12.1</td>
</tr>
<tr>
<td>CK (IU/L)‡</td>
<td>108 (76 to 167)</td>
<td>88 (64 to 127)</td>
<td>102 (75 to 160)</td>
<td>145 (98 to 213)</td>
</tr>
<tr>
<td>Creatinine (μmol/L)†</td>
<td>74.4 (29.6)</td>
<td>73.8 (20.3)</td>
<td>71.2 (16.8)</td>
<td>76.4 (41.0)</td>
</tr>
<tr>
<td>SBP (mm Hg)†</td>
<td>126.3 (20.3)</td>
<td>123.8 (19.8)</td>
<td>127.4 (19.5)</td>
<td>128.3 (20.7)</td>
</tr>
<tr>
<td>DBP (mm Hg)†</td>
<td>81.9 (11.9)</td>
<td>79.3 (11.5)</td>
<td>83.3 (11.0)</td>
<td>83.8 (12.2)</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>29.9</td>
<td>23.8</td>
<td>30.3</td>
<td>35.4</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)†</td>
<td>5.4 (1.1)</td>
<td>5.7 (1.0)</td>
<td>5.4 (1.0)</td>
<td>5.1 (1.0)</td>
</tr>
<tr>
<td>Glucose (mmol/L)†</td>
<td>5.7 (1.7)</td>
<td>5.5 (1.2)</td>
<td>6.3 (2.3)</td>
<td>5.6 (1.8)</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, DBP, systolic/diastolic blood pressure; Hypertension, systolic / diastolic blood pressure ≥140/90 mm Hg and/or treated; CK, creatine kinase; *Includes participants with “other” ethnicity; †Mean (SD); ‡Median (IQR); §High glucose in South Asians as previously reported.43

RESULTS

The dataset contained 1444 subjects.17 Crude CK activity ranged from 14 to 5783 IU/L (median 111 IU/L). Mean BMI was 27.2 kg/m² (SD 5.1). Exclusion of outliers and log transformation of the data as described in the method section reduced the non-Gaussian distribution characteristics of positive skewness to a z-score of 1.5.21 We also excluded 1 subject without data on BMI and 62 subjects with controlled hypertension. The latter are thought to have lower CK activities compared to uncontrolled hypertension.21 Hence, 1342 subjects were analyzed. The characteristics of the analyzed subjects are listed in
Table 1. Mean BMI increased from the first through the third log CK tertile, with values of resp. 26.4 (5.1), 27.2 (5.2), and 27.9 (5.0) kg/m² ($p<0.05$ for tertile I vs II and III, $p=0.51$ for tertile II vs III). Correlation coefficients for CK and other parameters that correlated significantly with BMI are shown in Table 2. In univariable analysis, BMI increase per log CK increase was 2.57 kg/m² (95% CI: 1.46 to 3.69). When we adjusted for age, sex, ethnicity, educational level, and serum creatinine in linear regression analysis, CK was the main predictor of BMI with a beta coefficient of 3.06 (95% CI: 1.79 to 4.33) (Table 2). The assumptions of the linear regression model, including normality, linearity, and homoscedasticity, and no collinearity were met. In particular, the correlation between CK and creatinine was low (R 0.38). Finally, the sequence of variable entry had no impact on the outcomes (data not shown).

Table 2. Correlation coefficients and linear models for BMI.

<table>
<thead>
<tr>
<th>Correlation Coefficients*</th>
<th>Multivariable Regression $\beta$ (95% CI)$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>18.2</td>
</tr>
<tr>
<td>Log CK</td>
<td>0.15</td>
</tr>
<tr>
<td>Sex$^\dagger$</td>
<td>0.16</td>
</tr>
<tr>
<td>Educational level$^\mathsection$</td>
<td>0.09</td>
</tr>
<tr>
<td>Ethnicity$^{</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>0.11</td>
</tr>
<tr>
<td>Creatinine$^{**}$</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* One-tailed Spearman rank correlation; $P<0.01$ for all variables. Creatinine, a non-enzymatic metabolite derived from creatine in skeletal muscle and therefore a substitute measure of muscle mass, was not significant in univariable and multivariable regression analysis. $^+$The $\beta$-coefficients are for 1-unit increase in the continuous variables age, BMI, and log CK; $^\dagger$With higher BMI in women; $^{||}$With higher BMI at a lower level of education; $^{||}$Black vs non-Black people; $^{**}$With higher BMI at lower serum creatinine levels.
Creatine kinase predicts obesity in the general population

In skeletal muscle, creatine kinase (CK) buffers and transports ATP produced by anaerobic glycolysis and mitochondrial oxidative phosphorylation to sites of energy consumption, including myofibrils (myosin-ATPase) and membrane ion pumps (Ca\(^{2+}\)-ATPase). Based on the existing evidence we propose that the CK-system has a key regulatory function in processes that direct the entry and oxidative catabolism of glucose and fatty acids in muscle cells. Type II fibers display high cytosolic CK activity, closely coupled to glycolysis, while the mitochondrial capacity for oxidation of glucose and fatty acids is relatively low. Thus, high cytosolic CK activity may be associated with limited cellular glucose and fatty acid uptake and utilization, and predispose to the storage of lipid as fat tissue. In contrast, type I fibers contain less cytosolic CK activity, relatively more mitochondrial CK activity, and a greater capacity for oxidation of glucose and fatty acids in the mitochondrion.
Table 3. Characteristics of skeletal muscle fiber types.

<table>
<thead>
<tr>
<th>Type II</th>
<th>Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CK</td>
<td>Low CK</td>
</tr>
<tr>
<td>Predominantly glycolytic</td>
<td>Predominantly oxidative</td>
</tr>
<tr>
<td>Mitochondria poor</td>
<td>Mitochondria rich</td>
</tr>
<tr>
<td>Capillary rarefaction</td>
<td>High density of capillaries</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Burst exercise</td>
<td>Endurance capacity</td>
</tr>
<tr>
<td>Low GLUT-4 expression</td>
<td>Higher GLUT-4 expression</td>
</tr>
<tr>
<td>Insulin Resistant</td>
<td>Insulin sensitive</td>
</tr>
<tr>
<td>Less glucose uptake</td>
<td>High glucose uptake</td>
</tr>
<tr>
<td>Glucose and fatty acid stored as lipid</td>
<td>Glucose and fatty acid utilisation</td>
</tr>
<tr>
<td>Obesity prone</td>
<td>Lean</td>
</tr>
<tr>
<td>Hypertension prone</td>
<td>Normotension</td>
</tr>
</tbody>
</table>

CK, creatine kinase; GLUT-4, insulin-dependent glucose transporter protein 4.

**DISCUSSION**

The main finding of this study is that serum CK is strongly associated with BMI in a random population sample. This association was independent of age, sex, ethnicity, and serum creatinine, as a measure of muscle mass.

Serum CK activity is thought to reflect CK activity from striated skeletal muscle, in particular high CK type II fibers (Table 3).\textsuperscript{20-23} These fibers are well known to be associated with obesity and weight gain. As these fibers tend to be glycolytic and insulin resistant with less capacity for oxidation of fatty acids compared to type I fibers, this is thought to lead to storage of fatty acids as lipid instead of utilization.\textsuperscript{17-19,27}

The association of CK and fiber type distribution with obesity may be explained by the central regulatory function of the enzyme in key metabolic processes that direct the entry and oxidative catabolism of glucose and lipids in skeletal muscle fibers (Figure). The CK-system couples cellular ATP-producing with ATP-consuming processes, by catalyzing the reversible transfer of a high-energy phosphate moiety (Pi) between creatine and adenosine diphosphate, via the reaction:

\[
\text{MgADP + CrP + H}^+ \leftrightarrow \text{MgATP + Cr.}
\]
ATP generated by glycolysis and oxidative phosphorylation, is transported as phosphocreatine to subcellular locations of ATP utilization, such as myofibrills and membrane ion pumps, where ATP is regenerated. In type II fibers, high (cytosolic) CK activity, functionally coupled to glycolysis, ensures rapid resynthesis of ATP for burst contractions. On the other hand, the mitochondrial content and vascularisation is low in these fibers, with low expression of the insulin dependent transporter protein GLUT-4. This is associated with limited capacity for uptake and oxidation of fatty acids and glucose. Therefore, high skeletal muscle CK activity and type II fiber predominance may promote storage fatty acids and glucose as lipid in adipose tissue rather than uptake and oxidation in skeletal muscle, which may lead to obesity. In agreement with this, it was shown that skeletal muscle CK activity was 25.7% higher in obese subjects. In addition, CK activity of type IIa fibers of healthy young men was reported to correlate negatively with metabolic rate. In contrast, type I fibers contain less CK, are more reliant on mitochondrial metabolism, contain more capillaries, and are well fit for endurance exercise. These fibers utilize fatty acids as the major energy source, with a higher glucose uptake (Table 3), leading to less storage of fatty acids as lipid. In support of this, animal studies showed that inhibition of the CK-system lead to a shift from type II to type I fiber predominance, mitochondrial proliferation, increased oxidative enzyme activities, and 10% weight loss, increased GLUT-4 protein content, and improved glucose tolerance.

One strength of this study is the multiethnic sample with people of European, South Asian, and African ancestry. High skeletal muscle and serum CK activities are found in black people, a population subgroup with a higher risk for obesity compared to white people. However, the association of CK with BMI was independent of ethnicity in our analyses. This implies that CK may have potential use as a biomarker for obesity risk beyond skin color or ethnicity.

Second, we standardised for exercise. Serum CK activity is elevated up to 3 days with regular exercise, and up to a week after strenuous eccentric exercise, where the muscle lengthens and contracts at the same time against an external load. This leads to disruption of muscle fibers, and highly elevated serum CK, to up to 10.000 IU/L during a week or longer. However, none of the participants stated to be involved in such eccentric exercise. Still, we cannot exclude an exercise induced component in the CK values, but this would have led to an underestimation of the association between CK and BMI.
Limitations include the cross-sectional design of this study, which implicates causal inferences cannot be made. Furthermore, we cannot completely exclude increasing muscle mass with increasing BMI levels in this population. However, this is unlikely, as we found no significant association between serum creatinine within the normal range (excluding subjects with kidney failure), as a substitute measure of muscle mass, and BMI in our population.

In summary, we showed that serum CK activity is independently associated with obesity in a large population sample. As CK has been previously linked to hypertension,11,13,21 the high CK phenotype might be hypertension and obesity prone, as the muscle characteristics that underlie high serum CK, with predominance of high CK type II muscle fibers, include low vascularisation and low capacity for glucose and fatty acid oxidation, leading to higher peripheral resistance and insulin resistance. Further studies are needed to assess whether high CK in obesity is an epiphenomenon, or part of a causal pathway leading to obesity and to assess the role of CK and energy metabolism in the main risk factors for cardiovascular disease.
References


High blood pressure in women with uterine fibroids

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*both authors contributed equally

Submitted
ABSTRACT

**Background** Women are at greater risk of premature cardiovascular death than men, with hypertension as a main risk factor. In search for female-specific risk factors, we assessed the independent association between uterine fibroids and hypertension.

**Method** In a hospital-based setting, we collected data on blood pressure in black and white patients admitted for surgery for uterine fibroids compared with women with other gynaecological surgery and population controls. Main reasons for exclusion were lack of blood pressure data and malignancy. We used multivariable binary logistic regression analysis to assess whether women with uterine fibroids were more likely to have high blood pressure, adjusted for age, body mass index and ethnicity.

**Results** We included 241 women with uterine fibroids (126 black), 308 women who underwent surgery for other gynaecological reasons (37 black), and 606 population controls (360 black), with a mean age of 43.4 (SD6.6), 41.3 (11.2), and 45.0 (6.6) y respectively, and mean BMI of 27.4 (5.3), 25.5 (5.4), and 28.0 (5.6) kg/m$^2$. High blood pressure was found in 43.6, 28.6, and 24.3% of the women with fibroids, other surgery, and population controls respectively. Women with fibroids were more likely to have high blood pressure after adjustment for age, BMI, and ethnicity with an odds ratio of 2.7 (95% CI, 1.9 to 3.9).

**Discussion** High blood pressure occurs more frequently in women with symptomatic uterine fibroids than in women with other gynaecological surgery and population controls, independent of age, BMI and ethnicity. Further research should establish whether this association is related to “growth prone” factors, and whether women with fibroids should be counselled for cardiovascular disease.
**INTRODUCTION**

Cardiovascular disease (CVD) is the leading cause of death for women, and more women than men die of heart disease each year. Importantly, women are less likely than men to receive aggressive diagnosis and treatment of cardiovascular disease, and nearly two-thirds of women who die of cardiovascular disease suddenly were not aware of her condition. Therefore, there is a need for strategies to fill the gaps in cardiovascular health care and health outcomes that exist between men and women.

Hypertension is a major risk factor for cardiovascular morbidity and mortality in women. The prevalence rate of hypertension equals that of men with more than 25% of women worldwide affected. Therefore, detecting and treating hypertension is a main measure to prevent premature cardiovascular death in this population subgroup. In search for sex-specific risk factors for cardiovascular disease, we assessed the occurrence of hypertension in women with uterine fibroids.

**METHODS**

**Study design and population**

Research procedures were approved and exempted from informed consent by our local institutional review board. In this retrospective analysis, we enrolled black and white women, age 18 to 60 y, who underwent surgery for symptomatic uterine fibroids at the department of Gynaecology in the Academic Medical Center, Amsterdam, the Netherlands, from January 2008 to December 2011. We compared this group of women with women who underwent gynaecological surgery for other reasons and population controls. Population controls were 606 self defined black and white women selected from participants in the “Surinamese in the Netherlands: Study on EThnicity and health” (SUNSET) Study: a stratified random population sample of 3000 noninstitutionalized persons (1000 white European and 2000 Surinamese-Dutch persons), aged 35 to 60 y, living in Amsterdam. The methods of this study have been described in detail elsewhere. We excluded women without blood pressure data, and women with a previous diagnosis of a malignant (gynaecological) tumor.

**Data retrieval and analysis**

We collected data on ethnicity, date of birth, height, weight, medical history, use of (anti-hypertensive) medication, and blood pressure as measured during pre-assessment for
surgery. Included women were contacted by telephone to verify self identified ethnicity. Blood pressure in women with uterine fibroids and surgical controls was measured once by a nurse at the outpatient clinic during pre-assessment with a Datascope Accutor Plus monitor. Blood pressure in the population controls was measured with an oscillometric automated digital BP device (Omron M4 oscillometric device: Omron Healthcare Europe BV, Hoofddorp, the Netherlands), with the mean of two readings used in the analyses. High blood pressure was defined as systolic pressure ≥140, or diastolic pressure ≥90 mm Hg, or receiving antihypertensive drugs. Body mass index (BMI) was calculated as weight (kg) divided by the height (rounded to the nearest centimeter) squared.

**Statistical analysis**

The primary outcome was the odds ratio of the occurrence of high blood pressure in women undergoing surgery for symptomatic uterine fibroids compared to women who underwent gynecological surgery for other reasons and population controls, after adjustment for age, BMI, and ethnicity. Secondary outcomes were differences in the occurrence of high blood pressure in women who underwent total uterine extirpation versus fibroid enucleation, as a surrogate measure of the severity of the condition; the occurrence of high blood pressure in women with fibroids compared to controls in different age categories (<40 versus ≥40 y); body weight categories (BMI ≤30 versus BMI >30 kg/m²), and black versus white women.

Sample size calculations for the primary outcome were based on a previous study comparing hypertension prevalence in women undergoing hysterectomy for uterine fibroids with hysterectomy for other reasons. For a multivariable logistic regression analysis with three other predictors, we calculated that 167 women were needed enter the study (alpha 0.05, power 80%).

Crude differences in hypertension prevalence between groups were performed using the Chi-squared test. We used binary logistic regression analysis to assess whether women with uterine fibroids were more likely to have hypertension, adjusted for age, BMI, and ethnicity. A one-tailed $p$ value of 0.05 or less was considered statistically significant. Data were analyzed with SPSS statistical software package for Windows, version 19.0 (SPSS Inc., Chicago, II, U.S.A.). Data in squared brackets are standard deviations unless stated otherwise.
RESULTS

We included 241 women with uterine fibroids, 308 women who underwent surgery for other gynaecological reasons, and the 606 population controls. The characteristics of the included women are shown in Table 1. Notably, women admitted for fibroids were more often black compared to women admitted for other surgery, respectively 52.3 vs. 12.0%. The population sample was stratified for ethnicity and 59.4% of the included women were black.

Table 1. Patient characteristics.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Surgery for uterine fibroids</th>
<th>Other gynaecological surgery</th>
<th>Population controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of women</td>
<td>241</td>
<td>308</td>
<td>606</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>43.4 ± 6.6</td>
<td>41.3 ± 11.2</td>
<td>45.0 ± 6.6</td>
</tr>
<tr>
<td>BMI (kg/m²)*</td>
<td>27.4 ± 5.3</td>
<td>25.5 ± 5.4</td>
<td>28.0 ± 5.6</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>27.0</td>
<td>15.6</td>
<td>31.5</td>
</tr>
<tr>
<td>Black ethnicity (%)</td>
<td>52.3</td>
<td>33.8</td>
<td>40.6</td>
</tr>
<tr>
<td>SBP (mm Hg)*</td>
<td>133.2 ± 18.3</td>
<td>126.6 ± 15.7</td>
<td>122.9 ± 21.1</td>
</tr>
<tr>
<td>DBP (mm Hg)*</td>
<td>81.4 ± 11.0</td>
<td>75.9 ± 10.8</td>
<td>79.9 ± 12.1</td>
</tr>
<tr>
<td>High blood pressure (%)</td>
<td>43.6</td>
<td>28.6</td>
<td>24.3</td>
</tr>
</tbody>
</table>

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; High blood pressure is defined as systolic pressure ≥140, or diastolic pressure ≥90 mm Hg, or receiving antihypertensive drugs. *Values are mean ± SD.

The prevalence of high blood pressure was significantly higher in women with fibroids compared to women admitted for other surgery and population controls, respectively 43.6%, 28.6% and 24.3% (p<0.001 for comparisons between women with fibroids and controls), with respectively 38.5, 51.1, and 22.4% of women with high blood pressure using antihypertensive drugs (p=0.006 for the difference between women with fibroids and population controls, p=0.078 for the difference between fibroids and other surgery; p<0.001 for the difference between women admitted for other surgery and population controls) (Figure 1). In women who underwent a total uterine extirpation for fibroids, high blood pressure was more common compared to women who underwent fibroid enucleation, respectively 52.7 and 35.7% (p<0.001). The occurrence of high blood pressure was age-dependent as expected (Figure 2, panel A) and higher in obese and
black women (Figure 2, panel B and C). For white women, high blood pressure prevalence was 42.1% in women with and 21.4% in women without fibroids (\( p < 0.001 \)). For black women, this was 45.2 and 31.0% in women with and without fibroids respectively (\( p = 0.003 \)) (Figure 2).

In univariable logistic regression analysis, the odds ratio for having high blood pressure with surgery for uterine fibroids was 2.23 (95% CI, 1.66 to 3.00). After adjustment for age, BMI, and ethnicity in multivariable logistic regression analysis undergoing surgery for fibroids was the main predictor of having high blood pressure, with an odds ratio of 2.71 (95% CI, 1.89 to 3.89) (Table 2).

**Figure 1. Prevalence of high blood pressure and antihypertensive treatment in women with fibroids versus controls.**
Vertical bars represent the prevalence of high blood pressure (systolic/diastolic blood pressure ≥140/90 mm Hg or antihypertensive treatment) and the percentage of those subjects on antihypertensive treatment in women with fibroids, other gynecological surgery, and population controls. The prevalence of high blood pressure is significantly higher in women with fibroids compared to women admitted for other surgery and population controls. The percentage of women with high blood pressure using antihypertensive drugs was higher in women admitted for surgery compared to population controls. *\( p < 0.001 \) compared to population controls and women admitted for other surgery; **\( p < 0.001 \) and ***\( p = 0.006 \) compared to population controls.
Figure 2. Prevalence of high blood pressure in women with fibroids versus controls divided in age groups (A), non obese and obese (B), and white and black ethnicity (C).
Vertical bars represent the prevalence of high blood pressure in women with and without fibroids in different age categories (panel A), non obese (BMI <30 kg/m²) and obese (BMI ≥30 kg/m²) (panel B), and for white and black ethnicity (panel C). *p<0.01, **p<0.001 versus women without fibroids.

Table 2. Odds ratios for hypertension.

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Odds ratio (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroids</td>
<td>2.71 (1.89 to 3.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Black ethnicity</td>
<td>1.18 (1.05-1.32)</td>
<td>0.004</td>
</tr>
<tr>
<td>Age*</td>
<td>1.10 (1.08-1.13)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI*</td>
<td>1.10 (1.07-1.13)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

BMI, body mass index. * for increasing age and BMI.

**DISCUSSION**

The main finding of this study is that women admitted for surgery for fibroids are 2.7 times more likely to have high blood pressure compared to women admitted for other gynaecological surgery and population controls, independent of age, BMI, and ethnicity.

Although uterine leiomyomata are the most common pelvic benign tumors, little is known about cardiovascular health implications of this condition. In the recent largest rapport on the prevalence, symptoms, and management of uterine fibroids including more than 20,000 women, data on the prevalence of hypertension or cardiovascular disease were not reported. In addition, although current guidelines
for prevention of CVD in women emphasize that conditions associated with increased CVD risk should be recognized and that those women should be screened for other risk factors, uterine fibroids as a risk factor for hypertension is not mentioned. This is in spite of the fact that there are numerous anecdotal, retrospective, cross-sectional, case-control, and prospective cohort studies where this association is shown, including a greater occurrence in black women.

Several hypotheses for the association between hypertension and uterine fibroids have been forwarded, including surveillance bias, a common association with obesity, sex steroids, and hormone replacement therapy. Furthermore, it was proposed that the uterus with fibroids might affect the renin angiotensin system, due to extrarenal excretion of angiotensin forming enzymes or increased renal renin excretion, which is thought to be due to ureteric obstruction by the enlarged uterus or concurrent leiomyomata. However, evidence supporting this theory is lacking.

The most common hypothesis forwarded is that high blood pressure and fibroids share a common "growth prone" pathophysiology, of cardiac, smooth vascular and smooth uterine tissue. Several hormones (angiotensin II, insulin, ovarian steroids) and growth factors (transforming growth factor β1, epidermal growth factor, insulin like growth factor), implicated in proliferation and remodeling of smooth muscle, were postulated to link uterine fibroids with hypertension. The enzyme creatine kinase is another potential candidate. Creatine kinase, in particular the B isoenzyme is known to provide ATP to neoplasms. In addition, CK has been causally implicated in hypertension. Therefore, we propose that the ATP buffering CK system might be a common intracellular pathway of cellular growth responses in hypertension and uterine fibroids. The enzyme catalyzes the reversible transfer of a high-energy phosphate group from phosphocreatine to ADP, creating ATP. CK is tightly bound near ATPases involved in ion transport, smooth, cardiac, and skeletal muscle contractility and growth, where it serves to rapidly provide ATP for ATPases. As the enzyme directly provides ATP for pressor responses as well as for muscle hypertrophy, the high creatine kinase state might enhance vascular contractility as well as growth responses, promoting hypertension. Interestingly, relatively high CK activity is found in uterine fibroid tissue compared to adjacent myometrium. Clearly, more studies are needed in this field.

The main strength of this study is that we present a large sample of women of European and African ancestry and include surgical and population controls. In
addition, we showed that the association between hypertension and uterine fibroids is independent of common risk factors.

We acknowledge that this study has several limitations. First the retrospective design of the study includes a risk for selection and information bias, and temporal relationships cannot be assessed. Furthermore, we used single blood pressure measurements to define high blood pressure. Possibly, blood pressure levels were increased due to stress regarding the upcoming surgical procedure. This would have led to an overestimation of the prevalence of hypertension in women with fibroids. However, we also found a significantly prevalence of hypertension in women with fibroids compared to surgical controls, with equal blood pressure readings. Finally, the prevalence of uterine fibroids in the population sample is not known. However, if the association between blood pressure and fibroids is also present in the population, this would probably have led to an underestimation of the difference in hypertension prevalence between women with and without fibroids.

In summary, women with fibroids are around three times more likely to have hypertension compare to surgical and population controls, independent of age, BMI, and ethnicity. As hypertension and cardiovascular disease pose a considerable threat to women worldwide, increased physician and patient awareness on hypertension and increased risk for cardiovascular disease in women with fibroids is needed.
REFERENCES


PART 2

Therapeutic implications
Creatine kinase is associated with failure of hypertension treatment

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Journal of Hypertension, in press
ABSTRACT

Background Failure of hypertension treatment is a major clinical issue because of the high prevalence and the associated mortality risk. We have reported evidence that creatine kinase (CK) increases blood pressure through greater sodium retention and cardiovascular contractility, by rapidly providing ATP for these functions. Therefore, we hypothesized that high CK is associated with failure of antihypertensive treatment.

Method We analyzed a cross-sectional, random multi-ethnic sample of the general population (N=1444), aged 34-60y. The primary outcome was the independent association between resting serum CK and treated uncontrolled hypertension in the population, using multinomial logistic regression analysis.

Results Hypertension prevalence was respectively 26.8; 30.8, and 41.2% for the lowest (<88 IU/L) through the highest population CK tertile (>145 IU/L); (p<0.001). Treatment failed in 72.9% of subjects within the highest CK tertile vs 46.7% with low CK (p=0.004). In logistic regression analysis, CK was the main predictor of treatment failure (adjusted OR 3.7; 95% CI 1.2 to 10.9), independent of age, sex, BMI, fasting glucose, ethnicity, or education level.

