

Application of Magnetic Resonance for non-invasive phenotyping of mice with altered creatine metabolism



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Declaration of own work

This thesis is entirely my own work with the exception of the following parts:

Chapter 3:

- The calibration curve for creatine measurement by ^1H -MRS versus HPLC (section 3.3.2) was done by Dr Schneider

Chapter 4:

- All the surgeries for haemodynamic measurements were performed by Miss Debra Medway. However, I provided support during these examinations especially for tissue harvesting. The analysis of the haemodynamic measurements was performed by myself.
- Solution state NMR in section 4.3.2.1 was performed by Dr Helen Atherton (University of Cambridge).
- Adenylate kinase assay in section 4.3.2.4 was done by Dr Dunja Aksentijevic.
- Histology to measure myocytes cross-section area in section 4.3.2.5 was done by Miss Victoria Thornton.
- Finally, in section 4.3.3, experimental design and data acquisition (with the exception of HPLC) were performed by Dr Craig Lygate and Miss Debra Medway.

Chapter 5:

- Measurement of ribose-5-P concentrations in the myocardium in section 5.2.2.3 was done by Dr Dunja Aksentijevic.
- Preliminary study looking at the effect of heart failure on TAN pool (section 5.2.2.1) was done by Miss D. Medway for the surgical part and Mr L. Sebag-Montefiore for HPLC measurements.

All chapters:

- All mouse breeding and genotyping was done by Miss Debra Medway.

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Abstract

Changes in myocardial energetics have been implicated in the pathophysiology of heart failure (HF). However, the precise contribution of creatine (Cr) / phosphocreatine (PCr) / creatine kinase (CK) energy buffer and transfer remains unclear. The aim of this thesis was to study the effects on murine cardiac function of both impairment and enhancement of creatine metabolism.

In order to longitudinally follow the cause and effect relationship of myocardial creatine concentration, a non-invasive method of quantification was required. Cardiac Cr levels measured *in vivo* by ^1H -MRS were therefore compared with gold-standard invasive HPLC and found to correlate over a wide-range ($r^2=0.91$). ^1H -MRS was reproducible for measuring Cr levels in the heart, brain, and skeletal muscle.

The cardiac phenotype of a novel model of creatine depletion, the AGAT^{-/-} mouse, was characterized using *in vivo* MRI, ^1H -MRS and LV catheterisation, under conditions of gradually reducing Cr concentrations; zero Cr; and attempted phenotype rescue with dietary Cr. For the first time in the heart, the rate of Cr turnover was quantified (~3 % per day) and demonstrated that cardiac function was preserved even when creatine levels reduced by ~70-90%. Total absence of myocardial Cr induced impairment of inotropic and lusitropic cardiac function and reduced inotropic reserve. Cardiac dysfunction was only partially rescued by replenishment of the Cr pool, suggesting this to be a consequence of long-term adaptations to chronic low Cr.

Finally, we tested the hypothesis that combined elevation of myocardial creatine and ribose would be beneficial in a mouse model of chronic HF by increasing cardiac energy availability. Despite an increase in myocardial ribose concentration, this did not prevent loss of total adenine nucleotides (TAN), and there was no improvement in post-infarct LV remodeling or function. Future studies are needed to explore alternative approaches for maintaining TAN in combination with total creatine.

Aut viam inveniam aut faciam

Hannibal

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Abbreviations

AcCoA: acetyl coenzyme A
ADP: adenosine diphosphate
AGAT: L-arginine : glycine amidinotransferase
AGAT^{-/-}: AGAT knockout
AK: adenylate kinase
AN: adenine nucleotide
AMP: adenosine monophosphate
ANP: atrial natriuretic peptide
ANT: adenine nucleotide transporter
 α -SA: α -skeletal actin
ATP: adenosine triphosphate
 β -GPA: beta-guanidinopropionic acid
BNP: brain natriuretic peptide
 β -MHC: β -myosin heavy chain
bpm: beat per minute
BW: body weight
B₀: main magnetic field
CHESS: chemical shift selective (a method of water-suppression)
CHF: congestive heart failure
CK: creatine kinase
CO: cardiac output
CV: coefficient of variation
CVD: cardiovascular disease
Cr: creatine
CRLB: Cramer-Rao lower bound
CrT: creatine transporter
CrT-OE: creatine transporter over-expressor transgenic mouse
 ΔG_{ATP} : Gibbs free energy
2,3-DPG: 2,3-diphosphoglycerate
DNA: deoxyribonucleic acid
dNTP: deoxynucleotide triphosphate
ECG: electrocardiogram
EDV: end-diastolic volume
EF: ejection fraction
EMCL: extra-myocellular lipids
FFA: free-fatty acid
FLASH: fast-low angle shot
FWHM: full-width at half maximum
GA: guanidinoacetate
GAMT: guanidinoacetate N-methyl transferase
GAMT^{-/-}: GAMT-knockout mouse
GAPDH: glyceraldehyde-3-phosphate-dehydrogenase
Gd-DTPA: gadopentetate dimeglumine
GE: gradient echo
GTP: guanosine triphosphate
G-6-PD: glucose-6-phosphate dehydrogenase
HEP: high-energy phosphate
HF: heart failure
¹H-MRS: proton magnetic resonance spectroscopy
HPLC: high performance liquid chromatography
HR: heart rate

id: inner diameter
 IMCL: intra-myocellular lipids
 IMP: inosine monophosphate
 I/R: ischaemia/reperfusion
 ISF: isoflurane
 LV: left ventricle
 LVM: LV mass
 LVESP: left ventricular end-systolic pressure
 LVEDP: left ventricular end-diastolic pressure
 MR: magnetic resonance
 MRI: magnetic resonance imaging
 MRS: magnetic resonance spectroscopy
 MRSI: magnetic resonance spectroscopic imaging
 MT: magnetisation transfer
 NA: number of averages
 NMR: nuclear magnetic resonance
 od: outer diameter
³¹P-MRS: phosphorus magnetic resonance spectroscopy
 PCr: phosphocreatine
 PCR: polymerase chain reaction
 P-GA: phosphorylated guanidinoacetate
 PGK: 3-phosphoglycerate kinase
 Pi: inorganic phosphate
 PRESS: point resolved spectroscopy
 PRPP: 5-phosphoribosyl-pyrophosphate
 q.i.d: *quater in die* (four times a day)
 RF: radiofrequency
 RNA: ribonucleic acid
 rpm: revolutions per minute
 RR: respiratory rate
 RT-qPCR: real-time quantitative polymerase chain reaction
 RV: right ventricle
 SERCA: sarco/endoplasmic reticulum Ca²⁺-ATPase
 SNR: signal-to-noise ratio
 STEAM: stimulated echo acquisition mode
 SV: stroke volume
 T₁: spin-lattice relaxation time
 T₂: spin-spin relaxation time
 TAN: total adenine nucleotide
 TE: echo time
 TEP: total exchangeable phosphates
 Tg46 and Tg55: CrT-OE strains
 t.i.d.: *ter in die* (three times a day)
 TMA: tri-methyl ammonium
 TR: repetition time
 TSP: sodium-3-(tri-methylsilyl)-2, 2, 3, 3-tetradeuteriopropionate
 VDAC: voltage-dependent anion channel
 VOI: volume of interest
 WEFT: water eliminated Fourier transform (a method of water-suppression)
 WS: water-suppression / water-suppressed
 WT: wild-type

Chapter 1

INTRODUCTION

Everyday, the heart consumes in the form of adenosine triphosphate (ATP) more energy than any other organ, an amount estimated between 6 and 35 kg of ATP for the human heart^{1, 2}. Of these, only 20 to 25 % of it is actually converted to mechanical work, most of it (60-70 %) being lost through heat production. The heart requires ATP for contraction *sensu stricto* (myosin ATPase) but also relaxation (calcium uptake by the sarcoendoplasmic reticulum calcium ATPase) and activity of membrane transport system (mainly Na⁺/K⁺ pump)³.

Importantly, the demand for energy is extremely dynamic to adapt to the wide-ranging haemodynamic requirement of the body. However, intracellular ATP levels are maintained constant in the healthy heart, which implies a tight matching between ATP demand and supply⁴. Given the intracellular compartmentation between sites of energy production (mitochondrion) and sites of energy utilisation (e.g. sarcomere), how can energy be made available quickly enough to adapt to a sudden increase in demand? How are ATP levels maintained sufficiently high in the vicinity of sarcomeres to ensure thermodynamically favourable conditions for enzymatic activities?

Since the failing heart is defined by its inability to supply sufficient blood flow to cover the need of the organism⁵, could this incapacity to meet energy requirement be the consequence of reduced energy availability or be a mismatch between energy demand and supply? Furthermore, could this represent a therapeutic target in heart failure?

These are the key questions that will be addressed in this introduction, and which more generally form the scope of this thesis.

1.1 Cardiac high energy phosphate metabolism

1.1.1 ATP, “the energy currency of the cell”

All living organisms convert chemical energy to ATP, which is then used to drive nearly all biological reactions requiring energy such as active transport of ions, cell signaling, protein synthesis or muscle contraction.

ATP contains two high-energy phosphate bonds, which can be hydrolysed releasing energy. Gibbs free energy (ΔG_{ATP}) released from the hydrolysis of 1 mole of ATP to adenosine diphosphate (ADP) and inorganic phosphate (Pi) and available to drive ATP-requiring reactions is given by the equation below⁴:

$$\Delta G_{\text{ATP}} = \Delta G_{\text{ATP}}^0 - RT \ln \frac{[\text{ATP}]}{[\text{ADP}][\text{Pi}]}$$

Equation 1: Free energy change of ATP hydrolysis (ΔG_{ATP}) according to the reaction: $\text{ATP} \rightarrow \text{ADP} + \text{Pi}$. ΔG_{ATP}^0 is the standard free energy change of ATP hydrolysis (-0.5 kJ/mol under standard conditions where each substrate and product is 1 mol/L, standard temperature, pH 7 and 1 mM Mg^{2+}), R is the gas constant (8.3 J/mol/K) and T is the temperature in Kelvin.

$[\text{ATP}]/[\text{ADP}][\text{Pi}]$ is the phosphorylation potential of the cell. The higher it is, the more exergonic the reaction of ATP hydrolysis is. Maintaining a high $|\Delta G_{\text{ATP}}|$ is essential for normal myocyte function. Small changes in the free energy of ATP hydrolysis would impede the activity of intracellular ATPases⁶⁻⁸. For example, $|\Delta G_{\text{ATP}}|$ is 55-60 kJ/mol in normal

rodent heart⁹. Function of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) is impaired when $|\Delta G_{\text{ATP}}|$ falls below 52; the myosin ATPase at 46 kJ/mol, and the Na^{+} - K^{+} -ATPase at 50 kJ/mol¹⁰.

1.1.2 From ATP production to ATP utilisation

The heart is a metabolic omnivore able to use a wide variety of substrates, depending on availability, inotropic state, and hormonal levels rather than an intrinsic preference of the myocardium. In the fasted state, the main myocardial fuel is free fatty acids (FFA) (60 to 90 % of the generated ATP in the human heart), whereas carbohydrates and lactate account for 10 to 40 %. However, after a meal rich in carbohydrates, when blood glucose and insulin levels are high, glucose become the major substrate and can account for up to 50 to 75 % of ATP produced^{3, 11, 12}.

The glucose, taken up by cardiomyocytes by the glucose transporter GLUT4 (and to a lesser extent GLUT1) is then converted in 2 molecules of pyruvate by the process of glycolysis, which generates directly 2 molecules of ATP and 2 molecules of NADH. Pyruvate, formed in the cytosol, is then transported in the mitochondrion, where it is oxidatively decarboxylated to acetyl-CoA (AcCoA)^{3, 11, 12}.

FFAs enter the cell via the fatty acid transporter protein before being esterified in the cytosol in acyl-CoA. The latter is then transported into the mitochondrion using a carnitine carrier system. Following β -oxidation, AcCoA and a large quantity of ATP are generated (4 ATP per oxidation cycle).

AcCoA, which is at the confluence of fat and carbohydrate metabolism, is then oxidised in the Krebs cycle to generate further ATP (10 ATP per acetyl-CoA molecule).

Finally, the ATP generated in the mitochondrion need to be transferred to the sites of utilisation. This is realised through a spatially arranged intracellular enzymatic network, which catalyses the transfer of high-energy phosphoryl bonds from the site of production to the site of utilisation. This energy circuit involves mostly creatine kinase (CK) (89 % of total myocardial phosphotransfer in the dog heart) but also adenylate kinase (10 %) and to a lesser extent glycolytic enzymes (3-phosphoglycerate kinase (PGK) / glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)) and contributes to efficient intracellular energetic communication, matching ATP production with energetic requirements^{13, 14}.

1.1.3 The creatine kinase network: a temporal and spatial buffering system

1.1.3.1 The creatine kinase reaction

In tissues with high and fluctuating energy demand, creatine kinase catalyses the reversible transfer of the high energy γ -bond from ATP to the guanidine group of creatine (Cr) to form phosphocreatine (PCr) and adenosine diphosphate (ADP)¹⁵:



Equation 2: Creatine kinase reaction.

Due to the small size and relatively low polarity, Cr and PCr can diffuse more readily than ATP and ADP molecules; thus PCr can serve as energy carrier. In addition, PCr can build up as much higher concentration, allowing for a higher intracellular flux of high energy phosphate¹⁵.

1.1.3.2 A highly compartmentalized system with multiple roles

CK exists in different isoforms with specific subcellular locations. The heart contains one sarcomeric mitochondrial (mt-CK isoform) and three cytosolic dimeric isoforms (MM-, MB-, BB-CK with M standing for Muscle and B for Brain). In the normal mouse heart, CK activity is mostly accounted by mt-CK (41 % of total CK activity) and MM-CK (55 %) isoforms, while the contribution of MB (3 %) and BB (1 %) is small¹⁶. The differential localization on a subcellular level of these different isoforms play an essential role for the functioning of the CK network¹⁷ (Figure 1-1). The mitochondrial isoform situated in the inter-mitochondrial space is coupled to the adenine nucleotide transporter (ANT), accepting ATP produced from oxidative phosphorylation. The PCr thus generated can leave the mitochondrion for the cytosol via the voltage-dependent anion channel (VDAC). In the cytosol, PCr/Cr (ratio of about 2:1) and ATP/ADP (ratio > 100:1) are kept in equilibrium by a soluble fraction of cytosolic CK. It has been suggested that the sites of energy production and utilisation are linked by successive near equilibrium CK reactions in the cytosol. According to this model of metabolic wave propagation, the incoming PCr triggers a propagation of the high-energy phosphoryl-bond through sequential CK reactions and could be imaged as the propagation of energy between the balls of a Newton's cradle. This process,

also referred to as “walking without moving” would allow a much faster propagation of the energy than by simple diffusion of PCr^{13, 18, 19}.

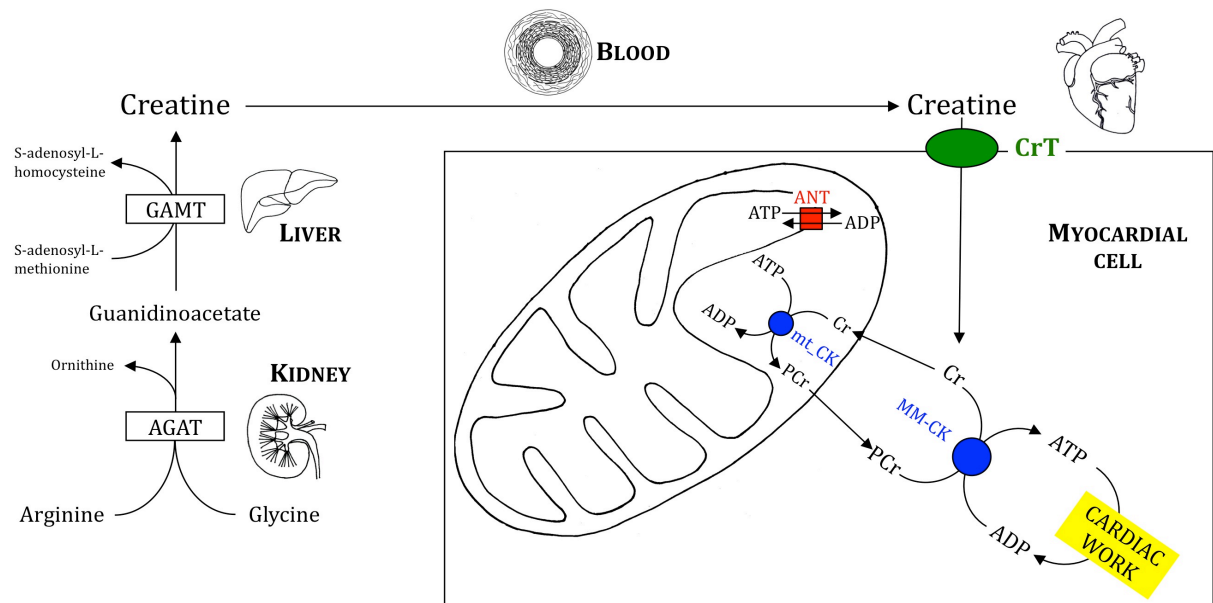


Figure 1-1: Creatine de novo biosynthesis pathway and CK shuttle. ATP produced inside the mitochondrion is transported to the intermembrane space by the Adenine Nucleotide Translocase (ANT). The mitochondrial isoform (mt-CK) of creatine kinase is coupled to ANT and transfers the high-energy phosphoryl-bond from ATP to creatine. PCr thus formed diffuses to the cytosol, where its high-energy phosphoryl group enters a chain of near equilibrium reactions catalysed by the muscle isoform of CK (MM-CK) and culminating in regeneration of ATP from ADP.

Thanks to the properties of PCr (see section 1.1.3.1) and the high intra-cellular compartmentation of the CK isoforms, multiple roles have been proposed for the Cr/PCr/CK system^{15, 17, 20, 21}:

1. The first main function widely accepted is a “temporal energy buffer”. PCr plays a role of energy reserve and is available for immediate regeneration of ATP hydrolysed during intense cardiac work, and contributes to keeping ATP and ADP concentrations almost constant in the healthy heart.

2. The second function is an “energy transport / shuttle” system. As seen above, distinct CK isoenzymes are functionally coupled to sites of energy production (e.g. mt-CK in

the intermembrane space) and consumption (e.g. cytosolic CK bound to the myofibrillar M-line²² and the sarcoplasmic reticulum²³. PCr serves as energy carrier between these sites.

3. The third role of the CK system is to keep ADP levels low, hence keeping a high $|\Delta G_{\text{ATP}}|$ and, as such, preventing inactivation of cellular ATPases, but also loss of adenine (see 1.1.4)^{20, 21}.

4. The Cr/PCr/CK system also plays a role in proton buffering. During high-energy demand, hydrolysis of large amount of ATP would lead to liberation of protons. As protons are a substrate of the CK reaction in the direction of ATP synthesis, it prevents acidification of the cell^{20, 21}.

5. Finally, the tight coupling between mt-CK and ANT at the mitochondrial levels allow for ATP generated to be immediately used for the CK reaction in the intermembrane space. This favours effective functioning of the F_0F_1 -ATPase and decreases generation of reactive oxygen species¹⁷.

1.1.4 Adenylate kinase and loss of purines

Adenylate kinase (AK) helps maintain intracellular ATP in concert with the CK shuttle. It is activated by high intracellular ADP levels and catalyses the following reaction:



Equation 3: adenylate kinase reaction

The AK system is a complementary phosphotransfer network, which contributes to 10-15 % of the total ATP turn-over rate in the normal heart^{14, 24}. Its major function is to make available the energy of the β -phosphoryl bond. This has complex consequences as it contributes to ATP preservation but can also lead to purine loss. Indeed, adenosine monophosphate (AMP) formed by the reaction can be deaminated to inosine monophosphate (IMP) and ammonia by AMP deaminase. IMP and AMP can then be dephosphorylated by 5'-nucleotidase to inosine and adenosine, which can freely diffuse across the cellular membrane²⁰. This phenomenon contributes to the decrease in the total adenine nucleotide pool observed in the failing heart²⁵ (see 1.1.6). On the contrary, a functioning CK system, by maintaining low ADP levels, prevents this loss.

1.1.5 Creatine metabolism

Creatine, a β -amino-acid, is either taken up from alimentary sources (mostly meat) or is biosynthesized.

De novo synthesis is a two step process during which the substrates arginine and glycine are transformed to creatine following the action of two enzymes: L-arginine : glycine amidinotransferase (AGAT) and guanidinoacetate N-methyl transferase (GAMT) (Figure 1-1). AGAT is predominantly expressed in pancreas and kidney, whereas expression of GAMT is mostly localized in pancreas and liver. In addition, expression or activity of these enzymes has been detected in other organs, albeit at lower levels^{15, 26}. With regards to the cardiovascular system, a developmental study showed that blood vessels express AGAT whereas the heart does not have detectable levels of either enzymes²⁶. However, another recent study suggested that AGAT expression and activity was detectable in both human and

rat hearts and was reversibly increased in patients with heart failure²⁷. However, as no distinction was made between fibroblasts and cardiomyocytes, the relevance of this finding is unclear, especially without the concomitant presence of GAMT. It is also possible that AGAT might be involved in other biochemical pathways. Therefore, to this date, expression of AGAT and GAMT – even more concomitant expression – by the cardiomyocytes has never been demonstrated and it is commonly considered that the heart cannot synthesize creatine and hence relies exclusively on creatine uptake from the plasma.

Myocytes take up creatine from the bloodstream using a specific chloride- and sodium-dependant creatine co-transporter (CrT)²⁸. This is the only known mechanism for Cr to enter the cell and it occurs against a very large concentration gradient. Expression of this CrT has been described in many tissues including skeletal muscle, heart, smooth muscle, fibroblasts, neurons, red blood cells and macrophages¹⁵.

1.1.6 High-energy phosphate metabolism in heart failure

Changes in high-energy phosphate (HEP) are a hallmark of heart failure (HF). A decrease in Cr has consistently been observed in the failing human and animal heart regardless of the aetiology^{16, 29-32}. This drop occurs at a very early stage of HF, sometimes even at a compensated stage of cardiac hypertrophy before a true cardiac dysfunction develops^{31, 33}. In human patients and multiple animal models (dog, rat, pig, turkey, Syrian hamster), left ventricular (LV) creatine levels typically drop by 30-50 %^{29, 34-39}, while in mouse heart, consistently decrease by 11-16 % in two different models of HF¹⁶. As cardiomyocytes

cannot synthesize creatine, and as Cr is only lost by passive diffusion, this lower Cr content is the consequence of a reduction in creatine uptake³², probably mediated by a decrease in CrT protein content. Although a decrease in CrT protein levels⁴⁰ has been shown in patients with HF, the result has since been questioned as the anti-CrT antibody used has since been found to cross-react with other common proteins⁴¹. However, more recently, a reduction of expression of CrT gene was found by Lygate et al.¹⁶ in a mouse model of HF. It has been suggested that the progressive loss of Cr during the development of HF could be an adaptative process by minimising the expected increase in ADP, thus maintaining ATP/ADP ratio and the free energy of ATP hydrolysis^{33, 42}.

Associated with these changes in CK reaction substrates, a decrease in CK activity and in CK flux is observed in HF^{16, 29-31, 38}. This decrease in CK activity is the consequence of a reduction in both mt-CK and MM-CK activities^{16, 34, 36}. These changes impair phosphoryl transfer capacity increasing the cost of mechanical work and decreasing contractile reserve^{37, 38, 43}. Finally, a correlation between the maximum achievable CK reaction velocity (CK activity x [Cr]), and cardiac function was found in the mouse and hamster hearts, suggesting a tight coupling between CK changes and progression of heart failure^{16, 44}.

At the early stages of HF, ATP levels are maintained at the expense of PCr. Therefore, a reduction in PCr/ATP ratio is observed in the hypertrophied and failing human and animal myocardium⁴⁵⁻⁴⁸. This parameter, which can be measured by phosphorus magnetic resonance spectroscopy (³¹P-MRS), is considered as one of the most important indicator of the metabolic status of the human heart as it can be measured non-invasively by ³¹P-MRS⁴⁹. It has been shown to be a better prognostic indicator in patients with dilated cardiomyopathy than the traditionally used New York Heart Association class or LV ejection fraction (EF)⁵⁰. However, this marker is only used as a surrogate of absolute measurement in the absence of

a widely available method to non-invasively quantify PCr and ATP. In the presence of reduced ATP levels, this factor will underestimate the true extent of HEP depletion.

Indeed, as HF progresses, PCr fails to keep the total adenine nucleotide (TAN = ATP + ADP + AMP) pool adequately phosphorylated, hence an increase in ADP and AMP levels, which results in a loss of adenine nucleosides through sarcolemmal diffusion (see 1.1.4) and a decrease in ATP levels in severely failing hearts^{33, 36, 37, 51}. This slow but steady reduction in TAN and ATP correlates with disease progression³³. However, even in severe HF, ATP levels decrease by no more than 30 %^{1, 33, 43}, which corresponds to a critical tipping point (decrease of TAN pool by 30 %) below which the energy state of the myocardium is severely compromised⁵².

A few adaptations to these alterations in the CK energy transport system have been described. A change in the relative distribution of the different CK isoenzymes towards a more foetal profile with an increase in BB isoform has been shown^{16, 39} and is suggested to be compensatory due to its higher affinity for ADP⁵³. However, absolute concentrations of BB-CK remain so low (< 3 % of total CK activity in the infarcted mouse heart) that it is unlikely to have functional consequences¹⁶.

Compensatory changes in the other phosphotransfer networks have been described. In the pacing dog model of HF, Dzeja et al.¹⁴ showed a doubling in the contribution of AK-mediated phosphotransfer (from 10 % of the total ATP-turnover in the normal myocardium to 21 % in the failing heart), despite a decrease in AK activity. However, the compensation for the decrease in CK flux (from 89 % to 40 %) was only partial, in spite of an overall maintained ATP turnover rate, suggesting the existence of other compensatory – less efficient – pathways such as diffusion or glycolysis. In the mouse heart, our laboratory showed in two different models of heart failure unchanged AK activity but an increase in the

activity of glycolytic phosphotransfer enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) & 3-phosphoglycerate kinase (PGK)⁵⁴. The PGK shuttle plays a role in intracellular ADP removal¹³; the negative correlation between PGK and CK in the mouse heart suggest a compensatory role of PGK in maintaining ATP/ADP ratio high. The fact that in these two models AK activity was not increased indicate that there is a limit to how much AK fluxes can compensate for the reduction in CK phosphotransfer. Hence, these glycolytic pathways could represent an important compensatory mechanism in the failing heart and further studies are required to determine how this translates in terms of ATP flux⁵⁵.

1.2 The high burden of heart failure and the necessity for new therapeutic targets

The changes in myocardial energetics described above suggest that the failing heart may be energy-starved. This is further supported by the observation that energy-sparing drugs improve the prognosis of HF, whereas energy-costly treatments are deleterious⁵⁶. Therefore, modulating energy metabolism could provide new – and much needed – therapeutic alternatives for the treatment of HF.

Due to its prevalence and poor prognosis, heart failure is a substantial societal burden. Multiple causes can lead to this complex clinical syndrome, characterised as the inability of the heart to supply sufficient blood flow to meet the needs of the body. In the United Kingdom and Western world, hypertension and ischaemic heart disease are the two

commonest causes of HF^{5, 57}. In South America, it is Chagas disease, the result of parasitic trypanosoma infection, which nevertheless still results in typical impairment of HEP metabolism⁵⁸.

In Britain, HF affects at least one person out of 100, and this number is anticipated to rise in the next few years due to the combined effect of improved survival in patients with cardiovascular disease (CVD), and an ageing population⁵. Despite improvement in patient care with the use of pharmacological agents (such as β -blockers or angiotensin-converting enzyme (ACE) inhibitors) or resynchronisation therapies, the prognosis remains poor with about 30 % of patients dying within one year of initial admission for HF⁵.

In addition, the direct and indirect cost of CVD for the society is extremely heavy, constituting 17 % of overall national health expenditure in the United States of America⁵⁹. This is higher than for any other disease group⁶⁰ and is expected to worsen over the next few decades⁵⁹.

Consequently, there is an urgent need for new, more effective therapies for HF and correction of energetic imbalance is one approach that has currently not been exploited. However, this will require a better understanding of high-energy phosphate (HEP) metabolism. Central to this will be the creation and subsequent cardiac phenotyping of genetically modified mouse models to study the consequences of targeted augmentation and ablation of the CK system.

1.3 Magnetic resonance (MR) for murine cardiac phenotyping

1.3.1 The need for a non-invasive technique

More than 20 years ago, the first cardiac-specific transgenic mouse was generated ⁶¹. Since then, the ability to manipulate the mouse genome has revolutionized the way we study the pathophysiology of cardiovascular disease and cardiac energetics is no exception.

Using pharmacological inhibitors of HEP metabolism or loss-of-function transgenic mice, the consequences of an impaired HEP transfer can be studied. Mice models could also be the key to test whether increasing energy fluxes is beneficial in heart failure. However, mouse research is hampered by technical barriers to fully characterize these models *in vivo*. In particular, since the CK system is principally thought to be functionally important under conditions of high workload, it is critical to assess cardiac function and metabolism at physiologically-relevant workloads, which are not obtainable *ex vivo*.

Magnetic resonance (MR) is an ideal tool to achieve this, since it can uniquely measure structure, function and biochemical metabolites *in vivo* longitudinally. However, its application on mouse heart is a technical challenge and requires a highly specialised set-up.

1.3.2 Cardiac magnetic resonance imaging (MRI)

High-resolution cine-MRI is the most widely used application in murine cardiac MR⁶²⁻⁶⁶. Fast, 2D spoiled gradient echo (GE) sequences with very short repetition times (TR) allow the acquisition of data from the same slice at multiple time points of the cardiac cycle. The number of frames per cardiac cycle depends on the sequence timing and heart rate (HR) of the mouse⁶⁷.

This technique allows for morphological and functional examination of the heart. In addition to the thoroughly validated LV mass measurement^{64, 65, 68, 69}, cine-MRI can also quantify wall thickness, wall thickening, LV volumes, and subsequently stroke volume (SV), cardiac output (CO) and ejection fraction (EF)^{67, 70, 71} (see Table 2-2). Finally, it permits measurement of infarct sizes on surgical models of MI; using the multi-frame ability of the technique, infarcts are easily recognized as a zone of akinesis usually accompanied by a thinning of the myocardium^{67, 72}. In order to detect more accurately the infarcted area, gadolinium contrast-enhanced techniques can also be used at least in the acute stage of myocardial infarction⁷³.

Recent developments have focused towards reducing scan times, improving hardware (e.g. use of multi-array coils⁷⁴) or optimizing data acquisition using compressed sensing⁷⁵. Using these techniques, global cardiac function examination can now be completed in a few minutes versus 15-30 minutes with more traditional methods. This is particularly useful for haemodynamically compromised mice, for example with HF, to reduce anaesthetic exposure or increase the number of parameters measured in one examination.

More advanced techniques can also be used to assess wall motion⁷⁶⁻⁷⁹ and myocardial perfusion^{80, 81}. These techniques, which allow for a more thorough examination of cardiac function, will not be detailed further.

1.3.3 Cardiac magnetic resonance spectroscopy (MRS)

Magnetic resonance spectroscopy (MRS) is the only available technique to non-invasively study tissue metabolism without the need to inject radiolabelled tracers, as is required with single-photon emission computed tomography (SPECT) or positron emission tomography (PET)^{49, 82}. The most frequently used nuclei in cardiac MRS are phosphorus (³¹P) and proton (¹H), as they provide insight in energy and creatine metabolism.

1.3.3.1 Phosphorus magnetic resonance spectroscopy (³¹P-MRS)

³¹P-MRS has been used on human heart to study high-energy phosphate metabolism in numerous cardiac diseases such as myocardial infarction⁸³, dilated cardiomyopathy^{50, 84} or hypertensive heart disease^{45, 85}. In these studies, PCr/ATP ratio has been used as the main indicator of the metabolic status of the heart (see Singhal et al.⁴⁹ for an extensive review). In animal models, ³¹P-MRS has mostly been performed on the isolated heart⁸⁶⁻⁹⁰. However, it has widely contributed to the understanding of the CK system (see 4.1). It allows quantification of ATP (with its three characteristic resonances: α -ATP, β -ATP, γ -ATP), PCr and Pi resonances, which are well resolved due to the high chemical shift between these species (Figure 1-2). Intracellular pH can also be calculated from the chemical shift between Pi and PCr, which is pH-dependent^{82, 91}. Finally, chemical exchange between PCr and ATP, and thereby flux through the CK reaction can be measured by magnetization transfer^{92, 93}.

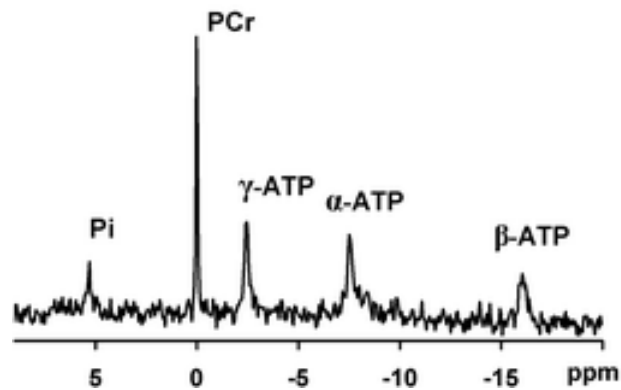


Figure 1-2: ^{31}P -MR spectrum of an isolated, perfused wild-type mouse heart. PCr, the three phosphate groups of ATP (γ -, α -, and β -ATP) and inorganic phosphate (Pi) can be clearly identified. This spectrum is reproduced from the review by ten Hove and Neubauer⁸².

In vivo application of ^{31}P -MRS is limited due to the relatively low MR-sensitivity of the phosphorus nucleus (about 15 times less sensitive than ^1H nucleus). It has been described in the canine^{47, 94-96}, rat⁹⁷ and even mouse⁹⁸ heart. However, in the rodent experiments cited above, the examination was performed on open-chest with the surface coil lying immediately below the heart to increase signal-to-noise (SNR) and avoid contamination from chest skeletal muscle. To date, non-invasive ^{31}P -MRS in the mouse heart has only been described by a limited number of groups world-wide⁹⁹⁻¹⁰¹. Moreover, all these *in vivo* experiments are limited by the low spatial resolution and contamination by signal arising from chest wall, liver or blood.

Compared to spectra from isolated hearts, two additional resonances appear *in vivo*: a resonance overlapping with Pi, which has been assigned to the 2,3-diphosphoglycerate (2,3-DPG) and is due to blood contamination, and a signal arising from serum phospholipids (phosphodiesteres). This renders measurement of intracellular pH difficult *in vivo*^{49, 82}.

1.3.3.2 Proton magnetic resonance spectroscopy (^1H -MRS)

Despite the high abundance of hydrogen nuclei, ^1H -MRS has not been as widely used both in human or animal hearts, partly because most of the information on high energy metabolism can be obtained from ^{31}P -MRS but also due to the technical difficulties associated with the development of the technique⁴⁹ (for a detailed discussion of the difficulties involved, see 3.1.). However, it allows for measurement of total creatine pool, such that combination of ^1H -MRS and ^{31}P -MRS would provide a more complete profile of the Cr/PCr/CK system. Furthermore, it also provides information on triglyceride metabolism (Figure 1-3). This technique will be described in detail in Chapter 3.

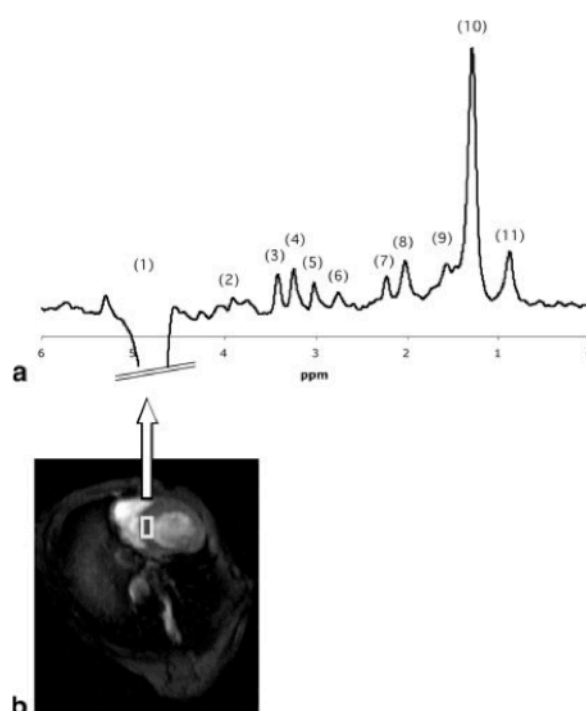


Figure 1-3: **a:** Cardiac volume-selective ^1H -spectrum from a voxel positioned in the septum of a wildtype mouse as shown in **(b)**. The resonances were assigned as residual water signal (peak (1)), (2) (P)Cr – CH_2 , (3) taurine, (4) carnitine, (5) (P)Cr – CH_3 , (6) unassigned, (7) glycerides (C_αH), (8) unassigned; (9) glycerides (C_βH), (10) glycerides ($-\text{CH}_2$)_n, (11) glycerides terminal methyl. The residual water signal is the result of imperfections in water suppression. This spectrum is reproduced from Schneider et al.¹⁰².

1.3.3.3 Other nuclei

Less widely used nuclei include ^{23}Na , ^{13}C , ^{19}F or ^{87}Rb ⁴⁹. ^{23}Na has, for example, been applied on isolated rat hearts to evaluate myocardial viability using a shift reagent to discriminate between intra and extracellular sodium¹⁰³. ^{13}C -MRS is limited by the very low natural abundance of ^{13}C . However, ^{13}C -labelled metabolites or hyperpolarized labelled metabolites (which increase signal by at least 10,000 fold) have been successfully used to study the tricarboxylic acid cycle^{104, 105}.

1.3.4 Technical challenges of murine cardiac MR

Due to the very small size of the mouse heart ($\sim 1/2000^{\text{th}}$ the mass of human heart) and the high heart rate (HR) (~ 600 beats per min (bpm)), specific hardware and techniques developed for the mouse heart are necessary⁶⁷. Even though examination using 1.5 T human whole-body system have been described⁶⁹, higher-field magnets with high performance gradients are typically used to improve SNR, and thus, spatial resolution and scan time⁷¹. Therefore, most studies have been performed at 4.7 – 11.7 T⁷¹, but with a tendency to always higher magnetic field strength, murine cardiac images have been acquired at up to 17.6 T⁷⁷. However, the use of high-field strength is not trivial due to an increase in magnetic field inhomogeneities and a greater susceptibility artefact⁷¹.

During cardiac MR, mice need to be anaesthetised for the purpose of immobilisation. Isoflurane (ISF) is commonly the anaesthetic of choice due to its ease of administration, its rapid onset and recovery and its reproducibility in attaining adequate anaesthetic depth; most importantly, it causes minimum cardiac depression^{106, 107}. It is vitally important to monitor

and maintain body temperature, as due to their small body size, mice are prone to hypothermia, which not only jeopardizes their health but also impacts on cardiac function^{67, 71}. Cardiac and respiratory signals are acquired for physiological monitoring and gating purpose. Most cardiac MR sequences use electrocardiographic (ECG) signal to synchronize acquisition with a specific period of the cardiac cycle, thus limiting motion artefacts and/or allowing examination of changes occurring during the cardiac cycle^{67, 71}. Finally, specific adaptations of pulse sequence traditionally used in patients are required for use at high magnetic field and on fast-beating heart⁶⁷.

1.4 Scope of this thesis

It remains a matter of debate whether the changes observed in the Cr/PCr/CK system are causative or adaptative. Therefore, the aim of this thesis was to contribute to the understanding of creatine implication in the pathophysiology of heart failure.

One way to address the role of creatine is to study models of reduced creatine levels to determine to what extent this directly affects cardiac function. Therefore, in **chapter 4**, I have studied the consequences of creatine depletion using a knockout mouse model of Arginine:glycine amidinotransferase (AGAT^{-/-}). This is the first description of this model and uses *in vivo* MRI, ¹H-MRS, and LV catheterisation to characterise the cardiac phenotype under conditions of gradually reducing creatine, zero creatine, and attempted phenotype rescue with dietary creatine.

However, loss of function models are often difficult to interpret due to wide-ranging adaptations and biochemical redundancy. A more direct approach is to determine whether augmenting energetics is beneficial by testing in an appropriate model. As argued by Ingwall¹⁰⁸, there are too few studies aimed at improving energy metabolism and testing whether this delays the development of HF. These strategies could prove to be valuable in enhancing cardiac function in patients with heart failure. Hence, in **chapter 5**, I sought to enhance cardiac energetics by increasing both total creatine and total adenine nucleotide pools and I assessed whether these interventions are beneficial in a surgical model of heart failure secondary to myocardial infarction.

As a prerequisite to longitudinal studies, a method to non-invasively measure creatine in murine models was required. Consequently, in **chapter 3**, after *ex vivo* validation of the technique, I assessed whether ¹H-MRS could be used to reliably measure myocardial creatine levels. I then developed a set-up, which allows a broader evaluation of the creatine status of the mouse by measuring creatine levels within a single anaesthesia in the three main creatine containing organs: heart, brain and skeletal muscle.

Chapter 2

GENERAL MATERIAL

&

METHODS

This chapter describes general techniques used in multiple chapters of this thesis. Specific methods will be described in the relevant chapters.

2.1 Mouse husbandry and genotyping

2.1.1 General husbandry

All mice were kept in a specific pathogen free environment, with controlled humidity and temperature (20 °C to 22 °C) and a 12 hours light-dark cycle. With the exception of AGAT^{-/-} (see chapter 4), all mice were fed standard chow (which is naturally creatine-free) and water *ad libitum*. All experiments were approved by the institutional ethical review committee and conform with the UK Home Office Guidance on the Operation of the Animal (Scientific Procedures) Act, 1986 (HMSO).

C57BL/6J mice were bought from a commercial breeder (Harlan, UK), whereas transgenic animals were bred on-site.

2.1.2 Transgenic mice generation

2.1.2.1 Creatine-transporter overexpressing mice (CrT-OE)

Transgenic mice over-expressing the creatine transporter (CrT-OE) have been created by our laboratory⁸⁷. Two strains of this mouse model were used in this thesis (the Tg46 and Tg55

strains) (see relevant sections for more details). Both strains have been backcrossed to C57BL/6J for more than 10 generations.

All animals used were generated by mating a male transgenic to a female C57BL/6J, thus producing 50 % of transgenic and 50 % of wild-type (WT) littermates at each generation.

2.1.2.2 Arginine:glycine amidinotransferase knockout mice (AGAT^{-/-})

Arginine:glycine amidinotransferase knockout mice (AGAT^{-/-}) were generated by Prof. D. Isbrandt's laboratory (Hamburg) by homologous recombination in embryonic stem cells. Mice heterozygous for the knockout allele (AGAT^{+/-}) were backcrossed for at least 8 generations onto a C57BL/6J background.

All mice used were bred by intercrossing AGAT^{+/-} thus generating a mix of knockout (AGAT^{-/-}), heterozygous (AGAT^{+/-}) and WT littermates (AGAT^{-/-}). After weaning and genotyping, knockout mice were housed separately from heterozygous and wild-type littermates to prevent creatine ingestion via coprophagia.

2.1.3 Genotyping

All transgenic animals were weaned and identified by ear biopsy at 21 days.

Ear biopsies were then digested in 500 µL lysis buffer (100 mM EDTA, 50 mM Tris-HCl (pH 8), 1 % SDS, 200 mM NaCl) supplemented with 3.3 µL Proteinase K (20 mg/mL) (Bioline, UK). After overnight incubation at 25 °C, DNA was purified by isopropanol/ethanol precipitation. After addition of 600 µL isopropanol, samples were mixed by inversion, then spun at 13,000 rpm for 10 min at room temperature. Supernatant was

discarded and pellet washed and re-spun in 500 μ L of 70 % ethanol. After removal of the supernatant, the pellet was air-dried at room temperature for 5 min. Finally, the DNA was resuspended overnight in 300 μ L T0.1E.

Polymerase chain reaction (PCR) was then performed on the genomic DNA using Immolase DNA Polymerase (Bioline, UK). Reaction mix, primers sequences and PCR programs used for the genotyping of CrT-OE and AGAT^{-/-} mice is given in Table 2-1. Final products were analysed by electrophoresis on a 1 % agarose gel supplemented with ethidium bromide (50 μ g of ethidium bromide per 100 mL of gel).

	CrT-OE	AGAT ^{-/-}
MASTER MIX COMPOSITION	10 X Immobuffer 2.5 μ L dNTPs (8 mM solution (2mM of each)) 2.5 μ L MgCl ₂ (50 mM) 0.75 μ L Primer Forward (100 μ M) 0.5 μ L Primer Reverse (100 μ M) 0.5 μ L Immolase 0.5 μ L Water 14.75 μ L	10 X Immobuffer 2.5 μ L dNTPs (8 mM solution (2 mM of each)) 1.63 μ L MgCl ₂ (50 mM) 0.6 μ L Primers (F and R mix: 2 μ M each) 6 μ L Immolase 0.3 μ L Betaine (5 M) 5 μ L DMSO 1.25 μ L Water 2.72 μ L
PCR CONDITIONS	Initial denaturation: 95 °C, 10 min Denaturation: 95 °C, 30 sec Annealing: 67 °C, 30 sec Extension: 72 °C, 30 sec 30 cycles	Initial denaturation: 94 °C, 5 min Denaturation: 94 °C, 60 sec Annealing: 65 °C, 60 sec (- 0.5 °C at each cycle) Extension 72 °C 30 sec 10 cycles Denaturation: 94 °C, 60 sec Annealing: 60 °C, 60 sec Extension: 72 °C, 30 sec 35 cycles
PRIMERS	Forward: 5'-gcattctcatcttcaacatcgta-3' Reverse: 5'-tcacagatcctcttctgagatgag-3'	Forward: 5'-agccctctatttccctttcatt-3' Reverse: 5'-ttccactgcgtcattctcgttaa-3'

Table 2-1: Genotyping protocol for CrT-OE and AGAT^{-/-} mice.

2.2 Magnetic resonance – General considerations

2.2.1 MR system

All nuclear magnetic resonance (NMR) experiments were carried out on a 9.4 T (400 MHz) MR system (Agilent Technologies, USA) with a 210 mm diameter horizontal bore, a VNMRs Direct Drive™ console and actively shielded gradient system (600 mT/m, rise time 180 µsec, outer diameter (od) 205 mm, inner diameter (id) 120 mm) used for ³¹P-MRS and a gradient insert (1000 mT/m, rise time 130 µs, od 115 mm, id 60 mm) used for ¹H-MRS and cine-MRI.

2.2.2 Animal preparation

After induction of anaesthesia in an anaesthetic chamber (4 % isoflurane (ISF) in 100 % oxygen), mice were positioned prone in a purpose-built animal cradle and maintained at 1.5-2 % ISF throughout the duration of the experiment. ECG signal was obtained with electrodes inserted sub-cutaneously in both forelimbs. Respiratory function was detected with a pressure pad positioned under the abdomen. Physiological parameters (respiratory and cardiac signals) were monitored and processed with an in-house-built gating device¹⁰⁹. Temperature was maintained constant at ~37 °C using an air-heated blanket.

2.2.3 Cine-MRI and analysis

2.2.3.1 Data acquisition

Cine-MRI was performed as described in Schneider et al.⁶⁷ A quadrature-driven bird cage coil (id 33 mm – Rapid Biomedical, Rimpur, Germany) was used to transmit/receive the magnetic resonance signals. Localizer scans and scouting for the short axis were carried out. The coil was then tuned and matched prior to each experiment and shimming performed over the cardiac volume using an automatic shimming technique as previously described¹¹⁰. After calibration of the pulse, seven to nine contiguous cine slices were acquired in short axis orientation to cover the entire heart from base to apex. The parameters used were as follow: TR (repetition time) = 4.6 ms, TE (echo time) = 1.79 ms, 15° flip angle, thickness = 1 mm, field of view: 25.6 x 25.6 mm, resolution for high-resolution scans used in chapter 5: matrix size = 256 x 256, number of averages (NA) = 2 & resolution for low-resolution scans used in chapter 4: matrix size = 128 x 128, NA = 1. The number of frames acquired per slice was determined by the respective heart rate but was usually in the range of 15-30 frames. The acquisition of the cine-MRI data set *per se* lasted about 10 (low resolution) to 25-30 minutes (high resolution) and the whole experiment (from anaesthesia induction to recovery) less than one hour. Cardiac and respiratory physiology was monitored throughout the whole length of the experiment to insure the right depth of anaesthesia is reached and maintained (HR~400-600 bpm, RR~20-60 respiratory movements per min). The time required for localisation scans, tuning and matching and shimming allowed for anaesthesia equilibration before cine-MRI itself was acquired.

2.2.3.2 Data analysis

General segmentation

Images were reconstructed using purpose-written IDL-software (Research Systems International, Crowthorne, Berkshire, UK) before segmentation with Amira 4.1.2-1 (Mercury Computer Systems, Berlin). End-diastolic and end-systolic frames were selected according to maximal and minimal ventricular volume. Myocardial volumes in systole and diastole, Left Ventricular End Systolic and Diastolic Volumes (ESV and EDV respectively) were measured by summing up the respective volumes measured for each slice. Myocardial mass was subsequently calculated using the mean of myocardial volume in systole and diastole and considering a myocardial density of 1.05 g/mL. Functional parameters were then calculated (Table 2-2). The parameters assessed during cine-MRI are summarized in Table 2-2.

Parameter	Method of assessment	Unit
Heart rate (HR)	Directly (ECG)	bpm (beats/minute)
Respiratory rate (RR)	Directly (pressure pad)	/minute
End systolic volume (ESV)	Segmentation of cine-MRI images	μL
End diastolic volume (EDV)	Segmentation of cine-MRI images	μL
Mean left-ventricular mass (LVM)	Segmentation of cine-MRI images and correction for myocardial density Average of masses estimated from systolic and diastolic frames	mg
Stroke volume (SV)	EDV-ESV	μL
Ejection fraction (EF)	$(\text{SV}/\text{EDV}) \times 100$	%
Cardiac output (CO)	SV x HR	mL /minute

Table 2-2: Typical physiological parameters assessed during a cine-MRI experiment (modified from Schneider *et al.*⁶⁷).

Measurement of infarct size

Infarct size was measured using ImageJ (version 1.44 for Mac). For each slice, the length of the infarcted area and the total circumference were measured in diastole, mid-way between the endocardium and the epicardium. The infarct size (in % of total LV) was calculated by dividing the sum of all infarct lengths by the sum of all LV circumferences^{67, 111}. In order to distinguish between infarcted and healthy myocardium using cine-MRI images, it was necessary to assess changes between systole and diastole using the multi-frame capability of the technique. The infarcted area is characterized by a thinning of the myocardium and an akinesia, whereas a thickening is observed in systole in healthy myocardium⁶⁷.

2.3 Haemodynamic measurements

2.3.1 General principles

Measurements were performed in closed-chest, spontaneously breathing mice as described by Nemoto et al.¹¹². After induction with 4 % ISF in 100 % O₂, anaesthesia was maintained with 2 % ISF during the surgical procedure. Animals were positioned supine on a controlled heating pad. A Millar cannula was inserted in the right carotid and advanced in the left ventricle under ultrasound monitoring. A stretched polyethylene cannula was inserted in the right jugular for administration of dobutamine. The mouse was then maintained at 1.5 % ISF and allowed to equilibrate for at least 15 minutes. Data were continuously recorded using a Powerlab 4SP Chart recorder (AD instruments). The parameters measured are summarized in Table 2-3.

At the end of the experiment, the mouse was sacrificed; organs were excised, weighed and the left ventricle frozen in liquid nitrogen and stored at – 80 °C until further analysis.

Parameter	Unit
Heart rate (HR)	bpm (beats/minute)
Left ventricular systolic pressure (LVSP)	mm Hg
Left ventricular end-diastolic pressure (LVEDP)	mm Hg
Rate of pressure development (dP/dt _{max})	mm Hg/s
Rate of pressure decline (dP/dt _{min})	mm Hg/s
τ or tau	ms

Table 2-3: Typical parameters studied during invasive assessment of cardiac haemodynamic function by LV cannulation.

2.3.2 Study-specific differences

Haemodynamics protocol performed on AGAT^{-/-} mice (Chapter 4)

Due to the very small size of these animals (~15 g), a 1F ultra-miniature pressure only Millar mikro-tip catheter (SPR-1000, Millar instruments, Houston, Texas) was used. Protocol consisted of recordings at baseline followed by infusion of low and high dose dobutamine (4 and 16 ng/g/BW/min respectively) through the jugular vein. Previous studies have determined 16 ng/g/BW/min dose to elicit maximal contractile response.¹¹³ Data were analysed manually using Chart 5 (AD Instruments) from at least 12 cardiac cycles; cardiac cycles acquired during respiratory events were discarded. The pressures were corrected for the baseline drift measured at the end of the experiment.

Haemodynamics protocol performed on infarcted mice (Chapter 5)

A 1.4 F ultra-miniature pressure-volume Millar mikro-tip catheter (SPR-839, Millar instruments) was used and recordings were performed at baseline and high dose dobutamine (16 ng/g BW/min). Data were then analysed automatically using the PVAN software version 3.0 (Millar Instruments, Houston, Texas) over at least 20 cycles, discarding cardiac cycles acquired during respiratory events. The pressures were corrected for the baseline drift measured at the end of the experiment.

2.4 High performance liquid chromatography (HPLC)

Creatine concentration was measured by HPLC as previously described¹¹⁴. Frozen myocardial tissue was powdered in a percussion mortar cooled in dry ice. An aliquot of ~10 mg of tissue was homogenised in 550 μ L of 0.4 N perchloric acid at 0°C. An aliquot of 100 μ L was removed for protein quantification. The homogenate was neutralized with potassium hydroxide (KOH) before centrifugation for five minutes at 5500 rpm at 4°C, filtration of the supernatant through a 0.45 μ m filter and injection in the column (Supelco, LC-18-T reverse phase column, 25 cm x 4.6 mm, 5 μ m pore size). The column was run by a Jasco PU-2089Plus Pump and detection of the signal performed by a Jasco UV-2075Plus UV-detector. The signal was analysed with Azur v.3.0 (Datalys).