Conclusion CK is associated with failure of antihypertensive therapy. Further investigations concerning the causal relationship for CK in hypertension might help improve treatment strategies for difficult to treat hypertension.
BACKGROUND

A substantial proportion of treated hypertensive patients does not achieve blood pressure control.\textsuperscript{1-4} In general, these subjects tend to be obese, older, or have diabetes and end organ damage. However, many patients have uncomplicated, primary hypertension, and it is not well explained why these subjects respond poorly to drug therapy, even when patients’ adherence and physicians’ therapeutic inertia are taken into account.\textsuperscript{1-4}

The enzyme creatine kinase (CK) is thought to enhance pressor responses through rapid regeneration of ATP, as the enzyme catalyses the reversible transfer of the high-energy phosphate moiety (P) between creatine and ADP:
\[
\text{MgADP + PCreatine + H}^+ \leftrightarrow \text{MgATP + Creatine}
\]
The rate of transfer of the phosphoryl group by CK is greater than the maximum rate of ATP generation by oxidative phosphorylation and glycolysis together, ensuring rapid resynthesis of ATP.\textsuperscript{4-11} Cytosolic CK is tightly bound in the immediate proximity of ATP-utilizing enzymes such as Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and Ca\textsuperscript{2+}-ATPase, and myosin light chain kinase and myosin ATPase at the contractile proteins. Here, ATP synthesized by CK is preferentially used to fuel highly energy-demanding processes such as sodium retention, cardiovascular contractility, as well as remodeling of arteries.\textsuperscript{3,5-13}

Serum CK was found to be a main independent predictor of blood pressure in the general population, independent of age, sex, body mass index (BMI), or ethnicity, with a crude systolic blood pressure increase of 14 mm Hg per log CK increase, without evidence of muscle damage.\textsuperscript{3,5,14,15} Importantly, high tissue CK precedes hypertension,\textsuperscript{11,16,17} and antihypertensive therapy lowers high tissue CK in animal models,\textsuperscript{16,17} while incubation of human resistance arteries with a CK inhibitor reduces vascular contractility.\textsuperscript{18} Finally, tissue CK is high in population subgroups with high hypertension risk,\textsuperscript{5,19,20} including in skeletal muscle, cardiac muscle, and vascular muscle.\textsuperscript{20} Taking this evidence on enhanced pressor responses with high CK into account, we proposed that serum CK activity after rest is associated with failure of hypertension treatment in the general population.
**METHOD**

**Study population**

The study population has been previously analysed for the association between serum CK and blood pressure, but in that report, treated hypertensives were excluded from the analyses.\(^5\) This group of treated hypertensives is the focus of the current report. The institutional review committee approved the study and the participants gave written informed consent. Further methods have been previously described in detail.\(^5\)

In brief, we included random population sample of 1444 subjects aged 34 to 60 years, and living in Amsterdam. Cardiovascular risk factors, the use of antihypertensive drugs, socioeconomic status, and self-defined ethnicity were assessed through a questionnaire.

We instructed participants to abstain from heavy exercise for 3 days before visiting our hospital for a physical examination. Walking, driving a car, and normal daily activities were allowed. Physical examination included height, weight, and blood pressure levels. Blood pressure was measured by a trained observer with an Omron M4 oscillometric device (Omron Healthcare Europe BV, Hoofddorp, the Netherlands) in a quiet room with the subject seated. An appropriately adjusted cuff size was used on the nondominant arm, which was supported at heart level.

To account for blood pressure variability, blood pressure was calculated as the mean of the first 2 consecutive readings, with a maximum of 5 mm Hg difference, as recommended by the Dutch Institute for Healthcare Improvement.\(^21\) This method results in lower blood pressure readings with smaller standard deviations.\(^22\) Laboratory studies included serum CK activity after 3 days of rest,\(^5\) estimated with automated analyzers (Roche/Hitachi Systems, Roche Diagnostics, Indianapolis, Ind) according to procedures recommended by the International Federation of Clinical Chemistry.\(^23\)

**Definitions**

Subjects were classified as follows: treated controlled hypertension (taking antihypertensive drugs and blood pressure <140 mm Hg systolic and <90 mm Hg diastolic); treated uncontrolled hypertension (taking antihypertensive drugs and blood pressure ≥140 mm Hg systolic or ≥90 mm Hg diastolic); untreated high blood pressure (no antihypertensive drugs and blood pressure ≥140 mm Hg systolic or ≥90 mm Hg diastolic); and normal blood pressure (no antihypertensive drugs and blood pressure <140 mm Hg systolic and <90 mm Hg diastolic).
Primary outcome measure
The primary outcome was the independent association of resting serum CK with failure of hypertension treatment in the general population.

Statistical analyses
We used the sample size calculation method for multinomial logistic regression of Peduzzi et al.,24 and calculated that with 8 predictors planned (age, sex, BMI, ethnicity, CK, fasting glucose, cholesterol, and education level), and for the response variable, hypertension categories, 25%-30% hypertensives expected, of which half would be treated (12.5% of the population), and half adequately treated (6.5% of the population), the minimum number of cases required was 1333.

Since the distribution of serum CK was known to be skewed to the right,25 we planned to exclude outliers and establish the empirical 97.5 percentile point of CK, to discard values that were abnormally high.5,25 We also planned to exclude subjects without data on blood pressure levels or CK activity.

To assess whether treated uncontrolled hypertension was associated with serum CK, we first calculated the difference in mean CK between normotension, treated controlled hypertension, and treated uncontrolled hypertension. After testing the assumption of a normal distribution, we used one-way analyses of variance (ANOVA) statistics to establish differences in CK between these blood pressure categories, with a Tukey HSD post test. Furthermore, to assess a potential dose-effect relationship, we used the Kruskal-Wallis test to assess differences between low to high CK tertiles within these blood pressure categories.

We used multinomial, multivariable logistic regression analysis to assess whether an increase in serum CK levels increased the likelihood to be categorized as treated uncontrolled, independent of other known predictors of blood pressure or treatment status. In a parsimonious approach, we first analyzed known predictors of blood pressure treatment status in a univariable multinomial logistic regression model, before including those predictor variables that were significant at \( p < 0.05 \) for at least one blood pressure category in multivariable multinomial logistic regression analysis, using the backward procedure when needed to formulate the best model. Blood pressure categories of normotension, treated controlled, treated uncontrolled, and untreated blood pressures were entered in the analysis as a four-group categorical outcome variable. Using these four categories, we defined normotension as the comparison category and determined separate relative risk ratios for predictor variables for each
category of the blood pressure status, except the comparison category. Relative risk ratios, the exponential beta coefficient, represented the change in the odds of being in the blood pressure category of treated-controlled, treated-uncontrolled, and untreated, versus the normotension associated with a one unit change on the predictor variable.

We ensured that the following assumptions were met for the multivariable multinomial logistic regression, adequate sample size related to the number of predictors, independent cases in the sample (no repeated observations in one individual, no paired or clustered individuals), no strongly correlated independent variables (multicollinearity), and linearity of predictor variables and log odds. To meet the assumption of linearity of independent variables and log odds, we reanalyzed the data, and categorized all the continuous independent variables to ordinal levels before including them in the model.

Finally, we addressed the question whether altering the inclusion criteria had a major effect on the results of the analyses. We reanalyzed the data, excluding participants using statins, because these drugs may cause an increase in CK activity; and excluding those with renal failure, because this condition has been associated with higher serum CK activities. Where applicable, we considered a two-sided \( p \) value of <0.05 to be significant. Data within parentheses are standard errors, and within square brackets are 95% confidence intervals, unless otherwise specified. Statistical analyses were performed with SPSS statistical software package for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

**RESULTS**

Table 1 summarizes the characteristics of the participants stratified for blood pressure status. Blood pressure was normally distributed. Crude CK activity ranged from 14 to 5783 IU/L (median 111 IU/L), with a distribution highly skewed to the right (z score for skewness 228.5), as previously reported. We excluded 3 outliers and 36 participants with CK activities above the 97.5 percentile. The data were still skewed to a significant degree (z-score for skewness: 22.3). Subsequent log transformation of the data to the base of 10 reduced the non-Gaussian distribution characteristics of positive skewness to a z-score of 1.5.
Creatine kinase is associated with failure of hypertension treatment

Table 1. Estimations of parameters within treatment groups.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Hypertension</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
<td>Controlled</td>
<td>Uncontrolled</td>
<td>Untreated</td>
</tr>
<tr>
<td>N</td>
<td>942</td>
<td>62</td>
<td>94</td>
<td>307</td>
</tr>
<tr>
<td>Male, %</td>
<td>36.9</td>
<td>25.8</td>
<td>40.4</td>
<td>53.1*</td>
</tr>
<tr>
<td>Black people, %</td>
<td>37.7</td>
<td>48.3</td>
<td>50.0*</td>
<td>48.1*</td>
</tr>
<tr>
<td>Age, y</td>
<td>44.2 (0.2)</td>
<td>47.0 (0.9)*</td>
<td>49.0 (0.6)*</td>
<td>47.9 (0.4)*</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>116.1 (0.4)</td>
<td>124.6 (1.2)</td>
<td>154.7 (1.8)</td>
<td>148.8 (0.8)</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>76.0 (0.2)</td>
<td>81.4 (0.7)</td>
<td>97.9 (1.0)</td>
<td>95.0 (0.5)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.4 (0.2)</td>
<td>29.4 (0.8)*</td>
<td>30.1 (0.5)*</td>
<td>28.5 (0.3)*</td>
</tr>
<tr>
<td>CK, IU/L†</td>
<td>126.8 (2.5)</td>
<td>124.3 (10.9)</td>
<td>159.7 (9.4)*</td>
<td>144.7 (4.7)*</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.6 (0.1)</td>
<td>6.8 (0.4)*</td>
<td>6.1 (0.2)*</td>
<td>5.8 (0.1)</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>5.4 (&lt;0.1)</td>
<td>5.4 (0.1)</td>
<td>5.3 (0.1)</td>
<td>5.4 (0.1)</td>
</tr>
<tr>
<td>University degree (%)</td>
<td>26.0</td>
<td>20.7</td>
<td>16.1*</td>
<td>21.3</td>
</tr>
</tbody>
</table>

Data are mean (SE) unless indicated otherwise; *Significant predictor of hypertension category (as compared to normotension) in univariable multinomial logistic regression. †after exclusion of outliers.

Table 2. Multivariable predictors of hypertension categories.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Hypertension Categories</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated Controlled</td>
</tr>
<tr>
<td>Log CK</td>
<td>0.28 [0.08 to 1.00]</td>
</tr>
<tr>
<td>Age</td>
<td>1.06 [1.01 to 1.11]*</td>
</tr>
<tr>
<td>BMI</td>
<td>1.08 [1.01 to 1.13]*</td>
</tr>
<tr>
<td>Black ethnicity</td>
<td>2.26 [1.17 to 3.38]*</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.18 [1.08 to 1.30]*</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.96 [0.49 to 1.89]</td>
</tr>
<tr>
<td>Education level</td>
<td>1.51 [0.91 to 3.77]</td>
</tr>
</tbody>
</table>

Multivariable, multinomial logistic regression analysis. Creatine kinase (CK) was the main and only specific predictor of treatment failure in the general population. Other predictors did not discriminate between treated controlled and treated uncontrolled hypertension. Data are the odds ratios (95% CI) of being classified in one hypertension category in the population, compared to the reference category normotension. Black ethnicity was compared to non-black ethnicity, and results on education level represent primary education vs tertiary education (university degree). *p<0.05. CK, creatine kinase. BMI, body mass index. Glucose, fasting serum glucose. Model goodness-of-fit: Pearson 3702.887, df 3873, p=0.96; Deviance 2109.062, df 3873, p=1.00; Nagelkerke's pseudo R square 0.23; Model chi square (24) 272.59 (p<0.001)
Hypertension risk was the highest with high CK, respectively 26.8; 30.8; and 41.2% of the subjects within the first (CK<88 IU/L) through the third (CK>145 IU/L) population CK tertile were hypertensive ($p<0.001$ for differences between all groups). In addition, subjects with treated, but uncontrolled hypertension, had significantly higher CK, 157.9 IU/L (9.4), vs 124.3 (10.9) IU/L in controlled hypertension, and 126.8 (2.5) in normotensives. ($p<0.001$ for differences between groups; and in the post test, between treated uncontrolled vs controlled and vs normotension, Figure 1).

**Figure 1. Population mean CK levels in normotension and treated hypertension.**
Values depict mean creatine kinase (CK) activity (SE) in serum after rest in a random population sample, with significant differences between groups in ANOVA ($p<0.001$). Treated Controlled, and Treated Uncontrolled refer to hypertensive subjects. *In the post test, CK levels in Normotension and Controlled Hypertension did not significantly differ. †‡CK in Treated Uncontrolled Hypertension was significantly higher than Normotension and Treated Controlled Hypertension ($p<0.001$).

There was no significant difference in the treatment rates between low and high CK tertiles (respectively 34.1 and 30.1%; $p>0.05$). However, importantly, treatment failed in 72.9% of subjects within the highest CK tertile vs 46.7% within the lowest CK tertile ($p<0.004$ for differences between CK tertiles; Figure 2).

Using univariable multinomial logistic regression analysis, we assessed the association of age, BMI, sex, ethnicity, fasting glucose, CK, cholesterol, and education level with the blood pressure categories of normotension, treated controlled hypertension, treated uncontrolled hypertension, and untreated hypertension. Except for serum total
Creatine kinase is associated with failure of hypertension treatment

Cholesterol, these predictors showed a significant association (at \( p < 0.05 \)) with at least one hypertension category.

![Figure 2. Hypertension control rates within CK tertiles.](image)

Members of the population with high creatine kinase (CK) had the highest hypertension rates. Treatment failed in the majority of hypertensive subjects with high CK (>70%), while it was successful in the majority of hypertensive subjects with low CK. Hypertens, hypertensive subjects in the population (%); Contr. HT., Controlled hypertension, as a percentage of all hypertensive subjects; Contr.Tr., Controlled hypertension, as a percentage of treated hypertensives (Control defined as systolic <140 and diastolic <90 mm Hg). Low, Med. and High CK are the lowest (<88 IU/L) through the highest (>145 IU/L) serum CK tertile after 3 days of rest (\( p < 0.001 \) differences between tertiles).

The univariable odds ratio for log CK in the categories treated controlled, treated uncontrolled, and untreated hypertension, was respectively 0.62 [0.21 to 1.80], 5.06 [2.12 to 12.10], and 2.85 [1.67 to 4.84]. We further quantified these results in multivariable multinomial logistic regression analysis. CK was the only specific, independent predictor of treatment failure (OR 3.67 [1.23 to 10.91]). Age, BMI, and black ethnicity were not useful to discriminate among hypertension treatment categories, neither were fasting glucose (which increased the odds of being treated), or male sex (which increased the odds of receiving no treatment; Table 2). The model had a good overall fit with an overall accuracy rate of 68.9%, and a -2 Log Likelihood of 2109.06 in the final model.
with a model chi-square of 272.6 (df 24; \( p < 0.001 \)), supporting a significant relationship between the predictor variables and blood pressure treatment status in this model.

Reanalyzing the data, categorizing all the continuous independent variables to ordinal levels before including them in the model to assess the assumption of linearity of independent variables and log odds, did not change the direction of the outcomes. We also reanalyzed the data excluding participants using statins, and those with renal failure, which did not change the outcomes (data not shown).

\[ 
\text{Creatine~P} \quad \text{Creatine} \quad \text{L-arginine} \\
\text{CK/Calcium ATPase} \quad \text{NO} \\
\text{ATP} \quad \text{ADP} \quad \text{cGMP} \quad \text{RhoA/Rho kinase} \\
\text{Ca}^{2+} \quad \text{SER} \quad \text{MLCP} \\
\text{CK/Myosin LC Kinase} \quad \text{Myosin} \quad \text{cGMP} \quad \text{Myosin-P + Actin} \\
\text{CK/Myosin ATPase} \quad \text{Myosin} \quad \text{ATP} \\
\text{ADP} \quad \text{ADP} \quad \text{ATP} \\
\text{Vascular Contractile Response} 
\]

**Figure 3. Creatine kinase enhances vascular contractility.**

This is a schematic representation of the main intracellular regulatory pathways of vascular smooth muscle contraction, based on Brewster et al.\textsuperscript{3,5} Creatine and nitric oxide (NO) share a common precursor in L-arginine. Creatine kinase (CK) is colocalized with Ca\textsuperscript{2+}-ATPase and myosin ATPase, and evidence suggests the enzyme is also colocalized with myosin light chain (LC) kinase, to rapidly supply these enzymes with ATP using creatinephosphate (Creatine~P)\textsuperscript{3,5,12,15-18} NO, RhoA/Rho kinase, and calcium-dependent pathways are intracellular effectors of blood pressure-regulating systems that converge on metabolic processes fueled by CK.\textsuperscript{3,5} Thus, high CK activity might lead to greater vascular contractility, partly through a lack of bioavailability of L-arginine for nitric oxide synthesis.\textsuperscript{5} cGMP, guanosine cyclic 3',5'-(hydrogen phosphate); MLCP, myosin light chain phosphatase. SER, sarcoendoplasmic reticulum.
DISCUSSION

Creatine kinase was the main and only specific variable associated with treatment failure in the general population, independent of age, sex, BMI, fasting glucose, or ethnicity. To our knowledge, this is the first report linking CK with treatment failure.

There are several possible explanations for this association between CK and treated uncontrolled hypertension. Creatine kinase activity in tissue and serum is known to be higher in men, in obese people, and in the black subpopulation, and has been associated with blood pressure. Since patients with higher mean blood pressure levels are often more difficult to treat, the results could merely reflect the association of serum CK with high blood pressure reported earlier. In the Dutch setting, according to national guidelines treatment of uncomplicated hypertension is only imperative at systolic pressure levels ≥180 mm Hg. This selection is probably related to the finding of higher mean blood pressures in participants from our population study with treated hypertension, than those with untreated hypertension (Table 1). However, since treatment failure was independently, strongly, and specifically associated with serum CK, with a clear dose-dependency, a causal relationship between CK and resistance to treatment cannot be excluded.

There is no evidence that hypertension directly increases serum CK. Clearance of serum CK is not altered with higher blood pressure levels, and there is no evidence that circulating CK is derived from the luminal surface of vascular endothelial cells, or that higher blood pressure levels causes cardiovascular muscle damage and increased serum CK activity. Importantly, normal CK isoenzymes are reported in subjects with relatively high serum CK activity and uncomplicated hypertension. A common cause of elevated serum CK activity levels is exercise, but participants were instructed to refrain from exercise for 3 days before the test. This period of 3 days used in this study should have substantially reduced the effect of exercise on serum CK, but CK activity can be elevated up to 3 weeks after eccentric muscular activity (where muscle contracts and lengthens at the same time). None of the participants stated to have been involved in such vigorous exercise, and if so, this would have led to an underestimation of the association between serum CK activity and treatment failure in this study.

In the absence of overt muscle damage, serum CK at rest is thought to be derived from tissue. Proportional to the level of tissue CK activity, normal tissue loses a small fraction of cytosolic CK into the interstitial space, which is transported through
lymphatic vessels into the blood stream.\textsuperscript{33} Hence, in resting subjects without muscle damage, serum CK is considered a measure of tissue CK levels.\textsuperscript{5,20,25,34}

Existing evidence indicate that an association between the level of tissue CK and treatment failure is biologically plausible.\textsuperscript{5} CK functions to rapidly regenerate ATP at subcellular locations of high energy demands.\textsuperscript{3,5-18} The enzyme is reported to enhance vascular contractility (Figure 3), and there is evidence CK facilitates renal salt retention, through providing ATP to basolateral Na\textsuperscript{+}/K\textsuperscript{+}-ATPase as a final common and rate limiting step.\textsuperscript{3,5,12,13,15-18} High tissue CK activity, which may be constitutive, induced, or both,\textsuperscript{5} might thus attenuate responses to antihypertensive therapy through enhanced contractile responses and salt retention.\textsuperscript{3,5,15,16,18} Experimental evidence shows that the activity of the enzyme is already elevated in relatively young spontaneously hypertensive rat’s myocardium and aorta, before the development of hypertension in the early normotensive phase, and increases further after hypertension occurs.\textsuperscript{3,10,16,17} Increasing CK is also seen in cardiac tissue of animal models of acute pressure overload of the left ventricle.\textsuperscript{11} This high tissue CK activity might enhance ATP buffer capacity and contribute to the greater cardiovascular contractility.\textsuperscript{3,5,35}

Our data are also in consensus with experimental evidence of lowering of the high tissue CK levels upon blood pressure lowering in the spontaneously hypertensive rat,\textsuperscript{16,17} and reduction of human vascular contractility with inhibition of CK.\textsuperscript{18} On the other hand, in heart failure in humans and animals, decreased total cardiac CK activity, and a reduction in the flux through the CK reaction are typical findings.\textsuperscript{36} Thus, the aggregated data provide evidence that the level of tissue CK activity modulates the function and dysfunction of cardiovascular system, with elevated CK associated with hyperfunction and decreased CK with hypofunction of the cardiovascular system.\textsuperscript{5,18}

The main strength of this study is the finding that CK shows a dose-dependent association with treatment failure, in accord with existing data on CK enhancing pressor responses.\textsuperscript{3,5-18} However, the cross-sectional design precludes causal inferences, for which prospective studies are needed. Furthermore, in this study we focussed on the real world outcome of the likelihood of hypertension treatment failure in the general population, with high CK as the hitherto unknown factor that enhances pressor responses. Details of medication type, dose, participants’ compliance, or physicians’ therapeutic inertia, well known to affect blood pressure control, were not available. But these factors are unlikely to have explained the strong independent association of treatment failure with CK, a hidden factor increasing the propensity toward higher blood pressures. Also, we cannot exclude that subclinical cardiovascular damage contributed
to the results. However, we consider this option unlikely as both normotensive and hypertensive people with relatively high CK activities are shown by us and others to have normal isoenzyme patterns.\textsuperscript{15,30,37}

Studies on the pathophysiology of failing hypertension treatment are scarce.\textsuperscript{38} To our knowledge, this paper is the first showing that creatine kinase, the enzyme that regenerates ATP for pressor responses, significantly increases the likelihood of being classified as hypertension treatment failure in a real-world setting of a multi-ethnic population, independent of age, sex, BMI, fasting glucose, or ethnicity. CK was the main and only specific independent predictor of failure of antihypertensive treatment. Prospective analysis on this association between CK and treatment failure is needed for causal inferences, and to decide whether CK could serve as a new, clinically useful biomarker for difficult to treat hypertension.
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Creatine kinase is associated with failure of hypertension treatment


Creatine and creatine analogues in hypertension and cardiovascular disease

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**ABSTRACT**

**Background** The creatine kinase system, the central regulatory system of cellular energy metabolism, provides ATP in situ at ATP-ases involved in ion transport and muscle contraction. Furthermore, the enzyme system provides relative protection from tissue ischaemia and acidosis. The system could therefore be a target for pharmacologic intervention.

**Objectives** To systematically evaluate evidence regarding the effectiveness of interventions directly targeting the creatine kinase system as compared to placebo control in adult patients with essential hypertension or cardiovascular disease.

**Search methods** Electronic databases searched: Medline (1950 – Feb 2011), Embase (up to Feb 2011), the Cochrane Controlled Trials Register (issue 3, Aug 2009), Latin-American/Caribbean databank Lilacs; references from textbooks and reviews; contact with experts and pharmaceutical companies; and searching the Internet. There was no language restriction.

**Selection criteria** Randomized controlled trials comparing creatine, creatine phosphate, or cyclocreatine (any route, dose or duration of treatment except for short-term use during cardiac surgery) with placebo; in adult patients with essential hypertension, heart failure, or myocardial infarction.

**Data collection and analysis** The outcomes assessed were death, total myocardial infarction (fatal or non-fatal), hospitalisation for congestive heart failure, change in ejection fraction, and changes in diastolic and systolic blood pressure in mm Hg or as percent change.

**Results** Full reports or abstracts from 1164 papers were reviewed, yielding 11 trials considering treatment with creatine or creatine analogues in 1474 patients with heart failure, ischemic heart disease or myocardial infarction. No trial in patients with hypertension was identified. Eleven trials (1474 patients, 35 years or older) comparing add-on therapy of the creatine-based drug on standard treatment to placebo control in patients with heart failure (6 trials in 1226/1474 patients), or acute myocardial infarction (4 trials in 220/1474 patients) or 1 in ischemic heart disease (28/1474 patients) were identified. The drugs used were either creatine, creatine phosphate (orally, intravenously, or intramuscular) or phosphocreatinine. In the trials considering heart failure all three different compounds were studied; creatine orally (Gordon 1995, Kuethe 2006), creatine phosphate via intravenous infusion (Ferraro 1996, Grazioli 1992), and phosphocreatinine orally (Carmenini 1994, Maggi 1990). In contrast, the
acute myocardial infarction trials studied intravenous creatine phosphate only. In the ischemic heart disease trial (Pedone 1984) creatine phosphate was given twice daily through an intramuscular injection to outpatients and through a intraveneous infusion to inpatients. The duration of the study intervention was shorter for the acute patients, from a two hour intravenous infusion of creatine phosphate in acute myocardial infarction (Ruda 1988, Samarenko 1987), to six months in patients with heart failure on oral phosphocreatinine therapy (Carmenini 1994). In the acute patients the follow-up period varied from the acute treatment period (Ruda 1988) to 28 days after start of the symptoms (Samarenko 1987) or end of the hospitalization period (Zochowski 1994). In the other trials there was mostly no follow-up after discontinuation of treatment. Only three out of five trials in patients with acute myocardial infarction reported mortality outcomes, with no significant effect of creatine or creatine analogues (RR 0.94, CI: 0.46-1.93). In addition, there was no significance on the progression of myocardial infarction or improvement on ejection fraction, the main effect seems to be on improvement of dysrythmia.

**Conclusion** There is inconclusive evidence to decide on the use of creatine analogues in clinical practice. In particular, it is not clear whether there is an effect on mortality, progression of myocardial infarction and ejection fraction, while there is some evidence that dysrythmia and dyspnoea might improve. However, it is not clear which analogue, dose, route of administration, and duration of therapy is most effective. Larger studies are needed to confirm the observations.
BACKGROUND

The creatine kinase system, the central regulatory system of cellular energy metabolism, is central to cardiovascular function. Creatine kinase regulates, buffers and transports, via creatine phosphate and creatine, ATP produced by glycolysis and oxidative phosphorylation, to sites of energy consumption such as myofibrils and membrane ion pumps. The enzyme substrate creatine is normally found in meat and fish, but it is also synthesized in the human body from dietary amino acids. Synthesis begins in the kidney with arginine and glycine forming guanidoacetic acid. This product is methylated in the liver, forming creatine (methylguanidine-acetic acid). Normal creatine plasma levels are 40-100 micromoles/liter (mcM/L); levels are about 25 mcM/L in vegetarians. The total adult body pool is approximately 120-140 grams. About 95% of body stores are found in muscle; creatine is also found in the liver, kidney, sperm, brain, eyes and the nervous system.