Non-collagen protein was measured according to the method described by Lowry et al.¹¹⁵. An aliquot of 100 μ L of the above-mentioned homogenate was mixed with 100 μ L of NaOH and incubated at 60°C for two hours. After dilution at 1/50 (v/v) in water, one mL of the sample was mixed with one mL of Lowry reagent and left at room temperature for 20 minutes before adding 500 μ L of Folin's reagent. After 30 minutes at room temperature, the absorbance was measured at 750 nm (Ultrospec 2100Pro). All samples were measured in triplicate and calibrated against a standard curve obtained in the same conditions with five Bovine Serum Albumine (BSA) concentrations (range: 0-80 μ g/mL).

2.5 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.0c for Mac and GraphPad InStat version 3.1a for Mac. Differences were considered statistically significant for $p < 0.05$.

The tests used within this thesis were as follow:

- for two groups comparison, paired or unpaired Student t-test were used where appropriate;
- for multiple groups comparison, one-way or two-way ANOVA with or without repeated measures were used. Bonferroni correction for multiple comparison was used to compare all groups between each other, whereas Dunnett post-hoc test was employed to compare all groups vs a control/baseline group;
- for analysis of contingency tables, χ^2 -tests were used;
- for analysis of the relationship between two quantitative variables, simple linear regression were used. R^2 estimated the goodness of fit and an F test was used to test whether the slope was significantly different from zero.

Chapter 3

NON-INVASIVE ASSESSMENT OF CREATINE LEVELS BY ^1H -MRS

3.1 Introduction

To date, ^1H -MRS is the only available technique to assess directly, non-invasively and quantitatively metabolites such as creatine in various organs. However, it is a demanding and challenging technique with multiple pitfalls, which need to be recognised and avoided in order to get reliable results.

3.1.1 Challenges of ^1H -MRS^{82, 91, 116}

Due to the high natural abundance of proton in living beings (> 99.9 %) and the high intrinsic sensitivity to NMR of this nucleus, ^1H -MRS can be seen as a very powerful technique to assess a very wide range of metabolites. However, *in vivo* application of ^1H -MRS is a challenging technique for multiple reasons.

First, the concentration of metabolites of interest is more than 10,000 lower than that of water (mM range for most metabolites versus a tissular water concentration of circa 100 M). Consequently, it is necessary to suppress this dominant signal either during acquisition and/or at post-processing. Secondly, the range of chemical shift for ^1H -MRS is very narrow (less than 5 ppm), which requires excellent magnetic field homogeneity over the volume of interest in order to discriminate between the metabolites. Thirdly, it is of utmost importance to reliably localize a volume of interest within the subject body, without being subjected to interferences from nearby tissue. Fourthly, ^1H -MRS remains a very insensitive technique, which necessitates a compromise between signal to noise ratio (SNR) and time resolution to detect the low concentration metabolites. Finally, on top of these general considerations, the

technical challenges are increased in murine MRS and even more in cardiac MRS due to the small size of the animal and cardiac / respiratory motion.

3.1.2 Localized spectroscopy^{117, 118}

An accurate localization is a prerequisite for the acquisition of good quality spectra. Not only is it necessary to minimize “partial volume effects” (i.e. contamination of the signal arising from one compartment by the signal from another), but also to improve spectral resolution. Indeed, shimming, the process consisting of optimizing the magnetic field homogeneity, is easier to perform over a small volume. Consequently, a good spatial resolution gives narrower resonances, helps exclude broad unwanted resonances and improves water suppression efficiency.

Two types of spatial localization are principally used:

- the single voxel technique, which is the technique used in this thesis;
- the multi-voxel technique or magnetic resonance spectroscopic imaging (MRSI).

A voxel (or volume element) designs a unit volume of a tissue, from which the signal arises.

MRSI or chemical shift imaging allows the detection of localized spectra from a multidimensional array of locations. Therefore, this technique is particularly well-suited for metabolite mapping within an entire organ. However, it is a particularly challenging technique, which has for now mostly been applied to human and mouse brain^{119, 120}. It requires a good homogeneity of the magnetic field over a large volume to resolve the resonances and achieve good water suppression. Finally, it involves long scan times due to the inherently slow encoding process.

On the contrary, single voxel MRS is more widely used for ^1H -MRS. The two most commonly used localisation techniques (STEAM and PRESS) select the volume of interest (VOI) within a single acquisition by applying three sequential frequency-selective radiofrequency pulses along three orthogonal axes.

In the Stimulated Echo Acquisition Mode (STEAM) sequence, three orthogonal slice selective 90° pulses are applied, which result in formation of four spin echoes and a stimulated echo. The stimulated echo is the signal of interest.

The point-resolved spectroscopy (PRESS) sequence is a double spin-echo, in which the slice selective excitation (90° pulse) is followed by two slice-selective refocusing pulses (180° pulses). The second spin-echo contains the signal from the intersection of the three planes (Figure 3-1).

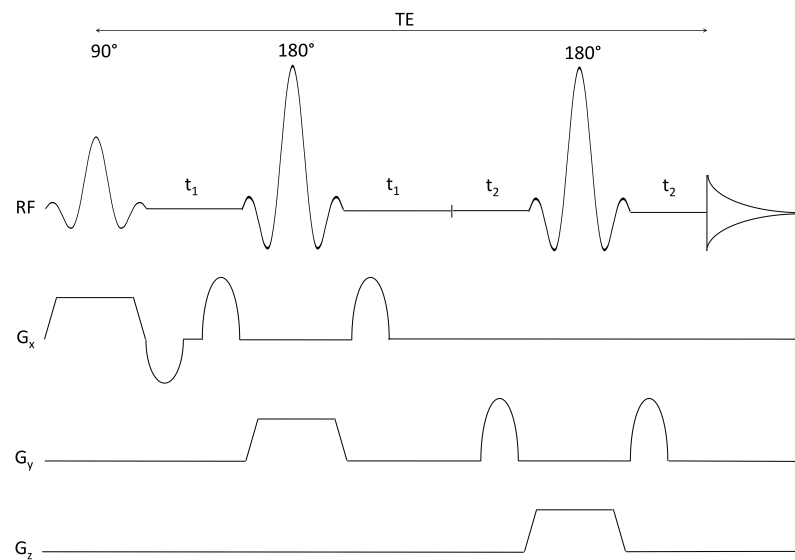


Figure 3-1: PRESS pulse sequence. Two pairs of crusher gradients surrounding the 180° are used to remove unwanted magnetization. The first 180° pulse is executed at a time t_1 after the 90° pulse and results in the formation of a first echo after a delay equals to $2t_1$. The second spin-echo refocuses this echo after a delay $2t_2$. Therefore the final spin-echo is formed at time $2t_1+2t_2$, which equals the total echo-time (TE) of PRESS. Adapted from de Graaf¹⁷.

STEAM was traditionally preferred on clinical scanners as, for a long time, it was the only technique allowing the use of short echo time. Moreover, since the technique uses only 90° pulses, a better delineation of the VOI is generally achieved with less profile contamination. Finally, it requires less radiofrequency (RF) power¹²¹. However, the main limitation of STEAM is its lower SNR. Due to the way the stimulated echo is generated, the SNR of STEAM is inherently half that of PRESS.

Nowadays, improved hardware and development of optimized pulse sequences brought the accuracy of the 180° slice profile to a similar standard as those obtained with STEAM¹²² and rendered the use of short echo-time possible even in PRESS^{123, 124}. Hence, PRESS is more and more used especially at high field on animal models, where power availability is not such a limiting factor. Indeed, in preclinical scanners, relative to human scanners, strong RF pulses may be used as concerns about potential side effects secondary to rapid tissue power absorption (expressed by the specific absorption rate or SAR) are more limited.

Whatever the technique employed, one needs to bear in mind that slice profiles will never be perfect and that background contamination is inevitable. Crushers are used to dephase unwanted coherences and improve the accuracy of spatial localisation. In addition, both techniques will suffer from a chemical shift displacement. The application of a frequency selective pulse in the presence of magnetic field gradient will select the targeted VOI only for on-resonance excitation. Metabolites off-resonance will be excited at slightly shifted voxel location. This artefact is more pronounced at higher magnetic field but can be minimized by using short RF pulses, thus increasing the spectral bandwidth of the pulse. As a matter of fact, it is always advised to use, when possible, a small voxel within a larger region of interest, which will reduce contamination from surrounding tissue. However, this needs to be carefully considered as it is done at the expense of SNR and/or experimental time.

3.1.3 *In vivo* cardiac MRS in the mouse

In vivo murine cardiac ^1H -MRS remains a cutting-edge technique. Whilst ^1H -MRS has been widely used on isolated rodent hearts¹²⁵⁻¹²⁷ and applied *in vivo* in dogs⁹⁵ and patients^{95, 128-130}, its use in mouse live heart was only described for the first time in 2004 by our group in an 11.7 T vertical bore magnet¹⁰². Since then, to our knowledge, only one group implemented the technique worldwide¹³¹. On top of the general ^1H -MRS challenges already described, specific hurdles have to be overcome. Cardiac and respiratory motion artefacts need to be avoided by using cardiac and respiratory gating. Moreover, the very small size of the mouse heart requires high spatial resolution and very sensitive coils. In these conditions, PRESS was the localization technique of choice due to the better SNR and the availability of strong gradients on our system, which minimizes voxel bleed. Moreover, cardiac spectroscopy has the advantage of being barely affected by signal bleed from surrounding tissues. Indeed, the contamination by blood is negligible as the water signal from moving blood is dephased by the strong gradients used for localisation. Due to this “black-blood” property of the PRESS sequence at high magnetic fields¹⁰², outer volume suppression is not required.

3.1.4 Spectral analysis and quantification

^1H -MRS can give a quantitative insight into organ metabolism. Indeed, in NMR the signal of each resonance is directly proportional to the concentration of the metabolite. There are three major steps in quantitative MRS: data acquisition (already described in details), data

processing and fitting and, finally, data calibration. These steps are summarised on a flow-chart in Figure 3-2.

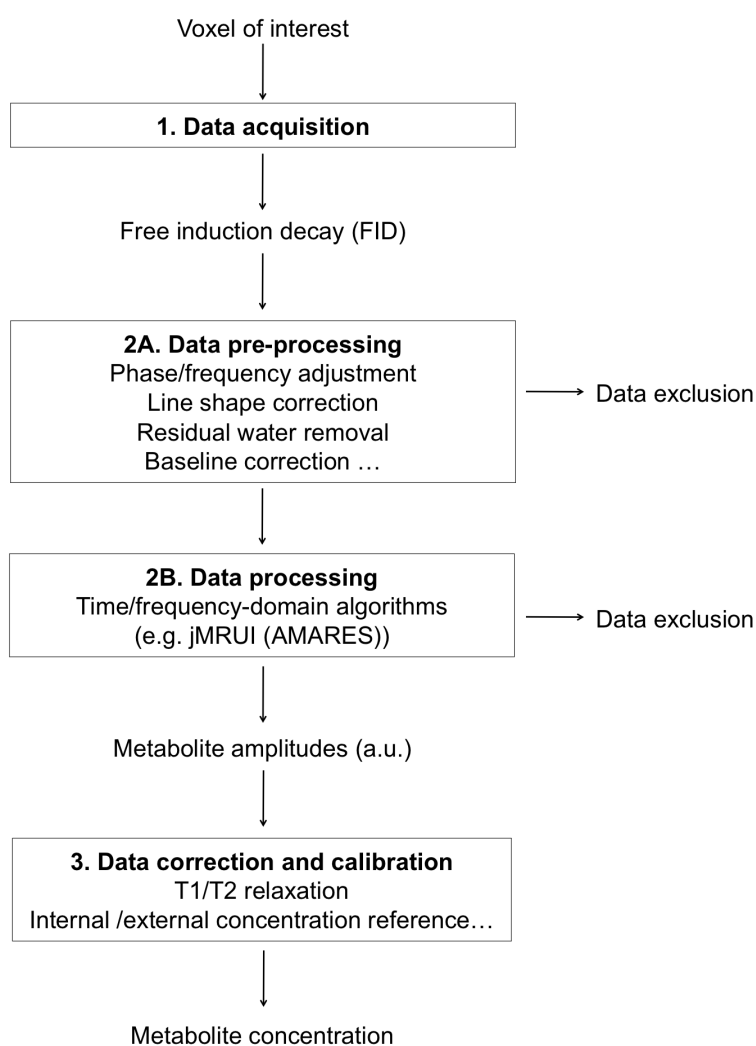


Figure 3-2: Flowchart for processing and quantification of NMR signals. Adapted from de Graaf⁴³²

Data processing and fitting

A few pre-processing steps are sometimes required to improve data quality before fitting. These can include phase, frequency or lineshape adjustments, removal of the residual water signal, correction for eddy current effects and baseline distortion^{132, 133}.

Quantification of spectral peaks can be performed in the time or the frequency domain. Both methods have advantages and drawbacks. However, even if the debate is still open regarding the best technique to use, they mostly give equivalent results¹³⁴.

Quantification in the time-domain can be done with AMARES within the jMRUI software¹³⁵, which allows inclusion of prior biophysical knowledge to improve the accuracy of the fitting. Quality of the fitting should then be assessed. Error estimates of the fit-values are given by the Cramer-Rao lower bounds (CRLBs). Kreis¹³⁶ proposed to reject the data if:

- the full-width at half maximum (FWHM) of metabolites is superior to 0.07-0.1 ppm;
- CRLBs (standard deviation of the amplitude) exceed 50 % of metabolite amplitude;
- there are unexplained features /artifacts in the residuals;
- the peaks are doubled;
- the lineshape is very asymmetric;
- there are outer volume ghosts or other artefacts.

Data calibration

The final step in metabolite quantification is the conversion of signal amplitude to biologically relevant data. There are two levels of quantification^{133, 137}:

- Relative quantification. This is the simplest method and compares the signal of the metabolite of interest to the signal of another metabolite usually from the same spectrum. In the brain, the metabolites of interest are generally compared to total creatine. Alternatively, water signal acquired from the same voxel, immediately before or after the metabolite spectra, can be used as an internal reference. This technique has the advantage of being easy to implement, as it does not require any correction. However, it supposes that the concentration of the reference metabolite remains constant, which may be incorrect, especially in pathological conditions. Results can be expressed by comparison to normal individual or in institutional units. However, one needs to be extremely careful when comparing results between different establishments due to possible variation in the level of saturation of metabolites. Finally, it has been shown that metabolite ratios exhibit higher coefficients of variation than absolute concentrations¹³⁸.
- Absolute quantification. With this technique, metabolite concentrations are expressed in standard biochemical units (molar or molal concentrations). It benefits from unambiguous interpretation but is more time-consuming and technically more challenging. The signal of interest needs to be calibrated against a reference of known concentration. Different strategies exist: external reference, replace-and-match method, water signal reference method or the principle of reciprocity. All these techniques have advantages and disadvantages and necessitate different correction factors (e.g. for B_1 inhomogeneities, relaxation times).

In this thesis, we used water signal as internal reference due to its ease of access (high concentration, acquisition from the same voxel as the metabolite of interest). However, a few assumptions are made when using water signal as reference, as listed by Christiansen et al.¹³⁹:

1. the metabolites and water are uniformly distributed within the voxel;
2. the water concentration is constant within the tissue even in pathological conditions;
3. the signal vary linearly with the concentration;
4. the signal vary with the volume size of the voxel in a homogeneous system;
5. the metabolite signal is fully MR-visible;
6. there is a high degree of volume selectivity i.e. the chemical shift between water and the metabolite of interest should be small to minimize the chemical shift displacement as described in 3.1.2. However, this pitfall can be overcome by acquiring water and metabolite spectra on their respective resonances, which we did in this thesis.

3.1.5 Is the entire pool of creatine MR-visible?

One factor that can bias spectral quantification is the partial NMR invisibility of some metabolites. Part of their pool may not contribute to the MR signal.

Various studies have shown a discrepancy between metabolite levels measured by NMR and by standard biochemical techniques. This is the case, for example, of lactate in rat and human brain cells¹⁴⁰ or creatine in the isolated rat heart¹²⁵. However, these findings are a matter of debate as Unitt et al.¹²⁷ in the isolated rat heart showed only a small difference

between the creatine measured by HPLC and NMR. Similarly, in't Zandt¹⁴¹ showed comparable results between ¹H-MRS and a spectrophotometric assay in mouse skeletal muscle.

Two main phenomena have been considered to explain this decrease in NMR visibility.

Compartmentation of creatine within the cell and binding to macromolecules or membranes could induce a decrease in its rotational mobility, hence shortening its spin-spin relaxation time (T_2). As a consequence, this pool of creatine will be NMR invisible with conventional MRS techniques, whereas the destructive nature of acid extraction for HPLC measurement will make all creatine detectable. Interestingly, the existence of two creatine pools has been suggested by experiments using isotopically labelled creatine in the rabbit heart¹⁴² already in 1961, and more recently in the trout fast-twitch skeletal muscle¹⁴³ and in isolated rat atria¹⁴⁴. In the latter case, Savabi¹⁴⁴ found that about 36 % of the creatine was bound to unknown intracellular components and could not readily participate in the phosphorylation process.

Moreover, magnetization transfer (MT) could explain a decrease in creatine visibility. The methyl resonance of creatine showed a strong signal intensity decrease upon off-resonance saturation in the rat¹⁴⁵ and human¹⁴⁶ brain, or in the skeletal muscle of the rat¹⁴⁷, mouse¹⁴⁸ or human¹⁴⁹. This MT effect is observable for all metabolites detectable by MRS but is particularly strong for the creatine resonance¹³² and involves both creatine and phosphocreatine¹⁵⁰. The exact mechanisms are not fully understood. Swanson et al.¹⁵¹ showed *in vitro* a MT effect from mobile water to lactate upon addition of bovine serum albumin, suggesting that immobilization is necessary for the induction of the MT effect. Kruiskamp and Nikolay¹⁴⁷ demonstrated that the role of exchangeable protons is negligible in the magnetic coupling of creatine protons in the skeletal muscle. Two main hypotheses remain: a direct intermolecular transfer from a pool of immobilized protons (not belonging to

creatine) to the creatine methyl protons or a chemical exchange of creatine molecules between a mobile and an immobile state. Using CK-knockout transgenic mice, a chemical exchange between a free, mobile and a CK bound motionally restricted creatine was ruled out¹⁴⁸. It is likely that the MT involves a cross-relaxation between total creatine and an immobile proton pool. The latter could consist of protons immobilised in structures such as high molecular weight globular proteins or in membrane proteins¹⁴⁸.

This MT needs to be taken into consideration during pulse sequence design. Indeed, some water suppression methods are more prone to the induction of partial saturation of the total creatine resonance via MT. Techniques involving a low amplitude presaturation of the water such as Water Eliminated Fourier Transform (WEFT)¹⁵² gave rise to strong MT effect, whereas a transient chemical shift selective (CHESS) water suppression element did not lead to detectable MT in the rat brain¹⁵³. However, it is anticipated that longer CHESS pulse blocks could cause unwanted decrease of the creatine resonance, which could explain the 30 % reduction in creatine amplitude observed by Schneider et al.¹²⁵ in the rat heart following CHESS water suppression¹⁴⁶.

Finally, MT experiments can be used to estimate the proportion of bound creatine by applying a two-pool exchange model in which magnetization from the mobile creatine methyl protons is transferred to an immobile pool of protons with solid-like relaxation properties. With this model, the proportion of bound creatine was estimated to be only 1 % in human skeletal muscle¹⁴⁹ and less than 3 % in the mouse and rat skeletal muscles^{148, 150}. This would imply that NMR invisibility of creatine is mostly accounted for by MT effects and not by the immobilization of a creatine pool.

3.1.6 Aims

As previously mentioned, ^1H -MRS is the only available technique to assess non-invasively creatine levels *in vivo*. Cardiac ^1H -MRS had already been described by our group on a vertical bore 11.7 T magnet; however, the value of MRS as a method to quantitatively measure creatine levels in the mouse had not been systematically assessed. Therefore, before applying the technique to phenotype animal models, it needed to be validated on our new horizontal bore 9.4 T magnet, in particular the quantitative aspects.

The aims of this chapter were:

1. To validate stability, repeatability and linearity of ^1H -MRS on phantoms.
2. To assess whether ^1H -MRS could be used to reliably quantify myocardial creatine levels in mice. The aim was to validate the technique against HPLC, the gold standard, over a wide range of myocardial creatine concentrations. It would then be possible to use it to non-invasively follow-up creatine levels in mice or to pre-select mice according to their myocardial creatine concentrations.
3. To contribute to the study of NMR creatine visibility in the heart. Whether or not MR can quantify the entire creatine pool is still a matter of debate, and a limited MR-visibility would have consequences for the interpretation of creatine estimations by ^1H -MRS.
4. And finally, to implement ^1H -MRS in the brain and skeletal muscle and to assess the repeatability of the technique in these organs and in the heart. Furthermore, we sought to determine the feasibility of measuring creatine levels in these three organs within a single anaesthesia. Ultimately, this would provide a tool yielding a wider

insight into creatine metabolism following pharmacological and/or genetic interventions.

3.2 Material and methods

3.2.1 General considerations

A quadrature-driven birdcage coil (i.d. 33 mm – Rapid Biomedical, Rimpfing, Germany) was used to transmit/receive the magnetic resonance signals. Prior to each experiment, the probe was tuned and matched manually. After localization of the voxel of interest, shimming was performed over a 4 mm diameter sphere using an automatic method as previously described¹¹⁰.

Pulses were then manually calibrated and spectra acquired using a PRESS sequence (TE = 8 ms and TR = 2 s, sinc pulses) with or without cardiac triggering/respiratory gating depending on the organ or phantom scanned. As water signal was used as an internal reference, two types of spectra were acquired successively: water-suppressed and unsuppressed. Water suppression was achieved using a CHESS module consisting of three frequency-selective RF pulses (bandwidth: 500 Hz) and doubling spoiler gradients.

3.2.2 Experiment specific methods

3.2.2.1 Phantoms experiments

Stability of the technique was assessed using a phantom consisting in a tube containing 40 mM creatine in water solution doped with 1 mM gadopentetate dimeglumine (Gd-DTPA) (Omniscan, Amersham Health). 256 scans without or with incomplete water-suppression were consecutively acquired on water-resonance without averaging (NA = 1) using an identical PRESS sequence as the one used *in vivo*. Respiratory-gating / ECG triggering was tested using electronically simulated signals. The experiment was repeated three times both for unsuppressed and water-suppressed scans.

To check the linearity of the technique, we used five phantoms of increasing creatine concentration (10, 20, 30, 40 & 50 mM creatine in water solution doped with 1 mM Gd-DTPA). For each concentration, we acquired spectra from 3 voxels and for each voxel, 3 water spectra on water resonance (NA=16) and 3 water-suppressed spectra on resonance with creatine (NA = 256) were obtained.

3.2.2.2 *In vivo* myocardial ¹H-MRS

Mice were prepared as previously described (see 2.2.2). The heart was positioned in the isocenter of the coil using localizer scans (cardiac-triggered and respiratory gated segmented fast low-angle shot (FLASH) sequence in the 3 orthogonal planes). Four contiguous, 1 mm thick axial slices were subsequently acquired and used to position the 2 μ L (2 x 1 x 1 mm) voxel of interest in the septal wall of the heart at the papillary muscle level (Figure 3-3).

Water (NA = 16) and water-suppressed scans (NA = 256) were then acquired interleaved and in triplicate using a cardiac triggered and respiratory gated PRESS sequence (TE = 8 ms, TR ~2 s (determined by the heart rate))¹⁰⁹. Steady state was maintained during respiratory events and water-suppression was calibrated for each mouse.



Figure 3-3: Localisation of the 2 µL PRESS voxel in the septal wall

3.2.2.3 Ex vivo ¹H-MRS on intact myocardium

Mice were sacrificed at the end of the *in vivo* experiment, the hearts were excised, and the right ventricular wall removed. After rinsing with saline, the left ventricle was split in two halves. The septum was embedded in fomblin® and four water-suppressed (NA = 64) and four unsuppressed (NA = 4) scans were immediately acquired from a 2 µL voxel using a non-gated version of the PRESS sequence. The second half of the left ventricle was frozen for further analysis (see 3.2.2.4).

3.2.2.4 ^1H -MRS on a myocardial extract

The frozen free-wall of the left ventricle was prepared using the same protocol as the one used for HPLC (see 2.4). HPLC was performed on an aliquot of this myocardial extract to measure creatine levels, whereas the remaining solution was subjected to ^1H -MRS. Three water-suppressed (NA = 6000) and three unsuppressed (NA = 16) spectra were acquired interleaved from an 8 μL voxel using the same PRESS sequence as for the *in vivo* and *ex vivo* in intact myocardium experiments. HPLC and MRS results were then standardized against the amount of proteins contained in the extract using a Lowry assay (see 2.4).

3.2.2.5 ^1H -MRS on brain and skeletal muscle

Mice were prepared as previously described. For brain spectroscopy, head immobilization was achieved using a bite bar and surgical tape to fix the head to the cradle. The left leg was taped to the cradle for the tibialis anterior to be parallel to the main magnetic field B_0 .

Localization of the voxel in both brain and skeletal muscle was done using a fast, multi-slice non-gated Spin Echo sequence.

In the brain, spectra were acquired from a 8 μl (2 x 2 x 2 mm) voxel localised in the thalamus, whereas a 4.5 μl (1.5 x 1.5 x 2 mm) voxel was used for the tibialis anterior (Figure 3-11). For each organ, three water-suppressed (NA=256) and three unsuppressed (NA=16) spectra were acquired interleaved using a non-gated PRESS sequence.

3.2.3 Data analysis

Spectra were fitted in the time-domain using the AMARES algorithm within the jMRUI software¹³⁵. The amplitude of the methyl resonance of the creatine for each water-suppressed spectrum was normalised to the amplitude of the water peak of the un-suppressed spectrum acquired immediately before.

Linear regressions were used to compare ¹H-MRS values and HPLC measurements. The goodness of fit (r^2) and the significance of the constant term of the linear regression (t-test) were calculated.

A Blandt-Altman plot was used to compare *in vivo* and *ex vivo* in intact myocardium MRS results.

3.3 Results

3.3.1 *Ex vivo* validation of the technique

Before its *in vivo* application, we validated the spectroscopic protocol on aqueous solutions of creatine, using a set-up similar to the one used *in vivo*, which includes the use of electronically simulated respiratory and cardiac signal to verify the gating of the PRESS sequence. We checked that the technique was stable and repeatable as phase and frequency stability is necessary to add the averages constructively. We also corroborated the linearity of the method - a necessary condition for its quantitative use.

3.3.1.1 Stability and repeatability of the technique

To test the stability and reproducibility of the technique, 256 scans without or with incomplete water-suppression (on water resonance for both) were acquired consecutively without averaging ($NA = 1$) on a water solution of creatine. This experiment was repeated three times in a row both for water-suppressed and unsuppressed scans.

The repeatability of the technique over 256 water-scans ($NA = 1$) with an incomplete or without water suppression (WS) is shown in Figure 3-4. The degree of water suppression was chosen for the signal to be still strong enough to be quantified to be able to assess whether the technique was stable when WS was used.

The frequency of the water peak oscillated over a range of less than 2 Hz (i.e. 0.005 ppm) for both suppressed and unsuppressed scans over the 256 repetitions (Figure 3-4c). The coefficient of variation of the amplitude for suppressed scans was 1.3 % and un-suppressed 0.8 % (Figure 3-4 a). Finally, the phase oscillated over a range of five and four degrees for suppressed and un-suppressed scans respectively (Figure 3-4 b).

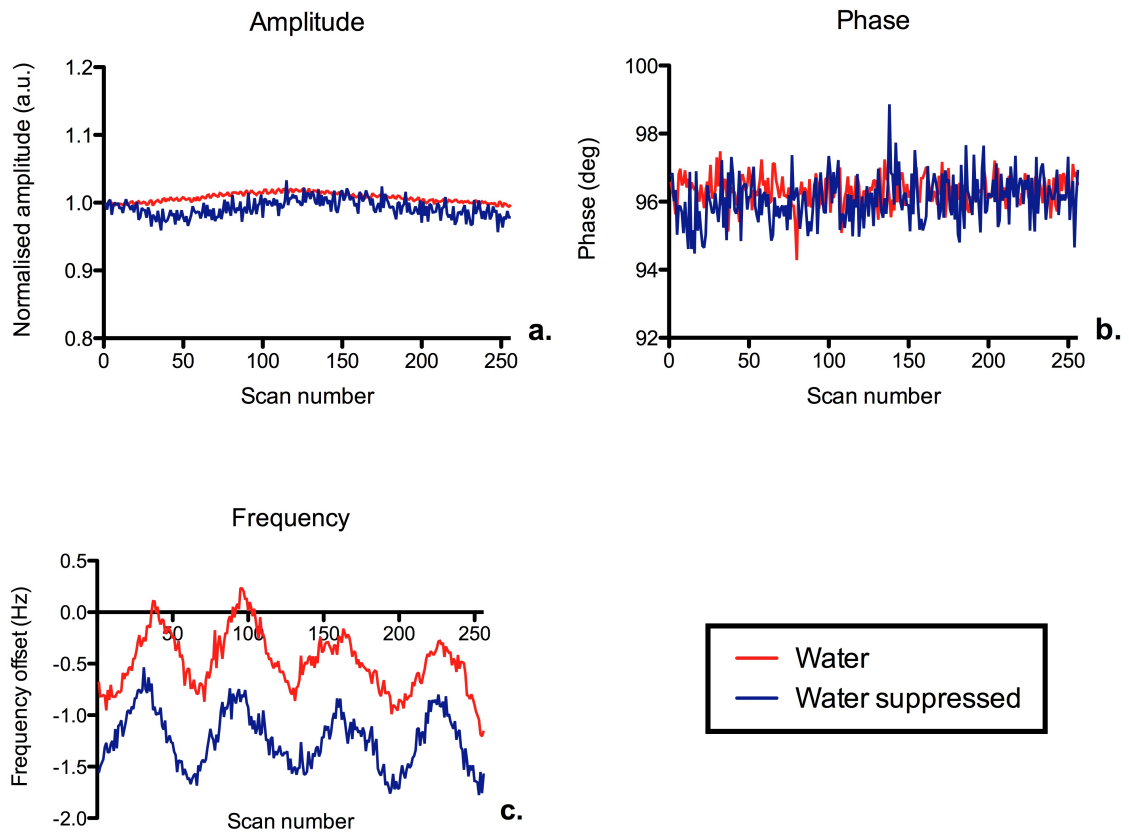


Figure 3-4: Stability of ^1H -MRS in terms of: (a) amplitude, (b) phase and (c) variation of frequency relative to the set-up frequency, for both un-suppressed (in red) and weakly suppressed (in blue) water signals. The amplitude of the signal for both suppressed and unsuppressed scans is normalised to the amplitude of the first scan of the first spectrum. One representative experiment is shown both for water and water-suppressed scans.

3.3.1.2 Linearity of the technique

To assess the linearity of the technique, phantoms consisting of a wide concentration range of creatine solution (10, 20, 30, 40 & 50 mM) and centred over the physiological myocardial concentration were used*. Moreover, to confirm the independence of the technique to voxel location, water-suppressed (NA = 256) and unsuppressed (NA = 16) spectra were acquired

* Creatine levels in the skeletal muscles of mouse¹⁵⁴, rat¹⁵⁵ and human^{156, 157} varies between 22 and 38 $\mu\text{mol/g}$ ww depending on the sources and between 13 and 28 for the murine¹⁵⁴, rat¹²⁵, canine⁹⁵ or human^{130, 158, 159} hearts. The mass percentage of water in skeletal and cardiac muscle is circa 73-76 %^{130, 156}. Consequently, from these data, we calculated intracellular Cr concentration to be around 20-50 mM.

interleaved from three different voxel (three spectra of each type per voxel) and for each concentration. The coefficient of variation due to changes in voxel location was 3.6 % for the lowest concentration solution and inferior to 2.0 % for the other concentrations. As seen on Figure 3-5, there is an excellent correlation between the amplitude of MRS data and the values of creatine concentration. Moreover, as expected, a doubling in creatine concentration results in a doubling in MR signal.

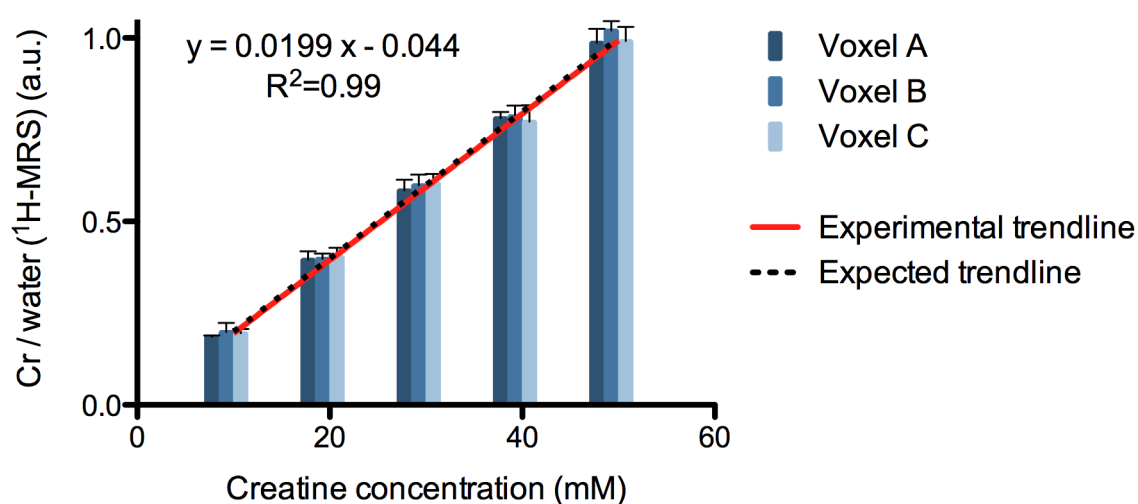


Figure 3-5: Correlation between the ratio of creatine/water amplitude assessed by ^1H -MRS and the creatine concentration of the solution for three different voxels. Three measurements were obtained for each voxel. The linear regression curve is shown alongside with the expected correlation.

3.3.2 Validation of absolute quantification of creatine *in vivo*

As the technique was stable and linear *ex vivo*, we applied it *in vivo*. To quantify myocardial creatine, we calibrated the amplitude of creatine signal (normalised with water) against the gold-standard (HPLC).

Seven CrT-OE mice (Tg55 strain) expressing a wide-range of myocardial creatine concentrations⁸⁷ and three wild-type (WT) littermate-controls were scanned. A representative spectrum is shown in Figure 3-6. At the end of the scan, the mice were culled and their hearts excised. Creatine was then measured in the LV by HPLC.

A good correlation ($r^2 = 0.91$) between creatine/water amplitude determined by ^1H -MRS and absolute creatine concentration (HPLC) was found (Figure 3-7) suggesting that this calibration curve can be applied for non-invasive creatine quantification.

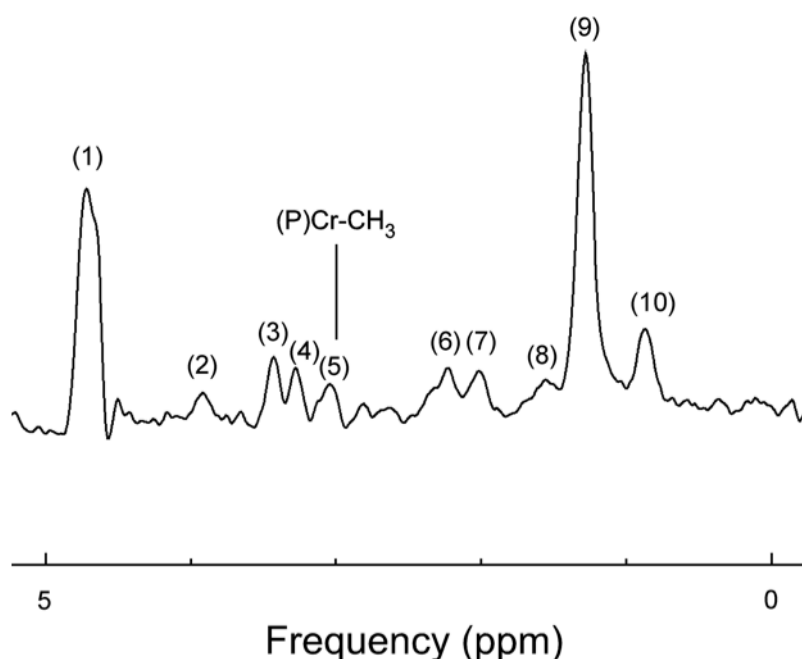


Figure 3-6: Representative ^1H -MRS cardiac spectrum from a wild-type mouse. The resonances were assigned as (1) water, (2) creatine- CH_2 , (3) taurine, (4) taurine/choline/carnitine, (5) creatine- CH_3 , (6) glycerides (CaH), (7) unassigned, (8) glycerides (CBH), (9) glycerides ($-\text{CH}_2$) $_n$, (10) glycerides ($-\text{CH}_3$).

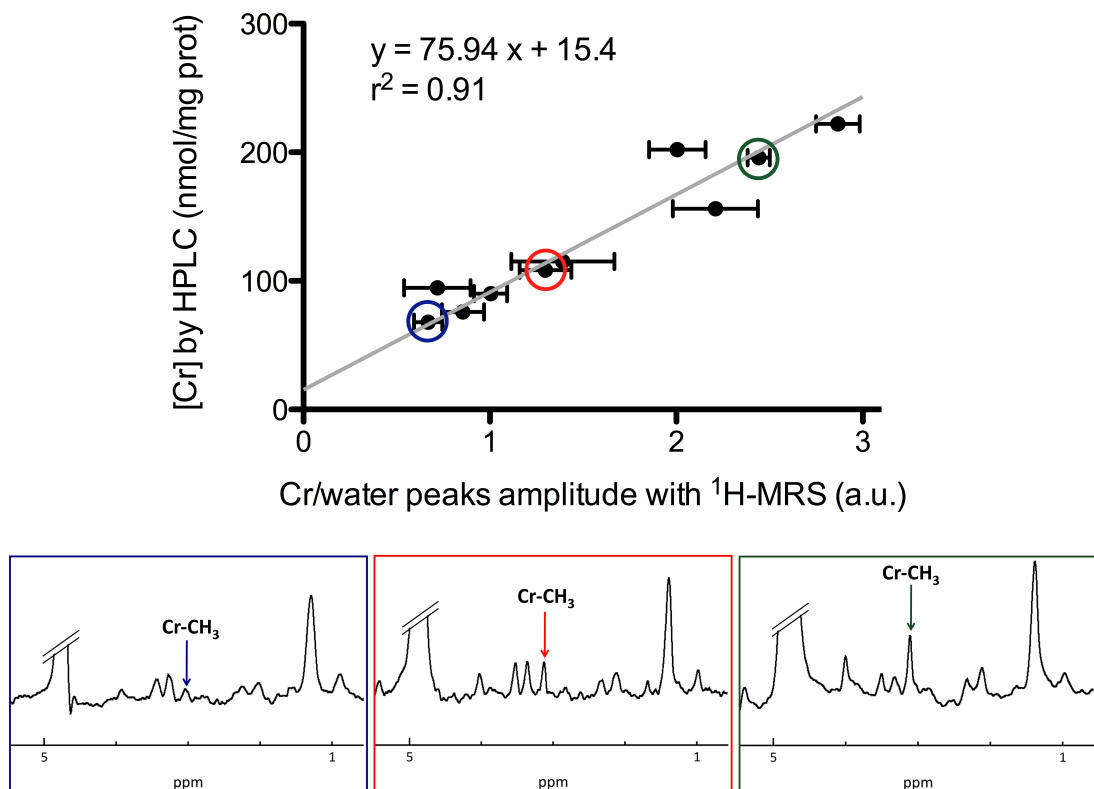


Figure 3-7: Correlation between the creatine/water ratio assessed by ^1H -MRS and the creatine concentration measured by HPLC in 3 WT and 7 CrT-OE mice. ^1H -MRS results are shown as mean $\pm$ SD (3 spectra per mouse). Three representative spectra from mice with physiological Cr levels (in blue), with moderately elevated Cr levels (in red) and with very high Cr levels (in green) are shown.

It is notable that this calibration curve does not go through zero but has a y-offset of 15 nmol/mg protein (which is about a quarter of WT creatine levels). Although this y-offset is not significantly different from zero ($p = 0.1$, 95% CI of y-offset = $[-27.0 ; 42.0]$), it hints at the possibility of a reduced MR-visibility for creatine. However, with the relatively low number of data points used in the calibration curve and the uncertainty in spectra fitting especially at low concentrations, it is not possible to draw a definitive conclusion at this stage.

3.3.3 Is the entire myocardial creatine pool MR-visible?

In order to investigate the MR-visibility question further, creatine was measured by ^1H -MRS under three different conditions: *in vivo*, *ex vivo* in the intact myocardium and in myocardial extracts. It was hypothesised that, both *in vivo* and *ex vivo* in the intact myocardium, part of the creatine is bound to proteins and, as such, cannot be detected. On the contrary, the acid extraction used to prepare the myocardial extracts will liberate creatine and render the whole pool MR-visible.

In vivo ^1H -MRS was performed in 3 WT and 11 CrT-OE mice (Tg55 strain). At the end of the experiment, the mice were sacrificed and the LV split in two halves. The septum of the LV was immediately scanned whereas the other half was frozen for later analysis. Finally, a myocardial extract was prepared from the frozen sample and HPLC and ^1H -MRS were performed in parallel on this solution.

3.3.3.1 Spectra quality and stability

Successful spectra were obtained from all 14 mice *in vivo* and for extracted proteins. However, *ex vivo* spectra on intact myocardium were not available in one mouse due to technical issues (spectra were acquired with a different TR). Representative spectra are shown in Figure 3-8.

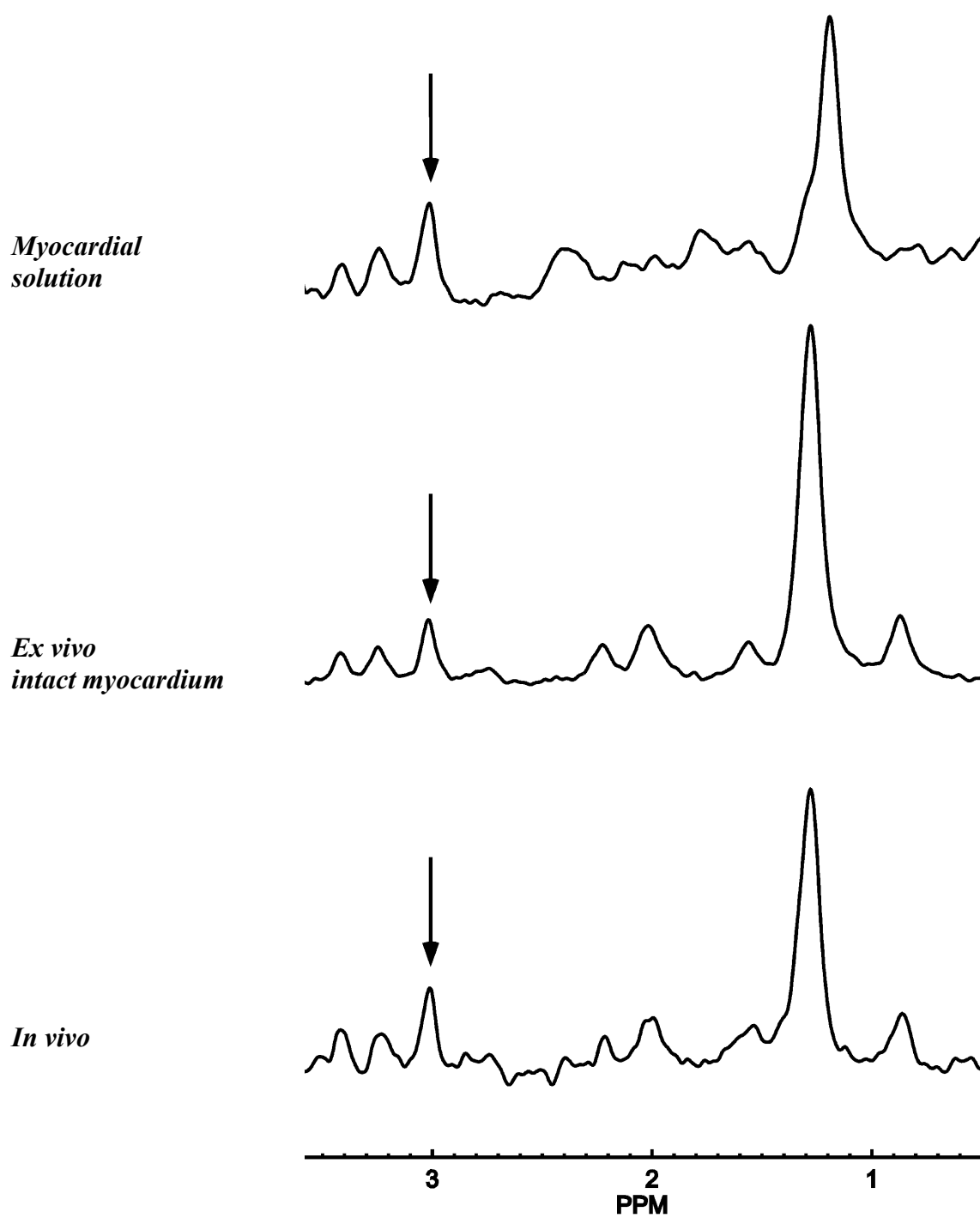


Figure 3-8: Representative spectra obtained from the same mouse in vivo (bottom), ex vivo on intact myocardium (middle) & on myocardial solution (top). The arrow at 3 ppm shows the methyl resonance of creatine.

The delay between cardiac excision and the acquisition of the last *ex vivo* spectrum on intact myocardium was 40 ± 18 minutes. During the acquisition of the four spectra, no change in creatine levels was observed ($p = 0.99$, repeated measures ANOVA).

Similarly, the creatine signal in the myocardial solution spectra was stable over the three consecutive acquisitions ($p = 0.66$, repeated measures ANOVA).

3.3.3.2 Correlation of creatine concentrations between the different techniques

There is an overall good agreement between *in vivo* and *ex vivo* creatine values in intact myocardium measurement as shown by a Bland-Altman plot¹⁶⁰ (Figure 3-9). This confirms the efficiency of cardiac triggering and respiratory gating for motion correction.

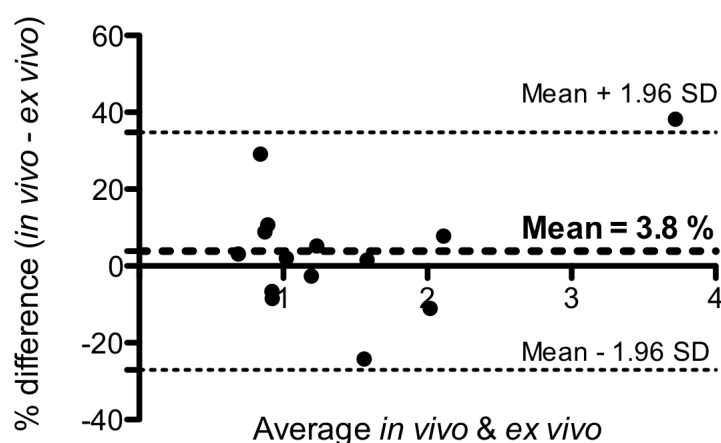


Figure 3-9: Bland-Altman plot comparing ¹H-MRS creatine/water ratio *in vivo* and *ex vivo*

Figure 3-10 shows the correlation between creatine levels assessed by MRS *in vivo*, *ex vivo* in intact myocardium, and in myocardial solution versus creatine concentration measured by HPLC. *In vivo*, *ex vivo* in intact myocardium and *ex vivo* in myocardial solution showed a y-offset of 16, - 22 and 5 nmol/mg protein respectively. None of the y-offset values were significantly different from zero.

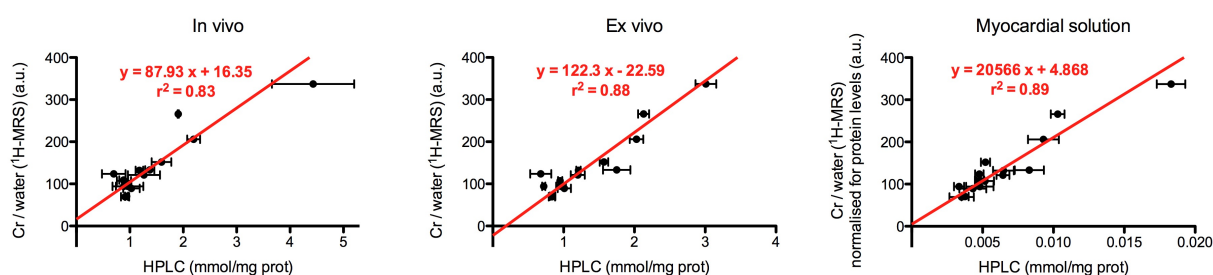


Figure 3-10: Correlations between ¹H-MRS creatine/water ratio and creatine concentrations by HPLC in vivo (a), ex vivo (intact myocardium) (b) and on myocardial solution (c).

The correlation plots between HPLC and MR measurements *in vivo*, *ex vivo* on intact myocardium and in myocardial solution confirm the good correlation between MR and standard biochemical measurements. However, the analysis of the y-offset is inconclusive. If part of the creatine was MR invisible *in vivo*, and *ex vivo* in intact myocardium, but released by acid extraction, the attended results would have shown a significant y-offset *in vivo* and in intact myocardium disappearing following myocardial extraction. As the y-offsets for *in vivo* and *ex vivo* (intact myocardium) are either side of zero, the presence of a significant MR-invisible creatine pool is unlikely.

3.3.4 Assessment of creatine metabolism in multiple organs

Organ-specific differences in creatine metabolism¹⁵⁴ necessitate the investigation / measurement of creatine concentrations in multiple organs, ideally within a single recovery MRS examination to reduce the number of animals and to increase statistical power. Therefore, the aim of this study was to evaluate the feasibility and reproducibility of a ¹H-MRS technique using a simple set-up, which does not require time-consuming mouse or coil repositioning.

With this aim in mind, it was necessary to ensure that ¹H-MRS was reproducible enough to reliably assess creatine levels with a single scan for each organ.

Therefore, the coefficients of variation were established for creatine measurement in the heart, brain and skeletal muscle, the three main creatine-dependent organs. With regards to cardiac MRS, intra-day or within-run reproducibility was assessed i.e. the reproducibility of successive measurements made without moving the subject. The hearts of 33 wild-type mice and 78 creatine transporter overexpressor mice (Tg46 strain) were scanned by the author for varied projects using the protocol previously described (i.e. 3 unsuppressed (NA = 16) and 3 water-suppressed scans (NA = 256) acquired interleaved (see 3.2.2.2)). For brain and skeletal muscle, both intra-day and inter-day (or between days) reproducibility were measured by acquiring 3 water-suppressed (NA = 256) and 3 unsuppressed (NA = 16) scans from the same wild-type mouse and from each organ on 5 different days over a two weeks period.

3.3.4.1 Descriptive analysis of the spectra

Representative spectra from all three organs are shown in Figure 3-11.

Typical linewidths achieved for the water resonance in the heart, brain and skeletal muscle were 30, 20 and 25-30 Hz respectively (i.e. less than 0.075 ppm).

- The tibialis anterior spectrum shows typical orientation dependant bulk susceptibility magnetic shift of the lipids, with a frequency shift between extra-myocellular (EMCL) and intra-myocellular lipids (IMCL) resonances of about 0.25 ppm. The chemical shift between these two resonances arising both from the CH₂ groups of lipids is the consequence of the geometrical arrangement of EMCL in flat structures along the muscle by opposition to IMCL, stored as lipid droplets within the myocytes. Moreover, splitting of the creatine methylene (3.9 ppm) and tri-methyl ammonium (TMA) / taurine (3.1–3.5 ppm) peaks is caused by dipolar coupling. Both phenomena confirm the orientation of tibialis anterior parallel to the main magnetic field B₀.¹⁶¹⁻¹⁶⁵.
- The quality of the brain spectra allows us to clearly resolve the methyl resonance of creatine from other metabolites. However, due to the short TE used, there is a moderate macromolecule baseline visible on the spectra.
- Finally, in the cardiac spectra, both methyl and methylene creatine resonances can be clearly identified as previously described¹⁰².