Myocytes use creatine (Cr) to make creatine phosphate (CrP) via creatine kinase. CrP is used to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). By using hydrogen ions to make ATP, creatine kinase also buffers intracellular hydrogen ions associated with lactate production and muscle fatigue during muscle contraction. There is evidence that the creatine kinase system protects the cardiovascular system from ischaemia and increases contractility. Cardiovascular implications are that high blood pressure is proposed to occur earlier and to be more severe with greater activities of this enzyme system. On the other hand, low creatine kinase activities are associated with heart failure. The system could therefore be a target for pharmacologic intervention in cardiovascular disease.

Methods

Objectives

To systematically evaluate evidence regarding the effectiveness of interventions directly targeting the creatine kinase system as compared to placebo control in adult patients with essential hypertension or cardiovascular disease.

Criteria for considering studies for this review

Types of studies

Randomized controlled trials. There was no language restriction. Abstracts and reviews were excluded. Studies could have taken place in any care setting (in-patient, outpatient,
day-care, or community). We did not include papers on the short-term use of creatine during cardiac surgery.

**Types of participants**

Adults (over 18 years of age) with cardiovascular disease (essential hypertension, heart failure or myocardial infarction) were considered.

**Types of interventions**

Studies examining agents directly interfering with the creatine kinase energy system, such as creatine, creatine phosphate or other creatine analogues versus placebo were considered. The intervention could be addressed by any route, in any dose and for any duration.

**Types of outcome measures**

The outcomes assessed were death, total myocardial infarction (fatal or non-fatal), hospitalisation for congestive heart failure, change in ejection fraction, and changes in diastolic and systolic blood pressure in mm Hg or as percent change.

**Search methods for identification of studies**

The Database of Abstracts of Reviews of Effectiveness (DARE) was searched for related reviews. The following electronic databases were searched for primary studies:

- The Cochrane Central Register of Controlled Trials (CCTR) (2011, issue 1)
- Bibliographic databases, including MEDLINE (2005 – January 2011), EMBASE (2010 – January 2011), Latin American and Caribbean Health Sciences Literature (LILACS) (to August 2009), and the Cochrane Hypertension Group Specialised Register (all years).
- The Hypertension Group Specialised Register includes controlled trials from searches of AGRICOLA, Allied and Complementary Medicine (AMED), BIOSIS, CAB Abstracts, CINAHL, Cochrane Central Register of Controlled Trials, EMBASE, Food Science and Technology Abstracts (FSTA), Global Health, International Pharmaceutical Abstracts (IPA), LILACS, MEDLINE, ProQuest Dissertations & Thesis, PsycINFO, SCIRUS, and Web of Science. Other sources were handsearching of references from textbooks and reviews; reference lists of all papers and relevant reviews identified; contact with experts and pharmaceutical companies; and searching the Internet, in this order. No language restrictions were used.

Search strategy used for key databases, with results (February 2011):

**Pubmed:** [(creatine OR cyclocreatine OR phosphocreatin* OR guanidino OR Neoton)ti] AND (heart OR myocard* OR hypertensi* OR blood pressure OR cardiovascular); limit to "clinical trial" and "human"; 131 papers, 12 eligible trials; seven included in this review.
**Embase:** (creatine or cyclocreatine or phosphocreatin$ or guanidino or Neoton).m_titl. AND (heart or myocard$ or hypertensi$ or blood pressure or cardiovascular).mp. AND clinical trial; 104 papers, 10 new eligible trials not found in Pubmed systematic search; of which four are included in this review.

**Lilacs:** (creatine OR cyclocreatine OR phosphocreatin$ OR guanidino OR Neoton)ti AND (heart OR myocard$ OR hypertensi$ OR blood pressure OR cardiovascular); 13 papers, no eligible trial that was not already found in Pubmed or Embase.

**Cochrane Library:** ((creatine OR cyclocreatine OR phosphocreatine OR phospho/ creatinine OR guanidino OR Neoton)ti) AND (heart OR myocard* OR hypertensi* OR blood pressure OR cardiovascular); 106 papers, no eligible trial that was not already found in Pubmed or Embase.

**Handsearch:** six new eligible trials, not found in any database with systematic searching. This was either because the clinical trial was absent from the database (EMBASE and Cochrane), or the “clinical trial” identifier was missing (MEDLINE). However, none of these papers fulfilled the inclusion criteria for this review.

Hence, in total, 11 trials were included, please see Figure 1.

**Data collection and analysis**
At least two reviewers independently assessed each eligible study unblinded. Risk of bias assessment was also performed independently by two reviewers. Disagreements were resolved through discussion. When there was no consensus between two reviewers, a third reviewer was asked for his or her opinion.

**Judgement of validity**
We included only randomized controlled trials.

**Data collection**
We developed a standard data abstract form for systematic collection of data on key trial characteristics, methodological quality, participants, comorbidity, intervention characteristics, drop outs, and outcomes. Two reviewers independently extracted data unblinded. Disagreements were resolved through discussion. We were unable to retrieve missing information.

**Analysis**
Statistical analysis was performed using Revman 5.1 software. Quantitative analysis of outcomes is based on intention to treat results (primary) and per protocol analysis (secondary). Our measure of effect for each study has been reported as relative risk for dichotomous data and weighted mean difference for continuous data.
**Heterogeneity assessment**

Chi square tests for heterogeneity were used to assess outcome data for computability with the assumption of a uniform risk ratio ($p>0.05$). If statistical heterogeneity was found across studies, the sources of the heterogeneity were to be explored and decision was made if studies should be aggregated. If so, the random effect model would then be used.

**Sensitivity analysis**

If applicable, data was to be reanalyzed using both fixed and random effect models and using log odds ratio versus risk ratio.

**Subgroup analysis**

Separate analysis of results were planned for patients with hypertension, myocardial infarction, and heart failure, and for gender and ethnicity if data was available.

**RESULTS**

**Trial retrieval**

Full reports or abstracts from 1164 papers were reviewed, yielding 11 trials considering treatment with creatine or creatine analogues in 1474 patients with heart failure, ischemic heart disease or myocardial infarction. No trial in patients with hypertension was identified. Most trials were in English (n=6), Italian language (n=3), other trials were in Polish (n=1), or Russian (n=1). A flow chart for trial retrieval and selection is provided in Figure 1.

**Description of included studies**

All trials were randomized controlled trials of add-on therapy of the creatine-based drug to standard treatment, versus placebo. The methods, participants, interventions and outcomes of the included studies are listed in table ‘Characteristics of included studies’. Eleven trials (1474 patients, 35 years or older) comparing add-on therapy of the creatine-based drug on standard treatment to placebo control. Four trials (220/1474 patients) considered patients in the acute stage of myocardial infarction, 8-11 six trials (1226/1474 patients) included patients with heart failure, 12-17 and one (28/1474 patients) considered patients with ischemic heart disease. 18 The drugs used were either creatine orally, 14,16 creatine phosphate (CrP) (orally, intravenously, or intramuscular). 8-11,13,15
or phosphocreatinine orally. In the heart failure trials all three different compounds were studied; creatine orally, creatine phosphate via intravenous infusion, and phosphocreatinine orally. The trials in patients with acute myocardial infarction only evaluated intravenous creatine phosphate. In the ischemic heart disease trial creatine phosphate was given twice daily through an intramuscular injection to outpatients and through an intravenous infusion to inpatients. The duration of the study intervention was the shortest for the acute myocardial infarction patients, ranging from a two hour to a 24 hr intravenous infusion of phosphocreatine.

In contrast, intervention periods in the heart failure trials ranged between ten days and six months. In the acute myocardial infarction patients the follow-up period varied from the acute treatment period to 28 days after start of the symptoms, between 24-30 days after start of the symptoms, or end of the hospitalization period. In the heart failure trials there was mostly no follow-up after discontinuation of treatment. Data in square brackets are standard deviations, unless otherwise specified.

**Description of excluded studies**

Sixteen studies were excluded because they were not randomised controlled trials. A table with characteristics of excluded studies is provided online.

**Risk of bias in included studies**

The results of the Risk of Bias assessment for each trial are outlined under Characteristics of the included studies, provided online. Figure 2 shows an overview of all trials. All trials were randomized controlled trials of add-on therapy of the creatine-based drug to standard treatment, versus placebo. Ten studies had a parallel design and two studies were crossover studies. Three trials mentioned method of randomization. Allocation concealment was adequate in two trials and was unclear in ten trials. Blinding was adequate in six trials and was unclear in two trials. In the remaining four studies blinding was not adequate. Incomplete outcome data were addressed in six trials. Ten trials were free of selective reporting, in two trials this was not clear. None of the 11 trials were free of “other bias”. Please see the Risk of bias in included studies table online for further details.
Figure 1. Flow chart.

Reports retrieved in databases N=1444

Duplicate reports N=280

Abstracts evaluated N=1164

Excluded trials N=810
No trials of guanidino compounds in cardiovascular disease

Full reports evaluated N=354

Excluded reports N=333
No randomized controlled trials of guanidino compounds in cardiovascular disease

Eligible trials N=21

Nonelectronic search N=5*
Handsearching, contact with authors

Total eligible trials N=26

Excluded trials N=810
No trials of guanidino compounds in cardiovascular disease

Total included trials N=11
Acute myocardial infarction (N=4), heart failure (N=6), ischemic heart disease (N=1)

Figure 2. Risk of bias graph.
This figure shows each risk of bias item is presented as percentage across all included studies.

Effects of interventions

Trials in patients with hypertension

No randomized clinical trials were identified in patients with hypertension.

Trials in patients with acute myocardial infarction

1. Mortality. Only two out of four trials in patients (n=134) with acute myocardial infarction reported mortality outcomes during hospitalisation, with no significant difference between creatine analogues and placebo. Samarenko 1987 reported a mortality rate of 10% of the patients in the CrP group (n=30) with 17% in the control group (n=30), that did not reach statistical significance with this small sample size ($p>0.05$). Zochowski 1994 reported a 2.1% mortality rate in the intervention group (n=47), according to the authors, this was due to thrombolytic treatment complications; and no deaths in the control group (n=27). Pooling this data yielded a point estimate for the relative risk of 0.94 (CI: 95% 0.42-2.09), with this small samples size (Figure 3).

2. Hospitalisation for congestive heart failure. None of the acute myocardial infarction trials reported hospitalisation for congestive heart failure.

3. Total myocardial infarction (fatal or non-fatal). Data were inconclusive, in that Pedone 1984-2 measured the progression of myocardial infarction scintigraphically in the intervention vs the placebo group between day 2-6 vs day 24-30 after the onset of symptoms. They found that the number of segments that were partially or totally revascularized between day 24-30, were 15/40 (38%) with creatine
phosphate (n=13) vs 5/46 (11%) with placebo (n=13). In addition they reported new abnormalities in 2/40 (5%) in the intervention vs 7/46 (15%) in the placebo group. However, Samarenko 1987 reported no significant differences in perfusion at day 27 or 28: the final size of the region of the myocardial perfusion defect was 20(2)% of the myocardium in the intervention group (n=30) vs 23(2)% in the control group (n=30) (p>0.10).

4. **Changes in diastolic and systolic blood pressure.** None of the acute myocardial infarction trials reported changes in blood pressure.

5. **Changes in ejection fraction.** None of the acute myocardial infarction trials reported changes in ejection fraction.

Figure 3. Phosphocreatine versus placebo. Outcome: mortality.

**Trials in patients with heart failure**

1. **Mortality.** None of the heart failure trials reported mortality outcomes.

2. **Hospitalisation for congestive heart failure.** None of the heart failure trials reported hospitalisation for congestive heart failure.

3. **Total myocardial infarction (fatal or non-fatal).** None of the heart failure trials reported total myocardial infarction.

4. **Changes in diastolic and systolic blood pressure.** None of the heart failure trials reported changes in blood pressure.

5. **Changes in ejection fraction.** Five trials reported ejection fraction as an outcome. Two out of five reported a significant effect of intervention. Carmenini 1994 reported a significant improvement from 35.9% ejection fraction at baseline to 40.5% after six months in the intervention group (n=39) that were reported to be significantly different (p<0.001) from the placebo group (n=40), although the
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Data of the placebo group were not shown. Ferraro 1996 reported a significant improvement with creatine phosphate (n=13) vs placebo after acute and short term treatment baseline: CrP 48(SD12)% vs placebo 50(12)%; acute: 53(12) vs 48(10)%; short term: 52(11) vs 48(12)%.[13] However, we could not reproduce the authors significant results. Out of the three remaining papers, two reported no significant effect of the intervention. Gordon 1995 after 10 days of treatment (no outcome data specified) and Kuethe 2006 after six weeks of treatment (Creatine 30(9)% vs placebo 29(8)%, n=13 in each group).[16,19] Finally, Maggi 1990 did not report the difference between phosphocreatinine vs placebo. In conclusion, no study showed clear effect on ejection fraction.[17]

Other outcomes as reported in publication of the included trials

The following outcomes were not prespecified in the review protocol, but were reported by authors.

Electrocardiographic findings

Reduction in dysrhythmia was found in the following trials. Three out of the four acute myocardial infarction (AMI) trials reported dysrhythmia as an outcome, with all three reporting a significant reduction in favour of the active intervention.[9-11] Ruda 1988 reported a reduction in the total number of ventricular premature beats (VPBs) in the creatine phosphate group (n=30) vs placebo (n=30) with Holter monitoring (24hr ECG) during the 2hr treatment (CrP 690 (179) vs placebo 2468 (737), p<0.02).[9] Samarenko 1987 showed a decreased frequency of ventricular extrasystoles in the intervention group (n=30) vs the control group (n=30) during Holter monitoring simultaneously with treatment on the first day (CrP 690 (179) vs placebo 2468 (737), p<0.02), without a significant decrease in supraventricular extrasystoles.[10] Zochowski 1994 showed a significant difference in percentage reduction of dysrhythmia (not further specified) in CrP treated patients (n=47) vs controls (n=27) during Holter monitoring of the first 24hrs after the AMI (CrP -96.3% vs placebo -29.7%), but did not mention a P-value.[11] Furthermore, a reduction in dysrhythmias was found in the single trial including patients with ischaemic heart disease and in two of the six heart failure trials.[15,17] Pedone 1984 reported a significant reduction in the number of VPBs in the CrP intramuscular group (n=10) and the CrP intravenously group (n=18) compared to placebo (resp. n=10 and n=18) with Holter monitoring on the 10th day (CrP im -31.4% p<0.05, CrP iv -33.4% p<0.001).[18] Grazioli 1992 showed a significant difference in percentage reduction of
VPBs in the CrP group (n=167) compared to placebo (n=150) after 15 days of treatment (CrP 68%, placebo 57%, \( p<0.05 \)). Maggi 1990 reported a reduction in the number of dysrhythmias and atrophicventricular blocks in the patients recieving creatine phosphate (n=30) compared to placebo (n=30) (CrP 50%, placebo 30%), but data on the exact time of measurement during the intervention period was not given.

**Dyspnoea and orthopnoea**
Four of the heart failure trials reported improvement in dyspnoea and orthopnoea. Carmenini 1994 reported a reduction in dyspnoea (38.3%), orthopnoea (37.8%) and cough (32.8%) after six months in favour of the phosphocreatinine treatment (n=39), which all significantly differed from placebo (n=40) (\( p<0.001 \)). In addition, Ferraro 1996 reported a significant improvement of symptoms in the creatine phosphate group (n=13) compared to the controls (n=13) (\( p<0.01 \)), but did not further detail this data. Furthermore, Grazioli 1992 reported significant differences in dyspnoea after the 45 day treatment with creatine phosphate measured with a self-monitoring four point scale: in the intervention group (n=508) 7% reported moderate to severe dyspnoea vs 23% in the control group (n=499) on the 45 day (\( p<0.001 \)). Finally, Maggi 1990 reported improvement on dyspnoea in favour of creatine phosphate (n=30) compared to controls (n=30), but not further detailed the data or mentioned the statistical significance. In contrast, Kuethe 2006 reported no significant differences on the Borg Scale of dyspnoea (measuring exhaustion on exercise) between creatine (n=13) and placebo (n=13), but did not provide further details.

**DISCUSSION**
High activity of the creatine kinase energy system has been associated with a greater risk to develop high blood pressure. On the other hand, too low activity might lead to heart failure and myocardial infarction. Although there is evidence that the creatine kinase system has a crucial role in the energy homeostasis, cardiovascular contractility, and ischaemic resistance, to our knowledge, the existing evidence on drugs directly targeting this system in humans has not been systematically reviewed previously. In this review, we found no trials of drugs targeting the CK system in patients with hypertension. The existing trials considered myocardial infarction and heart failure mainly. We found little evidence of a beneficial effect of creatine and creatine analogues in the prespecified outcomes, including ejection fraction and mortality. Pooling of the data resulted in a
point estimate of 0.94 for mortality without evidence of heterogeneity, but this outcome did not reach statistical significance. With no significance on the progression of myocardial infarction or improvement on ejection fraction, the main effect seems to be on improvement of dysrhythmia. Ventricular premature beats, ventricular extrasystoles and atrioventricular blocks were reported to be significantly reduced with the use of creatine phosphate or phosphocreatinine. Limitations of this review are that given the small sample size of the discussed trials and the clinical heterogeneity in patients, duration, mode of treatment (oral, intramuscular, or intravenous), and the drugs used (creatine, creatine phosphate, and phosphocreatinine), larger clinical studies are needed to confirm these observations. In particular, the potential effect on mortality needs further study with a larger sample size. The importance of the creatine kinase system for cardiovascular functions renders the system an interesting therapeutic target for drug intervention in cardiovascular disease.

CONCLUSIONS

Implications for practice
In our opinion, there is inconclusive evidence to decide on the use of creatine analogues in clinical practice. In particular, it is not clear whether there is an effect on mortality, progression of myocardial infarction and ejection fraction. While there is some evidence that dysrhythmia and dysnoea might improve. However, it is not clear which analogue, dose, route of administration, and duration of therapy is most effective.

Implications for research
Larger clinical studies are needed to confirm the observations of beneficial effects of supporting the creatine kinase system with creatine analogues in heart disease.

The importance of the creatine kinase system for cardiovascular performance under high energetic demands, renders the system an interesting target for therapeutic interventions in cardiovascular disease.
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The effect of the creatine analogue beta-guanidinopropionionic acid on energy metabolism: a systematic review

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*PLoS ONE* 8(1): e52879. doi:10.1371/journal.pone.0052879
Chapter 8

**ABSTRACT**

**Background** Creatine kinase plays a key role in cellular energy transport. The enzyme transfers high-energy phosphoryl groups from mitochondria to subcellular sites of ATP hydrolysis, where it buffers ADP concentration by catalyzing the reversible transfer of the high-energy phosphate moiety (P) between creatine and ADP. Cellular creatine uptake is competitively inhibited by beta-guanidinopropionic acid. This substance is marked as safe for human use, but the effects are unclear. Therefore, we systematically reviewed the effect of beta-guanidinopropionic acid on energy metabolism and function of tissues with high energy demands.

**Methods** We performed a systematic review and searched the electronic databases Pubmed, EMBASE, the Cochrane Library, and LILACS from their inception through March 2011. Furthermore, we searched the internet and explored references from textbooks and reviews.

**Results** After applying the inclusion criteria, we retrieved 131 publications, mainly considering the effect of chronic oral administration of beta-guanidinopropionic acid (0.5 to 3.5%) on skeletal muscle, the cardiovascular system, and brain tissue in animals. Beta-guanidinopropionic acid decreased intracellular creatine and phosphocreatine in all tissues studied. In skeletal muscle, this effect induced a shift from glycolytic to oxidative metabolism, increased cellular glucose uptake and increased fatigue tolerance. In heart tissue this shift to mitochondrial metabolism was less pronounced. Myocardial contractility was modestly reduced, including a decreased ventricular developed pressure, albeit with unchanged cardiac output. In brain tissue adaptations in energy metabolism resulted in enhanced ATP stability and survival during hypoxia.

**Conclusion** Chronic beta-guanidinopropionic acid increases fatigue tolerance of skeletal muscle and survival during ischaemia in animal studies, with modestly reduced myocardial contractility. Because it is marked as safe for human use, there is a need for human data.
INTRODUCTION

Creatine kinase (CK) is the central regulatory enzyme of cellular energy metabolism. The enzyme catalyses the reversible transfer of a phosphoryl group from ATP to creatine, creating ADP and phosphocreatine. The enzyme is mainly expressed in tissues with high energy demands, including skeletal muscle, heart, and brain. In the mitochondrial intramembraneous space, octameric CK facilitates the formation of phosphocreatine, which is transported to subcellular locations of ATP consumption. Here, ATP is rapidly regenerated in situ by cytosolic, dimeric CK (isoenzymes CK-MM, CK-MB, CK-BB). The CK-system thus functions as an effective cellular energy buffer and transport system, coupling mitochondrial ATP production to cytosolic ATP utilization, with an ATP regenerating capacity greater than from oxidative phosphorylation and glycolysis together.1-6

Despite the central role in energy metabolism, CK-knockout mice, deficient in either mitochondrial or cytosolic CK, are viable. Furthermore, CK-M deficient hindlimb muscles of mice show 2-fold higher aerobic energy generating potential, lack burst activity, but exhibit improved endurance, leading to a shift from type II to type I fiber predominance.7-9

Similar physiological alterations may be induced by beta-guanidinopropionic acid (βGPA or N-(aminoiminomethyl)-beta-alanine), an amino acid with a similar molecular structure as creatine, that reduces the flux through the CK reaction, by reducing cellular creatine uptake.10-12 βGPA is phosphorylated in cytoplasm, but both βGPA and phosphorylated βGPA are “inefficient substrates” for the CK reaction: in vitro Vmax values are <1% of the Vmax values of creatine and phosphocreatine.13,14 Furthermore, in contrast to creatine, βGPA is not utilised by mitochondrial CK.15 Thus, βGPA may modulate function of tissues with high energy demands. This amino acid can be freely obtained for human use. However, to our knowledge, the effects and potential side effects of βGPA are unclear, with some reports suggesting heart failure like complications with chronic us.16 Therefore, we conducted a systematic review to assess the effect of βGPA on energy metabolism.
METHODS

Outcomes
The primary outcome was the effect of βGPA on energy metabolism, function, and morphology of tissues with high and fluctuating energy demands.

Search strategy
We sought to identify all publications, published or unpublished, that considered the effect of βGPA on tissues with high energy demands in humans and animals. To identify relevant publications, we searched MEDLINE, EMBASE, the Cochrane Controlled Trials Register, and LILACS from their inception through March 2011. We also searched the Database of Abstracts of Reviews of Effects, Best Evidence, and Reviews in Progress (all from the United Kingdom). Finally, we searched for studies by using references from textbooks, narrative reviews, and systematic reviews; by contacting experts; and by searching the internet. We applied no language restriction.

Selection criteria
We included all controlled studies that considered the effect of βGPA in vivo or in vitro, in animal models or humans.

Data assessment
At least 2 reviewers independently assessed each eligible study. Disagreement was resolved through final discussion. Our primary outcome measure was the percentage difference in means. In addition, we assessed the weighted difference in means, using the random effects model. We assessed studies for heterogeneity in baseline animal characteristics, intervention (dose and duration), analytical methods, and expression of the different outcomes and decided whether we should aggregate studies.

We aggregated studies when the direction of the outcome was homogeneous. Furthermore, we used I^2 statistics to quantify the proportion of total variation in the estimates of the effect of intervention that was due to statistical heterogeneity and explored the sources of the heterogeneity.\(^{17}\) When the I^2 value is >75%, the variability in the effect estimates due to statistical heterogeneity is considered high, and the pooled results are less reliable [18]. We performed sensitivity analyses by reanalyzing data using fixed-effects models. Studies without numerical data were described. We predefined subgroups of different animal species, age (at baseline), muscle type, and
dose and duration intervention. Cut off points for age (at baseline), baseline weight (animal, skeletal muscle, and heart), and duration of intervention were determined post hoc based on available data. Statistical analyses were performed with the Cochrane Review Manager (RevMan) software, version 5 (Cochrane Collaboration, Oxford, United Kingdom) and with the SPSS statistical software package for Windows (Microsoft, Redmond, Wash), version 19.0 (SPSS Inc, Chicago, Ill).

RESULTS

Study retrieval
Full reports or abstracts from 259 references yielded 131 eligible papers in animals (n=120) and humans (n=11) (Figure 1: Study Flow Chart).

Methodological quality of included studies
None of the included studies reported randomization. Blinding or dropouts were not described. Studies used preset doses of βGPA and did not report whether they assessed the minimum dose needed to reach a maximum effect, although a dose-response curve was assessed in one study.19 All animal studies included a control group.

Heterogeneity analysis
Studies were heterogeneous regarding species (rats, mice, guinea pigs, turkey poultś, frogs, and rhesus monkeys), sex, skeletal muscle fiber type predominance (slow “type I” or fast “type II”), part of the heart (total heart, left, or right ventricle), βGPA dose, duration of intervention, and outcome measures. Because of the small numbers in other species, only studies in rodents were pooled.

I. Body weight and food intake
Of 117 papers in animals, 41 papers reported the effect of βGPA on body weight.16,20-59 Studies with weight outcomes included rats (median age 32, range 15 to 84) and mice (median age 77, range 21 to 210). The dose of βGPA ranged from 0.8 to 2.5%. The median duration of intervention was 56 days (range, 4 to 240). These studies showed an average weight decrease of 10.1% (SD 7.6) (Figure 2). In five studies with numerical information on ad libitum food intake, there was no evidence of reduced food intake (data not shown).12,20,35,56,60 One study proposed weight loss due a central hypophagic
effect, showing that βGPA (single intrathecal injection) resulted in a 9-fold increased Fos expression, a measure of hypothalamus activity, in rats. 

**Figure 1. Flow diagram.**
Flow chart of reports retrieved in databases. CK, creatine kinase; βGPA, beta-guanidinopropionic acid.