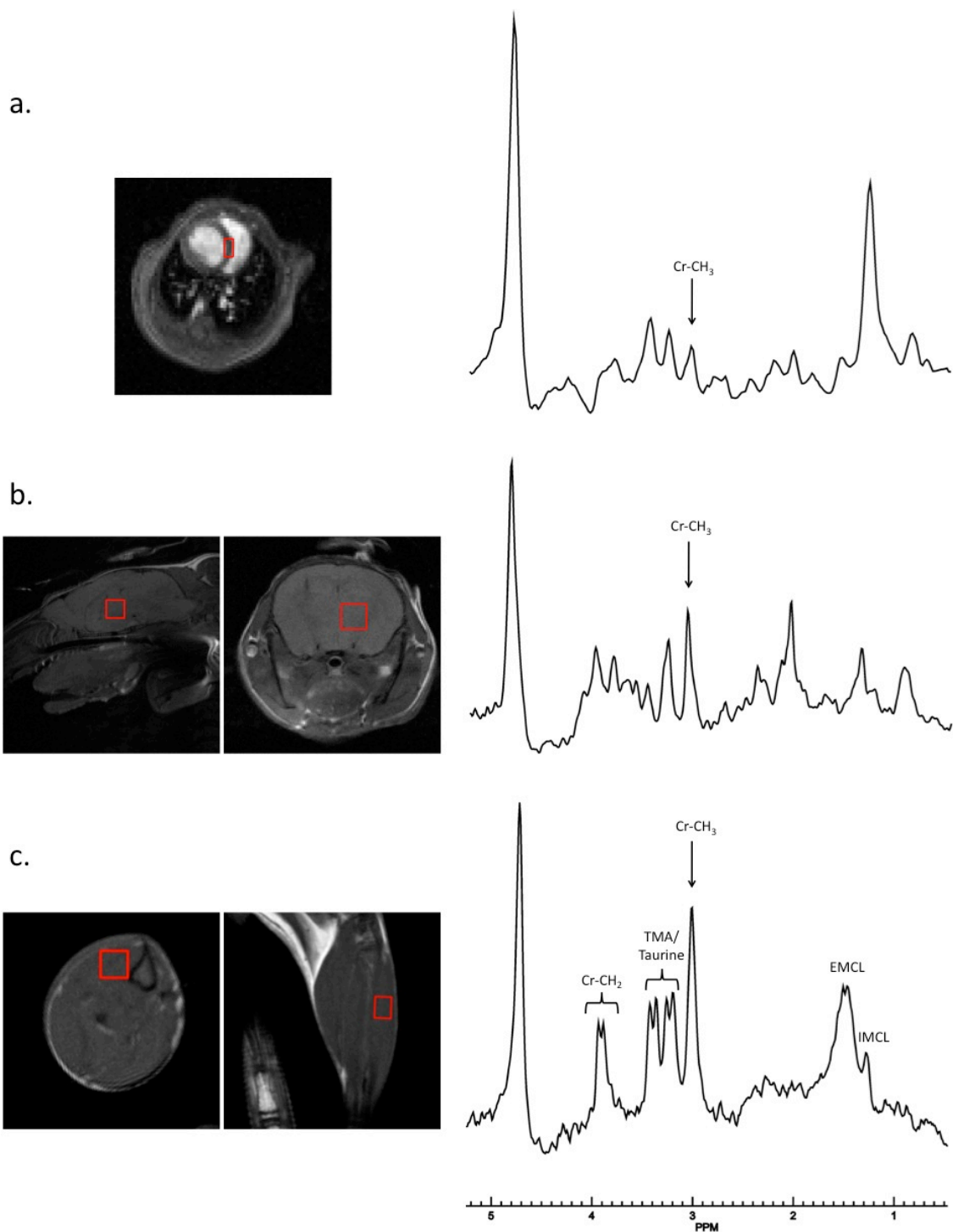


Figure 3-11: Representative spectra from the heart (a.), thalamus (b.) and tibialis anterior (c.) of WT mice. The arrows show the methyl resonance of creatine. The orientation-dependant features of the skeletal muscle (splitting of TMA / taurine resonances and frequency shift between EMCL and IMCL resonances) are shown.

3.3.4.2 Reproducibility of creatine measurement

The average coefficients of variation for the heart, brain and skeletal muscle are shown in Table 3-1. In brain and skeletal muscle, both intra-day and inter-day coefficients of variation are inferior to 7 %.

In the heart, the variability is higher (15 % in CrT-OE mice and 20 % in WT mice). The coefficient of variation (CV) is significantly higher in the WT mice than in CrT-OE ($p = 0.03$, t-test), which is explained by a higher CV in the measurement of creatine peak in the WT mice (20 ± 14 % in WT vs 15 ± 10 % in CrT-OE, $p = 0.03$, t-test). No difference in CV of the amplitude of water peaks was found. This suggests that the higher CV in the WT mice is due to the lower SNR.

For a change to be considered as significant between two successive scans, creatine levels need to vary by about 20 % compared to WT values both for brain and skeletal muscle. This is acceptable, since a change of more than 20 % has been considered by our laboratory as therapeutically relevant (unpublished data).

		Mean Cr/water peaks (a.u.)	CV (%)		SD _{total} (a.u.)	Sensitivity (a.u.)	Sensitivity (% of WT value)
			Intra- day	Inter- day			
Heart (septum)	WT (n=32)	0.74 ± 0.14	19.9 %				
	CrT- OE (n=78)	1.36 ± 0.23	15.1 %				
Brain (thalamus)	WT (n=1)	0.80	6.6 %	3.2 %	0.06	0.16	20 %
Skeletal muscle (tibialis anterior)	WT (n=1)	2.35	4.8 %	4.8 %	0.16	0.45	19 %

Table 3-1: Reproducibility of creatine measurements in the heart, brain and skeletal muscle. In the heart, the standard deviation of creatine levels in the mouse population studied here is given. Total standard deviation takes into consideration both intra and inter-day variation with $SD_{total} = \sqrt{(\sigma_{intra}^2 + \sigma_{inter}^2)}$ with σ_{intra}^2 and σ_{inter}^2 the intra and inter-day variances. The sensitivity of the technique is defined as the changes between two scans of the same animal, which will be considered as significant. $Sensitivity = 2\sqrt{2} \times SD_{total}^{166}$.

3.3.4.3 Application: MRS in multiple organs in a wild-type mouse

The aim of this reproducibility study was to assess whether creatine levels can be determined precisely in the three main creatine-dependant organs and in a limited amount of scans. We would like to estimate creatine levels in these different organs within single recovery anaesthesia, which would then give us a broad overview of general creatine metabolism in

the mouse. It would be of particular interest to assess whether, following pharmacological modulation of creatine levels for example, the changes are organ specific or not.

The variability in the brain and skeletal muscle being low, one MRS scan will give a precise enough answer, whereas in the heart the acquisition of three scans was deemed to be necessary due to the higher variability. We scanned the heart (three spectra), the brain (one spectrum) and the skeletal muscle (one spectrum) of a wild-type mouse within a single session. Between each organ, the mouse was repositioned in the scanner by simply advancing the whole cradle inside the volume coil, repositioning the voxel and reshimming. The whole procedure lasted about 2.5 hours, including preparation time. During this period the physiological parameters remained stable ($HR = 418 \pm 13$, $RR = 28 \pm 12$). The same mouse was rescanned the following day using the same procedure. The variations in the creatine measurements between the two sessions were of approximately 9.0 % for the heart, 2.5 % for the brain and 6.7 % for the skeletal muscle.

3.4 Discussion / Conclusion

^1H -MRS is the only available technique to assess non-invasively creatine metabolism. In this chapter, we investigated the use of ^1H -MRS to measure *in vivo* creatine levels in mice. After confirmation of the stability of the technique on phantoms, we applied it to the heart, skeletal muscle and brain.

3.4.1 Cardiac ^1H -MRS

Using a semi-quantitative method, we confirmed that creatine levels could be assessed reliably in the mouse heart by ^1H -MRS. Spectroscopic results correlated very well with the gold-standard HPLC over a wide-range of creatine concentrations from wild-type levels up to a three fold increase.

This technique has the advantage of being very simple, as contrary to the absolute quantification method, it does not necessitate any correction for B_1 inhomogeneities or relaxation times. However, it reposes on the postulate that water levels and relaxation times are constant in healthy mice but also in transgenic or pathophysiological models. Water signal is frequently used as an internal reference since pathological variations of water content are generally small. No change in water levels was found in the infarcted canine^{95, 158} and human heart¹⁶⁷, in the hypertrophied hyperthyroid rat heart¹⁶⁸ and in the hypertrophied human heart¹⁶⁷. However, when it comes to models with changes in creatine levels, the assumption might not hold true. Indeed, Murphy et al.¹⁶⁹ showed in the fibers from rat skeletal muscle that creatine uptake is accompanied by an increase in cellular water to maintain osmotic balance. Creatine ingestion has also been shown to increase body weight in human, which had been attributed to an increase in muscle water content¹⁷⁰. Finally, at the other end of the spectra of creatine levels, changes in body composition have been shown in creatine-deficient mice (GAMT knockout model)¹⁷¹. Moreover, this calibration curve has been validated on wild-type and CrT-OE mice. Consequently, it is difficult to extrapolate these results to mice with lower creatine levels, all the more as the calibration curve shows an important y-offset. To study metabolites concentrations in varied transgenic models, an absolute quantification would still be preferable¹⁰².

In conclusion, this technique allows us to reliably quantify Cr levels in heart with normal and elevated creatine concentrations. This technique renders possible the longitudinal follow-up of Cr levels (see Chapter 4) but also the pre-selection of mice according to their creatine levels (see Chapter 5).

3.4.2 MR visibility of creatine in the mouse heart

The existence of the y-offset on the calibration curve lead us to investigate whether the entire creatine pool is MR visible.

We compared creatine visibility in three different states: *in vivo*, *ex vivo* in intact myocardium and in myocardial homogenate. Our hypothesis was that creatine is not fully MR-visible due to the compartmentation and that acid extraction would release all creatine.

In vivo and *ex vivo* creatine values were in good agreement, confirming the efficiency of cardiac triggering / respiratory gating. However, the results were inconclusive regarding creatine visibility. We particularly failed to show any difference in the amplitude of y-offset between the three settings. Different reasons can explain this lack of conclusiveness. The whole study was based on differences in the y-offset of the linear regression curves. Results can mainly be influenced by the accuracy of measurement of each individual point and by the regular dissemination of the points.

Sources of variability in MRS are numerous. The main common reasons amongst the three settings are fitting errors aggravated by distorted lineshapes or poor water suppression. To this, we should add signal bleed and motion artefacts (*in vivo*) or finally frequency shift over the length of the scan (myocardial extract).

Uncertainties in HPLC measurements may have contributed to the poor quality of the results. Indeed, fitting of HPLC chromatographs is strongly influenced by a baseline effect, which is all the more prominent when the amount of available tissue is low. Moreover, HPLC data are normalised against protein concentration measured by a Lowry assay. Even if a standard curve is generated each time, errors can arise mostly from the approximation of the nonlinear standard curve to a straight line over the range of concentrations used¹⁷².

Besides, linear regression curves are strongly influenced by the spread of the data. We tried to get a broad and evenly distributed range of creatine concentration by using the Tg55 strain of CrT-OE, which compared to the Tg46 strain has a wider range of creatine levels. However, the number of high and very high over-expressors was quite limited and most Tg55 mice had only a mild to moderate increase in creatine levels.

Finally, the study was designed to prove that part of creatine is MR invisible. Our data are not in favour of this hypothesis.

Future work will be aimed at investigating further MR visibility focusing on the effect of water suppression. Indeed, over the last few years, attention has been drawn to techniques allowing acquisition of ¹H-MRS spectra without WS. Two approaches have been developed:

- standard acquisition of non water suppressed spectra with, at post-processing, either water removal and correction for sidebands¹⁷³, or estimation of water and metabolite resonances using a Bayesian probabilistic approach theory¹⁷⁴. To date, these methods have only been applied to phantoms and human brain for the first one and on mouse brain and skeletal muscle for the second.
- Two scan acquisition method during which in one of the two scans, metabolite signals are inverted, while water signal is unaffected. By adding or subtracting the two data sets, a metabolite spectrum or a water spectrum can be obtained, which was validated in the rat brain¹⁷⁵.

By comparing water-suppressed spectra acquired by our standard method and metabolite spectra acquired without water suppression, we will be able to determine whether CHESS contribute to the decrease in creatine signal by MT.

3.4.3 Reproducibility of *in vivo* ^1H -MRS

^1H -MRS is a powerful technique to assess non-invasively creatine metabolism *in vivo*. However, it is necessary to estimate the reliability and reproducibility of results before applying it as a quantitative tool. It is all the more important in longitudinal studies, where in the presence of a change, we need to be able to discriminate between a real pathophysiological or pharmaceutical variation and technique variability.

Our data confirm the good reproducibility of the technique with intra-session and between day CV of less than 7 % for both brain and skeletal muscle. These values are comparable to those published by other groups in the human or rodent brain and skeletal muscle using organ dedicated set-up, pulse sequences and hardware. For example, using a surface coil, de Feyter et al.¹⁷⁶ showed a CV for total creatine measurement in the rat tibialis anterior over 3 days of 7.6 to 14.3 %. Reproducibility of metabolite measurement has been more extensively assessed in human brain with most CV ranging from 5 to 20 %^{133, 136, 166, 177-179}. However, the reproducibility in the heart is slightly lower with an intra-day variability of about 15-20 %.

Within session variability arise from fitting errors mostly caused by a poor SNR, distorted baseline or non-conventional lineshape of metabolites. For brain and skeletal muscle, subject motion can be considered negligible through the use of general anaesthesia and of a set-up,

which does not allow head or leg movement. Factors contributing to between days variability are mostly an inaccuracy in VOI repositioning but also long term system instabilities^{136, 166}. Changes in creatine concentrations between days is very unlikely as less than 2 % of creatine pool is lost per day and there is no active efflux mechanism¹⁵. For our cardiac spectra, we would anticipate that the lower reproducibility is most likely explained by a lower SNR consequence of a smaller voxel size. This is also suggested by the fact that the CV is better in mice with high creatine levels. However, we cannot rule out an influence of cardiac and respiration motion despite the use of a cardiac triggered and respiratory gated PRESS sequence.

Importantly, the sensitivity of the technique enables to detect variations of 20 % or more from WT levels both in brain and skeletal muscle. This sensitivity is good enough to detect changes following pharmacological modulation that we would consider biologically relevant.

To conclude, we showed that ¹H-MRS is a reliable method to quantify creatine in the mouse heart. Consequently, it can be applied to non-invasively pre-select mice according to their creatine levels (chapter 5) or to follow creatine levels over time (chapter 4). We have also demonstrated that we can assess creatine metabolism in the three major creatine-dependent organs (heart / brain / skeletal muscle) within a single anaesthesia. This simple but robust set-up will be used as part of future work to follow creatine levels secondary to pharmacological intervention. Indeed, our laboratory recently showed in CrT-OE mice that a moderate elevation of creatine protects against ischaemia/reperfusion injury (unpublished data). In light of these promising results, our laboratory is seeking to increase pharmacologically creatine levels in mice either directly by using creatine pro-drugs such as

creatine chelates¹⁸⁰⁻¹⁸² or indirectly by modulating the activity of the CrT. We will confirm *in vivo* using ¹H-MRS whether or not these molecules could be promising therapeutic agents.

Chapter 4

CARDIAC PHENOTYPING OF A NOVEL MOUSE MODEL OF CREATINE DEFICIENCY: THE AGAT KNOCKOUT MOUSE

4.1 Introduction

To study the role of CK in cardiac function, two main strategies have been used: pharmacological modulation or transgenic models. The first approach has mostly been used before transgenesis revolutionized the study of biological systems. However, it has made invaluable contributions to the understanding of CK and still remains a relevant strategy.

4.1.1 Pharmacological modulation of CK system

4.1.1.1 Inhibition of CK

Iodoacetamide, a sulfhydryl group modifier, can irreversibly inhibit creatine kinase. Infusion in isolated rat heart reduced contractile-reserve in a dose-dependant manner¹⁸³. However, there is a great functional reserve of the CK system, such that contractile reserve was well maintained when CK activity was reduced by 74 % and only dramatically decreased in hearts with less than 10 % activity of CK¹⁸³.

By infusing low dose of iodoacetamide to isolated rat hearts, Tian et al.¹⁸⁴ managed to alter the levels in myocardial ADP without affecting ATP or Pi. Under these conditions, they showed impaired relaxation, confirming the importance of low ADP levels for normal cardiac function. Finally, a more recent study on isolated cardiomyocytes showed that iodoacetamide directly blunts high extracellular Ca^{2+} -induced increases in cardiomyocyte contractile reserve and diminishes the intracellular Ca^{2+} transient. This study further emphasized the role of creatine kinase in diastolic function¹⁸⁵. More recently, using a

³¹P-MRS magnetization transfer method, Xiong et al.¹⁸⁶ examined acute administration of iodoacetamide in the swine heart *in vivo*. Surprisingly, they did not observe any difference in ejection fraction with CK inhibition at baseline or at increased cardiac workload. However, haemodynamic parameters were only assessed at rest, and did not include markers of isovolumetric contraction and relaxation. Given that LVSP decreased after 60 minutes of iodoacetamide infusion, it would have been informative to also include load-independent measures of contractile function.

Finally, it is important to bear in mind that although iodoacetamide is often described as a rather selective CK inhibitor¹⁸⁷, this approach is still limited, as for all drug approaches, by off-targets effects such as, for example, a dose-dependent inhibition of GAPDH, which will impair glycolysis¹⁸⁴.

4.1.1.2 Use of Cr analogues

Guanidino compounds other than creatine can be taken up by the heart and phosphorylated by creatine kinase, replacing the PCr pool by synthetic phosphagens. A variety of these creatine analogs, with difference in their kinetic and thermodynamic properties¹⁸⁸, have been synthesized and used to study the physiological role of the CK system¹⁵. The most frequently used, β -guanidinopropionic acid (β -GPA), is a competitive inhibitor of creatine uptake by CrT¹⁸⁹ but is a poor substrate for CK (increased Michaelis constant and decreased maximum velocity)¹⁹⁰. Following β -GPA feeding, cardiac PCr stores were reduced by 80 %, which resulted in a decrease in ATP synthesis flux by 72 %¹⁹¹. Most studies have been performed

on the isolated rat heart following a period of β -GPA feeding of several weeks and showed a decrease in left ventricular developed pressure¹⁹¹⁻¹⁹⁴. *In vivo*, a decrease in systolic function has been shown at rest and under stress conditions¹⁹⁵, which reversed upon withdrawal of creatine analogue. Moreover, rats Cr-depleted by β -GPA cannot survive acute myocardial infarction^{194, 196}. Mortality occurs very early in the post-infarction period from malignant ventricular arrhythmias and progressive heart failure¹⁹⁶. These results support the concept that an intact PCr/Cr/CK system is essential in situations of acute cardiac stress.

When using this creatine analogue to study the Cr/PCr/CK system, one limitation is that β -GPA can still take part in the cytosolic CK reaction *in vivo*¹⁸⁸. However, as the CK reaction velocity is much slower, β -GPA is typically confined to the role of energy storage and not that of energy buffer. Furthermore, all experiments with creatine analogues must be interpreted with caution due to confounding side effects / toxicity in particular a decrease in body weight despite normal food intake^{194, 195, 197}. Our laboratory observed, in mice fed β -GPA, a precipitous drop in body weight by 20-30 % in less than a week (unpublished observation). These side effects not only limit long-term dosing for welfare reasons, but will have complex metabolic consequences that would be confounding.

Finally, only a partial depletion of creatine levels can be achieved. Reductions by 47 to 89 % in myocardial creatine levels have been described depending on dose and length of treatment^{191-193, 196-199}.

4.1.2 Transgenic models

4.1.2.1 Creatine kinase knockout mice

Functional phenotype of CK-deficient mice

The cardiac phenotype of mice deficient for the cytosolic muscle isoform of CK (M-CK^{-/-}) is relatively mild. No overt change in cardiac size or in contractile performance of isolated perfused hearts was noted^{200, 201}. Using cine-MRI, Nahrendorf et al.²⁰² showed that there is no significant change in cardiac anatomy and function *in vivo*.

The consequences of a deletion of the sarcomeric mitochondrial CK gene have mostly been studied in combination with a deletion of the cytosolic muscle isoenzyme (M/mt-CK^{-/-}). Initial cardiac phenotyping did not reveal signs of hypertrophy both in knockouts for the mitochondrial isoform (mt-CK^{-/-})^{90, 203, 204} and M/mt-CK^{-/-} mice^{201, 204, 205}. However, later studies described signs of hypertrophy both at anatomical (increase in LV mass by 13 to 72 %) and cellular (increase in myocyte cross-section area) levels in both strains while blood pressure remained essentially normal^{202, 206-209}. Interestingly, where cardiac hypertrophy was shown, there was no further increase in LV mass following exercise²⁰⁷ or myocardial infarction²⁰². With regard to cardiac function, no functional alteration has been shown in the isolated hearts of mt-CK^{-/-} and M/mt-CK^{-/-} at baseline, during increased workload, or at recovery^{90, 201}. However, Spindler et al.²⁰⁵ showed an increased susceptibility of these isolated hearts to ischaemic injury associated with a blunted post-ischaemic recovery.

In vivo a dilatation of LV with conserved ejection fraction has been observed by echocardiography²¹⁰ and cine-MRI^{202, 207}. A trend towards a blunted response to β -adrenaline stimulation has also been described in a study limited by the number of animals and the use of echocardiography²¹⁰; this finding was not confirmed by other authors²⁰⁷. Moreover, it needs to be emphasized that these imaging studies cannot assess isovolumetric contraction and relaxation function. Therefore, changes in the pressure-generating capacity of these hearts could have been overlooked.

Two points emerge from these studies and warrant further discussion. First, the very close phenotype between mt-CK^{-/-} and M/mt-CK^{-/-} would suggest that changes are mostly the consequence of the deletion of the mitochondrial isoenzymes. Secondly, there is a high phenotype variability: it is for example unclear whether or not these mice develop cardiac hypertrophy. Potential reasons could include difference in sex or age of the mice used but also a genetic drift. Indeed, all the studies were performed on a mixed C57BL/6 and 129Sv genetic background and using pure C57BL/6 as littermate controls. Therefore, our own laboratory backcrossed mt-CK^{-/-} on a pure C57BL/6J and showed no change in cardiac anatomy and function both at baseline and following dobutamine stimulation, using WT littermates as controls¹⁶.

Contribution of MRS to the study of high-energy phosphate metabolism in CK deficient models

MRS has been particularly used to assess high-energy phosphate metabolism in isolated hearts of CK deficient mice. Results are summarized in Table 4-1.

In M-CK^{-/-} hearts with only ~30 % of remaining CK activity, ³¹P-MRS revealed a conserved PCr/ATP ratio with normal absolute PCr and ATP levels^{200, 201}. pH, estimated from the chemical shift between P_i and PCr, and ADP levels, calculated through the CK equilibrium reaction levels, were normal^{200, 201}. Moreover, the high-energy phosphate profile of M-CK^{-/-} hearts followed tightly the changes observed in WT hearts during increased workload and recovery²⁰¹. Finally, using a saturation transfer experiment, CK flux was clearly detected in the M-CK^{-/-} hearts²⁰⁰, while this high-energy phosphate transfer from PCr to ATP – or vice versa – could not be observed by the same authors in skeletal muscle²¹¹. This could be explained by a higher contribution of mitochondrial CK to the total CK flux in the myocardium than in the skeletal muscle. Cardiac muscle cells have higher mitochondrial capacity than skeletal myocytes and a more oxidative character.

The importance of the mitochondrial isoform in energy transfer in the myocardium was confirmed by the fact that hearts lacking the mitochondrial isoform of CK (mt-CK^{-/-} or M/mt-CK^{-/-} mice) had a decrease in PCr levels by about 20 to 35 % at baseline^{90, 201}. The calculated cytosolic ADP concentration was higher, which would imply that the driving force for the ATPases (ΔG_{ATP}) is decreased even at low workload¹⁰⁸. Following increased cardiac work, PCr levels decreased more in M/mt-CK^{-/-} hearts than in WT or M-CK^{-/-} mice. In parallel, ADP levels doubled while it remained stable in WT and M-CK^{-/-}²⁰¹. Importantly, these experiments showed that, following increased workload, hearts deficient in mitochondrial CK are still able to hydrolyse PCr and resynthesize it following recovery^{90, 201}. Consequently, a bidirectional phosphotransfer across the mitochondrial membrane is maintained in the absence of mitochondrial CK. Moreover, hearts of the double knockout mice are still able to maintain ATP levels at increased workload by hydrolyzing PCr²⁰¹. This is not the case in skeletal muscle of adult double-knockout mice where the ability to use PCr

is markedly reduced following an ischaemic insult²⁰¹. This difference is most likely due to the higher proportion of the cytosolic brain isoform in the heart.

To conclude, these experiments using ³¹P-MRS highlights the importance of mt-CK in maintaining normal high-energy phosphates in the heart.

	Van Dorsten et al. ²⁰⁰	Saupe et al. ²⁰¹		Spindler et al. ⁹⁰
	M-CK ^{-/-}	M-CK ^{-/-}	M/mt-CK ^{-/-}	mt-CK ^{-/-}
[PCr]	⇒	⇒	↓	↓
[ATP]	⇒	⇒	⇒	⇒
[ADP]	⇒	⇒	↑	↑
pH	⇒	⇒	⇒	⇒
PCr/ATP	⇒	Not reported but must be ⇒	Not reported but must be ↓	Not reported but must be ↓
tCr	⇒	⇒	⇒	⇒
ΔG _{ATP}	Not reported but must be ⇒	⇒	⇒	↓

Table 4-1: Myocardial high-energy phosphate metabolites at baseline conditions (adapted from²¹²). ⇒ denotes no significant change vs WT controls, ↑ denotes increased value vs WT controls and ↓ denotes decreased values vs WT controls.

Biochemical and structural adaptations to CK deficiencies

The phenotype of creatine kinase deficient mice is surprisingly mild. Consequently, one may wonder whether adaptations occur to compensate for the loss of this key component of phosphotransfer.

Most studies have shown that the loss of one of the CK isoforms is generally not compensated by an increase in the remaining ones^{90, 213}. In mt-CK^{-/-}, a decrease in the M-CK isoenzyme has even been observed by Boehm et al.²⁰³. Knocking down both mt-CK and M-CK had no effect on the normal post-partum decrease in the B-CK isoform in adult heart²¹³. However, as previously stated, these studies did not compare the CK-deficient mice to WT mice from the same genetic background. In the heart of mt-CK^{-/-}, Lygate et al.²¹⁴ showed that M-CK was increased by 20 % compared to littermate controls.

Adaptations could include an increase in mitochondrial volume and cyto-architectural rearrangements. An increase in citrate synthase activity, suggestive of an augmentation of mitochondrial volume, has been shown by some authors^{207, 214} but not by others^{90, 215}. In the hearts of double knockout mice, a repositioning of the mitochondria closer to the myofibrils has been described²¹⁵. This suggests an attempt to reduce diffusion distances of ATP from the mitochondria to the contractile apparatus. This is supported by the studies from Kaasik, who showed a direct ATP/ADP channeling between the sites of energy production (mitochondria) and energy utilization (sarcoplasmic reticulum and myofilaments) in the heart and skeletal muscle^{215, 216}. It has been suggested in the skeletal muscle that alternative phosphotransfers could compensate for CK deficiency, namely adenylate kinase and glycolytic phosphotransfers²¹⁷. In heart, an up-regulation of AK has been found in some studies²⁰⁷ but not others²⁰³. Electron microscopic examination revealed a large amount of lipid droplets, located immediately adjacent to the intermyofibrillar mitochondria²¹⁸, which could suggest a change towards a more glycolytic metabolism. There is also evidence that, at least under basal conditions, glycolysis could replace the CK system in fueling the SR Ca²⁺-ATPase²⁰⁸.

4.1.2.2 Why study models of Cr depletion?

The study of CK-deficient mice confirmed that the Cr/PCr/CK system plays a role in cardiac metabolism. However, the relatively mild phenotype of these mice and the possibility of adaptations raise the question whether a new (better?) model could bring a different contribution to the study of CK system.

Spontaneous mutations of the enzymes involved in Cr synthesis (GAMT and AGAT) have been described in patients with neurological and musculoskeletal symptoms²¹⁹⁻²²². These patients have a systemic creatine depletion as shown for muscle and brain^{222, 223}.