**Subgroup analyses**
We performed subgroup analyses for species (i.e. mouse vs. rat), baseline weight (post hoc cut off based on available data: <300 grams, ≥300 g), and duration of intervention (post hoc cut off: ≤3 weeks; 3 to 10 weeks; >10 weeks), and found similar magnitudes and directions of the point estimate in these subgroups (data not shown).
The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention Mean Body Weight ± SD</th>
<th>Control Mean Body Weight ± SD</th>
<th>Weight, %</th>
<th>Weighted Mean Difference (95% CI)</th>
<th>Weighted Mean Percentage Change (%)</th>
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<tr>
<td>Lindström 2008</td>
<td>30 22.0 ± 5.5</td>
<td>30 26.0 ± 5.5</td>
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<td>-4.0 (-6.77 to -1.22)</td>
<td>-15.4</td>
</tr>
<tr>
<td>Mahanna 1979</td>
<td>19 24.5 ± 7.4</td>
<td>18 39.2 ± 13.2</td>
<td>7.8</td>
<td>-14.70 (-21.63 to -7.77)</td>
<td>-37.5</td>
</tr>
<tr>
<td>Movend 1994</td>
<td>20 35.3 ± 5.3</td>
<td>20 41.1 ± 4.8</td>
<td>18.1</td>
<td>-5.75 (-8.47 to -3.03)</td>
<td>-14.0</td>
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<tr>
<td>Otten 1986</td>
<td>7 29.0 ± 7.9</td>
<td>7 34.1 ± 8.5</td>
<td>5.6</td>
<td>-5.1 (-13.70 to 3.50)</td>
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<td>Zong 2002</td>
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<td>5 29.0 ± 4.5</td>
<td>10.5</td>
<td>-3.0 (-8.54 to 2.54)</td>
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<tr>
<td>Robinson 2002</td>
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<tr>
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<td>91</td>
<td>100.0</td>
<td>3.39 (-4.67 to -2.11)</td>
<td>-18.05</td>
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</tbody>
</table>

A

B

Chi-square test for heterogeneity = 14.90; P = 0.02; I² = 60%

Figure 2. The effect of βGPA on body weight.

Legend. The effect of βGPA on body weight of mice with a baseline body weight less than 50 grams (A), rats with a baseline bodyweight less than 300 grams (B) and more than or equal to 300 grams (C). This is a random-effects model. Squares are weighted mean differences in body weight. The size of the squares represents study weight and horizontal lines represent 95% CIs. Arrowheads depict data outside the scale. Black diamonds are pooled estimates. The weighted mean percentage change was calculated from the percentage change per study and the sample size. Results for the effect of βGPA on body weight of rats with a baseline body weight of more than 300 grams are not pooled because of heterogeneity in the direction of effect. * Male sex; † Female sex; ‡ Animal age ≤6 weeks; § Animal age >6 weeks; †† Dose of βGPA =1% in the diet or drinking water; ‡‡ Dose of βGPA =2% in the diet or drinking water; ** Dose of βGPA =other; ††† Duration of intervention ≤10 weeks; †††† Duration of intervention >10 weeks.

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### II. Skeletal muscle

Studies included rats (median age 33, range 21 to 84) or mice (median age 56, range 21 to 84). The dose of βGPA ranged from 1 to 2.5% (diet or intraperitoneal injection). The median duration of intervention was 56 days (range 14 to 240).

#### Creatine, phosphocreatine, and ATP

Included studies showed decreased creatine, phosphocreatine, total creatine (creatinine + phosphocreatine), and ATP levels of respectively 66.1% (SD 19.2), 79.7% (SD 21.6), 86.7% (SD 10.0), and 38.8% (SD 13.6) after βGPA (Figure 3 and Supplement 1). We excluded one study with conflicting results (unchanged creatine, as well as a 56% increase).

#### CK activity, reaction velocity and flux

Total muscle CK activity decreased by 28.6% (SD 7.2) (Figure 3). In contrast to total muscle CK activity, mitochondrial CK activity was unchanged, and densitometry analysis showed a 3-fold increase of mitochondrial CK (Supplement 1).

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>N</th>
<th>Mean Body Weight ± SD</th>
<th>Control</th>
<th>N</th>
<th>Mean Body Weight ± SD</th>
<th>Weight, %</th>
<th>Weighted Mean Difference (95% CI)</th>
<th>Weighted Percentage change (%)</th>
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<tr>
<td>Bergeron 2001</td>
<td>5.4, 17.00 (-16.33 to 50.33)</td>
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<td>457.0 ± 49.8</td>
<td>11</td>
<td>510.0 ± 29.9</td>
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<tr>
<td>Bowen 2003</td>
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<td>9</td>
<td>342.0 ± 18.3</td>
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<td>361.0 ± 89.4</td>
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<td>Clark 1994</td>
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<td>361.5 ± 33.3</td>
<td>10</td>
<td>391.3 ± 42.4</td>
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<tr>
<td>De Tata 1990</td>
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<td>Levine 1996</td>
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<td>397.0 ± 24.0</td>
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<td>Lorentzon 2007</td>
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<td>325</td>
<td>433.0 ± 90.0</td>
<td>23</td>
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<td>Meyer 1988</td>
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<td>6</td>
<td>315.0 ± 34.1</td>
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<td>5</td>
<td>330.0 ± 17.9</td>
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<td>Wakazuki 1995</td>
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<td>-33.70 (-57.11 to -10.29)</td>
<td>-7.9</td>
<td></td>
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</tbody>
</table>

Total | 236 | 252 |
Adenylate kinase and AMP-deaminase
One study reported a 165% (no SD available) increase in skeletal muscle adenylate kinase activity after βGPA, while AMP-deaminase activity was 70.1% (SD 19.1) reduced (data not shown).

Glycolysis
Glycolytic enzyme activities were reported to decrease after βGPA, including phosphorylase by 38.8% (SD 17.7), and lactate dehydrogenase by 16.2% (SD 10.6). Phosphofructokinase and α-glycerophosphate dehydrogenase showed non-significant changes in activity of resp. 4.0 (SD 7.0) and 21.4% (SD 3.6) (data not shown).

Mitochondria
Mitochondrial oxidative enzymes were reported to increase after βGPA, including citrate synthase (+20.7%, SD 18.5), succinate dehydrogenase (+71.2%, SD 44.5), hydroxyacyl-Coa dehydrogenase (+38.6%, SD 27.5), 2-oxoglutarate dehydrogenase (+130%, no SD available), and hexokinase (+23.8%, SD 34.5), whereas cytochrome oxidase increased by 23.2% (SD 10.4) in type II fiber predominant muscle and decreased by 9.1% (SD 5.7) in type I fiber predominant muscle (Supplement 1B). Other mitochondrial markers reported to increase after βGPA were cytochrome c protein level by 41.2% and adenine nucleotide transporter level by 133.8% (no SDs available) (data not shown).

Other enzyme systems
AMP-activated protein kinase (AMPK) protein levels were reported to increase in 6 studies, of which two provided a percentage change. These studies showed a 45% increased AMPK protein content and 20% increased AMPK mRNA in mice, but no difference in rats after βGPA.

Glucose uptake and insulin resistance
In general, studies reported a 64% (SD 55.0) increased skeletal muscle glucose uptake (data not shown). In line with this, GLUT-4 protein content, a sarcolemmal insulin responsive glucose transporter, increased in 4 studies of which one provided a percentage increase; 45% in type I and 33% in type II fiber predominant muscle. Consequently, studies showed an 94.3% (SD 43.5) increased skeletal muscle glycogen
Results for plasma glucose were more pronounced in diabetic than non-diabetic animals; fasting glucose levels were decreased by resp. 54.5 (SD 31.4) and 5.1% (SD 10.4) (Supplement 1). Insulin levels followed a similar trend, with decreases in fasting plasma insulin of resp. 78.9 (no SD available) and 26.6 % (SD 20.9) (Supplement 1).

**Cellular fatty acid metabolism**

One study reported that fatty acid transporter protein content increased by resp. 21.0% and 55.0% in type I and type II fiber predominant muscle of rats and plasma free fatty acid concentration by 66.7% after βGPA. In contrast, 2 other studies reported unchanged fasting plasma concentrations of fatty acids, triglycerides, and total lipids in rats, but numerical details were not provided.

**Muscle function: In vivo endurance capacity**

Endurance capacity was increased during normal workload in rats (age 21 days), as measured by swimming to exhaustion with 2.5% of body weight attached or in a treadmill run, whereas endurance capacity at higher workload (swimming to exhaustion with 5.0% of body weight attached) was significantly reduced, without providing numerical details.

**In situ muscle function**

When assessed under general anaesthesia in situ, resistance to fatigue was reported to improve after βGPA in 7 studies, of which two provided numerical details, including a 166.3% (SD 27.9) increased maximal length of time to maintain isometric muscle contraction in type I fiber predominant muscle, which was not significantly changed in type II fiber predominant muscle (-9.8% (SD 16.4)) and a 54.8% increased residual force in type II fiber predominant muscle after a fatiguing protocol (data not shown). Initial isometric peak force was not significantly decreased by 4.2% (SD 16.6) after βGPA (Supplement 1). In line with this, 6 studies reported a reduced potentiation of force after βGPA in type I and II fiber predominant muscle of rats, without providing numerical details.
**Isolated muscle function**

Isolated muscle peak twitch force and peak tetanic force showed no significant change after βGPA; an average change of resp. +0.19% (SD 35.6) and -7.7% (SD 27.5) (Supplement 1).\(^{23,24,36,43,51,62,64,82-84}\) With regard to muscle contraction, the maximum rate of tension development decreased by 27.0% (SD 21.8), time to reach maximal contraction rate increased by 70.8% (SD 75.4), and contraction time showed no significant changes (+9.8% (SD 9.7) (data not shown)).\(^{23,24,36,43,62,82-84}\) In line with this, a 43.8% (SD 14.3) decreased maximum rate of relaxation, an 80.8% (SD 57.7) increased time to reach maximum relaxation rate, and a 13.9% (SD 17.9) increased one-half relaxation time was reported (data not shown).\(^{23,24,36,43,62,82-84}\) Fatigue resistance of type II fiber predominant muscle, indicated by residual force in after train stimulation, was reported to increase in two studies, but five reported no significant difference, without providing details.\(^{23,24,36,62,82-84}\)

**Morphology**

In general, studies reported a shift from type IIb fibers to type IIa and I fibers in type II fiber predominant muscle and a shift from type IIa to type I fibers in type I fiber predominant muscle (data not shown).\(^{34,43,51,53,57,66,69,76,81}\) with a decrease in absolute and relative muscle weight of resp. 18.6 (SD 8.9) and 10.2% (SD 10.1) (Supplement 1).\(^{23-25,28,29,34,36,43,45,48-50,53,54,57,59,76,82}\) This apparent shift towards oxidative metabolism was accompanied by a 2-fold increase in mitochondrial DNA,\(^{20}\) and increased mitochondrial density,\(^{56,78}\) with occasional subsarcolemmal accumulation of enlarged, elongated, or irregular shaped mitochondria, containing paracrystalline inclusions.\(^{48,54,74,81,85,86}\) The paracrystalline inclusions, in some studies, were found to be condensed and crystallized mitochondrial CK.\(^{54,86}\)
The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

### Table 1: Study Characteristics

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Control</th>
<th>Weighted Mean Percentage Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bergeron 2001* ‡</td>
<td></td>
<td>†† ‡‡ ***</td>
<td>11 2.6 ± 0.7</td>
</tr>
<tr>
<td>Ecochard 2004 * ‡</td>
<td></td>
<td>†† ‡‡ ¶¶</td>
<td>15 1.5 ± 0.5</td>
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<tr>
<td>Ecochard 2004 * ‡</td>
<td></td>
<td>†† ‡‡ ¶¶</td>
<td>15 3.3 ± 1.0</td>
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<td></td>
<td>†† ‡‡ ***</td>
<td>7 3.2 ± 1.0</td>
</tr>
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<td>** ‡‡ ¶¶</td>
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<td>†† ‡‡ ¶¶</td>
<td>9 4.7 ± 0.6</td>
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<td>Mahanna 1979 * ‡</td>
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<td>†† ‡‡ ¶¶</td>
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<td>†† ‡‡ ***</td>
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<td>†† ‡‡ ***</td>
<td>6 3.9 ± 0.5</td>
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Total: 188 186 100.0 -2.44 (-2.74 to -2.14) -42.8

Chi-square test for heterogeneity = 220.81, P < 0.00001, I² = 89%

Figure 3. The effect of βGPA on the creatine kinase system of skeletal muscle.

Legend. The effect of βGPA on skeletal muscle creatine (A), phosphocreatine (B), ATP (C), and creatine kinase activity (D). This is a random-effects model. Squares are weighted mean differences in body weight. The size of the squares represents study weight and horizontal lines represent 95% CIs. Arrowheads depict data outside the scale. Black diamonds are pooled estimates. The weighted mean percentage change was calculated from the percentage change per study and the sample size. * Rat; † Mouse; § Female sex; || Animal age ≤6 weeks; ¶ Animal age >6 weeks; ** Type I fiber predominant muscle; †† Type II fiber predominant muscle; ††† Dose of βGPA =1% in the diet or drinking water; §§ dose of βGPA =2% in the diet or drinking water; |||| Dose of βGPA =other; ††† Duration of intervention ≤3 weeks; *** Duration of intervention 3 to 10 weeks; †††† Duration of intervention >10 weeks.
Sensitivity analyses

For all outcomes, reanalyzing the data by using fixed-effects models, resulted in similar magnitudes of effect and conclusions about statistical heterogeneity, although the confidence intervals were narrower, as expected.

Subgroup analyses

We performed subgroup analyses for species (rats and mice), sex, muscle type (type I and II fiber predominant muscle, βGPA dose, duration of intervention (≤3 weeks; 3 to 10 weeks; >10 weeks), and found similar magnitudes and directions of the point estimate in all study groups (data not shown).

III. Heart

Studies included rats, mice, and guinea pigs (median age 51 days, range 15 to 82 days), with βGPA 0.5 to 2% in the diet or through an osmotic minipump (1M, 2.5 μL/hour). The median duration of intervention was 56 days (range 20 to 116 days).

Creatine, phosphocreatine, and ATP

As in skeletal muscle, myocardial creatine, phosphocreatine, and total creatine decreased after βGPA; an average reduction of respectively 62.4% (SD 19.3), 82.5% (SD 8.3), and 68.9% (SD 17.2) (Figure 4).30,35,39,41,42,47,55,87-94 Myocardial ATP concentration...
The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

decreased by 17.8% (SD 18.6) in vivo (Figure 4) and 30 to 40% (no SD available) in vitro.\textsuperscript{30,39,46,47,52,55,87,88,90,92,94,95}

**CK activity, reaction velocity, and flux**
Total myocardial CK activity was not significantly changed (10.1% (SD 14.0)) after βGPA (Figure 4).\textsuperscript{30,35,46,52,55,74,90} In line with this, the cytoplasmatic isoforms CK-MM, CK-MB, and CK-BB remained unchanged in vivo, but changed in vitro by resp. -31.6 ($p<0.05$), -58.2 ($p<0.05$), and +26.3% ($p>0.05$) after incubation of cardiomyocytes.\textsuperscript{30,52,55,89} In addition, mitochondrial CK decreased by 16.2% ($p>0.05$) (Supplement 2).\textsuperscript{30,52,55,74} The flux through CK-reaction and the CK reaction velocity decreased by resp. 67.0% (SD 23.7) and 46.9% (SD 11.0) after βGPA.\textsuperscript{96-101}

**Adenylate kinase and glycolysis**
In contrast to skeletal muscle, myocardial activities of adenylate kinase and the glycolytic enzymes phosphofructokinase, phosphorylase, and lactate dehydrogenase were not significantly changed after βGPA by resp. +17.6, -8.7, 0.0, and -8.1% (SDs not available) (data not shown).\textsuperscript{74,90}

**Mitochondria**
Myocardial oxidative enzymes including citrate synthase, 2-oxoglutarate dehydrogenase, 3-hydroxyacyl-Coa dehydrogenase, hexokinase, succinate dehydrogenase, and cytochrome c oxidase activity were not significantly changed (resp. -6.6 (SD 5.8), +5.1, +1.0, -29.2, -33.0, and -3.7%, SDs not available) (Supplement 2).\textsuperscript{26,52,74,102}

**In vivo cardiac function**
During baseline performance βGPA resulted in unchanged left ventricular systolic pressure, cardiac output, and rate of tension development, with a 69.1% reduced left ventricular end-diastolic pressure,\textsuperscript{35} but two other studies reported a 37.7% (SD18.7) reduced left ventricle fractional shortening, an echocardiographic measure of left ventricular function.\textsuperscript{41,42} During high workload, studies showed unchanged peak left ventricular developed pressure and cardiac output during aortic occlusion, \textsuperscript{35} a 15.7% reduced left ventricular shortening during increased pacing,\textsuperscript{41} and a reduced peak left ventricular blood pressure and heart rate (no numerical details) (Supplement 2).\textsuperscript{58} Mortality rates after the induction of myocardial infarction were increased: 93.5 to 100% after βGPA vs. 0 to 46.6% in controls.\textsuperscript{41,47,91}
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The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

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**Total**

Chi-square test for heterogeneity = 20.83; P = 0.002; I^2 = 71%

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**Total**

Chi-square test for heterogeneity = 20.83; P = 0.002; I^2 = 71%

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**Total**

Chi-square test for heterogeneity = 4.34; P = 0.04; I^2 = 77%

Figure 4. The effect of βGPA on the creatine kinase system of the heart.

Legend. The effect of βGPA on myocardial total creatine (A), phosphocreatine (B), ATP (C), and creatine kinase activity (D). This is a random-effects model. Squares are weighted mean differences in body weight. The size of the squares represents study weight and horizontal lines represent 95% CIs. Arrowheads depict data outside the scale. Black diamonds are pooled estimates. The weighted mean percentage change was calculated from the percentage change per study and the sample size. * Rat; † Mouse; ‡ Male sex; § Female sex; || Animal age ≤6 weeks; ‡‡ Animal age >6 weeks; ** Dose of βGPA =1% in the diet or drinking water; †† Dose of βGPA =2% in the diet or drinking water; ‡‡‡ Dose of βGPA =other; §§ Duration of intervention ≤3 weeks; |||| Duration of intervention 3 to 10 weeks; ††† Duration of intervention >10 weeks
**Function of perfused hearts and isolated cardiac fibers**

Despite an average left ventricular developed pressure reduction of 24.9% (SD 17.2) and a 39.7% (SD 10.1) reduction in tension development after βGPA, cardiac output was unchanged (105.2% (SD 10.5), with unchanged heart rate and coronary flow.35,39,46,90,91,95,103 Left ventricular end-diastolic pressure was 81.3% increased (Supplement 1).46 Myocardial oxygen consumption was increased by 18.7% in the right ventricle,30 but unchanged in the left ventricle.90,95 At high workload, left and right ventricular pressure were resp. reduced by 14.3% (no SD available) and unchanged, with unchanged left ventricular cardiac output.46,90 In line with this, βGPA had no effect on resting tension, maximum tension, and tension at increasing calcium concentrations of isolated myocardial fiber bundles, although the time to reach original tension after a quick stretch was 37.3% increased, indicating a slowing of the cross-bridge turnover.39 No effect on maximal force, rate of tension development, and half relaxation time of isolated papillary muscles was reported.93

**Morphology**

As in skeletal muscle, studies reported a shift from fast to slow myosin isoforms, including a 22.3% (SD 13.6) decrease of fast isoforms with a 3.8-fold (SD 3.1) increase of slow isoforms.39,58 Wet heart weight, heart weight to body weight ratio (HW/BW),
wet left ventricle weight, and left ventricle to body weight ratio were not significantly increased after βGPA by resp. 7.3 (SD 9.6), 11.8 (SD 14.6%), 27.4 (SD 27.8), and 19.4% (SD 18.8), while one study reported a 25.4% decreased dry heart weight (Supplement 2). Mitochondrial density was unchanged after βGPA without visible inclusions, but perturbations in mitochondrial form and DNA were reported in two other studies including cylindrically shaped mitochondria with paracrystalline inclusions with condensed and crystallized Mi-CK after in vitro incubation with βGPA, and a 76% increased proliferation of left ventricle mitochondrial organelles with 22% increased mitochondrial DNA (densitometric values).

Sensitivity analyses
When we reanalyzed the data by using fixed-effects models, we found similar magnitudes of effect and conclusions about statistical heterogeneity.

Subgroup analyses
Subgroup analyses for animal species, sex, animal age, dose and duration of intervention (≤10 weeks; >10 weeks, defined post hoc based on available data) all showed similar magnitudes and directions of the point estimate (data not shown).

IV. Vascular smooth muscle

The creatine kinase reaction
No papers reported data on vascular creatine concentration. Regarding phosphocreatine, acute perfusion of porcine carotid arteries with βGPA 50 mM during 12 hours, resulted in a 19.4 to 22.4% decreased phosphocreatine concentration. In addition, phosphocreatine was 86% reduced in the rat portal vein after βGPA 2% during 63 days. Perfusion of porcine carotid arteries with βGPA resulted in a 55.4% decreased ATP concentration with glucose in the perfusion solution, compared to 32.4% with pyruvate in the solution. In the rat portal vein, ATP concentration was unchanged.

Vascular function
Spontaneous contractile activity or developed force of the rat portal vein was unchanged after βGPA. In pulmonary vascular smooth muscle, βGPA 2% during 4 months to rats (aged 56 days) had no significant effect on normoxic pulmonary arterial pressure,
maximum rise in pressure during hypoxia, or peak pulmonary arterial pressure in response to angiotensin 2, a pulmonary vasoconstrictor.\textsuperscript{107}

V. Brain and nervous system

\textit{Creatine, phosphocreatine, and ATP}

Brain creatine, phosphocreatine, and ATP levels decreased by resp. 25.9\% (SD 3.0), 26.9\% (SD 10.8), and 25\% (no SD available) after βGPA.\textsuperscript{70,108,109} This was reported in total brain, cortex and striatum of mice and rats (median age 28 days, range 21 to 112 days) after βGPA 1 to 2.5\% in the diet for 6 weeks or after daily intraperitoneal injection (0.2ml of 0.5M) for 12 weeks.\textsuperscript{70,108,109} In vitro, creatine concentration in hippocampal slices of mice was reduced by 50.0\% after βGPA (10mM for 3 hours).\textsuperscript{110} In line with this, a 67\% inhibition of creatine uptake was reported in rat telencephalum after incubation with βGPA (500 µM), showing that creatine transporter inhibition is also present in brain.\textsuperscript{111} However, in vivo, the rate of phosphocreatine decrease in brain was reported to be slower than in skeletal muscle (2\% of baseline concentration per week vs 5 to 10\% decrease per week in skeletal muscle).\textsuperscript{109}

\textit{CK activity, reaction velocity and flux}

In stark contrast with skeletal muscle and heart, CK activity of total brain and white matter were increased by resp. 77.2 (SD 28.4, \(p<0.05\)) and 50.0\% (SD not available, \(p<0.05\)) after βGPA (2 to 3.5\%) for 6 weeks to 4 months, but CK activity of grey matter was unchanged (-11.3\%, no SD available, \(p>0.05\)).\textsuperscript{74,112,113} Regarding mitochondrial CK activity, an increase of 53.7\% (no SD available, \(p<0.05\)) was found after βGPA in one study,\textsuperscript{74} with no change in mitochondrial CK immunoreactivity reported in one other study.\textsuperscript{114} However, CK reaction velocity and flux were resp. 65 and 75\% reduced after βGPA 2 to 2.5\% during 42 to 70 days.\textsuperscript{112,113}

\textit{Adenylate kinase}

AK activity of total brain and white matter were resp. increased by 105.9\% (no SD available, \(p<0.05\)) and decreased by 16.7\% (no SD available, \(p<0.05\)). In grey matter AK was increased by 42.9\% (no SD available, \(p<0.05\)).\textsuperscript{74,112}

\textit{Mitochondria}

A 2-fold increased activity of succinate dehydrogenase, was reported in 1 study in rats (21 days) after βGPA 3.5\%.\textsuperscript{74} Furthermore, mRNA of mitochondrial markers was increased, including cytochrome C in cortex and striatum (resp. 120 and 145\%,}
The effect of the creatine analogue beta-guanidinopropionic acid on energy metabolism: a systematic review

Other enzyme systems
AMPK activity was increased by 20% (no SD available) in the cortex and 42.5% (SD 24.7) in striatum of mice after βGPA.60,108

Functional effects: Seizures
During seizures induced with pentylenetetrazole, no significant changes in brain ATP were observed in either βGPA-fed mice (2% for 21 days) or controls (no numerical details). Moreover, in brain of βGPA-fed mice phosphocreatine increased during seizures compared to baseline (no numerical details), while in brain of control mice phosphocreatine decreased by 10 to 20%.113 In line with this, during seizures the flux through the CK reaction increased by 40% from baseline in the βGPA-fed mice, while in controls induction of seizures did not change the CK reaction flux.113 Thus, ATP stability was not impaired during a high energy demanding seizure state. Regarding seizure activity, one study reported spontaneous epileptiform discharges in rats (age 63 days) after βGPA (daily i.p. injections with 400 mg/kg during 7 days), but βGPA had no effect on seizure activity in a rat model of chronic epilepsy.114

Functional effects: Hypoxia
During and after hypoxia, no reductions in brain phosphocreatine and ATP compared to baseline were reported among βGPA-fed mice, while hypoxia resulted in phosphocreatine and ATP reductions of 25 to 30% in controls. Moreover, during hypoxia CK reaction flux increased among βGPA-fed mice (no numerical details), while hypoxia did not change CK reaction flux in controls.113 Importantly, βGPA resulted in decreased mortality during and after ischaemia; 8.3% versus 38.5% in controls (p<0.05).112 Thus, through adaptations in CK isoenzymes and ATP metabolic pathways, βGPA may protect the brain from ATP loss during hypoxic, and perhaps other, states of ATP deprivation.

Neurodegenerative diseases
In mice (aged 70 to 84 days) with Parkinson’s disease and the associated mitochondrial loss of function (mimicked via injections with a mitochondrial complex-1 inhibitor) βGPA (1%) increased neuronal mitochondrial density by 16.1% (p<0.05) and mitochondrial...
number per cell by 33.3% ($p<0.05$), with unchanged mitochondrial volume compared to a 29.1% reduction in controls. Thus, βGPA might have a protective effect on mitochondrial function.

**Morphology**

In contrast to skeletal muscle, no mitochondrial inclusion bodies were reported in brain after βGPA 3.5% for 3 to 4 months. However, as in skeletal muscle, mitochondrial density in neurons of the substantia nigra was 16.1% increased in mice after βGPA 1% for 32 days, and increased mitochondrial DNA content (no numerical details shown) was reported in striatum and cortex after βGPA during 10 weeks. Finally, mild proliferation of astrocytes was reported in mice (aged 16 weeks) after βGPA during 10 weeks, indicating neural development.

**IV. Kidney**

Data on the kidney were scarce, with two studies reporting a reduced creatine uptake of kidney cortex brush-border membrane vesicles of fetal, newborn, and adult rats after incubation with 20 to 1000 millimolar βGPA.

**V. Brown adipose tissue**

Rectal and tail temperature of resting rats was reported to decrease by resp. 3.0 (SD 0.4) and 20.1% (SD not available) after βGPA. Brown adipose tissue weight, influenced by reduced body temperature, was reported to increase by 3.4% (SD 6.0).

**VI. Tumor growth**

A 63% (SD 14.1) reduced proliferation of intraperitoneal Ehrlich ascites tumor cells and a growth delay of mammary carcinoma of 1.6 days (SD 0.3) was reported after βGPA (through intraperitoneal injection, intravenous injection, or the diet). In addition, reduced food intake due to expansion of the tumor was prevented by βGPA (132.0% of control, no SD available).

**VII. Humans**

**Uptake of βGPA and the effect on human cell lines and plasma**

Regarding intestinal absorption in humans, one study showed that βGPA was reported to have high affinity for transport by the human intestinal proton-coupled amino acid transporter (hPAT1) in vitro. In addition, effects of βGPA on human cell lines were
reported in 10 studies, including a 30 to 40% decrease in creatine and phosphocreatine content, without alterations in ATP after incubation of endothelial cells (5mM βGPA for 24 hours),\textsuperscript{122} and increased immunostaining of myoglobin, a marker for slow oxidative fibers after incubation of isolated myoblasts (20μM βGPA for 7 days).\textsuperscript{77}

In red blood cells, a 45% reduced creatine transport was reported after incubation with βGPA (1 mM).\textsuperscript{123} In these red blood cells, studies reported a reduced hexose monophosphate shunt activity, indicated by a 55.8% (SD 2.13) reduced glucose-6-phosphate dehydrogenase activity after incubation with βGPA (20 μg),\textsuperscript{124,125} and a 20% increased ascorbate cyanide test after incubation of healthy human plasma with βGPA (20 µM).\textsuperscript{126} In line with this, increased in vitro haemolysis was reported after adding high doses of βGPA (340 to 1770 μM) to plasma of healthy volunteers, without providing numerical details.\textsuperscript{127} Regarding white blood cells, a reduced mitogenic response of lymphocytes was found in the presence of βGPA (10 mM),\textsuperscript{128} while in neutrophils a 50% decreased ATP concentration and a 25% reduced activation was reported 3 hours after βGPA incubation (5 μM).\textsuperscript{129,130}

**DISCUSSION**

In this systematic review we show that βGPA is an effective inhibitor of the flux through the CK reaction. In skeletal muscle, chronic intervention with βGPA led to markedly reduced creatine, phosphocreatine, and ATP concentrations and reduced cytosolic CK activity, while mitochondrial CK activity was increased. This generally led to a shift from glycolytic to mitochondrial oxidative metabolism, resulting in a shift from type II to type I fiber predominance, increased glucose tolerance, and reduced skeletal muscle and body weight, comparable to alterations reported after endurance exercise.\textsuperscript{131-137}

The reduced body weight with unchanged food intake may be related to the fiber type shift, as an association between type II fiber predominance and body weight was previously reported.\textsuperscript{138-141} In addition, cellular fatty acid transporter protein concentration and fatty acid oxidative capacity were increased after βGPA, which may lead to reduced lipid storage.\textsuperscript{31} However, with limited evidence for unchanged food intake, weight loss may as well be a consequence of reduced intake.\textsuperscript{12,20,35,56,60}

Several compensating mechanisms for the reduced cellular phosphagen content may lead to the observed fiber type shift. First, activation of AMPK is thought to defend against energy deficiency in skeletal muscle by stimulating mitochondrial biogenesis and alternative oxidative ATP generating pathways, with increased glucose transport.
and fatty acid oxidation. In addition, through mitochondrial proliferation, the mean diffusion distance between mitochondria and contractile elements, the myofibrils, decreases. In this way, tissues may compensate for the attenuated spatial buffer capacity by the CK system. Furthermore, the AK system may compensate for decreased CK activity, by providing a comparable energy-shuttling system. Increased cytosolic ADP is converted to AMP via cytoplasmic AK, which is used by mitochondrial AK in the intermembranous space to stimulate oxidative phosphorylation.

In line with the shift towards oxidative metabolism, the changes in skeletal muscle performance were comparable with functional changes after endurance training, i.e. increased fatigue tolerance with mildly reduced peak performance. This is in accord with studies in mice with a genomic deletion of CK, resulting in a mildly symptomatic phenotype with reduced muscle peak performance, showing that CK is relevant but not indispensable.

In contrast to skeletal muscle, cytosolic CK activity, AK activity, and oxidative enzymes of heart tissue were unchanged after βGPA, with reduced mitochondrial CK activity. Consistent with this, βGPA was not phosphorylated by mitochondrial CK. This differential response in cardiac and skeletal muscle was also found in rodents after endurance training, in which myocardial mitochondrial respiration chain activity and citrate synthase activity were unchanged. As energy is mainly supplied by mitochondrial fatty acid respiration, the myocardium may have sufficient pre-existent oxidative capacity to supply the energy requirement induced by βGPA.

As in skeletal muscle, myocardial contractile performance was minimally reduced. During baseline performance, the isolated heart ejected an equal cardiac output in spite of a slowed and reduced left ventricular developed pressure. This finding may be explained by a slower ejection rate after βGPA, which may facilitate a more prolonged and complete left ventricular ejection in spite of the reduced ventricular pressure. In vivo left ventricular pressure was unchanged, suggesting that the intact rat cardiac and/or humoral compensatory mechanisms, such as catecholamines and sympathetic and vagal innervation, may be sufficient to maintain normal function.

In contrast with the functional effects on skeletal muscle, the myocardial effects were not comparable with the effects of endurance training. It is known that endurance training may result in eccentric myocardial hypertrophy, increased stroke volume and decreased heart rate, leading to unchanged or decreased cardiac output at rest. During high workload, however, the endurance trained heart is able to reach a much higher cardiac output than the untrained heart, which we did not find in these data.
Compared to the myocardium and skeletal muscle, brain concentrations of creatine, phosphocreatine and ATP were less decreased after βGPA. It was previously hypothesized that this was due to the existence of two compartments in the brain, one in which creatine is replaced by βGPA and one in which creatine is not replaced. Another explanation might be that brain cells can synthesise creatine, but this is disputed since humans and mice with an absence of brain creatine transporter do not appear to have creatine in the brain. Total brain CK activity was increased, mainly due to an increase in white matter. However, brain CK catalyzed reaction rates were markedly reduced, with increased mitochondrial function. These findings may as well be explained by brain compartmentalization. Grey matter is characterized by the presence of CK-B and Mi-CK isoenzymes, with relatively slow CK-catalyzed reaction rates, similar to smooth muscle. On the other hand, white matter, with ≈50% of the CK activity of grey matter, contains higher CK-reaction rates, and little or no Mi-CK, comparable to skeletal muscle. The effects of βGPA in brain may therefore reflect inhibition of cellular creatine uptake in predominantly white matter, with the reduced CK-catalyzed flux a property of grey matter. Intriguingly, βGPA protected brain tissue of mice from ATP loss during seizures and mortality during hypoxia, by yet unknown mechanisms.

The effect of βGPA on tissue composition and function seems reversible, as almost no residual changes in enzyme activities of type I and II fiber predominant muscle were reported one month after withdrawal and no residual changes in mitochondrial morphology after four months. Repletion with creatine after βGPA resulted in an even faster recovery of biochemical and functional alterations induced by βGPA.

A limitation of this review is the lack of human data, despite the over the counter availability of βGPA. Furthermore, the sample size of the included studies was often small, which resulted in considerable statistical heterogeneity for several outcomes. In summary, reversible inhibition of the CK-system with βGPA in animal studies modulated metabolism towards enhanced mitochondrial function and oxidative capacity in skeletal muscle, with less marked alterations in heart muscle, probably due to the fact that βGPA is not utilised by mitochondrial CK in the myocardium. These modulations led to an increase in endurance capacity and insulin sensitivity of skeletal muscle, minimally altered contractility in the heart, and a greater tolerance for cerebral energy deprivation during seizures and hypoxia. The variation in metabolic and functional adaptations of the tissues studied in this review, suggest a tissue specific regulation of energy metabolism, and physiology of the CK reaction. Notably, despite the fact that βGPA can be purchased for human use, there is lack of human data.


Supplement figures 1 and 2 are provided online: PLoS ONE 8(1): e52879. doi:10.1371/journal.pone.0052879
Creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat

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ABSTRACT

Background We have reported that creatine kinase is the main predictor of hypertension in the population and has a strong contribution to vascular contractility. Catalyzing the reaction: MgADP + PCreatine + H+ ↔ MgATP + Creatine, the enzyme provides ATP for sodium retention and vascular contractility. In this study, we hypothesized that inhibition of creatine kinase with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat.

Method Male, 16-weeks-old spontaneously hypertensive rats (N=16) were randomly assigned to a standard diet with or without beta-guanidinopropionic acid. Blood pressure was measured weekly by the non-invasive tail-cuff method. After 4 weeks we assessed the effect on vasodilatory responses of mesenteric arteries in a wire myograph.

Results Treatment with beta-guanidinopropionic acid significantly reduced systolic and diastolic blood pressure compared to controls, by 42.7 (SD 5.5) and 35.3 (4.8) mm Hg respectively (p<0.001). Beta-guanidinopropionic acid enhanced the vasodilatory response of rat mesenteric arteries to dinitrofluorobenzene, by 82.2% (p=0.008) a creatine kinase inhibitor. Moreover, incubation of isolated rat mesenteric arteries with 150 mg beta-guanidino-propionic acid induced a 25.7(4.4)% vasodilation, demonstrating a decreased vascular contraction potential by creatine kinase inhibition.

Conclusion To our knowledge, we are the first to show that creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure. Modulation of the creatine kinase-system might be a novel promising target for the treatment of hypertension.
BACKGROUND

Creatine kinase (CK) was proposed to affect pressor responses through rapid supply of ATP for energy demanding processes such as sodium retention, cardiovascular contractility, and remodelling of arteries.\textsuperscript{1-5} In line with this, serum CK was reported to be the main predictor of blood pressure in the population, independent of age, sex, body mass index, and ethnicity.\textsuperscript{2,6}

By catalyzing the reversible conversion of creatine into phosphocreatine, CK builds up large cellular reserves of phosphocreatine for temporal and spatial buffering of ATP, via the reaction: Creatine + MgATP $\leftrightarrow$ Phosphocreatine + MgADP. ATP, generated by glycolysis and oxidative phosphorylation, is transported as phosphocreatine to cytosolic ATP-utilizing enzymes, including myosin light chain kinase and myosin-ATPase at contractile proteins and Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase at cellular membranes. Here, ATP is regenerated by cytosolic CK and used for vascular contractility and sodium retention.\textsuperscript{1,4,7}

The flux through the CK reaction can be inhibited by beta-guanidinopropionic acid (βGPA), a naturally occurring creatine analogue that reduces the flux by reducing cellular creatine uptake (Figure 1). βGPA is phosphorylated in cytoplasm, but both βGPA and phosphorylated βGPA are inefficient substrates for the CK reaction: in vitro $V_{\text{MAX}}$ values are <1% of the $V_{\text{MAX}}$ values of creatine and phosphocreatine.\textsuperscript{8,9} Therefore, we hypothesized that βGPA reduces blood pressure in the spontaneously hypertensive rat (SHR), an animal model for essential hypertension.

METHODS

An expanded description of the Methods section is available in the Data Supplement.

Animals

Fourteen-week-old male SHR (N=16, mean body weight 316.9 g) were obtained from Charles River (Maastricht, the Netherlands) and handled according to a protocol approved by the Animal Ethical Committee of the University of Amsterdam, the Netherlands, in conformity with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The SHR were divided in 2 groups. Group 1 (N=8) received pure chow (pellets). Group 2 (N=8) received identical chow containing βGPA (3%) (Pure bulk Vitamins and Dietary Supplements, Roseburg, Oregon, USA) during 4 weeks. Based
on pilot studies in our laboratory showing limited food intake with βGPA during the first days of the experiment, daily food supply of controls was restricted during the first 10 days. Both groups received 15 grams of chow per day. Thereafter, food was supplied ad libitum. Food intake and weight were measured daily.

Figure 1. Mechanism of action of beta-guanidinopropionic acid.
Beta-guanidinopropionic acid (βGPA) enters the cell instead of creatine through competitive inhibition of the creatine transporter. βGPA is phosphorylated by cytosolic creatine kinase (CK), but is an ineffective substrate, leading to reduced flux through the CK reaction. βGPA cannot be phosphorylated by mitochondrial CK. Cr, creatine; CrP, phosphocreatine; CM, cellular membrane; MEM, mitochondrial outer membrane; MIM, mitochondrial inner membrane.

Tail-cuff blood pressure measurements
Standardized tail-cuff blood pressure measurements of conscious SHR were performed once weekly with the CODA™ system (Kent Scientific Corporation, Torrington, CT, USA) after a handling period of 2 weeks. The animals were restrained in a transparent animal holder and placed on a heating pad. Body temperature was measured regularly
to maintain a temperature of 35 to 37°C. The rat was left untouched and fixated for 5 minutes before placing the tail-cuffs. Then, the tail-cuffs were placed over the tail slightly below the tail base. Based on results from our pilot studies, in which we estimated the number of measurements after which the standard deviation remained constant (data not shown), we performed 10 repeated measurement cycles per rat per week. The mean of measurement cycles 6 to 10 was used for further analysis. During the measurements, care was taken to ensure minimal stress for the animals. The animals were held in the holder for a maximum of 20 minutes.

**Anesthesia, blood draw, and tissue harvesting**

At baseline and after 4 weeks blood was drawn from the tail vein of each rat for estimation of CK, creatinine, sodium, potassium, urea, glucose, total cholesterol, HDL, and triglycerides. After 4 weeks of intervention the animals were anesthetised with ketamine (90 mg/kg)–dexmedetomidine (0.125 mg/kg)–atropine (0.05 mg/kg) (KMA) through intraperitoneal injection for tissue harvesting. Mesenteric arteries (3rd order) were prepared for tension recording with a four-channel wire myograph (Danisch Myo Technology, Copenhagen, Denmark). In addition, aorta segments were mounted in an organ bath and force readout was recorded via a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia).

**Data analysis and statistics**

The primary outcome was the difference in blood pressure of SHRs after intervention with βGPA (3%) versus untreated SHRs. Based on treatment of adult SHRs with other antihypertensive drugs we expected a decrease of 16 mm Hg (SD 7) after 4 weeks of intervention, and calculated we needed 6 rats in each group with a 2-tailed α=0.05 and 1-β=0.80. Secondary outcomes were differences in vasodilatory responses of isolated mesenteric arteries in a wire myograph. For the myograph experiments, based on an expected mean SD of 0.6 with a biologically relevant decrease of 1 mN in E_{MAX}, we calculated 8 rats would be needed. Thus, we included 8 rats in each group, 16 rats in total. Data are presented as mean ± SEM with N being the number of individual rats. Peak contraction values during the myography experiments were determined and expressed as relative tension (mN*mm^{-1}). Comparisons between groups were performed using the Student’s t-test. When the data consisted of repeated observations in time, one-way Repeated Measures ANOVA was used to investigate between-group differences. Statistical analyses of myograph experiments were performed using Prism.
(Graphpad Prism Software, San Diego, CA, USA). Other analyses were performed using SPSS Statistical Software, version 18.0.

**RESULTS**

**Body weight, food intake, and general appearance**

All animals appeared healthy and displayed normally physical activity. Food intake was reduced during the first four days with βGPA (3%) compared to controls, despite the food restriction as described in the methods section. After four days food intake was similar in both groups (data not shown). Treatment with βGPA (3%) reduced body weight during the first week. After one week body weight increased with a similar rate in both groups: βGPA 338.3 (5.6) versus control 355.6 (8.8) g ($p=0.11$) (Figure 2, panel A).

![Figure 2. Body weight, systolic and diastolic blood pressure.](image)

Line graphs showing body weight (A), systolic (B), and diastolic blood pressure (C) of spontaneously hypertensive rats from 16 to 20 weeks of age, ie. from baseline to 4 weeks of intervention with βGPA (3%) (N=8) (●) or control chow (N=8) (□). Data are presented as mean values with standard error bars.
Systolic and diastolic blood pressure, and heart rate

At baseline systolic and diastolic blood pressure were equal in both groups. After 4 weeks systolic as well as diastolic blood pressure of SHRs treated with βGPA were decreased compared to control SHRs by 42.7 (5.5) (p<0.001) and 39/2 (4.1) mm Hg (p=0.004) respectively (Figure 2, panel B and C). Heart rate was similar in both groups at all time points (data not shown).

Heart weight and heart weight/ body weight ratio

Heart weight (βGPA 987.5 (93.4) vs control 937.5 (101.6) mg, p=0.72) and heart weight/ body weight ratio (βGPA 2.9 (0.3) vs control 2.7 (0.3), p=0.65) were not significantly different between groups.

Mesenteric arteries and aorta: vessel diameter, contractility, and vasodilatory responses

The diameter of βGPA treated vessels was increased compared to control vessels; 299.0 (9.1) vs 274.8 (5.5) µm respectively (p=0.039). Maximum contractility of isolated mesenteric resistance arteries, induced by norepinephrine (10 µM), was not significantly different between controls and treated animals, 3.9 (0.4) and 4.5 (0.2) mN*mm⁻¹ (p=0.201). After precontraction with KPSS and norepinephrine (10 µM), there were no significant differences between concentration-response curves of mesenteric arteries in response to methacholine (p=0.12) and sodium nitroprusside (p=0.33) between groups, although there was a trend for increased vasodilation by methacholine after βGPA (Figure 3, panel A). The vasodilatory response to the CK-inhibitor DNFB was enhanced by 82.2% after treatment with βGPA (Figure 3, panel B). Incubation with 150 mg βGPA induced a 25.7 (4.4)% vasodilation (p=0.008) in both groups (Figure 3, panel C). In the aorta the vasodilatory response to methacholine was increased by 27.6% (p=0.007), which was partially augmented by L-NNA (p=0.24) (Figure 4).

Biochemical analyses

Plasma levels at baseline and after 4 weeks of intervention with βGPA, including serum CK, sodium, potassium, urea, non fasting glucose, and triglycerides were not significantly different between groups (Table 1). Non fasting cholesterol and HDL were increased by βGPA. Plasma creatinine concentration was significantly reduced by 49.1% after βGPA. This finding shows that inhibition of cellular creatine uptake by βGPA is effective, as
intracellular creatine is nonezymatically converted to creatinine, which diffuses out of the cells and is excreted by the kidneys.11

Myocardial ATP concentration was reduced after 4 weeks of βGPA compared to controls; 1.6 (0.1) vs. 1.9 (0.1) pmol per μg protein (p=0.05). The ATP/ADP ratio was similar in both groups: 3.33 (0.4) and 3.42 (0.2) in treated and control SHRs respectively. As expected, the ATP content of skeletal muscle was significantly reduced after βGPA; 1.05 (0.04) vs 1.35 (0.07) pmol per mg protein (p<0.001).

As expected given the blood pressure decrease, relative renin mRNA in the kidney cortex was 1.6 fold increased after βGPA compared to controls 1.7 (0.2) vs. 1.1 (0.2) (p=0.07).

Figure 3. Cumulative concentration-response curves in mesenteric arteries of spontaneously hypertensive rats.
Relaxation induced by cumulative concentrations of DNFB (A), βGPA (B), methacholine (C), and sodium nitroprusside (SNP) (D) in isolated mesenteric arteries of SHR treated with βGPA (3%) (●) and control SHR (■). Data are presented as mean values with standard error bars, N=5-8.
Creatine kinase inhibition reduces blood pressure

Figure 4. Cumulative concentration-response curves in aortas of spontaneously hypertensive rats.

Relaxation induced by cumulative concentrations of methacholine, without incubation with LNNA (A) and after incubation with LNNA (10 μM) (B), in isolated aortas of SHR treated with βGPA (3%) (■) and control SHR (●). Data are presented as mean values with standard error bars, N=7.

Table 1. Plasma biochemical parameters.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Intervention</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>βGPA</td>
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<tr>
<td>Creatine kinase (IU/L)</td>
<td>113.5 (53.4)</td>
<td>109.4 (40.2)</td>
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<td>Creatinine (μmol/L)</td>
<td>22.0 (2.7)</td>
<td>22.8 (2.8)</td>
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<tr>
<td>Urea (mmol/L)</td>
<td>6.5 (1.0)</td>
<td>6.9 (1.1)</td>
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<td>Sodium (mmol/L)</td>
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<td>143.2 (1.5)</td>
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<td>Glucose (mmol/L) ‡</td>
<td>9.8 (1.2)</td>
<td>9.4 (1.0)</td>
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<tr>
<td>Total cholesterol (mmol/L) ‡</td>
<td>1.6 (0.15)</td>
<td>1.6 (0.10)</td>
</tr>
<tr>
<td>HDL (mmol/L) ‡</td>
<td>1.3 (0.11)</td>
<td>1.3 (0.56)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L) ‡</td>
<td>0.78 (0.25)</td>
<td>0.87 (0.19)</td>
</tr>
</tbody>
</table>

Plasma biochemical parameters of treated and control spontaneously hypertensive rats before and after 4 weeks of intervention with βGPA (3%). Data are means with standard deviation in square brackets * P<0.01 vs all other groups † Intracellular creatine is nonenzymatically converted to creatinine, which diffuses into plasma. ‡ βGPA inhibits cellular creatine uptake, leading to decreased formation of creatinine and decreased plasma creatinine levels. ‡ non fasting
**DISCUSSION**

In the present study we show that βGPA, an inhibitor of the CK reaction flux, effectively reduced systolic and diastolic blood pressure of SHR by 42.7 (5.5) and 39.2 (4.1) mm Hg respectively compared to controls. To our knowledge, this is the first report on the blood pressure lowering effect of βGPA, an effective inhibitor of the flux through the CK reaction. In agreement, previous studies showed that serum CK was the main predictor of blood pressure in the population and that inhibition of intravascular CK in isolated human resistance arteries reduced vascular contractility.\(^2,6,12\) Furthermore, high CK activity is found in the heart and the aorta of the SHR, prior to the development of hypertension.\(^13-15\)

Several underlying mechanisms may be involved in the blood pressure lowering effect of βGPA in the SHR. These mechanisms all start with the inhibition of cellular creatine uptake, leading to a reduced flux through the CK reaction, with associated decreases of phosphocreatine, the main cellular ATP buffer, and ATP levels itself.\(^8,9,16\) The effective inhibition of cellular creatine uptake in this study is evident, as serum creatinine concentration was reduced after βGPA, showing reduced intracellular conversion from creatine.\(^11\)

In vascular smooth muscle, CK probably functions as an energy transducer at the contractile proteins, myosin-ATPase and myosin light chain kinase, where it rapidly provides ATP for muscle contractions.\(^1-3\) The SHR is known to display greater vascular myosin-ATPase and myosin light chain kinase activity, with greater and faster shortening of arterial muscle, greater contractile responses to agonist stimulation, and reduced responses to relaxing agents compared to normotensive controls.\(^17,18\) Thus, inhibition of vascular CK by βGPA may decrease ATP supply to the contractile proteins, leading to reduced vascular contractility. In agreement with this, the mesenteric arteries of treated SHR in this study showed reduced residual contractility after the CK-inhibitor dinitrofluorobenzene, pointing to lower CK activity in these vessels. Moreover, in vitro acute incubation of mesenteric arteries with βGPA induced vasodilation in both groups. Even a small decrease in the contractility of vascular smooth muscle could have a profound effect on arterial pressure, as expressed in Poisseuille’s formula, blood flow and resistance in vivo are markedly affected by small changes in vessel caliber.\(^19\)

As a signaling molecule for vascular vasodilatory responses, the availability of nitric oxide (NO) may be enhanced by βGPA. High CK activity is thought to be associated with reduced NO biosynthesis, and NO-dependent functions, through reducing the
Creatine kinase inhibition reduces blood pressure

bioavailability of L-arginine. Creatine and NO are both synthesized from L-arginine, but
creatine synthesis demands nearly 10 times the flux of plasma L-arginine compared to
NO-synthesis. As cellular creatine uptake is inhibited by βGPA, there may be a relative
creatine excess, leading to increased availability of arginine for nitric oxide synthesis.

We studied NO-dependent relaxation in the mesenteric arteries via cumulative addition
of methacholine on noradrenaline contraction in a high K+ depolarizing buffer. The
mesenteric arteries displayed no significant influence of βGPA treatment on NO-induced
relaxation. A possible explanation for the lack of effect of βGPA on NO-availability in the
mesenteric arteries is the small measuring window for relaxation. By using a high K+
depolarizing buffer, the responses of endothelium-derived hyperpolarizing factor are
abrogated, which include the main relaxation potential of mesenteric arteries, leaving
mainly NO-dependent relaxation. In the aorta the NO-dependent vasodilatory response
to methacholine was improved by βGPA. This response was almost completely blocked
by LNNA, showing NO-dependency and pointing to increased NO availability after βGPA.