These genetic mutations were not recognized until relatively recently and therefore most of the known patients are children. Unfortunately, the cardiac phenotype is currently unknown, in part due to ethical considerations of obtaining informed consent from minors, with mental retardation. Mice models for these genetic defects seem particularly attractive as they allow the study of all potential effects of Cr without confining it to a role in high-energy phosphotransfer. Furthermore, contrary to β -GPA feeding, the effects of complete Cr depletion and of graded levels of creatine levels via dietary feeding can be studied.

Mice models of these two genetic defects have recently been created by Prof Isbrandt's laboratory by knocking out the GAMT and AGAT genes. These two models were made available to us.

4.1.2.3 Guanidinoacetate N-methyltransferase knockout mouse (GAMT^{-/-})

The GAMT^{-/-} mouse was generated by gene targeting in embryonic stem cells¹⁷¹. Biochemical characterization confirmed creatine deficiency and accumulation of guanidinoacetate (GA), the creatine precursor on the synthesis pathway. However, in the first descriptions of the model, residual creatine was detected in the serum and organs (27 % of WT levels in the heart), which was later attributed to creatine uptake through coprophagia in mice housed with heterozygous or wild-type littermates¹⁷¹. GAMT^{-/-} mice are viable; the most obvious symptom is a decrease in body weight becoming progressively more pronounced as the mice age. It is mostly secondary to lower body fat content¹⁷¹ but also to growth retardation⁸⁶. The origin of this change in body composition is unknown. Besides, a general skeletal muscle hypotonia has been described but no change at the microscopy level could be detected¹⁷¹. Again, MRS proved to be an invaluable tool in assessing high-energy phosphate metabolism. It showed that during ischaemic stress, GAMT^{-/-} skeletal muscle is able to use the phosphorylated GA (P-GA) for high-energy phosphoryl transfer at a similar rate as PCr in WT animals. However, the rate of recovery of P-GA is markedly slower²²⁴. In line with skeletal muscle, the cardiac phenotype is relatively mild. Under resting conditions, there was no change in cardiac size nor in systolic or diastolic function with the exception of a slightly lower LV systolic pressure^{86, 225}. However, following β -adrenergic stimulation, a decrease in contractile reserve was observed. These hearts were also more susceptible to ischaemia/reperfusion injury despite the ability to use P-GA⁸⁶. In conclusion, this model suggested that a high capacity Cr/PCr/CK system plays a limited role at low workloads but is necessary under stress conditions. However, limitations of this model need to be mentioned. As GA can take part in the high-energy phospho-transfer, it could have masked the full consequences of creatine depletion. Moreover, GA is not inert

and in patients with GAMT deficiency, it plays a role in the symptomatology due to its neurotoxicity²²⁶. GA has also been demonstrated to inhibit the Na⁺/K⁺-ATPase, which could have profound repercussions on cellular homeostasis²²⁷.

4.1.3 Aims

As discussed above, all the models used to study the CK system have limitations. A new mouse model deficient for the first enzyme of the Cr synthesis pathway, the AGAT knockout mouse (AGAT^{-/-}), was generated. It provides new opportunities to study the full consequences of creatine depletion without the main limitation of the GAMT^{-/-} model: accumulation of the creatine precursor GA. We hypothesized that cardiac function at baseline and during inotropic stress is compromised by decreased creatine levels and resolved upon restoration of creatine pool.

By using creatine-free and/or creatine-enriched diets, it is possible to create graded levels of myocardial creatine, which will enable this hypothesis to be tested.

1. In AGAT^{-/-} mice fed Cr since weaning, it is expected that following withdrawal from dietary sources of Cr, myocardial Cr levels will progressively decrease. Cr levels will therefore be followed non-invasively by ¹H-MRS in order to measure the rate of Cr loss from the heart and evaluate the consequences on cardiac function.
2. As we anticipated that cardiac phenotype will be more pronounced in mice never exposed to creatine, we thoroughly studied cardiac function in these mice and looked for potential structural and biochemical adaptations to the lack of Cr.

3. To assess whether any observed phenotype was solely due to the lack of Cr or if long-term (mal-) adaptations to low creatine contribute to impairment of cardiac function, the effects of restoration of creatine levels will be examined. Two time-points were chosen to explore these two hypotheses. As Cr uptake was anticipated to be extremely quick (preliminary data), one week of creatine feeding should fully replenish the Cr pool, thus allowing to evaluate the contribution to the phenotype of Cr deficiency *per se*. To estimate the influence of long-term adaptations and assess whether they can be reversed upon Cr feeding, a 7 weeks time-point was chosen, as this duration was sufficient to reverse all structural and functional consequences in a model of LV hypertrophy secondary to aortic banding²²⁸.

4.2 Material and methods

4.2.1 Animal generation and care

Arginine:glycine amidinotransferase knockout mice (AGAT^{-/-}) were generated by Prof D. Isbrandt's laboratory (Hamburg) by homologous recombination in embryonic stem cells. Mice heterozygous for the knockout allele (AGAT^{+/-}) were backcrossed for at least 8 generations onto a C57BL/6J background. Mice were housed, bred and genotyped as previously described (see 2.1) i.e. all mice used in this study were bred by intercrossing of heterozygous animals (AGAT^{+/-}). After weaning, AGAT^{-/-} mice were housed separated from heterozygous (AGAT^{+/-}) and wild-type (AGAT^{+/+}) littermates to prevent creatine ingestion through coprophagia.

AGAT^{-/-} mice on creatine-free diet were fed Teklad Global 19 % protein extruded rodent diet (Harlan, UK), which has 10 % more energy density than the maintenance diet used for most of our strains. This diet was chosen to reduce adverse effects associated with the genotype. Mice receiving a creatine-enriched diet were fed R/M-H complete maintenance diet for rats and mice, with addition of 5 mg/kg creatine monohydrate and blue food colouring (Ssniff, Soest, Germany). The detailed characteristics of these diets are shown in Appendix 2.

4.2.2 Myocardial creatine loss

Six adult AGAT^{-/-} mice (2 males, 4 females, age: 17-21 weeks) and fed creatine-supplemented food from weaning underwent baseline ¹H-MRS and cine-MRI to measure non-invasively myocardial creatine levels and cardiac function respectively. Creatine was withdrawn from the diet the following day and myocardial creatine levels and cardiac function assessed repeatedly over the following three months. The rate of creatine loss was estimated using a kinetic model of non-enzymatic degradation, according to the following equation: $[Cr]_t = [Cr]_{t=0} \times e^{-kt}$. In this equation, $[Cr]_t$ is the myocardial creatine level as estimated by ¹H-MRS at the time t (in days), $[Cr]_{t=0}$ is the creatine concentration before creatine withdrawal and k, the constant rate of creatine breakdown.

90 days after creatine withdrawal, cardiac function was evaluated invasively by haemodynamics. A group of 6 AGAT^{-/-}, fed creatine throughout the whole experiment, was used as control group for haemodynamic measurements. Organs were then harvested for weighing and biochemical analysis by HPLC.

4.2.3 Phenotyping of creatine naïve AGAT^{-/-} mice

AGAT^{-/-} (5M, 5F) and their littermate AGAT^{+/+} (5M, 5F) controls, aged 4 to 5 months and fed creatine-free diet, were used for this study. Cardiac function was assessed non-invasively using cine-MRI. Cardiac haemodynamic function by LV catheterisation at baseline and following dobutamine infusion at 4 and 16 ng/gBW/min was then assessed a few days later. Due to technical failure during haemodynamics acquisition, a consequence of the extremely small size of the animals, one female and two male knockouts had to be replaced to get even numbers of animals in each group. At the end of the protocol, mice were culled by cervical dislocation and organs were harvested for weighing and further biochemical analysis.

Diastolic and systolic wall stress was calculated according to Laplace law using cine-MRI and invasive haemodynamic data²²⁹. First, the average myocardial wall thickness (in mm) was deduced from the measured cine-MRI volumes and assuming a spherical shape of the LV according to the following formula:

$$W_i = \left(\frac{3}{4\pi} \right)^{1/3} \times \left[\left(LVV_i + LVM_i / 1.05 \right)^{1/3} - LVIV_i^{1/3} \right]$$

where i represents either diastolic or systolic values, LVV is the internal left ventricular volume (cavity) in μL , LVM is the left ventricular mass in mg and 1.05 account for the density of the myocardium.

Calculation of the end-diastolic inner radius (r_{dia}) and end-systolic inner radius (r_{sys}) (in mm) were also derived from cine-MRI volumes according to:

$$r_i = \left(LVV_i \times \frac{3}{4\pi} \right)^{1/3}$$

According to Laplace law and again assuming a spherical shape of the LV, diastolic and systolic wall stress were calculated according to:

$$WS_i = [P_i \times r_i] / [2W_i]$$

where WS_i is systolic or end-diastolic wall stress (in kdyn/cm^2), P_i is LV systolic or diastolic pressure (in kPa) and r_i is expressed in cm.

Finally, we used another group of mice ($n=8$ AGAT^{-/-} and $n=7$ AGAT^{+/+}, age: 5 to 6 months) to acquire conscious, unrestrained ECG using the AnonyMOUSE ECG screening tool (Mouse Specifics Inc., Boston)²³⁰. ECG were then analysed manually.

Body composition was measured in another group of 11 AGAT^{-/-} (5M, 6F) and 11 AGAT^{+/+} controls (6M, 5F) (age: 4-5 months) mice using an EchoMRI-100 quantitative magnetic resonance whole body composition analyzer (Echo Medical Systems, Houston, USA) as described in 4.2.5.

4.2.4 Creatine uptake in AGAT^{-/-} mice

5-6 months old AGAT^{-/-} – never exposed to dietary creatine – and their littermate wild-type controls underwent body composition. They were subsequently fed a 0.5% creatine-enriched diet for 1 week for one group (8 AGAT^{-/-} (5M, 3F), 7 WT (4M, 3F)) and 7 weeks for another group (11 AGAT^{-/-} (3M, 8F), 8 WT (4M, 4F)). At the end of this period of supplementation, body composition was reassessed and cardiac function quantified by

haemodynamics at baseline and following dobutamine infusion at 4 and 16 ng/gBW/min. Mice were then culled by cervical dislocation and organs harvested for weighing and further biochemical analysis.

4.2.5 Body composition analysis by T₂-relaxometry

We attempted to develop on our 9.4 T magnet a technique to measure body composition using a T₂-relaxometry method. This technique has been successfully used both at low and higher field to determine body composition quickly, accurately, precisely and noninvasively in conscious mice²³¹⁻²³³ and rats²³⁴. It is based on the separation of lean mass and fat according to their different T₂-relaxation properties. However, fitting of the T₂-relaxation curves is challenging and we did not manage to obtain results with sufficient reliability. Therefore, body composition was assessed using a low-field magnet exclusively dedicated to body composition analysis.

Body composition was assessed in AGAT^{-/-} mice using an EchoMRI-100 system for mice (Echo Medical Systems, Houston, Texas). Calibration using canola oil was performed daily. Conscious mice were restrained in a plastic holder and individually inserted into the low-field magnet. After a scan of about one minute duration, the following parameters were obtained: fat, lean, free water and total water mass. All body parts containing water (excluding bones, hair, etc.) contribute to the lean mass whereas free water mass is mostly due to urine. The total water mass is the summation of both.

4.2.6 Biochemical measurements

4.2.6.1 CK enzyme activity and isoenzyme composition

Total CK activity and the distribution of the different CK isoenzymes were measured in left ventricular tissue³⁰.

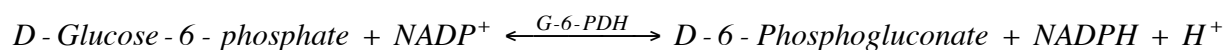
Sample preparation

5 to 10 mg of frozen powdered tissue was thawed in a drop of 4 °C homogenisation buffer (0.08 M K₂HPO₄, 0.02 M KH₂PO₄, 1mM EGTA and 1 mM β-mercaptoethanol, pH 7.4) and blotted on weighing paper (Macherey-Nagel, Germany) to measure its wet weight. Tissue was homogenised in a volume of buffer to obtain a final tissue concentration of 5 mg/mL. Homogenisation was performed using glass homogenisation vessels and an IKA Eurostar digital stirrer (1200 rpm for 10 to 20 s). Samples were kept at 4 °C during the whole extraction process. For later protein quantification using the method of Lowry et al.¹¹⁵, 50 µL aliquots were removed in triplicate, mixed with 50 µL of 4 % NaOH and incubated at 60 °C for 2 hours. Following removal of the aliquots, triton X-100 was added to the homogenate in the vessels at a final concentration of 0.1 %. Homogenates were vortexed briefly and left to settle on ice for 30 min to precipitate. The clear supernatant was then used for total CK assay, isoenzyme distribution and citrate synthase assay.

Total CK activity assay

Total CK activity was determined on each sample using the CK-NAC reagent (Thermo Fisher Scientific, Waltham, USA). The assay is based on the method first described by Oliver²³⁵, modified by Rosalki²³⁶ and employs the optimised conditions published by Szasz et al.²³⁷.

The enzymatic reactions involved in the assay are as follows:

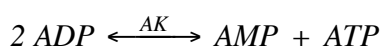


where HK stands for Hexokinase and G-6-PDH for Glucose-6-phosphate dehydrogenase.

The reduction of the nicotinamide adenine dinucleotide phosphate NADP^+ to NADPH in the final step results in a change in absorbance at 340 nm. The rate of change in absorbance is directly proportional to CK activity.

The CK-NAC reagent contains 100 mM Bis/Tris Buffer, 31.5 mM Creatine Phosphate, 5.3 mM AMP, 2.2 mM NADP, 2.1 mM EDTA, 10.3 μM AP_5A , 10.5 mM Mg^{2+} , 2.7 mM ADP, 21 mM D-Glucose, 21 mM N-acetyl-l-cysteine, > 3,000 U/L hexokinase (yeast), > 2,000 U/L glucose-6-phosphate dehydrogenase (leuconostoc).

N-acetyl-l-cysteine is a thiol compound, which is used to reactivate the CK enzyme prior to the reaction by reducing oxidised sulfhydryl compounds at the active site. AMP and P1,P5-di(adenosine 5')pentaphosphate (Ap5A) are added to inhibit interference caused by adenylate kinase (AK) activity. AK would otherwise interfere with the determination of CK activity by generating additional ATP via the following reaction:



The assay was carried out by diluting 20 µL of sample supernatant with 80 µL of homogenisation buffer. The reaction was performed in triplicate by mixing 1 mL of CK reagent to 20 µL of the diluted sample. After 3 min of incubation at 30 °C, absorbance at 340 nm was read at 20 s intervals for 5 min. The activity in U/L was calculated automatically from the change in absorbance using the following formula:

$$\text{Activity (U/L)} = \Delta_{\text{Abs/min}} \times \frac{TV \times 1000}{6.22 \times SV \times P}$$

Where:

TV = Total reaction volume in mL (1.02 mL)

SV = Sample volume in mL (0.02 mL)

6.22 = millimolar extinction coefficient of NADPH at 340 nm

P = cuvette pathlength in cm (1 cm)

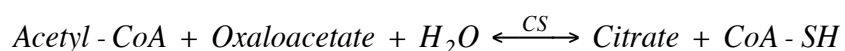
Isoenzymes distribution

The Rapid Electrophoresis (REP) system (Helena Biosciences Europe, UK) was used to separate CK isoenzymes by agarose gel electrophoresis and quantify their relative activity. Samples, run in duplicate, were prepared by diluting 80 µL of sample supernatant and 80 µL of 0.9 % NaCl; 75 µL of this mixture was then added to the REP sample tray. Samples were automatically loaded on an agarose gel in a Tris/Barbital buffer with sodium azide as preservative (REP CK Isoenzyme Kit). Following separation by electrophoresis, isoenzyme bands were revealed by incubation with a fluorescent substrate (CK8-reagent of the REP CK Isoenzyme Kit) reacting according to reaction (1) to (4) above. The reagent was supplemented with 1.7 µM AP₅A prior to incubation to inhibit contamination by AK. The isoenzymes bands were then visually inspected under UV light before relative intensities were assigned by automated scanning fluorometry. Since the specific activities of the isoenzymes are similar, their activities were assigned as a proportion of total CK activity²³⁸.

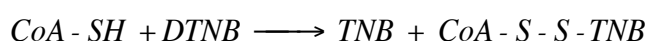
4.2.6.2 Citrate synthase assay

Citrate synthase catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid (OAA) to form citric acid. The assay is based on the following reactions:

Reaction catalyzed by citrate synthase (CS):



Colorimetric reaction:



Progress of the reaction is followed by observing spectrophotometrically the rate of production of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm²³⁹.

The reaction is run in duplicate. This is a two step-assay, for which the timing is essential. 50 µL of sample supernatant, prepared for CK activity measurement, is mixed with 850 µL of reagent (0.35 mM Acetyl-CoA, 0.12 mM DTNB, 0.15 M Tris base, pH 8.1) and left incubating for exactly 3 min at 25 °C. 100 µL of oxaloacetate solution (10 mM oxaloacetate, 1 M Tris base, pH 8.1) is then added and absorbance is immediately read at 412 nm at 2 s interval for 40 s. The activity in U/L was calculated automatically from the change in absorbance using the following formula:

$$Activity (U/L) = \Delta_{Abs/min} \times \frac{TV \times 1000}{13.6 \times SV \times P}$$

Where:

TV = Total reaction volume in mL (1 mL)

SV = Sample volume in mL (0.05 mL)

13.6 = millimolar extinction coefficient of NADPH at 412 nm

P = cuvette pathlength in cm (1 cm)

4.2.6.3 Adenylate kinase assay

The myocardial enzymatic activity of AK was measured as previously described^{54, 240}.

Briefly, frozen, ground heart tissue (1 mg/mL) was extracted with the buffer containing

150 mM NaCl, 60 mM Tris-HCl, 5 mM EDTA, 0.2% Triton X-100, and mini-protease inhibitor (Roche), pH 7.5. The AK activity was recorded at 30 °C, 340 nm in the assay buffer containing 100 mM potassium acetate (pH 7.5), 20 mM HEPES, 20 mM glucose, 4 mM MgCl₂, 2 mM NADP⁺, 1mM EDTA, 1 mM dithiothreitol, 9 U/mL hexokinase, 4 U/mL glucose-6-phosphate dehydrogenase, and 2 mM ADP.

4.2.7 Real-time quantitative PCR (RT-qPCR)

4.2.7.1 RNA extraction

Ribonucleic acid (RNA) was extracted from ~10 mg of powdered frozen left ventricular tissue. Samples were disrupted in 1 mL of Trizol Reagent (Invitrogen), by passing the lysate through a 20-gauge needle and syringe 5 to 10 times or until no tissue particles could be seen. After centrifugation (12,000 g for 10 min at 2-8° C), the clear homogenate supernatant, which contains RNA, was transferred to a fresh tube. After being left at room temperature for 5 minutes to allow for complete dissociation of nucleoprotein complexes, 0.2 mL of chloroform was added in each tube. The tubes were then shaken vigorously by hand for 15 seconds before another incubation step of 2 to 3 minutes at room temperature. After centrifugation at 12,000 g for 15 minutes at 2 to 8 °C, the colorless upper aqueous phase, containing RNA, was transferred into a fresh tube and RNA precipitation was performed with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Total RNA was eluted in 40 µL RNase-free water and purity was determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop technologies). The quality of RNA extracted was considered sufficient if its A₂₆₀/A₂₈₀ ratio was in the range 1.9-2.1.

4.2.7.2 One-step RT-PCR

Complementary deoxyribonucleic acid (cDNA) was synthesized from RNA of the above samples. PCR amplification was conducted in one step using the Quantitect SYBR Green RT-PCR Kit (Qiagen). A standard curve was run in parallel consisting of a five log serial dilution of pooled stock RNA template. All samples and standard dilutions were run in duplicate in a Rotor-gene 3000 (Corbett Research/Qiagen). The 10 μ L reaction mixture consisted in 2 μ L template RNA, 5 μ L 2 X Quantitect SYBR Green RT-PCR Master Mix, 0.5 μ M forward and reverse primers and 0.1 μ L Quantitect RT Mix. The primers used are shown in Table 4-2. The cycling conditions used were as follows: 30 min of reverse transcription at 50 °C and 15 min polymerase activation at 95 °C followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s, 72 °C for 20 s before holding at 60 °C for 15 s. On completion, specificity of the amplification was confirmed by exposing the PCR products to a temperature gradient from 60 to 90 °C for melt curve analysis.

4.2.7.3 Relative quantification

A cycling threshold, corresponding to the fluorescence intensity for which the reaction is in log linear phase of amplification, was automatically set by the Rotor-Gene 6.1 software using the standards. For each sample, relative quantification to the standard curve was done and results were normalised to the constitutive housekeeping gene product 36B4 (acidic ribosomal phosphoprotein P0) (primer Bank ID 6671569).

Gene	Reference	Sequence
ANP	241	(+) 5' - GTGTACAGTGCGGTGTCCAA - 3' (-) 5' - ACCTCATCTTCTACCGGCATC - 3'
BNP	242	(+) 5' - GAGGTCACTCCTATCCTCTGG - 3' (-) 5' - GCCATTCCTCCGACTTTTCTC - 3'
β -MHC	241	(+) 5' - GCATTCTCCTGCTGTTTCCTT - 3' (-) 5' - TGGATTCTCAAACGTGTCTAGTGA - 3'
α -SA	242	(+) 5' - CCCAAAGCTAACCGGGAGAAG - 3' (-) 5' - CCAGAATCCAACACGATGCC - 3'
CrT	Account number: BC049801	(+) 5' - TTCAACAACAACCTGCTACAAGG - 3' (-) 5' - ATGAAGCCCAGGATGGAGAA - 3'
36B4	242	(+) 5' - AGATTCTGGGATATGCTGTTGGC - 3' (-) 5' - TCGGGTCCTAGACCAGTGTTC - 3'

Table 4-2: Characteristics of RT-qPCR primers. (+) Sense (forward) primer. (-) Antisense (reverse) primer. ANP: Atrial natriuretic peptide; BNP: Brain natriuretic peptide; β -MHC: β -myosin heavy chain; α -SA: α -skeletal actin; CrT: creatine transporter, 36B4: acidic ribosomal phosphoprotein P0.

4.2.8 *Ex vivo* ^1H -NMR spectroscopy

A methanol-chloroform-water method was used to extract metabolites from the left ventricle of 3 AGAT^{-/-} and 3 AGAT^{+/+}. In brief, frozen tissues (~50 mg) were homogenised in 2:1 methanol-chloroform mixture (600 μL) using a tissue homogeniser and samples were sonicated for 15 minutes. A 1:1 chloroform-water mixture was then added (400 μL). Samples were centrifuged (4500 rpm, 20 minutes) and separated into the aqueous and organic layers leaving the resultant protein pellet. This extraction process was repeated on the protein pellet to maximise extraction efficiency. The aqueous layer (required for analysis) was dried under N_2 . The dried extracts were rehydrated in 600 μL D_2O buffered in

0.06 M sodium phosphate (pH 7.0) containing 0.25 mM (sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP)) and transferred to 5 mm NMR tubes.

NMR spectra were acquired on a Bruker Avance 500 MHz NMR Spectrometer operating at 500.13 MHz for the ^1H frequency and fitted with a TXI Inverse Automatic Tuning and Matching (ATMA) probe. All spectra were the result of the summation of 128 free induction decays (FIDs), with data collected into 32k data points over a spectral width of 16 ppm at 300 K. Spectra were collected using a solvent suppression pulse sequence based on a 1-dimensional NOESY pulse sequence to saturate the residual ^1H water proton signal (relaxation delay = 2 s, evolution time $t_1 = 3\ \mu\text{s}$, mixing time $\tau_m = 150\ \text{ms}$, solvent pre-saturation applied during the relaxation time and the mixing time). Prior to Fourier transformation, an exponential line broadening equivalent to 0.3 Hz was applied to the FIDs. Spectra were referenced to TSP at 0.00 ppm. Spectra for the 48 samples (24 samples for each tissue) were acquired in a random order.

NMR spectra were processed using ACD labs SpecManager 1D NMR processor Version 12 (ACD, Toronto, Canada). Spectra were phased and baseline corrected manually. Each spectrum was then integrated using 0.04 ppm integral regions between 0.5 and 10.0 ppm. The region around the water peak was removed (4.50 - 5.10 ppm) giving a total of 220 integral regions. The resulting data matrix was exported into Microsoft Excel 2010 and, to account for any difference in concentration between samples, each spectral region was normalised to the whole spectrum prior to further analysis.

Datasets were imported into the SIMCA-P 11.0 package for processing using both Principle Components Analysis (PCA). PCA is a data reduction technique that represents multivariate data in a reduced set of dimensions such that an overview of the data is permitted. The output from these analyses is in the form of score plots, which give an indication of the

differentiation of classes in terms of biochemical similarity, and loading plots, which indicate which spectral regions were important to the sample classification observed in the score plots. This allows identification of important metabolic perturbations in sample classes.

Pareto scaling was used, in which each variable was centred and multiplied by $1/(S_k)^{1/2}$, where S_k is the standard deviation of the variable. This scaling increases the importance of low concentration metabolites without significant amplification of the noise in the spectra.

R^2 and Q^2 were used as measures for the robustness of a pattern recognition model. R^2 is the fraction of variance explained by a component, and cross validation of R^2 gives Q^2 , which reveals the fraction of the total variation that can be predicted by a component. Both values are indicative of how good the overall model is. Typically a robust model has $R^2 > 50\%$ and $Q^2 > 40\%$. Coefficient lists rank the observations according to their contribution to the model. In order to confirm which metabolites contributed significantly to each model, the integral regions contributing most to the separation, as identified by the coefficient scores, were successively excluded from the analysis thus producing new models, the predictive power of which could be assessed using the new R^2 and Q^2 values.

4.2.9 Myocyte cross-section area

Heart samples were fixed in 10 % buffered formalin, processed and embedded with paraffin. 8 micrometre thick sections were prepared from the apex to the base. Sections were stained with Masson trichrome and examined under a light microscope. Images of 6 segments per heart were taken using a Nikon TE2000U microscope at 60x oil objective and 1.0x optical

zoom. Two random fields from each of the 6 segments per animal were analysed and approximately 150-200 myocytes per animal were measured. The quantification of myocyte cross-sectional area was determined using Image J software (National Institutes of Health).

4.2.10 Statistical analysis

Changes in body weight following creatine withdrawal were analysed using a one-way repeated measures ANOVA with Dunnet post-hoc test. Due to the presence of incomplete data sets (not all mice were studied at each time-point), a one-way ANOVA was used to assess changes in LV mass during the period of creatine withdrawal. Paired Student t-tests were used to measure differences before and at the end of the creatine withdrawal period.

Haemodynamic data were analysed using a repeated measures two-way ANOVA (with the following 2 factors: genotype (2 levels) and dobutamine concentration (3 levels)). Body composition data were analysed with a one-way ANOVA in creatine naïve animals and with a repeated measures two-way ANOVA in creatine rescue experiments (genotype (2 levels) and time (2 levels)). Statistical analysis of post-mortem data was done using a two-way ANOVA (with genotype and sex as factors). For all these data, a Bonferroni's post-hoc test was then performed.

All other data were subjected to a Student t-test unless otherwise stated.

4.3 Results

4.3.1 Myocardial creatine loss in AGAT^{-/-} mice

4.3.1.1 Myocardial creatine efflux

¹H-MRS allowed us to follow non-invasively and for the first time the rate of myocardial creatine depletion following dietary creatine withdrawal in six AGAT^{-/-} mice.

As shown on Figure 4-1, myocardial creatine levels decreased regularly from the day 0 to reach undetectable levels by ¹H-MRS by day 83. It should be noted that the number of animals per time-point varies. This is mainly due to the fact that due to legal restrictions on the number of scans we could perform per mouse, each individual animal could not be scanned at every time-point. Moreover, on a few occasions, we were unable to obtain spectra of sufficient quality (see 3.1.4.) for quantification.

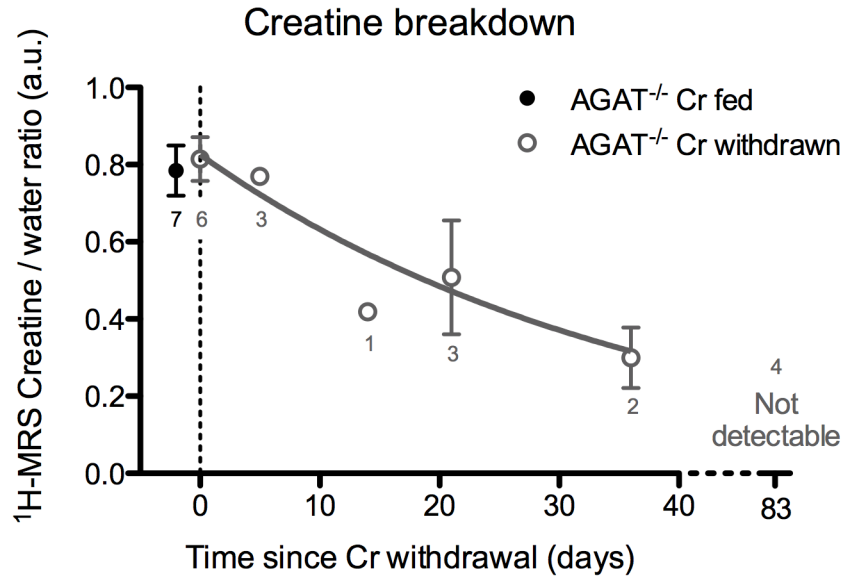


Figure 4-1: Myocardial creatine loss in AGAT^{-/-} mice following dietary creatine withdrawal. Cr concentrations (in a.u.) were determined by ¹H-MRS. The number of mice per time point is specified. Data were fitted using a kinetic model of non-enzymatic degradation, according to the following equation: $[Cr]_t = [Cr]_{t=0} \times e^{-kt}$.

The decrease in creatine levels between day 0 and day 36 (the last day we detected creatine) followed a clear exponential decay pattern with a calculated constant rate k of creatine breakdown of 0.027 ± 0.004 , which corresponds to a 2.7 % decrease per day. By day 83, no creatine resonance was observed (Figure 4-2). The experiment was aborted a week later (91 days after creatine withdrawal) due to the absence of detectable creatine and because of a drop in body weight, which had welfare implications (see below). When assessed by HPLC, the mean residual creatine concentration was 20.5 ± 3.9 nmol/mg protein (vs 73.6 ± 6.7 nmol/mg protein (¹H-MRS) before creatine withdrawal), when from the rate constant, we would have expected approximately 10 % of creatine to remain.

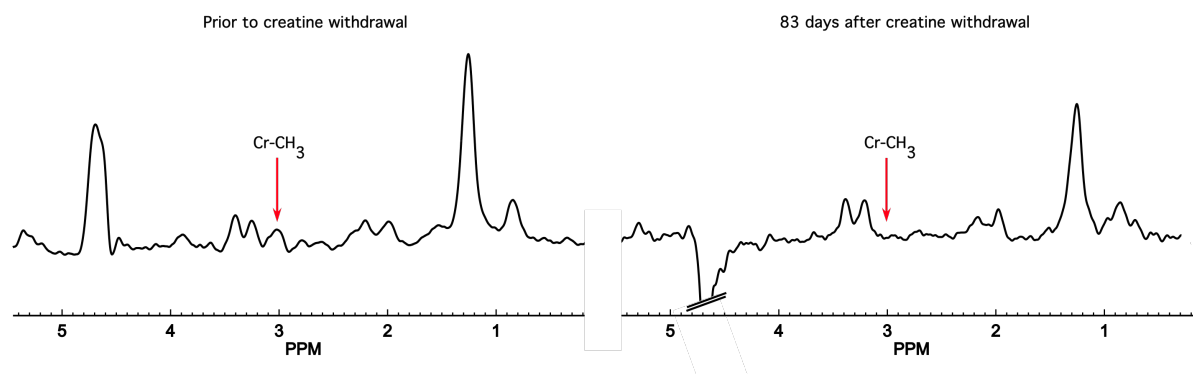


Figure 4-2: Representative spectra of the same mice before and after creatine withdrawal. No creatine resonance (at 3 ppm) can be detected 83 days after withdrawal.

4.3.1.2 Body weight

Body weight (BW) was carefully followed over the period of creatine withdrawal. The decrease in BW was negligible over the first two months. A marked inflection point can be seen around day 60 (which would correspond to ~20 % of starting creatine levels), after which a gradual daily decrease by about 0.5 % of initial BW was observed (Figure 4-3). After 91 days on a creatine-free diet, mice had lost 14 % of their initial BW (repeated measures one-way ANOVA with Dunnett post-hoc test, $p < 0.01$). At that point, the experiment was terminated for animal welfare reasons.

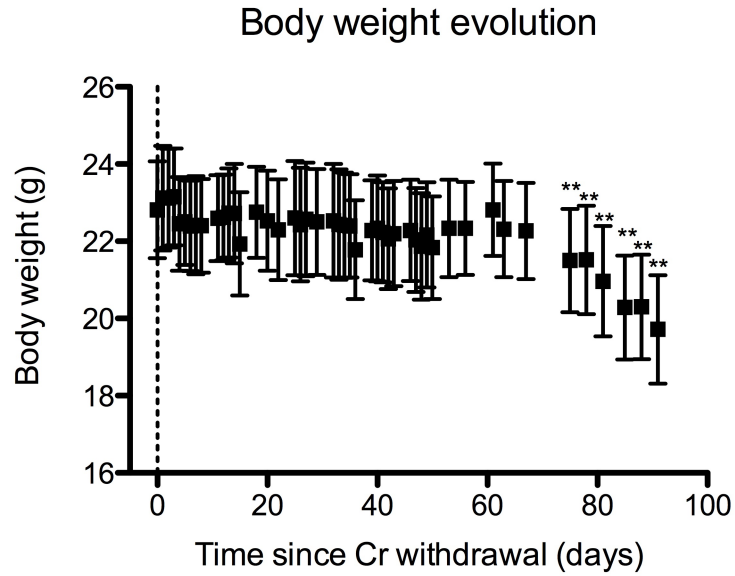


Figure 4-3: Evolution of body weight following dietary creatine withdrawal in 6 *AGAT^{-/-}* mice (2M, 4F). Data are expressed as Mean $\pm$ SE. ** denotes $p < 0.01$ vs initial BW ($t=0$) (one-way ANOVA with repeated measures and Dunnett post-hoc test).

4.3.1.3 Cardiac mass and function

Cardiac anatomy and function was followed non-invasively by cine-MRI. Despite a drop in myocardial creatine levels to 28 % of starting values, functional parameters (EF and CO) remained stable and within physiological range. However, myocardial mass decreased progressively (Figure 4-4) from the first time-point reaching a significant weight loss (> 8 %) at the final time-point ($p < 0.01$, paired t-test) (Table 4-3).

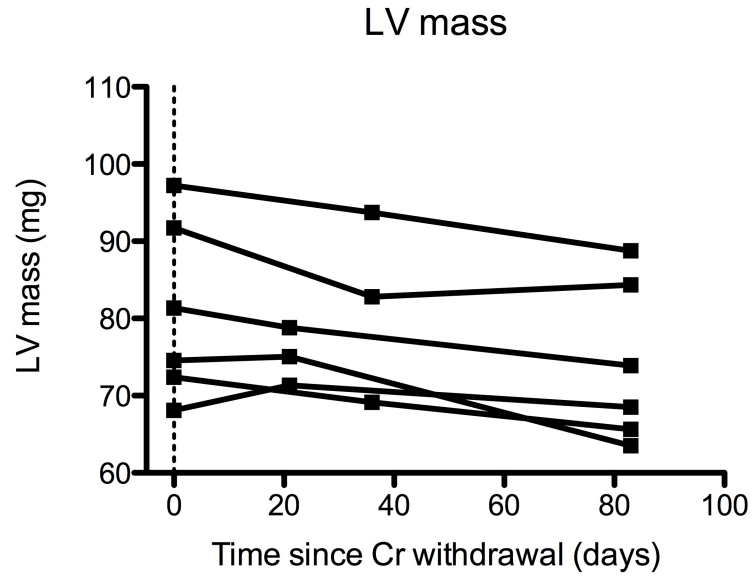


Figure 4-4: Evolution of LV mass following dietary creatine withdrawal in 6 $AGAT^{-/-}$ mice (2M, 4F). Due to incomplete data sets at each single point, repeated measures ANOVA could not be performed. Despite a clear decrease in LV mass in all but one mouse between the first and last time point, no difference between groups could be elicited using a one-way ANOVA.

	$AGAT^{-/-}$ pre-withdrawal (n=6)	$AGAT^{-/-}$ 83 days post-withdrawal (n=6)	Control group $AGAT^{-/-}$ Cr fed (n=6)
BW (g)	23 ± 3	20 ± 3 ***	24 ± 3
HR (bpm)	438 ± 9	454 ± 47	426 ± 26
EDV (μL)	51 ± 12	48 ± 4	58 ± 7
ESV (μL)	16 ± 6	13 ± 2	19 ± 6
EF (%)	70 ± 5	73 ± 4	67 ± 7
SV (μL)	35 ± 7	35 ± 3	39 ± 6
CO (mL/min)	16 ± 3	16 ± 3	17 ± 2
LV mass (mg)	81 ± 11	74 ± 10 **	85 ± 4
LV mass / BW (x 10 ⁴)	35 ± 3	36 ± 2	39 ± 4

Table 4-3: Cine-MRI parameters in $AGAT^{-/-}$ before and after Cr withdrawal. Data from the group used as control for haemodynamics analysis are also given and shows that the control group was well matched with the study group prior to withdrawal. ** denotes $p < 0.01$ for post-withdrawal vs pre-withdrawal (paired t-test).

4.3.1.4 Haemodynamics

The significant decrease in BW triggered the humane end-point in the animal licence, and since creatine could no longer be detected by ^1H -MRS, mice underwent invasive assessment of cardiac function at baseline and under stress conditions 91 days after creatine withdrawal (and one week after the last general anaesthetic).

The results are shown in Figure 4-5. There was no significant difference between both groups at baseline for LV pressures or for markers of contractility such as $\text{dP}/\text{dt}_{\text{max}}$ or relaxation such as $\text{dP}/\text{dt}_{\text{min}}$ or tau (τ), the isovolumetric constant of relaxation. Contractile reserve, an early marker of cardiac dysfunction was examined by infusing low and high dose dobutamine. Both groups showed a marked and significant response to this β -adrenergic stimulation with a positive chronotropic (increase in HR by 17 % and 13 % in Cr-withdrawn and Cr-fed respectively), inotropic (increase in $\text{dP}/\text{dt}_{\text{max}}$ by 40 % and 23 %) and lusitropic effect (decrease in tau by 20 % and 19 % and increase of $|\text{dP}/\text{dt}_{\text{min}}|$ by 21 % and 17 % in Cr-withdrawn and Cr-fed respectively). No difference between the two groups was found for any parameter.

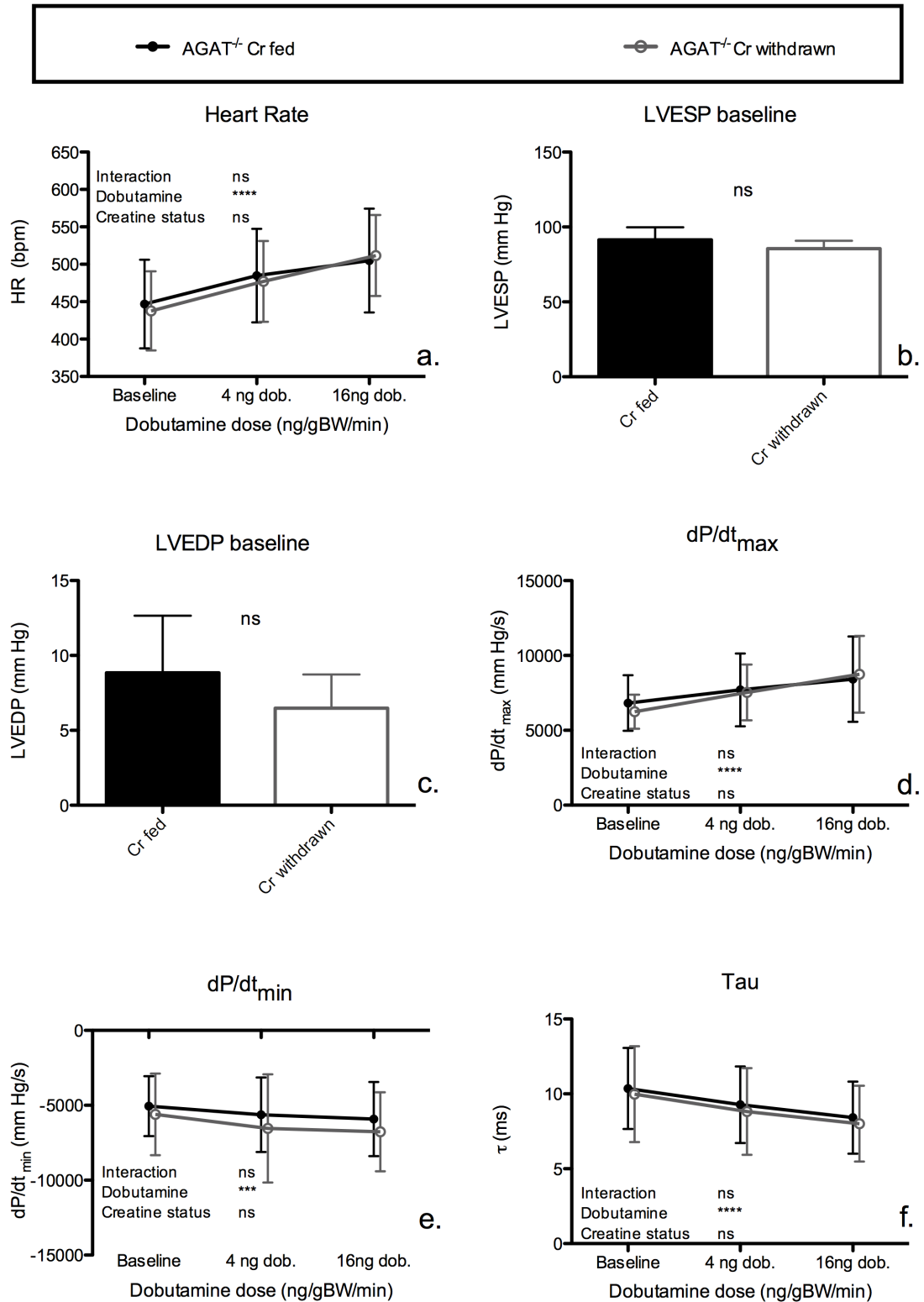


Figure 4-5: Heart rate (a.), baseline left ventricular end systolic pressure (LVESP) (b.), baseline left ventricular end diastolic pressure (LVEDP) (c.), dP/dt_{max} (d.), dP/dt_{min} (e.), tau (τ) (f.) in WT ($n=6$) and AGAT^{-/-} creatine-withdrawn mice ($n=6$). Values are mean $\pm$ SD. *** denotes $p < 0.001$ and **** $p < 0.0001$. The statistical tests used were t-test for baseline parameters and repeated measures 2-way ANOVA with Bonferroni's correction (Factors: Drug (3 levels) and genotype (2 levels)).

4.3.1.5 General macroscopic phenotyping

On post-mortem examination, organs appeared healthy and free of lesions in both Cr-withdrawn and Cr-fed mice. A significant decrease by 17 % of LV mass in Cr-withdrawn mice was observed, confirming the cine-MRI finding. However, there was no significant difference in the LV/body weight ratio. Finally, lung mass was 11 % lower in Cr-withdrawn mice (Table 4-4).

	AGAT^{-/-} Cr-fed (n=6)	AGAT^{-/-} Cr-withdrawn (n=6)	Difference with respect to WT (%)
Body weight (g)	24 ± 3	20 ± 3 *	- 17 %
Heart mass (mg)	120 ± 9	102 ± 13 *	- 15 %
LV mass (mg)	87 ± 7	72 ± 10 *	- 17 %
RV mass (mg)	20 ± 1	19 ± 4	- 5 %
Lung mass (mg)	137 ± 6	122 ± 10 **	- 11 %
Liver mass (g)	1010 ± 258	949 ± 212	- 6 %
Kidney (g)	281 ± 33	255 ± 53	- 9 %
Tibial length (mm)	17.9 ± 0.4	17.8 ± 0.3	0 %
LV mass / BW (x 10 ³)	3.6 ± 0.2	3.7 ± 0.3	0 %
Lung mass / BW (x 10 ³)	5.8 ± 0.2	6.2 ± 0.6	+ 8 %

*Table 4-4: Anatomical characteristics at post-mortem of AGAT^{-/-} mice fed creatine and of AGAT^{-/-} withdrawn from any source of creatine for 91 days. * denotes $p < 0.05$, ** $p < 0.01$ (t-test).*

4.3.2 Phenotyping of creatine naïve AGAT^{-/-} mice

This initial study demonstrated that loss of Cr from myocardium is a slow process since, after more than 3 months of dietary creatine withdrawal, measurable levels of Cr still remained in the myocardium. Continued study at longer time points was prevented by severe body weight loss. Therefore, in order to assess the consequences of true creatine deficiency,

it was decided to study the phenotype of creatine-naïve AGAT^{-/-} i.e. mice that have never been exposed to exogenous creatine since weaning.

4.3.2.1 Myocardial creatine concentration

Creatine levels were measured by HPLC in the myocardium of a random sample of the above-mentioned AGAT^{-/-} and littermates (2M, 3F for each group). Surprisingly, a peak was observed at the expected creatine retention time. This leads to an estimated creatine concentration in the knockout mice of 12.4 ± 0.9 nmol/mg protein, which still represents 18 % of control levels (creatin concentration in WT = 67.8 ± 5.2 , $p < 0.0001$).

As these mice had not been exposed to exogenous creatine in the 3 to 4 months since weaning, it was necessary to confirm the presence or absence of remaining creatine in the myocardium using another technique. Consequently, solution-state NMR was performed in a second group of mice comprising of 3 AGAT^{-/-} and 3 littermate controls. Spectra showed unambiguously the presence of both methyl and methylene resonances of creatine in hearts from WT but not in knockout mice (Figure 4-6). Moreover, a 1.3 fold increase in taurine levels in the creatine deficient hearts was measured ($p = 0.03$). This result suggests that HPLC has poor accuracy for measuring very low levels of myocardial Cr and confirms that Cr-naïve animals were in fact creatine-free.

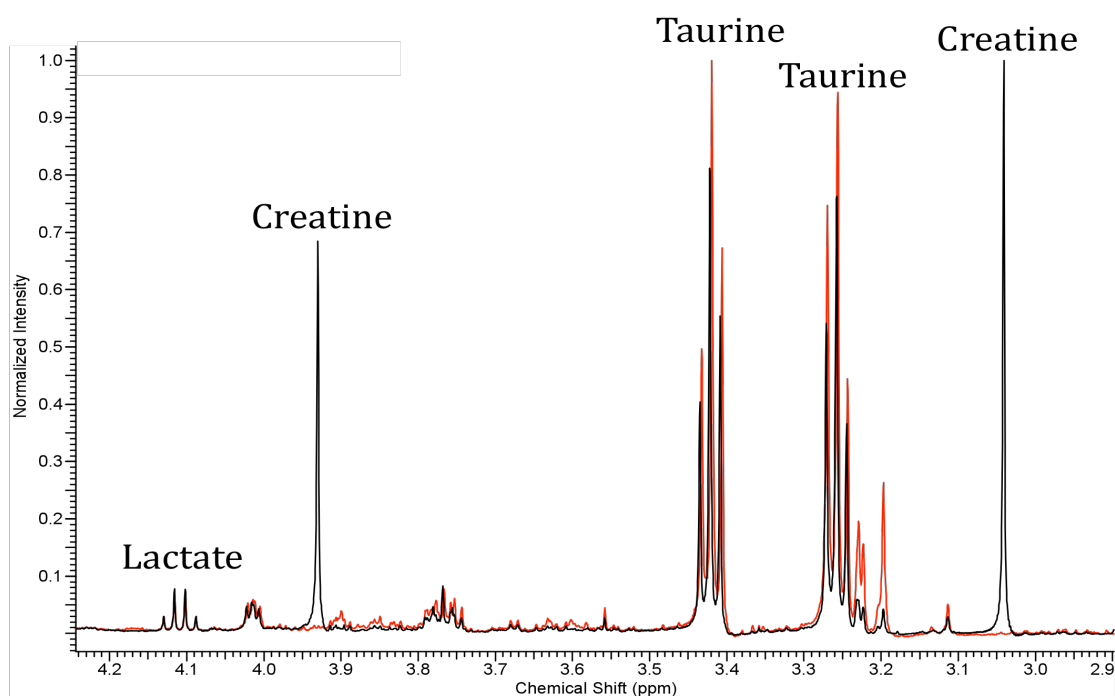


Figure 4-6: Solution state ¹H-NMR spectra containing the resonances characteristic of taurine and creatine, which were found to be discriminatory in differentiating WT from AGAT^{-/-} mice (WT is in black and AGAT^{-/-} in red). No creatine resonance is visible in knockout mice.

4.3.2.2 In vivo cardiac function

Cardiac function was assessed by two different methods: non-invasively at baseline using cine-MRI (Table 4-5) and invasively at baseline and following adrenergic stimulation by LV haemodynamics (Figure 4-8). As male and female mice behaved in the same way, they were grouped for further analysis.

Cine-MRI

In vivo MRI (Table 4-5) did not detect major changes in cardiac function. The ejection fraction was normal in the AGAT^{-/-} mice ($71 \pm 7\%$ versus $73 \pm 7\%$). The volume of the left ventricular cavity was smaller in the knockout group both in diastole and in systole, which

parallels the smaller LV mass (Figure 4-7). There was a significant decrease in cardiac output (15 ± 2 versus 12 ± 2 mL/min, $p < 0.05$), consequence of the concomitant decrease of HR and stroke volume; neither of the latter two parameters being significantly different from wild-type when analysed individually.

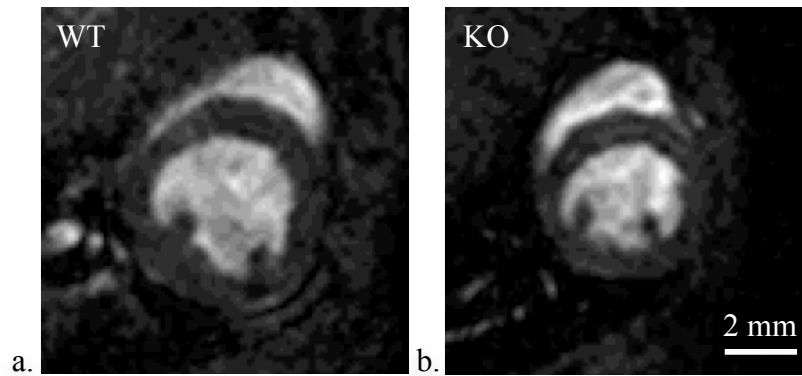


Figure 4-7: In vivo end-diastolic MR images from (a.) a WT and (b.) an $AGAT^{-/-}$ (KO) mouse. The scale of 2 mm applies to both panels. The $AGAT^{-/-}$ heart is clearly smaller.

	WT (n=10, 5M, 5F)	$AGAT^{-/-}$ (n=10, 5M, 5F)
Body weight (g)	28 ± 6	14 ± 1 ****
Heart rate (g)	411 ± 48	387 ± 44
EDV (μ L)	51 ± 7	43 ± 5 *
ESV (μ L)	15 ± 4	11 ± 3 *
SV (μ L)	35 ± 5	32 ± 7
EF (%)	71 ± 7	73 ± 7
CO (mL/min)	15 ± 2	12 ± 2 *
Cardiac index (mL/(min.g))	0.52 ± 0.07	0.85 ± 0.14 ***
LV mass (g)	82 ± 13	63 ± 5 ***
LV mass / BW ($\times 10^3$)	2.9 ± 0.3	4.4 ± 0.3 ****

Table 4-5: Cine-MRI parameters in $AGAT^{-/-}$ mice creatine-naïve and wild-type littermate controls. * denotes $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ (t-test).

***In vivo* haemodynamics at baseline and during inotropic stimulation**

Results of haemodynamic measurements are shown in Figure 4-8. At baseline, heart rate was significantly lower in AGAT^{-/-} (452 ± 37 bpm in WT vs 357 ± 60 in AGAT^{-/-}, $p < 0.01$). Markers of systolic function (LVESP, $dP/dt_{\max}$) were significantly lower in the transgenic group with a decrease in $dP/dt_{\max}$ by 39 % ($p < 0.01$, two-way ANOVA, Bonferroni's correction). Relaxation was also impaired with a significant difference in $dP/dt_{\min}$ (-4166 mmHg/min vs -7590 mmHg/min, $p < 0.01$). Finally, baseline LVEDP was increased (4 ± 3 vs 7 ± 3 , $p < 0.05$) but remained within physiological limits.

β -adrenergic stimulation resulted in a similar stepwise increase in HR by 20 % in WT ($p < 0.0001$) and 27 % in transgenic mice ($p < 0.001$) between baseline and high-dose dobutamine. In wild-type mice, dobutamine induced marked changes in $dP/dt_{\max}$ both at 4 and 16 ng/gBW/min (increase by 27 % ($p < 0.0001$) and 39 % ($p < 0.0001$) vs baseline respectively). The contractility increased in the AGAT^{-/-} group but in a more discrete way (non significant increase by 20 % at low-dose dobutamine and significant increase by 30 % ($p < 0.05$) at high-dose dobutamine). However, maximal contractility remained significantly lower in AGAT^{-/-} mice, and dobutamine did not induce significant changes in $dP/dt_{\min}$.