Furthermore, renal CK is thought be involved in sodium retention, as the enzyme
provides ATP for sodium reabsorption at tubular basolateral Na+/K+-ATPases. As
hypertension in the SHR is partly salt-sensitive, inhibition of renal CK by βGPA
may decrease sodium reabsorption in the renal tubulus. Whether this contributed to the
blood pressure decrease by βGPA remains to be determined.

Finally, it is reported that in striated skeletal muscle, inhibition of CK by βGPA
induces a shift from type II to type I fiber predominance with increased oxidative
metabolism, which is already found after 3 weeks of intervention with βGPA (1%) in
the diet. Type I fiber predominance of skeletal muscle is associated with increased
capillary density and lower peripheral resistance and lower blood pressures. In
agreement with this, skeletal muscle type II fiber predominance is associated with
hypertension. Moreover, evidence indicates a slow-to-fast fiber type transition
prior to the development of hypertension in SHR compared to their normotensive
counterparts. Thus, although beyond the scope of this study, evidence suggest that
βGPA treatment may have altered skeletal muscle characteristics of the SHR leading to
lower blood pressures.

All experimental animals appeared healthy and normally active. However, some
studies reported myocardial hypertrophy after βGPA in normotensive rodents. We
cannot exclude that βGPA may induce cardiomyopathy and heart failure, although
heart weight and heart weight to body weight ratio were unchanged. Moreover, as the
SHR is known to develop hypertensive cardiomyopathy between the age of 6 and 24
months, reducing blood pressure with βGPA may even prevent this complication of hypertension. As the SHR has high CK activity, we used a relatively high dose of βGPA of 3% in the diet compared to 1 to 2% in previous studies that investigated the effect of βGPA on skeletal muscle or heart function. We found a reduced myocardial ATP content with this relatively high dose. However, meta-analysis of previous studies showed that myocardial ATP content of normotensive rodents was not significantly reduced after lower doses of βGPA. Thus, a lower dose may be more selectively inhibiting vascular and kidney CK activity, as these activities are only 9 to 13% of myocardial CK. In dose finding experiments (unpublished data), we found that βGPA 1% reduced blood pressure of SHR.

This study has several strengths and limitations. One strength is that we standardized the tail cuff blood pressure measurements, according to previous pilot studies. The animals were all handled thoroughly during two weeks prior to the experiment and we took care that there was no difference in baseline blood pressure between both groups. All blood pressure measurements were performed under identical conditions. However, we cannot exclude stress from the restrainer. This is a limitation of the tail cuff method. Second, as pilot experiments showed weight loss with βGPA during the first experimental days, all animals were housed individually in order to assess individual food intake accurately.

**Perspectives**

It is well known that current antihypertensive agents are far from satisfactory, as more than 50% of patients does not achieve blood pressure control despite treatment. Especially certain population subgroups with relatively high tissue and serum CK activity, such as black people, suffer a greater burden of hypertension, with a higher prevalence of its complications, including stroke, heart failure, and hypertensive renal disease. Moreover, hypertension in blacks remains more difficult to treat. Therefore, new classes of antihypertensive drugs that act via new mechanisms are urgently needed. The results of this study show that inhibition of the CK-system with βGPA in SHR markedly reduces blood pressure. Together with previous reports on the association of CK with blood pressure, vascular contractility, and treatment failure, these results implicate that interference in the CK-system may be a novel promising target for antihypertensive treatment.
Creatine kinase inhibition reduces blood pressure

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Supplemental data to creatine kinase inhibition with beta-guanidinopropionic acid reduces blood pressure in the spontaneously hypertensive rat

I. Oudman
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Submitted
Methods

Materials
Beta-guanidinopropionic acid was obtained from Purebulk Vitamins and Dietary Supplements (Roseburg, Oregon, USA). Before using βGPA in the experiments, purity was tested with NMR (VU, Amsterdam, Netherlands), showing a purity of > 99%. In addition, the substance was tested for the presence of cyanide compounds (Omegar Laboratory, Amsterdam), which were not present (<1.0 mg/kg). The cyano-group in cyanamide, one of the compounds used in the formation of βGPA, provides a possible source of cyanide. Methacholine (MCH), norepinephrine (NE), phenylephrine (PHE), sodium nitroprusside (SNP), amlodipine, dinitrofluorobenzene (DNFB), N^G-nitro-L-Arginine (L-NNA), and RNAlater were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Blood sampling
At start of the experimental protocol and after 4 weeks of the diet, blood was drawn from the tail vein of each rat for estimation of CK, creatinine, sodium, potassium, urea, glucose, total cholesterol, HDL, and triglycerides. Prior to the first blood draw, rats were accustomed to laboratory handling procedures. Rats were anesthetized with isoflurane (3%) and oxygen (1l/min), and placed on a warming pad. Blood was drawn from the tail vein, placed on ice immediately, and centrifuged at 4ºC within one hour, according to the blood sampling method described for minimizing CK variability in rodents.11

Anesthesia and tissue sampling
Animals were anesthetised with ketamine (90mg/kg)–dexmedetomidine (0.125 mg/kg)–atropine (0.05 mg/kg) (KMA) through intraperitoneal injection. After exposing the abdominal and thoracic region of the rat, the heart, liver, kidney, musculus quadriceps femoris, and brain were rapidly excised, immediately rinsed in cold PBS buffer, snap frozen in liquid nitrogen, and stored in -80ºC. The frozen heart and the lungs were weighted. The cortex of the right kidney was cut into pieces of 5 mm³ and the samples were incubated overnight in RNAlater at 4ºC. The next day the RNAlater was removed and the samples were stored at 80ºC.
Resistance artery preparation and tension recording

Mesenteric arteries were carefully excised and immediately placed into cold (4°C), oxygenated physiological salt solution (PSS) consisting of (in mmol/L) 118.2 NaCl, 24.8 NaHCO₃, 4.6 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2 CaCl₂, 0.26 EDTA, 50 HEPES, and 5.6 glucose. Vessels were dissected under a microscope and cleaned of adherent adipose and connective tissues. Three to four segments of 2 mm were mounted on 40-μm stainless steel wires. The segments were then transferred into organ baths of a four-channel wire myograph (Danish Myo Technology, Copenhagen, Denmark). The myograph bath contained PSS at 37°C aerated with carbogen (5% CO₂/95% O₂). Then the vessels were subjected to a normalization procedure according to Mulvany and Halpern (1977). The individual circumference was adjusted to 90% of the value that the particular vessel would have had at a transmural pressure of 100 mm Hg. Afterwards, the arteries were equilibrated for 20 minutes. The buffer was replaced every 10 minutes. Maximum contractility was induced in duplicate with norepinephrine (10 μM) in KCl (125 mM) – substituted PSS (KPSS). After washing and 20 minutes of rest, the vessels were precontracted with the α₁-adrenoreceptor agonist phenylephrine (10 μM). After a steady level of contraction force was attained, one concentration (10 μM) of the endothelium-dependent vasodilator methacholine was added to assess the endothelial integrity. After arteries had been washed and reequilibrated in PSS, cumulative concentration-response curves for methacholine (1 nM to 0.1 mM), sodium nitroprusside (1 nM to 0.1 mM), amlodipine (10 nM to 1 μM), and βGPA (25 mg to 150 mg) were performed. Finally, the irreversible CK inhibitor dinitrofluorobenzene (DNFB 1 μM to 1mM) was added. Stock solutions of reagents (except DNFB) were prepared fresh daily in distilled water and diluted serially for use in myograph baths. DNFB was prepared as a 10-1 M stock solution in DMSO and further diluted daily in DMSO upon use in the myograph bath.

Aorta preparation and tension recording

The thoracic aorta was carefully excised and immediately placed in Krebs-Henseleit buffer (in mmol/L) 118.0 NaCl, 4.6 KCl, 25.0 NaHCO3, 1.2 MgSO4, 1.8 CaCl2, 1.1 KH2PO4 and 5.6 glucose at room temperature, aerated with carbogen, pH 7.4. The aorta was cut into 2 mm segments, and mounted between two stainless steel hooks in an organ bath, containing 5 mL aerated Krebs buffer at 37°C. The segments were attached to a force transducer, and force readout was recorded via a PowerLab data acquisition system (AD Instruments, Castle Hill, Australia). The aorta segments were equilibrated for 1 hour at
an isotonic resting tension of 10 mN, which was maintained throughout the experiment. Then, the preparations were contracted twice for 10 min with a depolarizing high K\textsuperscript{+} Krebs-Henseleit solution (40 mmol/L NaCl was replaced by 40 mmol/L KCl) with intermediate washing steps of 20 min intervals. Subsequently, the vessels were pre-contracted with the α1-adrenoceptor agonist phenylephrine (1 μM). After reaching a steady level of >60% contraction compared with previous K\textsuperscript{+}-induced depolarization contraction, the endothelium-dependent vasodilator methacholine (10 μM) was added to assess the endothelial integrity. After washing, again 40 mmol/L K\textsuperscript{+} was added to the vessel segments to obtain the maximal contractile response. After the aortas had been washed and reequilibrated during 30 minutes, a cumulative concentration-response curve methacholine (1 nM to 0.1 mM) were performed. To assess nitric oxide dependent vasodilation, one segment was incubated with the nitric oxide inhibitor L-NNA (10 μM) during 30 minutes prior to the addition of methacholine (1 nM to 0.1 mM).

**Biochemical analysis**

All plasma analyses were performed on a Modular Cobas 8000 (Roche Diagnostics, Darmstadt, Germany). Plasma levels of creatine kinase, glucose, total cholesterol and triglycerides were measured by enzymatic spectrofoctometric methods; high-density lipoprotein – cholesterol colorimetric/ spectrofotometric; plasma creatinine and urea with kinetic/spectofotometric methods; and sodium and potassium were estimated with Indirect Ion-Selective Electrode methods.

For the ATP determinations of heart and quadriceps muscle, tissue was grinded in liquid nitrogen, the powder transferred to 200 μl of 0.4 M perchloric acid (HClO\textsubscript{4}), centrifuged at 10,000 x g at 4°C, and neutralized with 5 M potassium bicarbonate (K\textsubscript{2}CO\textsubscript{3}). The supernatant was removed from the pelleted cell fragments and immediately placed on ice for 10 minutes. After centrifugation at 10,000 x g at 4°C, the supernatant was assayed by HPLC. The pelleted cell fragments were stored with 0.2 M sodium hydroxide (NaOH) and used for protein determination by the BCA assay (Pierce).

For estimation of transcriptional activity for renin mRNA of the kidney cortex, total RNA was isolated from frozen-thawed rat kidney cortex, using Trizol reagent (GibcoBRL), and was quantified using micro-spectrophotometry (NanoDrop Technologies, Wilmington, USA). Thereafter, cDNA was synthesized from extracted RNA using QuantiTect Reverse Transcription Kit (USA, Qiagen), in which genomic DNA is removed by genomic DNA wipeout buffer before RT reaction. Quantitative real-time PCRs were conducted in a total volume of 20 μL and 10 ng of cDNA was used for each real-time
PCR reaction using a Step-One cycler Applied Biosystems (UK, Applied Biosystems), and the SYBR ® Green PCR Master Mix (UK, Applied Biosystems, catalogue no: 4385610) as per manufacturer's recommendations. The Intron-spanning oligonucleotide primers for qPCR were designed with NCBI (Primer-BLAST). The following cycling conditions were used: [95°C for 10 min, (95°C for 15 s, 60°C for 60 s) × 40 cycles]. β-actin, B2M and HPRT-1 DNA quantitation was performed in parallel on all samples in order to determine the actual input amount of cDNA and were used as endogenous references to normalize variations in DNA recovery and amplification efficiency.

A 2-fold dilution series was created from a random pool of cDNA from our sample groups. The PCR efficiency and correlation coefficients ($R^2$) of each primer pair were generated using the slopes of the standard curves. The efficiencies were calculated by the formula: efficiency (%) = ($10^{(-1/slope)} - 1$) * 100. For a correct interpretation of the real-time PCR results, all data has been normalized which is achieved by calculating the geometric mean of the three stable reference genes.
Chapter 9B

Acute effect of beta-guanidinopropionic acid and creatine: study protocol

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ABSTRACT

Background Beta-guanidinopropionic acid (βGPA), a creatine analogue, can be obtained without prescription on the internet and used by sportsmen to increase muscle mass and endurance capacity. However, to our knowledge, there are no published data on the effects and side effects of this substance. In contrast, creatine is used to improve short-duration/high-intensity exercise. Although many studies assessed the effect of creatine on muscle performance, none of those studies reported the effect on hemodynamic parameters.

Objective In this study, we will assess 1) the tolerability of acute oral administration of βGPA 100 mg versus placebo in healthy male volunteers and 2) if tolerated, βGPA 100 mg during 1 week. In phase 2 we will compare βGPA with placebo and creatine 5 g.

Methods Study design: Randomized, double-blind, placebo-controlled, study.
Study population: Healthy male volunteers, 18-50 years old. 1) 16 subjects; 2) 24 subjects.
Intervention: 1) Oral administration of βGPA 100 mg or placebo. 2) One week of daily oral administration of βGPA 100 mg, creatine 5 g, or placebo.

Main study parameters/endpoints Tolerability for βGPA assessed with a questionnaire; hemodynamic parameters, including blood pressure, heart rate, cardiac output, and total peripheral resistance; biochemical parameters in serum, including creatine kinase, βGPA, creatine, glucose, insulin, sodium, potassium, creatinine, and urine, including βGPA, creatine, creatinine, urea, sodium, potassium, after acute oral administration of βGPA and one week of intervention with βGPA or creatine.
**BACKGROUND**

Beta-guanidinopropionic acid (βGPA or N-(aminoiminomethyl)-beta-alanine; C₄H₉N₃O₂) can be obtained without prescription on the internet for human use. This amino acid is a naturally occurring creatine analogue, a structural isomer to creatine (C₄H₉N₃O₂), which has an identical molecular formula (Figure 1).¹ It is used by sportsmen to induce greater endurance capacity and promote weight loss.

The amino acid βGPA can be generated in vivo via transamidation of β-alanine.²,³ The physiological concentration (without external supplementation) in human plasma is reported to range from 0.02 to 1.40 µmol/liter.⁴⁻⁶ Clearance is probably renal, as the structural isomer creatine is cleared renally as well and increasing plasma concentrations were reported in chronic renal failure, ranging from 0.18 to 180 µmol/liter.⁴,⁷,⁸

![Figure 1. Molecular structures of βGPA and creatine.](image)

BGPA acts as a competitive inhibitor of cellular creatine uptake, attenuating the flux through the creatine kinase (CK) reaction.¹⁻⁹ CK catalyzes the rapid and reversible transfer of a phosphate group from creatine phosphate to ADP, thereby forming creatine and ATP.¹⁰ The flux through the CK reaction is linearly related to the concentration of creatine.¹¹ βGPA is also phosphorylated by CK in cytoplasm, but both βGPA and phosphorylated βGPA are “inefficient substrates” for the CK reaction: in vitro Vmax values are <1% of the Vmax values of creatine and phosphocreatine.¹²,¹³ Therefore, βGPA may modulate the energy status of tissues, from fast activity bursts to slow endurance performance (Table 1).
Table 1. Characteristics of skeletal muscle fiber types.

<table>
<thead>
<tr>
<th>Type II</th>
<th>Type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>High CK</td>
<td>Low CK</td>
</tr>
<tr>
<td>Predominantly glycolytic</td>
<td>Predominantly oxidative</td>
</tr>
<tr>
<td>Mitochondria poor</td>
<td>Mitochondria rich</td>
</tr>
<tr>
<td>Capillary rarefaction</td>
<td>High density of capillaries</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Burst exercise</td>
<td>Endurance capacity</td>
</tr>
<tr>
<td>Low GLUT-4 expression</td>
<td>Higher GLUT-4 expression</td>
</tr>
<tr>
<td>Insulin Resistant</td>
<td>Insulin sensitive</td>
</tr>
<tr>
<td>Less glucose uptake</td>
<td>High glucose uptake</td>
</tr>
<tr>
<td>Glucose and fatty acid stored as lipid</td>
<td>Glucose and fatty acid utilisation</td>
</tr>
<tr>
<td>Obesity prone</td>
<td>Lean</td>
</tr>
<tr>
<td>Hypertension prone</td>
<td>Normotension</td>
</tr>
</tbody>
</table>

CK, creatine kinase; GLUT-4, insulin-dependent glucose transporter protein 4. BGPA has been shown to induce a shift from type II to type I fiber predominance, comparable with the effects of endurance exercise.

Animal studies, in which rats or mice received βGPA (1 to 2%) in the diet, showed skeletal muscle fiber type and metabolic enzyme transitions qualitatively similar to the adaptations of endurance training. These alterations included a transition from type II to type I muscle fiber type predominance, with a concomittant increase in markers of oxidative metabolism, improved glucose tolerance, increased plasma membrane fatty acid transporter expression, and a commensurate decrease in glycolytic potential. Furthermore, it was anecdotally reported in animal studies that chronic intervention with βGPA (1%) in the diet resulted in hemodynamic alterations comparable with the effect of endurance training. Importantly, in these rodent studies, the animals appeared healthy after administration of very high doses of βGPA of more than 1% daily in the diet for more than 8 weeks (i.e. 200 milligrams βGPA in 20 grams food daily to an animal weighting 200 to 300 grams, or 600 to 1000 mg/kg animal weight), and the effect was reversible within 1 month after withdrawal. However, despite its human use, there is a lack of scientific data on the effects of βGPA on hemodynamic and biochemical parameters in humans. To our knowledge there are no US FDA reports on any side effect (www.fda.gov).
Creatine, the structural isomer of βGPA, is one of the most popular dietary supplements in the world. In contrast to βGPA, it is used to improve sport performance during short-duration/intensity exercise. Numerous studies have assessed the effect of creatine on muscle performance in healthy athletes and on health improvement a variety of clinical conditions. However, there is lack of data on the effect of creatine on hemodynamic parameters. Therefore, we will assess the effects of βGPA and creatine on biochemical and hemodynamic parameters.

**OBJECTIVES**

**Phase 1: βGPA in healthy male volunteers versus placebo**

The primary objective will be to assess the tolerability of oral administration of a single dose of βGPA. Other objectives include the assessment of the acute effect of oral administration of a single dose of βGPA on hemodynamic parameters, including blood pressure, heart rate, cardiac output, and total peripheral resistance, and to assess the acute effect of βGPA on biochemical parameters.

**Phase 2: βGPA in healthy male volunteers (if Phase 1 is uneventful) versus placebo and creatine**

The primary objective will be the assessment of tolerability of one week of intervention with βGPA. Other objectives will be to assess the effect of one week of oral administration of βGPA and creatine on hemodynamic parameters, including blood pressure, heart rate, cardiac output, and total peripheral resistance, and biochemical parameters.

**METHODS**

We will include 40 male volunteers (16 in phase 1, 24 in phase 2). Exclusion criteria include glucose, lipid spectrum, thyroid, kidney, or liver abnormalities, history of cardiovascular disease including TIA and stroke; CK-increasing drugs including statins; neuromuscular or endocrine disorders; vasculitis; HIV infection; infectious hepatitis; and bleeding disorders.

In phase 1 the volunteers will be randomly assigned by a computer-generated random number system to receive a single dose of βGPA (100 mg) or an identical looking placebo. The participants and the investigator will be blinded. Treatment allocation will be coordinated by an independent investigator. We will proceed to phase
2 if 100 mg is confirmed to be the NOAEL DOSE (no more adverse effects than placebo; safety measures we took to calculate this dose included calculation of HED using a close allometric relationship, and application of the tenfold safety factor). If 100 mg is not NOAEL we will assess, depending on the adverse effects observed (no overt or surrogate toxicity present), a single dose of 50, 10, or 1 mg/day, in that order in Phase 1. If 1 mg is not NOAEL, we will terminate the study for further ex vivo tests. If the NOAEL dose is established in phase 1, we proceed to phase 2. In phase 2 the volunteers will be randomly assigned by a computer-generated random number system to receive βGPA in the NOAEL DOSE, creatine 5 gram, or placebo during 1 week. As the capsules of βGPA and creatine can not be made identical, we will use the double-dummy design. All subjects take two sets of treatment: βGPA 100 mg or the equivalent placebo and creatine 5 g or the equivalent placebo. The participants and the investigator will be blinded.

**Test products: βGPA**

In accord with the definition for food supplements in the legislation of the European Union, we consider βGPA as well as creatine food supplements, as both substances are naturally occurring amino acids with a physiological effect (please see background). βGPA is a white, crystalline tasteless powder, soluble in water. βGPA powder is ordered at SeqChem (Sequoia Research Products). There are no reports or bans on this product or the company to our knowledge, presented on the FDA website using the FDA search engine, or online with search engine Google, as for February the 20th 2012. βGPA, creatine, and identical placebo capsules will be manufactured by the Pharmacy & Pharmacology department of the Slotervaart Hospital, Amsterdam. This department is GMP certificated (ISO 9001:2001). βGPA is sold in the U.S. and not in European countries. According to the legal guidelines of the European Union, criteria of international organs, generally accepted criteria, or national criteria are approved when a supplement is not listed in the legislation of the European Union. According to the U.S. FDA guidelines: We first qualified the supplier by establishing the reliability of the supplier, with the methods as mentioned above. Next, the substance was tested for purity, and for cyanide compounds. Cyanide was not expected to be present. However, the cyano-group in cyanamide, one of the compounds used in the formation of βGPA, provides a possible source of cyanide. We established in our tests in Amsterdam a purity of more than 99% (detection limit) and a cyanide level lower than 1 p.p.m (detection limit) (Supplement 1 and 2). Cyanide occurs in many food items, with high concentrations in cassava roots, almonds, and apricot kernels ("marsepein")
containing up to 1000-3000 mg/kg (1 part per thousand) (www.vwa.nl). The maximum allowed level of cyanide in food items is found in Annex II of Guideline 88/388/EEC (http://ec.europa.eu/food/fs/sfp/addit_flavor/flav09_en.pdf) and is 1 mg/kg in food or drinks, with the exception of the Dutch treats “noga” and “marsepein” or similar products, where 50 mg/kg is allowed (EEC, 1988; www.vwa.nl and http://ec.europa.eu/food/fs/sfp/addit_flavor/flav09_en.pdf). Because of concerns with these high levels, the Dutch Food Consumer Product Safety Authority has thereafter established a maximum daily cyanide intake of 0.05 mg/kg/day (www.vwa.nl; http://www.vwa.nl/actueel/nieuws/nieuwsbericht/10782), this is a total intake of 3.75 mg/day in a 75 kg man. With 100 mg GPA with <1 p.p.m. cyanide, the contribution to the daily intake in a 75 kg man will be <0.0001 mg/day or <0.00001 mg/kg/day.

**Dose calculation**

We used the FDA guidance on Estimating the Maximum Safe Starting Dose in Initial Clinical Trials in Adult Healthy Volunteers. This guidance outlines a process for deriving the maximum recommended starting dose (MRSD) for first-in-human clinical trials in adult healthy volunteers, and recommends a standardized process by which the MRSD can be selected. The purpose of this process is to ensure the safety of the human volunteers.\(^\text{28,32}\)

**NOAEL determination**

In animal studies βGPA was administered through the diet in concentrations of 1% or more during 8 weeks without apparent adverse effects.\(^\text{13-16}\) In animals weighting 200 grams, eating estimated 20 grams per day, we calculated a “no observed adverse effect level” of 1000 mg/kg/day. Furthermore, in a patent application, Meglasson et al. recommended a human dose of 1 to 500 mg/kg/day based on his research in mice and rhesus monkeys.\(^\text{33}\) In this paper rhesus monkeys weighting 9 kg were treated with oral βGPA 48 mg/kg/day (432 mg per monkey per day) during 2 weeks without apparent adverse events.

**Conversion of the NOAEL to HED**

We converted the oral NOAELs in rats and monkeys (resp. 1000 mg/kg/day and 48 mg/kg/day) to human equivalent oral doses (HED) based on an algorithm proposed by the FDA based on body surface area.\(^\text{32}\) This algorithm proposes a conversion factor from rat to human of 0.16 times the rat dose; and of monkey to men of 0.32 the monkey
dose (in mg/kg/day; for a man of 60 kg) resulting in HEDs of resp. 160 mg/kg/day and 15 mg/kg/day for a man of 60 kg.

**Safety factor**

A safety factor should be applied to the HED to increase assurance that the first dose in humans will not cause adverse effects. The use of the safety factor is based on the possibility that humans may be more sensitive to the toxic effects of a substance than predicted by the animal models, that bioavailability may vary across species, and that the models tested do not evaluate all possible human toxicities, or cannot be expressed by animals or easily measured, such as headache or nausea. We conservatively chose 15 mg/kg/day oral dose for our final calculations of the human dose, because this is the lowest dose, and because of the closer allometric relationship between monkey and man.\(^{32}\) FDA advises a safety factor of at least 10. Based on an average weight of a male volunteer of 75 kg, we calculated a starting oral dose for the phase 1 study of 75*1.5 mg/day=112.5 mg/day. We will start with 100 mg/day.

**Creatine**

For creatine, we will use an oral dose 5 g per day. For healthy omnivoric males the average daily rate of creatine synthesis is estimated to be 1.3 g.\(^{34}\) Most studies on creatine supplementation, in order to increase sport performance, use a loading dose of 20 gram creatine during 5 days to increase muscle creatine content by 20%. Thereafter, a maintenance dose of 3-5 grams is used. With this dose no side effects are reported. Thus, we will use a dose of 5 gram, as it would be a sufficient dose to increase the daily rate of creatine synthesis, and this dose is frequently used by sportsmen.\(^{35,36,37}\)

**STUDY MEASURES**

**Phase 1**

The participants will be asked to come to the hospital for visit 2 after an overnight fast. After baseline measurements they will receive 1 capsule of βGPA 100 mg, or placebo. Furthermore, the participants will receive a questionnaire to assess health status at 1h, 2h, 6h, 12h, 24h and after 1 week after the ingestion of βGPA. We have no data on the speed of the resorption of βGPA (C4 H9 N3 O2). However, the acute effects of the ingestion of the structural isomer creatine (C4 H9 N3 O2) on plasma creatine concentration have been reported.\(^{38}\) We consider the creatine data useful for our estimation. In the report
by Schedel et al, it was shown that the peak plasma level after the oral ingestion of 20 g creatine occurred after 2.5 hours. Six hours after the ingestion of 20 grams of creatine, plasma concentration was 50% of the maximum concentration. Thus, we will assess hemodynamic and blood and urine laboratory parameters during the first 8 hours after ingestion of βGPA, after 12, and after 24 hours. A timeline is shown in Figure 2.

**Systemic cardiovascular hemodynamics and laboratory studies**

(At baseline and at t=30, t=60, t=90, t=120, t=180, t=240, t=360, t=480 minutes, t=12h, t = 24h). Supine and sitting resting blood pressure; heart rate, cardiac output and total peripheral resistance will be measured, using a Bmeye Nexfin blood-pressure monitor for continuous non-invasive finger arterial blood pressure measurement, with an adjusted cuff size on the non-dominant arm, at heart level.

Laboratory studies will include serum βGPA, resting serum CK (after 3 days without heavy exercise), glucose, insulin, lipid profile, creatine, creatinine, liver enzymes (ASAT, ALAT, gamma GT), TSH (subclinical hypothyroidism as a cause of high CK), sodium, potassium, timed urine collection (βGPA, creatine, creatinine, urea, sodium, potassium).

![Figure 2. Time line during 24 hours after the ingestion of a single dose βGPA or placebo.](image)

Study time line in phase 1. After the baseline measurements the participant is asked to ingest a capsule with βGPA 100 mg or placebo. Hemodynamic and biochemical parameters are recorded during 24 hours after the ingestion.
**Phase 2**

The participant will come to the hospital for assessment of medical history, physical examination including weight and height, blood pressure, an electrocardiogram, and parameters of systemic cardiovascular hemodynamics, and fasting laboratory blood and urine studies. The participants will be asked to come to the hospital for visit 2 after an overnight fast. After baseline measurements they will receive the first capsule with βGPA 100 mg, creatine 5 g, or placebo in the hospital. They will receive capsules for 2 days and they will be instructed to consume the capsule on each day within 5 minutes before breakfast on a fixed time. On day 4 and day 7 the participant will be asked to come to the hospital after an overnight fast. At those visits the participants will ingest the capsules in the hospital. The participants will receive a questionnaire to assess tolerability at home during the week of intervention and 2 weeks after the intervention. At the hospital visits on days 4 and 7 of the intervention period the investigator will assess tolerability with a questionnaire. On days 4 and 7 of the intervention period, we will assess hemodynamic and laboratory parameters. A timeline is shown in Figure 3.

![Figure 3. Time line during phase 2.](image)

Study time line for 1 participant. After the baseline measurements the participants will be randomized between βGPA, placebo, creatine 5 g during one week with a follow-up period of two weeks.
Systemic cardiovascular hemodynamics and laboratory studies
At baseline and at day 4 and 7 of the intervention supine and sitting resting blood pressure; heart rate, cardiac output and total peripheral resistance will be measured, using a Bmeye Nexfin blood-pressure monitor for continuous non-invasive finger arterial blood pressure measurement, with an adjusted cuff size on the non-dominant arm, at heart level, ambulatory 24-hour blood pressure monitoring. Labatory studies will include serum βGPA, resting serum CK (after 3 days without heavy exercise), glucose, insulin, lipid profile, creatine, creatinine, liver enzymes (ASAT, ALAT, gamma GT), TSH (subclinical hypothyroidism as a cause of high CK), sodium, potassium, urine collection (creatinine, creatinine, urea, sodium, potassium) at baseline; and serum βGPA, serum CK, glucose, insulin, creatine, creatinine, liver enzymes (ASAT, ALAT, gamma GT), sodium, potassium, timed urine collection (βGPA, creatine, creatinine, urea, sodium, potassium) at day 4 and 7 of the intervention.

Data analysis and statistics
The main study parameter is the tolerability of βGPA after oral administration in healthy male volunteers versus placebo. Other parameters are hemodynamic parameters (Resting blood pressure; heart rate, cardiac output and total peripheral resistance, measured non-invasively) after oral administration of βGPA in healthy male volunteers versus placebo and creatine. Biochemical parameters, including resting serum CK, βGPA, creatine, creatinine, glucose, insulin, liver enzymes (ASAT, ALAT, gamma GT), sodium, potassium, timed urine collection during 24 hours (standard urine assessment, sodium, potassium, βGPA, creatine, creatinine, urea).

This is a first-in-man study with βGPA, with allometric data available from other species. According to the EMEA guidelines we will include 8 subjects in each arm to assess tolerability of a single dose of βGPA versus placebo and we will include 8 subjects to assess tolerability of βGPA versus placebo during one week.28
REFERENCES


Chapter 10

Summary and discussion
SUMMARY

An introduction for this thesis is provided in Chapter 1. The creatine kinase system is thought to play a key role in the intracellular energy homeostasis, by coupling of cellular ATP-producing with ATP-consuming processes. High activity of the enzyme was previously linked to hypertension. In this chapter we first discuss the pathways via which generalized high CK activity, with effects on skeletal muscle, heart, blood vessels, and the kidney may increase hypertension risk. In addition, we hypothesize that high activity of the enzyme may be involved in the pathogenesis of other clinical conditions that frequently coexist with hypertension.

PART I: CLINICAL CONDITIONS

The studies in the first part of this thesis mainly focus on associations between CK, blood pressure, and other clinical conditions that increase hypertension risk.

In Chapter 2 we assessed whether subjects with high serum CK activity display reduced urinary sodium excretion, as a reduced ability to excrete sodium may lead to higher blood pressures. In the kidney, sodium reabsorption is primarily driven by basolateral Na⁺/K⁺-ATPase, which exchanges intracellular sodium for potassium. The ATP for this highly energy demanding process is provided by colocalized CK. Therefore, high serum CK activity, as an indirect measure of renal CK, may be associated with reduced sodium excretion after a salt load, via increased availability of ATP for sodium reabsorption. In healthy male participants, younger than 50 years, with normotension or untreated hypertension, on 7 days of low sodium (<50 mmol/d) followed by 3 days of high sodium (>200 mmol/d), we examined differences in 24-h urine sodium excretion after a high salt diet between participants with low and high CK activity. We showed that serum CK was negatively correlated with 24-h urinary sodium excretion after a high salt diet; a correlation coefficient of -0.41 (95% CI, -0.62 to -0.16). Sodium excretion was 416.6(23.6) mmol/day in the lowest compared to 257.9(31.3) mmol/day in the highest CK tertile (p<0.001). Under the assumption that serum CK reflects tissue CK, the reduced sodium excretion with high serum CK activity may imply that CK has a contribution to sodium reabsorption in the renal tubules. Further studies should focus on the role of CK in the kidney in renal sodium handling.

The association of CK with blood pressure was also proposed to be the result of high CK activity in resistance arteries, where it regenerates ATP for vascular contractility.
Chapter 3 describes the results of a study on the possible association between human microvascular CK and blood pressure. Resistance-sized arteries from omental fat donated by consecutive women during uterine fibroid surgery were isolated. Microvascular CK isoenzyme mRNA was assessed using quantitative real-time PCR. The study shows that normalized CKB copy numbers, ranging between 5.18 and 24.43, were strongly correlated with blood pressure, with correlation coefficients for systolic and diastolic blood pressure of 0.64 (95% CI, 0.14 to 0.88) and 0.88 (0.64 to 0.96) respectively. This is the first evidence for an association between human microvascular CK gene expression and blood pressure that adds to the existing evidence on the potential role of CK in the enhancement of vascular contractility and pressor responses.

Hypertension occurs more frequently in obese people and high tissue and serum CK activity are more common in this group. We proposed that high CK activity in skeletal muscle could be involved in the pathogenesis of hypertension as well as obesity. In skeletal muscle highest CK activity is found in fast type II fibers. These fibers are particularly fit for short-term high-intensity exercise fuelled by CK and anaerobic glycolysis, with a low capacity for uptake and oxidation of fatty acids and glucose. Consequently, high skeletal muscle CK activity, as with a predominance of type II fibers, may promote storage of fatty acids and glucose as lipid in adipose tissue rather than uptake and oxidation in skeletal muscle, leading to obesity. Therefore, as a first assessment of a possible link between CK and obesity, we studied in Chapter 4 whether serum CK activity is associated with body mass index (BMI) in the general population. We analyzed a cross-sectional multi-ethnic population sample of 1444 subjects, aged 35 to 60. In multivariable linear regression analysis we showed that log serum CK is the main predictor of BMI, with an increase in BMI of 3.1 kg/m² (95% CI, 1.8 to 4.3 kg/m²) per log CK increase after adjustment for age, sex, ethnicity, educational level, and serum creatinine as a measure of muscle mass. Under the assumption that serum CK activity reflects tissue CK activity, the findings of this study may suggest that high CK activity in skeletal muscle increases obesity risk. As it is known that high CK activity in skeletal muscle is associated with capillary rarefaction, leading to increased peripheral resistance and higher blood pressures, the high CK phenotype might be hypertension and obesity prone. Further studies are needed to assess whether high CK in obesity is an epiphenomenon, or part of a causal pathway leading to obesity.

Several previous reports suggest that hypertension and obesity share a common association with uterine fibroids, the most common pelvic benign neoplasm. Although women are at greater risk of premature cardiovascular death than men, with
hypertension as a main risk factor, risk factors for hypertension in women are relatively understudied. Therefore, Chapter 5 describes the results of a retrospective cohort study on the prevalence of hypertension in women admitted for surgery for fibroids. We included 241 women with uterine fibroids (126 black), 308 women who underwent surgery for other gynaecological reasons (37 black), and 606 population controls (360 black), with a mean age of 43.4 (SD6.6), 41.3 (11.2), and 45.0 (6.6) y respectively, and a mean BMI of 27.4 (5.3), 25.5 (5.4), and 28.0 (5.6) kg/m$^2$. High blood pressure was found in 43.6, 28.6, and 24.3% of the women with fibroids, other surgery, and population controls respectively ($p<0.001$ for comparisons between women with fibroids and controls). Women with fibroids were more likely to have high blood pressure after adjustment for age, BMI, and ethnicity with an odds ratio of 2.7 (95% CI, 1.9 to 3.9). The most common hypothesis forwarded for the association between uterine fibroids and hypertension is that they share a common growth pathophysiology of smooth vascular and smooth uterine muscle. As CK is known to provide ATP for smooth muscle growth, high CK activity may stimulate growth responses of uterine and vascular smooth muscle, leading to a predisposition for hypertension and uterine fibroids. Furthermore, the high CK phenotype could explain the common association of fibroids and hypertension with obesity. Further research is needed to establish the role of CK as a common growth promoting mediator in the pathways leading to hypertension and uterine fibroids.

PART II: THERAPEUTIC IMPLICATIONS

In this part we focused on the CK-system as a possible therapeutic target in hypertension and cardiovascular disease and the effect of CK inhibition on tissues with high energy demands.

If high CK activity promotes sodium retention and vascular contractility, hypertension may be more difficult to treat in subjects with high CK. Therefore, we investigated in Chapter 6 whether serum CK activity after rest is associated with failure of hypertension treatment in the general population. We analyzed a multi-ethnic sample of the general population (N=1444), aged 34-60y. Hypertension prevalence was respectively 26.8, 30.8, and 41.2% for the lowest (<88 IU/L) through the highest population CK tertile (>145 IU/L); ($p<0.001$). Treatment failed in 72.9% of subjects within the highest CK tertile vs 46.7% with low CK ($p=0.004$). In logistic regression analysis, CK was the main predictor of treatment failure (adjusted OR 3.7; 95% CI, 1.2 to 10.9), independent of age, sex, BMI, fasting glucose, ethnicity, and education level.
Further study should focus on prospective analysis of this association, in order to assess causal inferences and whether CK could serve as a biomarker for difficult to treat hypertension.

In **Chapter 7** we systematically reviewed the evidence regarding the effectiveness of interventions directly targeting the CK system as compared to placebo in adult patients with essential hypertension or cardiovascular disease. We searched MEDLINE, EMBASE, LILACS, and the Cochrane Controlled Trials Register without language restriction and included only randomized controlled trials comparing creatine or creatine analogues with placebo in patients with essential hypertension, heart failure, or myocardial infarction. The outcomes assessed were death, total myocardial infarction, hospitalizations for congestive heart failure, change in ejection fraction, and changes in diastolic or systolic blood pressure. Full reports or abstracts from 1164 papers yielded 11 trials in 1474 patients with heart failure, acute myocardial infarction, or ischemic heart disease. The drugs used were either creatine, phosphocreatine, or phosphocreatinine. In patients with heart failure no study showed a clear effect on ejection fraction. In patients with acute myocardial infarction, two out of four trials reported mortality outcomes, with no significant effect of creatine or creatine analogues (RR 0.73, 95% CI, 0.22 to 2.45). The main effect of the interventions seems to be on improvement of dysrhythmia, although there is some evidence that dyspnoea might improve in patients with heart failure. In conclusion, it is not clear whether intervention in the CK-system has an effect on mortality, progression of myocardial infarction, ejection fraction, or blood pressure in patients with hypertension and cardiovascular disease.

In **Chapter 8**, we performed a systematic review on the effect of the CK inhibitor beta-guanidinopropionic acid (βGPA) on function and morphology of tissues with high energy demands. BGPA attenuates the flux through the CK reaction by competitive inhibition of cellular creatine uptake. This amino acid is marked as safe for human use, but the effects and side effects are not clear. We searched the electronic databases Pubmed, EMBASE, the Cochrane Library, and LILACS from their inception through March 2011. We retrieved 131 publications, mainly considering the effect of chronic oral administration of βGPA (0.5 to 3.5%) on skeletal muscle, the cardiovascular system, and brain tissue in animals. BGPA decreased intracellular creatine and phosphocreatine in all tissues studied. In skeletal muscle, this effect induced a shift from glycolytic to oxidative metabolism, increased cellular glucose uptake, and increased fatigue tolerance. In heart tissue this shift to mitochondrial metabolism was less pronounced. Myocardial contractility was modestly reduced, including a decreased ventricular
developed pressure, albeit with unchanged cardiac output. In brain tissue adaptations in energy metabolism resulted in enhanced ATP stability and survival during hypoxia. In conclusion, chronic βGPA increases fatigue tolerance of skeletal muscle and survival during ischaemia in animal studies, with modestly reduced myocardial contractility. The findings of this review show that CK is important in cellular energy metabolism but not indispensible.

As high CK activity has been linked to higher blood pressures, inhibition of the CK-system may have a blood pressure lowering effect. Therefore, we investigated in Chapter 9A whether treatment with βGPA reduces blood pressure in the spontaneously hypertensive rat, an animal model for essential hypertension, with known high CK activity in heart and blood vessels prior to the development of hypertension. Male, 16-weeks-old spontaneously hypertensive rats (N=16) were assigned to a standard diet with or without βGPA. Blood pressure was measured weekly by the non-invasive tail-cuff method. After 4 weeks the effect on vasodilatory responses of mesenteric arteries was assessed in a wire myograph. Treatment with βGPA significantly reduced systolic and diastolic blood pressure compared to controls, by 42.7 (5.5) (p<0.001) and 35.3 (4.8) mm Hg (p=0.004). The vasodilatory response to the CK-inhibitor dinitrofluorobenzene was enhanced by 82.2% after treatment with βGPA (p=0.008). Moreover, incubation of isolated rat mesenteric arteries with 150 mg βGPA induced a 25.7(4.4)% vasodilation, demonstrating a decreased vascular contraction potential by CK inhibition. To our knowledge, we are the first to show that CK inhibition with βGPA reduces blood pressure. The results suggest that inhibition of the CK-system may be a promising new target for antihypertensive treatment. Therefore, we developed a protocol for the first-in-man study in healthy men with βGPA compared to its analogue creatine and placebo in Chapter 9B. The main outcomes will be tolerability, and changes in hemodynamic and biochemical parameters, including blood pressure and serum CK activity. A dose of 100 mg was calculated from animal studies as the human equivalent dose. This study is expected to show that there are no serious side effects with this low dose of βGPA.

DISCUSSION

Hypertension affects nearly 1 billion people worldwide and is one the most powerful contributors to cardiovascular morbidity and mortality. The prevalence of hypertension and its associated morbidity is higher in black people of African descent, but, despite extensive research, it is not fully explained why those ethnic differences exist. Thirteen
years ago it was proposed that higher CK activity in cardiovascular muscle and other tissues with high energy demands in black people could be a genetic factor contributing to the excess burden of hypertension in this group.\(^3\) Subsequently, a considerable amount of evidence substantiating this hypothesis was gathered. The general aim of this thesis was to build further evidence for an association between CK and pressor responses. Furthermore, our aim was to assess whether high tissue CK activity contributes to the greater occurrence of other conditions that frequently coexist with hypertension in people of African descent, such as obesity and conditions with smooth muscle proliferation involved.

**Clinical conditions**

As an elevated arterial blood pressure is achieved either by constriction of arterioles causing diminished volume capacity or through sodium retention causing fluid overload, we have further explored the role of CK in these two pathways. First, we have shown that subjects with high CK activity significantly excrete less sodium after a high salt diet. Under the assumption that serum CK reflects tissue CK, this finding may imply that high CK activity promotes sodium reabsorption in the renal tubules. Second, in line with the potential role of CK in the enhancement of vascular contractility, it was shown that transcriptional activity of the enzyme in isolated human resistance arteries strongly correlates with blood pressure. These findings may suggest that a genetically determined high CK phenotype, including high CK in the kidney and vasculature, contributes to the greater susceptibility of black people for renal sodium retention and attenuated vasodilatory responses.\(^4,5\) However, several issues need further exploration. First, the association of CK with renal sodium handling should be studied in more detail. Although serum CK is known to reflect tissue CK,\(^6\) it is not clear whether this is an accurate reflection of CK activity in the kidney. Direct assessment of CKB isoenzyme levels in the kidney in relation to sodium handling should provide a better insight into this relation. Second, regarding vascular CK, it is not completely clear whether upregulation of CK at the transcriptional level is associated with increased protein expression and whether this upregulation is a consequence of the hypertensive state. However, taken all the evidence together, including the strong association of CK mRNA with blood pressure, increased vascular contractility with high CK in resistance arteries as previously reported,\(^7\) high CK activity in heart and aorta prior to the development of hypertension in rodents,\(^8,9\) and the reduced ability to excrete sodium, a genetically programmed high CK phenotype is likely to have a contribution to vascular contractility
and sodium retention leading to higher blood pressures. Subsequently, the hypertensive state may lead to upregulation of CK transcriptional activity to meet the increased cardiovascular energy demand, which is substantiated by rodent studies showing increased transcriptional activity and protein expression of the enzyme in conditions of ventricular pressure overload. Clearly, further study is needed to unravel the relation between CK mRNA, CK protein expression, and hypertension.

As a first indication of a possible link between CK and obesity, we showed that serum CK activity is associated with body mass index, which is in line with the thrifty nature of skeletal muscle containing high CK activity. Furthermore, we reported that hypertension occurs more frequently in women with uterine fibroids and proposed that CK could be a common growth prone mediator in the proliferation of uterine and vascular smooth muscle. These findings suggest that a genetically determined high CK phenotype, including high CK activity in skeletal muscle, heart, the kidney, and smooth muscle of the vasculature and other organs, may provide a link between hypertension and other conditions that frequently coexist. However, it is clear that the association between CK and obesity needs further exploration. Furthermore, assessment of CK in uterine and fibroid tissue in relation to blood pressure may provide further insights in the involvement of CK in the pathophysiology of fibroids and hypertension.

Implications for therapy

Successful treatment of hypertension is difficult despite the availability of several classes of antihypertensive drugs. Treatment fails in nearly half of hypertensive patients, including many patients with uncomplicated hypertension. We have reported that serum CK was the main and independent predictor of antihypertensive treatment failure in a cross-sectional population study. Obviously, prospective analysis is needed before causal inferences can be made. However, as current antihypertensive agents are far from satisfactory, especially in population subgroups with high CK activity, and CK is known to provide ATP to subcellular ATPases affecting blood pressure as the final intracellular step before pressor responses occur, drugs targeting the CK system may be effective for the treatment of hypertension. We have described the promising results of our study in hypertensive animals. Inhibition of the CK-system with βGPA significantly reduced systolic and diastolic blood pressure compared to controls, as well as CK-dependent microvascular contractility. Importantly, we have reported in our systematic review that chronic βGPA treatment had minor side effects in animals, showing that CK is important in cellular energy metabolism but not indispensable. Therefore, inhibition the CK-system may be a novel target for antihypertensive treatment. However, before
Summary and discussion

this can be implemented in clinical practice, several issues need to be addressed. First, the effect of a longer duration of βGPA treatment as well as different doses in hypertensive animals is not yet studied. Further rodent studies should focus on these aspects of βGPA treatment. Second, research should be extrapolated to humans. Our first-in-man study with βGPA should make clear whether βGPA is tolerated in healthy men and what the effects are on biochemical and hemodynamic parameters. If βGPA is well tolerated, as expected, the next step may then be the assessment of the blood pressure lowering efficacy of βGPA in hypertensive patients.

**Figure 2. Clinical implications of the high creatine kinase phenotype.**

This figure depicts the proposed mechanism through which the high creatine kinase (CK) phenotype with high activities in skeletal muscle, heart, kidney, and smooth muscle, may lead to hypertension. In the kidney, high CK activity may lead to increased sodium retention through increased ATP availability for Na⁺/K⁺-ATPase, leading to a higher cardiac output. In the cardiovascular system, high CK activity is thought to provide ATP to enzymes involved in contractile responses, including myosin ATPase, Ca²⁺-ATPase, and myosin light chain kinase (MLCK), leading to increased peripheral resistance of blood vessels. Furthermore, CK may promote vascular and uterine smooth muscle proliferation, explaining the common occurrence of uterine fibroids and hypertension. The vicious circle between hypertension and vascular smooth muscle hypertrophy is not depicted. Finally, in skeletal muscle coupling of CK to anaerobic glycolysis is associated with limited mitochondrial fatty acid and glucose oxidation capacity, promoting obesity. In addition, high CK activity in these fibers is associated with capillary rarefaction and increased peripheral resistance.
CONCLUSION

In conclusion, generalized high CK activity could be a plausible biological factor that explains the higher blood pressures, as the result of increased vascular tone and enhanced sodium retention, as well as the greater occurrence of obesity and hypertrophic conditions in black people (Figure). However, it should be noted that these associations are thought to be independent of ethnicity. It could be questioned whether the existing evidence proves that high CK activity can cause hypertension. As suggested in the introduction of this thesis, in the context of human evolution, the high CK phenotype could have been particularly beneficial for our ancestors: high cardiovascular contractility, the ability to retain sodium in the kidneys, and the enhanced capacity of skeletal muscle for short bursts of running and storage of carbohydrates as lipid may have increased survival rates without causing hypertension. Importantly, environmental changes only started 5000 years ago, with increasing access to high-caloric and sodium-rich food. This short time interval makes it plausible that some of us may still be genetically programmed for survival in a different environment, leading to a predisposition for hypertension and obesity in times of salt and food abundance and lack of physical activity.

The evidence in this thesis on the relation between CK and blood pressure includes association studies. An association confirms that two conditions coexist and temporality is not shown. However, the hypothesis was not refuted in those studies. On the contrary, we have shown that CK is associated with sodium excretion and have reported a strong association of vascular CK with blood pressure. Notably, we have shown that hypertension can be treated by inhibition of the CK-system, which strongly contributes to the evidence for a causal role of CK in the development of hypertension. Thus, although proving causality is difficult, the findings in this thesis call for further exploration of the pathways via which CK affects blood pressure. Eventually, this may lead to recognition of the high CK state as a condition that increases hypertension risk.
REFERENCES

Nederlandse samenvatting

**Hoofdstuk 1** is een inleiding van dit proefschrift. Het creatine kinase (CK) systeem speelt een belangrijke rol in de cellulaire energiestofwisseling door het koppelen van cellulaire ATP (energie) productie met ATP verbruik. Het enzym komt in grote mate voor in organen die veel energie verbruiken, zoals de skeletspieren, het hart, de hersenen en in mindere mate de bloedvaten en de nieren. In deze organen fungeert het CK systeem als een ATP buffer- en transportsysteem, zodat voldoende ATP beschikbaar is voor spiercontractie en het transport van ionen, twee processen waar veel energie voor nodig is. Relatief hoge activiteit van het enzym komt vaak voor, maar vindt men voornamelijk bij mannen, mensen met overgewicht, en mensen van Afrikaanse afkomst. Eerder onderzoek heeft aangetoond dat er een relatie bestaat tussen CK activiteit en bloeddruk. De gedachte is dat relatief hoge activiteit van het enzym zorgt voor verhoogde beschikbaarheid van ATP voor het samenknijpen van de bloedvaten enerzijds en het vasthouden van zout in de nieren anderzijds. Dit zijn twee factoren die kunnen leiden tot een hogere bloeddruk. Dit kan verklaren waarom een verhoogde bloeddruk vaker voorkomt bij mensen met relatief hoge CK activiteit, zoals mensen van Afrikaanse afkomst. In dit hoofdstuk wordt eerst beschreven via welke mechanismen hoge CK activiteit met een effect op skeletspieren, hart, bloedvaten en de nieren, kan leiden tot een hogere bloeddruk. Tevens wordt beschreven hoe hoge activiteit van het enzym een mogelijke link kan zijn tussen een verhoogde bloeddruk en andere aandoeningen die vaak samen met hypertensie voorkomen.

**DEEL 1: KLINISCHE CONDITIES**

In het eerste deel van dit proefschrift worden de resultaten van een aantal studies beschreven, waarin de nadruk ligt op associaties tussen CK, bloeddruk en andere klinische condities die het risico op hypertensie verhogen.

In **Hoofdstuk 2** hebben we onderzocht of mensen met een hoge CK activiteit in het bloed (serum) minder natrium uitscheiden in de urine. In de nier wordt het grootste gedeelte van het gefiltraceerde natrium teruggeresorbeerd. De drijvende kracht hiervoor wordt geleverd door Na*/K*-ATPase, een enzym dat natrium de cel uit pompt en kalium de cel in. De grote hoeveelheid ATP die nodig is voor dit proces wordt geleverd door CK. Daarom zou een hoge CK activiteit kunnen leiden tot verhoogde beschikbaarheid van ATP voor de terugresorptie van natrium, wat samengaat met minder natriumexcretie.
Bij gezonde mannelijke vrijwilligers, jonger dan 50 jaar, met een normale bloeddruk of met onbehandelde hypertensie, die gedurende 7 dagen een laag natrium (<50 mmol/d) en vervolgens 3 dagen een hoog natrium (>200 mmol/d) dieet volgden, hebben we het verschil in 24-uurs natrium uitscheiding tussen hoog en laag CK onderzocht. Dit onderzoek toont aan dat de 24-uurs natriumuitscheiding na een dieet met veel zout lager is bij hoge CK activiteit. Deze bevindingen kunnen betekenen dat CK een rol speelt bij de terugresorptie van natrium in de nieren.

De associatie tussen CK en bloeddruk kan ook verklaard worden door hoge CK activiteit in bloedvaten, waar het de beschikbaarheid van ATP voor de contractiliteit vergroot. **Hoofdstuk 3** beschrijft de bevindingen van een studie naar de mogelijke associatie tussen CK genexpressie in humane vaten en bloeddruk. In dit hoofdstuk wordt aangetoond dat de genexpressie van het CK-B isoenzym in humane weerstandsvaten sterk gecorreleerd is met zowel de systolische als de diastolische bloeddruk. Deze bevinding is het eerste bewijs voor een sterke associatie tussen CK genexpressie in humane vaten en bloeddruk en draagt bij aan het reeds bestaande bewijs voor een mogelijke rol van CK in de contractiliteit van bloedvaten.

Hypertensie en hoge CK activiteit komt vaker voor bij mensen met obesitas. Mogelijk speelt een hoge CK activiteit in de skeletspieren een rol bij het ontstaan van hypertensie en obesitas. De hoogste CK activiteit in het lichaam vindt men in snelle type II spiervezels in de skeletspieren. Deze spiervezels zijn bovenal geschikt voor korte explosieve inspanning. De energie hiervoor wordt voornamelijk geleverd door CK, terwijl de mogelijkheden voor opname en oxidatie van vetzuren en glucose beperkt is. Het gevolg is dat hoge CK activiteit in de skeletspieren, zoals bij een dominantie van type II vezels, de opslag van vetzuren en glucose als vet kan bevorderen, wat mogelijk leidt tot overgewicht. In **Hoofdstuk 4** hebben we daarom onderzocht of serum CK activiteit na drie dagen rust geassocieerd is met body mass index (BMI) in de populatie. Dit om de mogelijke relatie tussen CK en obesitas te onderzoeken. In een steekproef van de populatie van 1444 mensen, bestaande uit verschillende etnische groepen, hebben we aangetoond dat serum CK de voornaamste voorspeller is van BMI, onafhankelijk van leeftijd, geslacht, etniciteit, opleidingsniveau en serum kreatinine als maat voor spiermassa. In de veronderstelling dat serum CK na drie dagen rust een afspiegeling is van weefsel CK, kunnen de bevindingen van dit onderzoek impliceren dat hoge CK activiteit in de skeletspieren geassocieerd is met een verhoogd risico op obesitas. Hoge CK activiteit in de skeletspieren is ook geassocieerd is met een hogere perifere weerstand, door de aanwezigheid van relatief weinig capilaires en een hogere
bloeddruk. Dit zou kunnen betekenen dat iemand met hoge CK activiteit een verhoogde kans heeft op het krijgen van zowel hypertensie als obesitas. Verder onderzoek moet uitwijzen of er inderdaad een causaal verband is tussen CK en obesitas.

Eerder onderzoek suggereert dat zowel hypertensie als obesitas geassocieerd zijn met uterine myomen. Bij vrouwen komen deze gynaecologische goedaardige tumoren vaak voor. Ook is bekend dat het risico op vroegtijdig overlijden aan hart- en vaatziekten voor vrouwen groter is dan voor mannen en dat hypertensie een van de voornaamste risicofactoren hiervoor is. Ondanks dit gegeven zijn risicofactoren voor hypertensie bij vrouwen relatief onderbelicht. Hoofdstuk 5 beschrijft de resultaten van een retrospectieve cohort studie waarin we het voorkomen van hypertensie hebben onderzocht bij vrouwen die waren opgenomen voor een operatie vanwege myomen. We hebben deze resulaten vergeleken met het voorkomen van hypertensie bij vrouwen die waren opgenomen voor een andere gynaecologische operatie en populatiecontroles. We hebben 241 vrouwen met myomen, 308 vrouwen die een gynaecologische operatie ondergingen vanwege andere redenen en 606 populatiecontroles geïncludeerd. Een hoge bloeddruk kwam voor in 43.6% van de vrouwen met myomen, in 28.6% van de vrouwen met een andere gynaecologische operatie en in 24.3% van de populatiecontroles. Vrouwen met myomen hadden vaker een hoge bloeddruk (systolisch/diastolisch ≥ 140/90 mm Hg) na correctie voor leeftijd, BMI en etniciteit. Mogelijk zorgt een gemeenschappelijke groeifactor voor zowel groei van glad spierweefsel van de baarmoeder als van glad spierweefsel van bloedvaten. Het is bekend dat CK betrokken is bij de groei van glad spierweefsel, doordat het enzym de nodige ATP voor dit proces verschafte. Daarom is het mogelijk dat hoge CK activiteit de groei van glad spierweefsel in de baarmoeder en de bloedvaten stimuleert, wat kan leiden tot het ontstaan van zowel hypertensie als myomen. De hoge CK activiteit zou dan ook de gezamenlijke associatie met obesitas kunnen verklaren.

DEEL II: BEHANDELING

Dit deel van het proefschrift beschrijft de resultaten van studies waarin het CK systeem als mogelijk doel voor behandeling van hypertensie en hart-en vaatziekten werd onderzocht. Tevens werd het effect van CK inhibitie op weefsels met veel energieverbruik onderzocht.

Omdat hoge CK activiteit samengaat met verminderde natriumuitscheiding en verhoogde vaatcontractiliteit, is hypertensie mogelijk moeilijker te behandelen bij hoge
Nederlandse samenvatting

Hoofdstuk 6 beschrijft de resultaten van onderzoek naar de associatie tussen serum CK en het falen van antihypertensieve behandeling in de populatie. In een steekproef van de populatie van 1444 mensen met een verschillende etnische achtergrond, 34 tot 60 jaar oud, hebben we aangetoond dat de behandeling van hypertensie faalde in 72.9% van de deelnemers in het hoogste CK tertiel versus 46.7% in het laagste tertiel. In dit onderzoek hebben we tevens laten zien dat CK de voornaamste voorspeller is van het falen van antihypertensieve behandeling, onafhankelijk van leeftijd, geslacht, BMI, nuchter glucose, etniciteit of opleidingsniveau. Prospectief onderzoek zal moeten aantonen of er een causaal verband is tussen CK en het falen van antihypertensieve behandeling.

Hoofdstuk 7 is een systematische review waarin we het effect van interventie in het CK systeem hebben onderzocht in vergelijking met placebo bij volwassen patiënten met essentiële hypertensie en hart- en vaatziekten. We zochten in MEDLINE, EMBASE, LILACS en het Cochrane Controlled Trials Register naar gerandomiseerde placebo gecontroleerde studies die het effect van creatine of creatine analogen met placebo hebben vergeleken in patiënten met essentiële hypertensie, hartfalen of een myocardinfarct. De uitkomsten waren sterfte, totaal myocardinfarct, veranderingen in ejectie fractie en veranderingen in systolische of diastolische bloeddruk. Elf studies voldeden aan de inclusiecriteria. De gebruikte middelen waren creatine, phosphocreatine of phosphocreatinine. Bij patiënten met hartfalen lieten de geïncludeerde studies geen duidelijk effect op ejectiefractie zien. Bij patiënten met een myocardinfarct rapporteerden twee van de vier studies mortaliteitsdata, zonder duidelijk effect van creatine of creatine analogen. Het voornaamste effect lijkt verbetering van dysritmie te zijn. Op grond van bovenstaande bevindingen kunnen we concluderen dat het niet duidelijk is wat het effect van interventie in het CK systeem is op mortaliteit, progressie van een myocardinfarct, ejectie fractie en bloeddruk in patiënten met hypertensie of hart- en vaatziekten.

Hoofdstuk 8 is een systematische review, waarin we het effect van de CK-remmer beta-guanidinopropionzuur (βGPA) op de functie en morfologie van organen met hoog energieverbruik hebben onderzocht. βGPA, een creatine analoog, remt de cellulaire creatine opname competitief. Men zegt dat dit aminozuur veilig is voor gebruik bij mensen, maar de effecten en bijwerkingen zijn niet duidelijk. We hebben in PubMed, EMBASE, de Cochrane Library en LILACS gezocht naar studies die het effect van βGPA op organen met een hoog en wisselend energieverbruik beschreven. We hebben 131 publicaties gevonden, die voornamelijk het effect van chronische toediening van βGPA
(0.5 tot 3.5%) onderzochten op de skeletspieren, het cardiovasculaire systeem en het brein in dieren. Door behandeling met βGPA daalden de intracellulaire creatine en phosphocreatine concentratie in alle organen. In skeletspieren leidde dit tot een toename van de mitochondriële oxidatieve stofwisseling met een verhoogde glucose opname en een verbeterend uithoudingsvermogen. In het hart was deze toename minder duidelijk. De contractiliteit van het hart nam in lichte mate af door behandeling met βGPA, maar het hartminuutvolume was onveranderd. In het brein leidden de veranderingen in de energiestofwisseling tot verhoogde ATP stabiliteit en overleving tijdens hypoxie. Concluderend laten de bovenstaande resultaten zien dat chronische toediening van βGPA aan dieren het uithoudingsvermogen van skeletspieren vergroot en de overleving tijdens ischemie bevorderd, met enigszins verminderde contractiliteit van het hart. Deze bevindingen impliceerden dat het CK systeem een belangrijke maar niet onmisbare rol speelt in de cellulaire energiestofwisseling.

Omdat hoge CK activiteit geassocieerd is met een hogere bloeddruk, zou remming van het CK systeem een bloeddrukverlagend effect kunnen hebben. In Hoofdstuk 9A hebben we daarom onderzocht of βGPA de bloeddruk verlaagt in de spontaan hypertensieve rat, een diermodel met hypertensie. Mannelijke 16 weken oude spontaan hypertensieve ratten (N=16) kregen gedurende vier weken een dieet met of zonder βGPA. De bloeddruk werd wekelijks gemeten aan de staart met de tail-cuff methode. Behandeling met βGPA leidde tot een aanzienlijke daling van zowel de systolische als diastolische bloeddruk in vergelijking met controloren: respectievelijk -42.7 (SD 5.5) en -35.3 (4.8) mm Hg. De bloeddrukdaling door behandeling met βGPA suggereert dat remming van het CK systeem een veelbelovend nieuw mechanisme voor de behandeling van hypertensie kan zijn. Daarom is door ons een protocol ontworpen voor de “first-in-man” studie met βGPA. De studieopzet wordt beschreven in Hoofdstuk 9B. De primaire uitkomstmaat is de verdraagzaamheid van acute behandeling met βGPA. Tevens zal het effect op hemodynamische (o.a. bloeddruk) en biochemische parameters worden onderzocht. Gebaseerd op experimenten met βGPA bij dieren hebben we een equivalente dosis voor mensen van 100 mg berekend. De verwachting is dat dit onderzoek geen serieuze bijwerkingen laat zien. Hopelijk zal deze studie uiteindelijk gevolgd worden onderzoek naar de effecten van βGPA bij hypertensieve patiënten.
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Dankwoord

Iemand vroeg me laatst of ik niet ook een dankwoord wilde schrijven met teksten zoals “bedankt voor de vele gezellige kopjes koffie, borrels, wintersportweekenden enzovoort”. Uit enig onderzoek in het AMC in de tijd die mij nog restte voor het drukken van het proefschrift, blijkt dit echter lastig uitvoerbaar: Om met de koffie te beginnen, ik heb uit betrouwbare bron vernomen dat patiënten en werknemers in het AMC dagelijks door in totaal 300 automaten van een koffie worden voorzien. Op de afdeling vasculaire geneeskunde op F4-zuid, waar de onderzoekers gedurende enige jaren vele uren per week doorbrengen in kleine ruimtes, staan er al meerdere binnen handbereik, in het kader van de efficiëntie veelal naast de toiletten. Daarom is het niet verwonderlijk dat vele contacten worden gelegd en onderhouden onder het genot van een kop koffie. Onder andere heb ik vele kopjes koffie gedronken met de vrijwilligers nadat zij meerdere malen nuchter naar het ziekenhuis kwamen voor verscheidene bloeddrukmetingen en bloedafnames zonder daar een vergoeding voor te krijgen. Daarom wil ik als eerste alle deelnemers bedanken voor hun hulp en betrokkenheid bij het onderzoek.

Toen ik op de afdeling vasculaire geneeskunde in het AMC begon had ik weinig onderzoekervaring. De eerste dagen in het AMC bestonden dan ook uit omschakelen van intensief patiëntencontact naar een dag achter de computer doorbrengen om zoveel mogelijk te lezen over een geheel nieuw onderwerp. Aan het eind van de dag vroeg ik me dan wel eens af wat ik nou precies gedaan had buiten koffie drinken. Daarom wil ik vervolgens degene die mij alle kneepjes van het vak heeft getracht te leren, mijn copromotor, Lizzy Brewster, bedanken. Lizzy, in de afgelopen drie jaar heb ik met bewondering jouw enorme passie voor het onderzoek naar etniciteitverschillen en de rol van creatine kinase in het ontstaan van hypertensie gevolgd. Het is bijzonder hoe je van een idee op eigen kracht bent gekomen waar je nu bent. Bedankt voor alles, ik heb veel van je geleerd! Ook wil ik hier Harry Büller, mijn promotor, bedanken. Harry, je bent niet betrokken geweest bij de dagelijkse begeleiding. Echter, je gedrevenheid, oplossingsgerichte aanpak van veel zaken, maar ook je humor en het vermogen tot relativeren zijn aanstekelijk. Mijn dank is groot!

Dankwoord

De hypertensie groep: Gert, Bert-Jan en Liffert, bedankt voor alle adviezen tijdens de wekelijkse hypertensiebesprekingen, maar ook de enorm leuke week in Paramaribo. Ik ben onder de indruk van jullie grote hoeveelheid aan kennis. Bas, Nanne, Daan, Fouad en Niels, veel dank voor het meedenken met onderzoeksproblematiek en alle gezelligheid. De congressen in Oslo, Milaan en Londen waren mooi. Marianne, bedankt voor je hulp bij de metingen, je vrolijkheid, en voor het feit dat ik altijd even mijn spreekwoordelijke ei bij jou kwijt kon. De toekomstige artsen: Doortje, je enthousiasme over veel zaken was aantrekkelijk tijdens het schrijven van de review. Fares, bedankt dat ik mee kon schrijven met jouw werk over CK mRNA, maar ook voor het delen van je kennis over het omgaan met de Surinaamse deelnemers. Yentl, het stuk over myomen en hypertensie is helemaal aan jou te danken. Ik ben benieuwd wat hier nog uit voort komt. En Inna, het was onmogelijk geweest zonder jou het gedeelte over CK en zout voldoende af te krijgen voor dit proefschrift. Vele fietstochten heb je afgelegd door Zuid-Oost. Ik ben heel blij dat jij degene was die reageerde op mijn advertentie.

Ook het doen van dierproeven was voor mij een geheel nieuw terrein. Gelukkig deden we deze proeven in samenwerking met de afdeling Farmacologie. Stephan Peters en Astrid Alewijnse, heel veel dank voor het feit dat ik altijd bij jullie binnen kon lopen met vragen en voor alle hulp bij het opzetten en uitvoeren van de dierproeven. De sfeer was altijd goed. Het is zonde dat de afdeling er niet meer is. Na uren in een donker ruimte bloeddrukken te hebben gemeten bij groeivertraagde ratten was het heerlijk om het laboratorium binnen te lopen waar de kalme bedrijvigheid van vaatproeven aan de gang was met op de achtergrond de goede meezingers van radio 3fm. Rob en Leon, veel dank voor al jullie hulp en het meedenken bij de vaatproeven, gaan we nog een keer naar zo’n goede film? Verder wil ik graag alle co-auteurs bedanken. Rene, bedankt voor je hulp bij de hartjes en spieren, zonder jou was een deel waarschijnlijk op de kast beland. Professor Joseph Clark, thank you for your comments on the review, animal studies, and the abstracts.

Tussen het bloeddruk meten door moest er soms een sprint worden getrokken naar het vasculaire trialbureau om patiënten te zien. Deze combinatie verliep niet altijd soepel. Het laatste jaar was ik hier bijna dagelijks te vinden vanwege de zoutstudie en ik kan niet anders zeggen: Trees, Linda, Elsa, Hans, Michelle, Remco, Johan, Mia, Liesbeth en Nanet bedankt voor alle hulp en de betere koffie! Henriette, bedankt voor de hulp bij het regelwerk rondom de promotie.
En dan de borrels en wintersportweekenden. Ook in dit geval is het niet verwonderlijk dat velen die mij voorgingen bij het schrijven van een dankwoord, regelmatig het woord borrel in de pen hebben genomen. Op de introductiedag vertelde een oud-collega mij wat een fantastische afdeling de vasculaire geneeskunde is, niet alleen een uitblikker op wetenschappelijk vlak, maar ook elke week een borrel dan wel feest. Op onze etage zitten ongeveer 25 onderzoekers, de oud-onderzoekers niet mee gerekend, dus reken maar uit: Ongeveer 10 promoties, 25 verjaardagen, meerdere congressen en dan nog 52 vrijdagen per jaar met een aanzienlijke kans dat 1 van de 25 voorstelt om het weekend op een toepasselijke manier in te luiden. En dan heb ik de wintersportweekenden niet eens genoemd. Daarom wil ik hierbij mijn medepromovendi Aart, Andrea, Ankie, Anne, Barbara, Bas, Brigitte, Carlijne, Corien, Daan, Danka, Diederik, Dirk-Jan, Elise, Fouad, Hans, Joost, Julian, Katrijn, Kees, Lily, Loek, Maartje, Mandy, Maayke, Marjet, Mathilde, Max, Meeike, Michiel, Nanne, Niels, Onno, Renée, Roeland, Ronne, Ruud, Sara, Stephano, Suthesh en Whitney bedanken voor alle memorabele momenten. Veel succes allemaal! In het bijzonder: Joyce, ik hoop dat je in een Excel schema bijhoudt hoe vaak je uitgebreid bent bedankt in een proefschrift (zo niet dan weet ik nog wel iemand die je de kneepjes van het vak kan leren). Het is niet meer dan terecht. Je bent de spil van de afdeling bij wie iedereen altijd aan kan kloppen voor hulp, je zorgt dat iedereen altijd in de juiste kamer terecht komt, en je bent daarbij ook nog eens een ontzettend leuke, gezellige dame! Dank voor alles.

Zoals voor velen was mijn eerste plek in het hartoor. Een plek met daglicht waar vriendschappen zijn begonnen. Ties en Josien, met jullie heb ik denk ik de langste tijd in 1 ruimte doorgebracht, eerst in het hartoor, toen F4-142. Na Onno's vertrek naar het volgende station richting het hoogleraarschap maakte Paulien de 4 m² compleet. Deze kamer lag op de route naar Joyce en fungeerde regelmatig als een tussenstation voor onze collega's. Dit maakte dat mij van hogerhand meerdere malen werd geadviseerd zoveel mogelijk dagen thuis te werken. Echter, het was een mooie tijd in F4-142, waar gesprekken over de goede dingen des levens werden afgewisseld met zeer welkome suggesties op onderzoeksgebied. Jullie boden altijd een luisterend oor voor de serieuze zaken en onderzoeksperikelen, maar ook soms voor de goede roddels. Josien, eerder is opgeschreven dat we swingend onze promotie doorgaan. Misschien is dat deels wel waar. Bedankt voor alles, je staat altijd klaar om iedereen die het vraagt te helpen met een lach op je gezicht. Paulien, jij kan mensen het juiste duwtje in de rug geven. Je hebt me regelmatig geholpen dingen in het juiste perspectief te plaatsen. Ties, ik ken niemand die enerzijds zo perfectionistisch kan zijn en anderzijds zo goed op de hoogte
is van de kapsels van G&G op Nu.nl. Heerlijk om zeker te weten dat jij er altijd al was met je krant en (hoe kan het anders) koffie als ik aankwam, hoe vroeg het ook was. Ons promotiefeest wordt een knaller en ik hoop dat er nog velen volgen! Na het emotionele vertrek van Ties leek het ons een verstandig plan om voor de laatste maanden van een promotietraject te verhuizen naar een wat rustigere locatie. De laatste verhuizing ging naar kamer F4-146 om orde te brengen in de chaos (of chaos in de orde, waarom, waarom...). Fleur, je verstaat de kunst om ook als het even tegenzit een lach op het gezicht van mensen te toveren. Je bent enorm ambitieus, maar ook altijd aan voor wat gekkigheid. Ik ben blij dat ik je heb leren kennen. Samen met Sophie vorm je een goed team. Sophie, niet alleen de Jordaan loopt met je weg. Dames, ik kijk nu al uit naar onze wintersportvakantie!

Het volgen van een promotietraject betekent niet dat het leven daarbuiten stilstaat...integendeel. Daarom wil ik de kans niet voorbij laten gaan om ook een aantal mensen te bedanken die niet letterlijk met de totstandkoming van dit proefschrift te maken hebben, maar het leven wel mooi maken. Dus al mijn vrienden en vriendinnen bedankt voor het feit dat jullie er zijn! Het kan niet anders dan dat ik hier ook twee woorden schrijf: ‘BVD HOOG’. Dan ‘de groep’: wat begon op het mentorweekend in 2002 is uitgegroeid tot een combinatie van lieve, knappe en (een beetje) gekke mensen. Ook al ziet niet iedereen elkaar even vaak, ik hoop jullie niet uit het oog te verliezen. In het bijzonder: Yuri, je hebt voor alles een oplossing. Bedankt voor al je zorgzaamheid. Ik weet zeker dat je gaat komen waar je wilt zijn. Jasper, niet alleen bedankt voor het dak boven mijn hoofd, jouw vrolijke aanwezigheid op de Wolvenstraat maakt me altijd aan het lachen. Marc, jij maakt de beste grappen van iedereen. Ik ben benieuwd of Pepijn je gaat evenaren. Kim, we kennen elkaar al 18 jaar en ik weet zeker dat we die jaren meer dan gaan verdubbelen. Ik ben trots op je. Je mooie dochter Nina gaat later vast net zulke mooie vakanties en avonden hebben als wij altijd hadden. Vera, Evelien, Mina en Judith, wat een tijden! Veer, ondernemer, met niemand kan ik uren- en nachtenlang doorpraten over zulke uiteenlopende onderwerpen. Ik vind het heel knap hoe je het doet. Het is fijn om te weten dat jouw deur altijd openstaat. Mien, ik zal maar niet zeggen hoe we elkaar hebben ontmoet, maar ik ben blij dat het is gegaan zoals het is gegaan. Eef, ik ken niemand die zoveel dingen tegelijkertijd kan doen en dan nog altijd klaar staat voor iedereen. Juud, hoe bijzonder dat jij en Eef nu de hartenbrekers van de volgende generatie op de wereld hebben gezet. Mijn paranimfen, Martina en Eva, ik had niemand anders hiervoor kunnen vragen. We zijn vriendinnen vanaf het eerste jaar van de studie en ik weet dat we dat altijd zullen blijven. Eef, ik zal onze reis naar Australië nooit
vergeten. Nog steeds toveren de herinneringen een glimlach op mijn gezicht, zo ook bij
dit schrijven. Ook krijg ik geen genoeg van je soms cynische humor waar ik altijd heel
hard om moet lachen. Je wordt een fantastische neuroloog, de volgende ben jij! Tien,
kanjer, toen de meesten van ons nog aan het bedenken waren wat we eigenlijk wilden
met ons leven was jij al bijna huisarts. Ik bewonder je altijd aanwezige vrolijkheid en
positiviteit, zelfs in mindere tijden.

Lieve pappa, mamma, oma, Sander en Daisy, tot slot wil ik jullie bedanken. Wat een
geluk heb ik met jullie. Ik weet dat jullie er altijd voor mij zullen zijn.
About the author


Inge Oudman, daughter of Elly Oudman-Verwaal and René Oudman, was born in Haarlem on August 15, 1983. After completing her secondary education at the Murmellius gymnasium in Alkmaar in 2001, she went on to study Medicine at the VU University Amsterdam. During her studies, she was active as a teaching assistant in various courses and worked at the nursing ward of the neurology department. In 2006 she finished her degree with a research project at the Women’s and Children's Hospital in Adelaide, Australia. To her delight, she also got the opportunity to do two internships in Paramaribo, Surinam. After obtaining her medical degree in 2008 Inge worked as a physician in the department of Internal Medicine at the Slotervaart Hospital in Amsterdam. Before starting her PhD in 2010, she worked as a physican in the care of homeless people. In April 2013 she is starting her residencies in Internal medicine at the AMC Amsterdam.

De paranimfen,
Eva Strijbis en Martina Meijboom
Portfolio

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2012  Oudman I, Kewalbansing PV, van Valkengoed IGM, Clark JF, Brewster LM. Creatine kinase predicts obesity in the general population. PLOS One, under revision.


ourses

2012  Scientific Writing in English (Graduateschool AMC)
2011  Biostatistics (Graduateschool AMC)
2011  Epidemiology (Graduateschool AMC)
2010  Laboratory animal science course (University of Utrecht)
2010  Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers
2010  Good Clinical Practice (Graduateschool AMC)
POSTER AND ORAL PRESENTATIONS


2012  Oudman I, Clark JF, van Montfrans GA, Brewster LM. Creatine kinase and hypertension: from theory to new treatment modalities. Poster presentation, European Society of Hypertension, Londen, United Kingdom. Poster award, €500,-.

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2012  Increased prevalence of hypertension in women with uterine fibroids.
2011  Creatine kinase and blood pressure. Preliminary results animal studies.
2010  Creatine kinase and blood pressure. Study proposal.