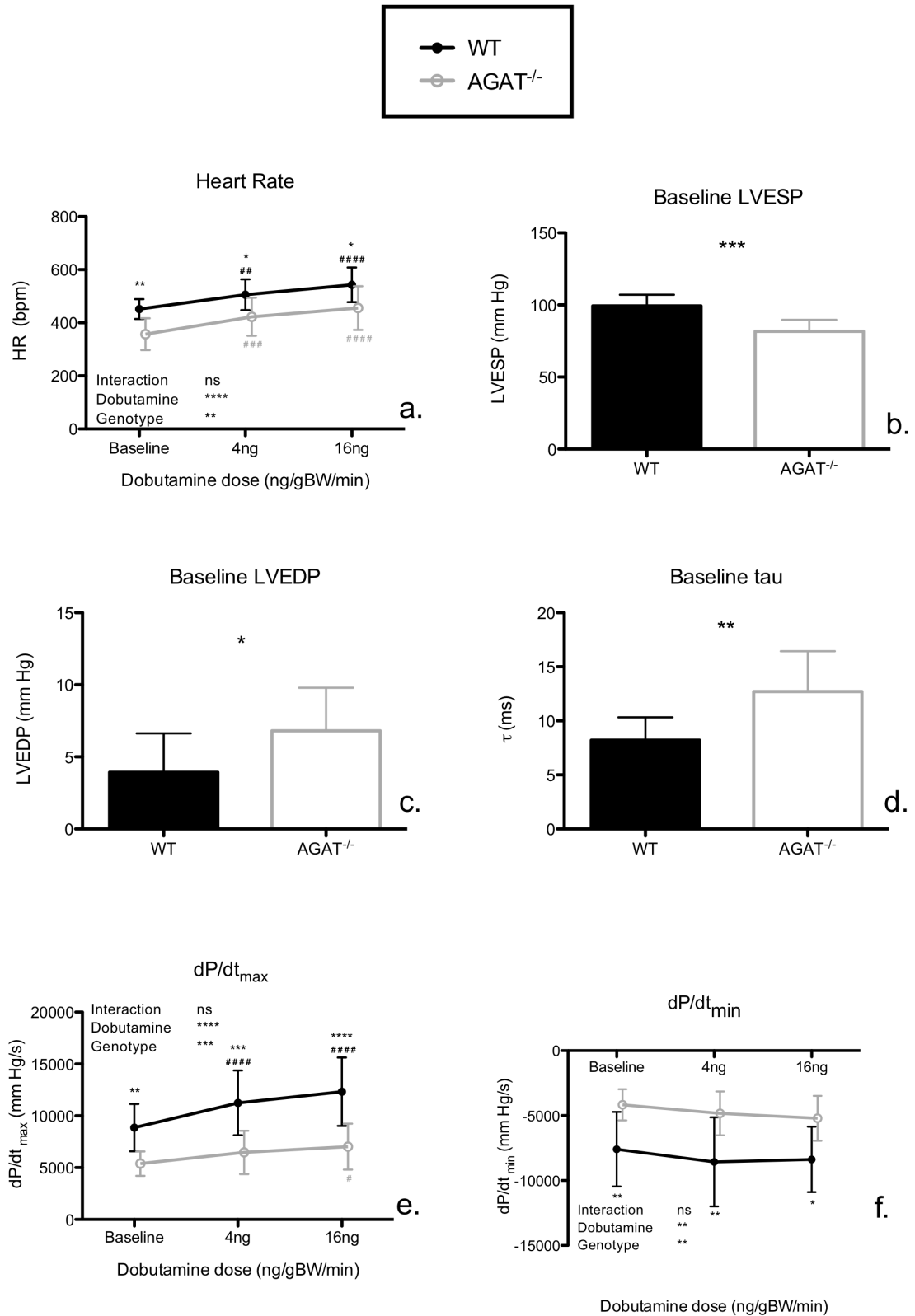


Figure 4-8: Heart rate (a.), baseline left ventricular end systolic pressure (LVESP) (b.), baseline left ventricular end diastolic pressure (LVEDP) (c.), tau (d.), dP/dt_{max} (e.), dP/dt_{min} (f.) in WT (n=10) and AGAT^{-/-} creatine-withdrawn mice (n=10). Values are mean ± SD. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs WT at the same dobutamine concentration. ## denotes $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$ vs baseline for the same genotype (t-test or 2-way ANOVA with Bonferroni's correction).

The main characteristics of cardiac phenotype of the AGAT^{-/-} mice were confirmed in a group of mice exposed to minute amount of creatine through coprophagia (Table 4-6).

	WT never exposed to Cr (n=10)	AGAT^{-/-} never exposed to Cr (n=10)	WT exposed to minute amount of Cr through coprophagia (n=10 at baseline and n= 9 for dobutamine)	AGAT^{-/-} exposed to minute amount of Cr through coprophagia (n=10 at baseline and n=9 for dobutamine)
[Cr] (HPLC) (nmol/mg prot)	68 ± 5	12 ± 1	73 ± 11	18 ± 5
Baseline haemodynamic parameters				
HR (bpm)	452 ± 37	357 ± 60 **	459 ± 63	383 ± 48 **
LVESP (mm Hg)	99 ± 8	82 ± 8 ****	101 ± 10	81 ± 7 ***
LVEDP (mm Hg)	4 ± 3	7 ± 3	9 ± 6	8 ± 4
dP/dt _{max} (mm Hg/s)	8859 ± 2286	5387 ± 1172 **	9117 ± 1905	6467 ± 746 **
dP/dt _{min} (mm Hg/s)	-7590 ± 2868	-4166 ± 1201 **	-8072 ± 1867	-5594 ± 916 **
Tau (ms)			8.3 ± 1.6	9.7 ± 2.0
Aortic pressure – systolic (mm Hg)			101 ± 5	84 ± 7 ****
Aortic pressure – diastolic (mm Hg)			66 ± 4	55 ± 8 **
Following dobutamine infusion (16 ng/gBW/min)				
HR (bpm)	543 ± 37	456 ± 82 *	512 ± 53	449 ± 47 *
LVESP (mm Hg)	102 ± 6	83 ± 12 ****	96 ± 5	86 ± 6 *
LVEDP (mm Hg)	6 ± 3	7 ± 3	7 ± 3	8 ± 4
dP/dt _{max} (mm Hg/s)	12322 ± 3305	7020 ± 2216 ****	11287 ± 2857	6782 ± 1273 ****
dP/dt _{min} (mm Hg/s)	-8378 ± 2522	-5212 ± 1731 *	-8108 ± 1669	-4924 ± 1531 ***
Tau (ms)			7.0 ± 1.8	10.2 ± 3.5 *
Aortic pressure – systolic (mm Hg)			93 ± 5	82 ± 6 **
Aortic pressure – diastolic (mm Hg)			54 ± 7	54 ± 6

Table 4-6: Summary of the haemodynamics data of mice exposed to minute amount of Cr through coprophagia and of Cr-naïve mice already described above. * denotes $p < 0.05$ vs the respective control, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (2-way ANOVA, Bonferonni correction). Values are mean ± SD. Creatine levels have been measured by HPLC with the limitations mentioned above.

Wall stress

AGAT^{-/-} had smaller hearts and lower LVSP. We therefore calculated wall stress to determine whether these changes were proportional. Using cine-MRI and haemodynamics data, LV thickness and wall stress were calculated assuming a spherical shape of LV. Data are shown in Table 4-7.

	WT systole	AGAT^{-/-} systole	WT diastole	AGAT^{-/-} diastole
Wall thickness (mm)	1.3 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	0.7 ± 0.1**
Wall stress (kdyn/cm ²)	79 ± 12	64 ± 12*	7 ± 5	14 ± 5*

Table 4-7: Wall thickness and wall stress in systole and diastole. Data were calculated from cine-MRI and haemodynamics data assuming a spherical shape of LV (see 4.2.3 for details). * denotes $p < 0.05$ and ** $p < 0.01$ (t-test). Data are expressed as Mean ± SD.

Systolic wall stress was lower in AGAT^{-/-} mice. However, it was increased by two-fold in diastole, which reflects an increase in preload²⁴³. This change is mainly driven by the higher LVEDP in AGAT^{-/-} mice. However, the significance of elevated end diastolic wall stress is questionable, as it remains within the physiological range. A decrease in the calculated wall thickness both in systole and diastole (significant only in diastole) was also observed.

Conscious ECG

A significant difference in heart rate was observed under general anaesthesia. To determine whether this difference was due to an intrinsic difference or to a susceptibility to general anaesthesia, we performed conscious ECG. Heart rate in conscious, unrestrained mice was 684 ± 18 bpm in WT and 692 ± 42 in $AGAT^{-/-}$ ($p = ns$). There was no difference in the duration of PR and QRS intervals (Table 4-8).

	WT	AGAT ^{-/-}
HR (bpm)	684 ± 18 (n=7)	692 ± 42 (n=8)
PR (ms)	32 ± 1 (n=6)	31 ± 2 (n=6)
QRS (ms)	9 ± 1 (n=6)	9 ± 1 (n=6)

Table 4-8: Conscious ECG parameters. No significant difference between groups (*t*-test). Values are mean $\pm$ SD.

4.3.2.3 Body composition

The most striking phenotypic characteristic of $AGAT^{-/-}$ was a reduced body weight, which was 45 % lower in adult males and by as much as 53 % in females (Table 4-9 and Figure 4-9).

		WT (5M, 5F)	AGAT ^{-/-} (7M, 6F)	Difference with respect to WT (%)
Age (weeks)	M	22 ± 1	22 ± 2	
	F	22 ± 1	22 ± 2	
Body weight (g)	M	33 ± 5	15 ± 1 ****	- 55 %
	F	23 ± 3	12 ± 1 ****	- 47 %
Whole heart mass (mg)	M	134 ± 11	91 ± 7 ****	- 32 %
	F	102 ± 11	80 ± 5 ***	- 22 %
LV mass (mg)	M	93 ± 11	63 ± 3 ****	-32 %
	F	72 ± 8	57 ± 3 **	- 21 %
RV mass (mg)	M	26 ± 3	16 ± 1 ****	- 39 %
	F	21 ± 4	15 ± 2 **	- 30 %
Lung mass (mg)	M	139 ± 14	97 ± 7 ****	- 30 %
	F	124 ± 13	91 ± 9 ****	- 27 %
Liver mass (mg)	M	1354 ± 253	857 ± 245 **	- 37 %
	F	993 ± 157	617 ± 116 *	-38 %
Kidney mass (mg)	M	305 ± 32	223 ± 23 ****	- 27 %
	F	218 ± 30	178 ± 17 *	- 18 %
Tibial length (mm)	M	18.1 ± 0.5	17.3 ± 0.3 **	- 5 %
	F	17.8 ± 0.7	16.9 ± 0.2 **	- 5 %
LV mass / BW (x 10 ³)	M	2.8 ± 0.2	4.3 ± 0.5 ****	+ 53 %
	F	3.1 ± 0.3	4.7 ± 0.5 ****	+ 49 %
LV mass / tibial length (mg/mm)	M	5.1 ± 0.5	3.7 ± 0.2 ****	- 29 %
	F	4.0 ± 0.3	3.3 ± 0.2 **	- 17 %

Table 4-9: Post-mortem anatomical measurements. N = 13 AGAT^{-/-} (7M, 6F) and 10 WT (5M, 5F). * denotes $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (two-way ANOVA, Bonferroni's correction). Values are mean ± SD.

Different factors contribute to this lower BW. First, these mice showed growth retardation with a significant decrease in tibial length by about 5 % both in male and female ($p < 0.01$, 2-way ANOVA, Bonferroni's correction). Moreover, body composition analysis, performed in a separate group of mice at the same age, showed a reduction in absolute content of lean mass and total water by about 40-50 % (Figure 4-9 a.). However, relative to body weight, the

water content and lean mass were identical in knockout and WT animals (Figure 4-9 b.). Interestingly, there is an overall downsizing of all major thoraco-abdominal organs by ~20-40 %, which can partially explain the decrease in lean mass in absolute value (Table 4-9). However, as the decrease in organ mass is not as pronounced as the decrease in lean mass, it implies the presence of a particularly strong atrophy of the skeletal muscles.

Finally, an extreme reduction in body fat content both in absolute and relative values contributed to the remarkably low body weight of these 5 months old mice. Total fat mass represented only 15 % of the total body weight in AGAT^{-/-} mice versus 22 % in wild-type ($p < 0.05$, t-test) (Figure 4-9).

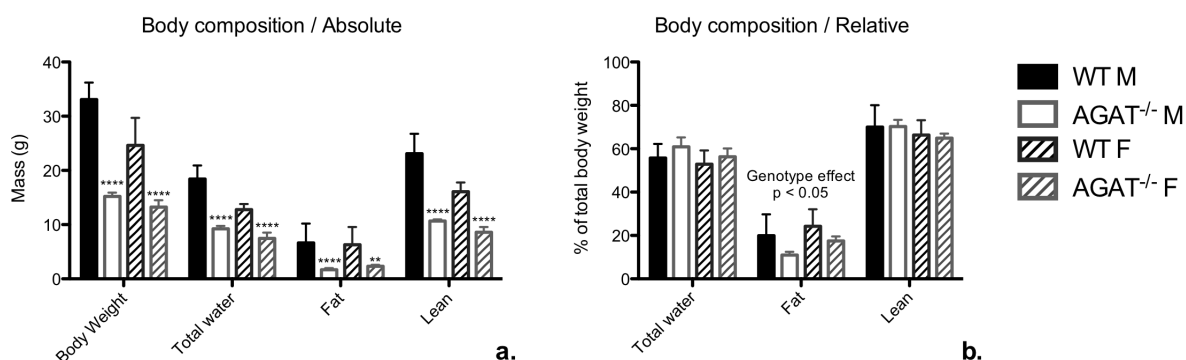


Figure 4-9: Body composition in AGAT^{-/-} in absolute values (in grammes) (a) and relative to body weight (in % of total body weight) (b). ** denotes $p < 0.01$ and **** $p < 0.0001$ (one-way ANOVA with Bonferroni's correction). Data are expressed as Mean $\pm$ SD.

4.3.2.4 Biochemical changes in the high-energy phosphotransfer system

Unless otherwise specified, all the biochemical analyses were performed on heart tissue from all the mice, which underwent cine-MRI and haemodynamics.

Activity of enzymes involved in energetic transfer

To determine whether changes in the energetic enzymes occurred secondary to lack of creatine, we measured the activity of the two main enzymes involved in the high-energy phosphotransfer activity: creatine kinase and adenylate kinase. We did not find any striking difference in total creatine activity, neither real changes in the relative proportion of the different isoenzymes (Figure 4-10 a. and b.). Similarly, there was no change in the total adenylate kinase activity (Figure 4-10 c.).

Potential changes in mitochondrial volume were evaluated by measuring citrate synthase activity, a mitochondrial matrix marker enzyme. There was no difference in activity between WT and knockout mice (Figure 4-10 c.).

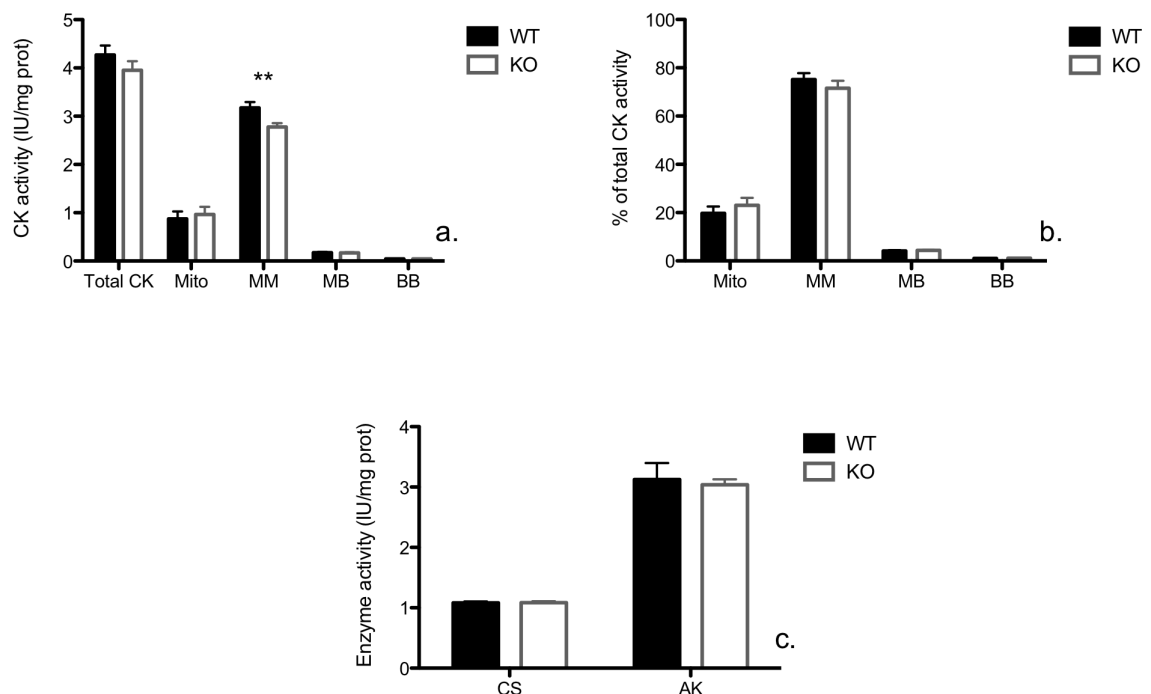


Figure 4-10: Creatine kinase, citrate synthase and adenylate kinase activities in the left ventricle. Panel a. shows activity of total CK and of the four isoenzymes. Panel b. shows the relative contribution of the different isoenzymes expressed as a percentage of total CK activity. Panel c. shows the activities of citrate synthase (CS) and adenylate kinase (AK). ** denotes $p < 0.01$ (t-test). Data are expressed as Mean $\pm$ SD.

Up-regulation of the creatine transporter

Finally, changes in the CrT gene expression were analysed by real-time RT-PCR and showed a significant up-regulation of the expression of the creatine transporter gene by 2.1 fold ($p = 0.02$, t-test) (Figure 4-11).

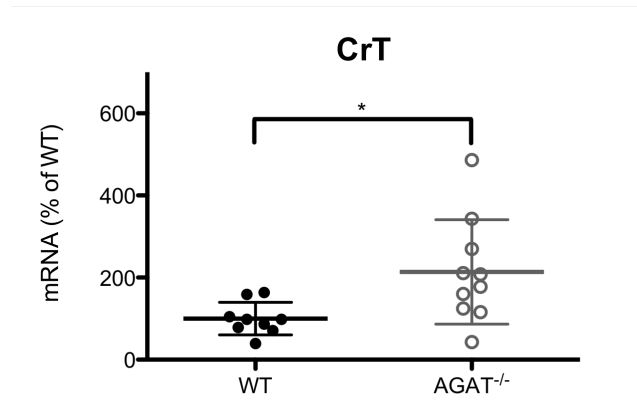


Figure 4-11: Relative mRNA expression of CrT (expressed in percentage of WT levels). * denotes $p < 0.05$ (t-test). Data are expressed as Mean $\pm$ SD.

4.3.2.5 Are the AGAT^{-/-} hearts hypertrophied?

In absolute terms, LV mass was 27 % lower in AGAT^{-/-}. However, there was an increase in the LV/Body weight ratio and a decrease in LV weight/tibial length ratio. These two ratios are frequently used as markers of cardiac hypertrophy. However, due to changes in body composition and size, it is unclear whether or not LV mass is proportionate to the haemodynamic requirements of the body.

Consequently, we quantified gene markers for LV hypertrophy to clarify whether the AGAT^{-/-} hearts are hypertrophied.

Gene expression of hypertrophy markers

Molecular markers of myocardial hypertrophy were measured in LV of WT and AGAT^{-/-} by RT-PCR. The expression of multiple members of the “hypertrophic gene programme” was determined due to known variability of expression in different models of hypertrophy²⁴⁴.

Differences in the expression pattern of these different genes were observed. Atrial natriuretic peptide (ANP) was over-expressed in AGAT^{-/-} mice by 4.2 fold ($p < 0.001$, t-test) whereas α -skeletal actin (α -SA) expression was 2.8 fold lower ($p < 0.05$, t-test). Changes in β -myosin heavy chain (β -MHC) and brain natriuretic peptide (BNP) were not significant (Figure 4-12). In brief, changes in hypertrophic markers were inconclusive and are therefore not in favour of cardiac hypertrophy, since a more consistent increase in all parameters would be expected.

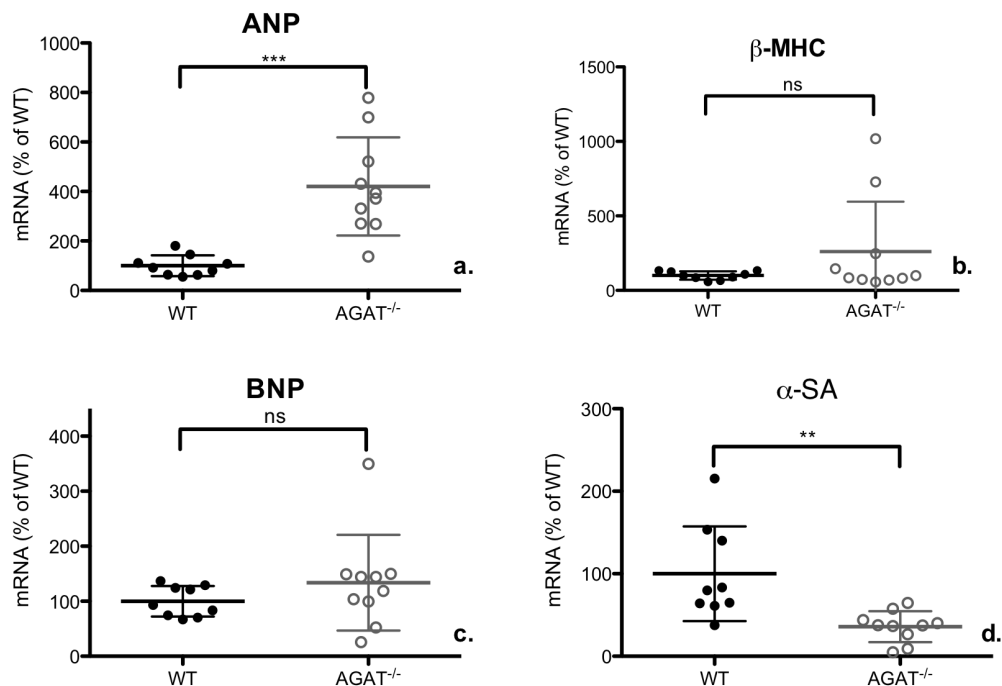


Figure 4-12: Relative mRNA expression of hypertrophic markers in LV tissue: ANP (panel a.), β -MHC (panel b.), BNP (panel c.) and α -SA (panel d.). Values are normalised to mean WT levels. ** denotes $p < 0.01$, *** $p < 0.001$ and ns non-significant. Data are expressed as Mean $\pm$ SD.

NB: One WT sample was discarded from analysis due to unspecific amplification.

Myocyte cross-section area

Absence of hypertrophy was confirmed by histological staining and quantification of cell morphometry. There was no difference in the myocyte cross-section areas between the AGAT^{-/-} and WT mice ($198 \pm 11 \mu\text{m}^2$ in WT vs 210 ± 70 in AGAT^{-/-}, $p = 0.79$, t-test) (Figure 4-13).

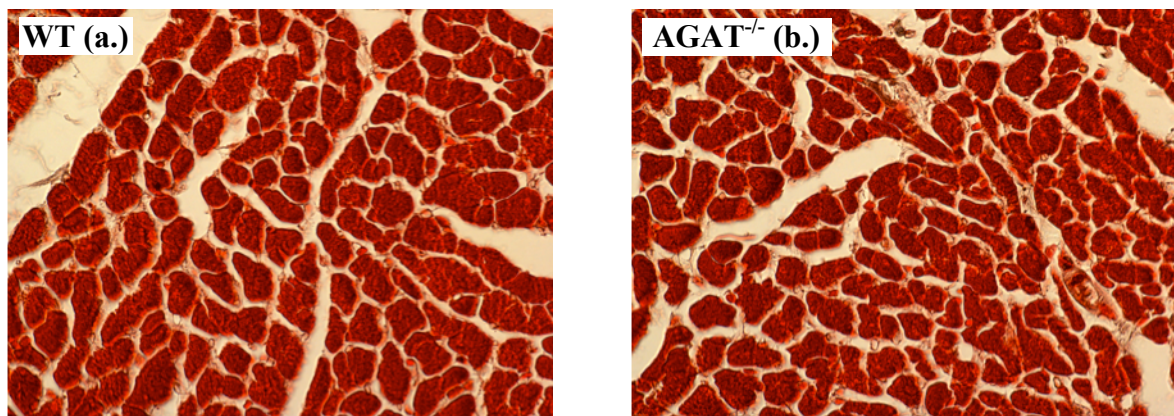


Figure 4-13: Histological analysis of heart sections from WT (a.) and AGAT^{-/-} (b.) mice stained with Masson trichrome.

4.3.3 Phenotype rescue by dietary creatine supplementation

The observed AGAT^{-/-} phenotype could be due to the absence of creatine *per se*, in which case it should rapidly resolve as creatine is introduced. Alternatively, the phenotype may be a consequence of long-term adaptations to chronic Cr depletion, in which case rescue by dietary creatine could be expected to take weeks. Parameters that do not resolve with long-term dietary creatine could reflect non-creatine role for the AGAT enzyme.

In order to determine this, one group of AGAT^{-/-} mice (and their WT controls) was fed Cr for 1 week while a second group was fed Cr for 7 weeks.

Two males AGAT^{-/-} (one in each group) were either euthanized or found dead a few days following the change in diet. Moreover, haemodynamic data were not available for one female knockout (7 weeks group) due to technical failure.

4.3.3.1 Myocardial creatine uptake

Creatine levels were measured in the left ventricle by HPLC. After one week of supplementation, creatine concentration had already reached normal levels (70 ± 2 in AGAT^{-/-} versus 74 ± 3 nmol/mg protein in WT, $p = ns$) (Figure 4-14).

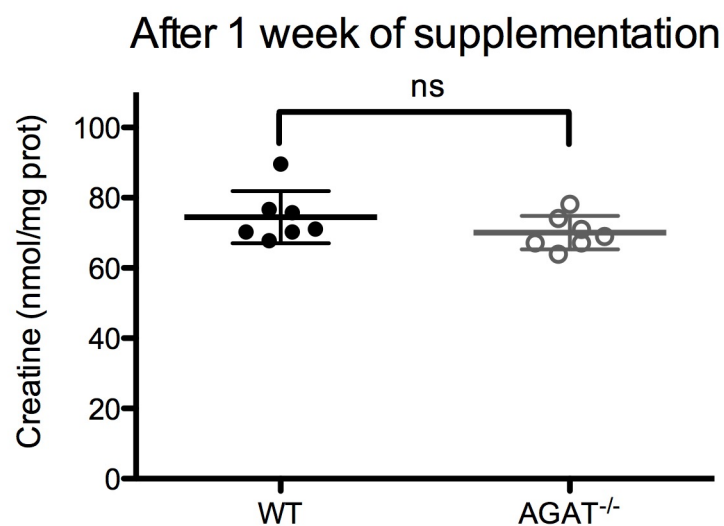


Figure 4-14: Myocardial creatine concentration after one week of dietary creatine supplementation. ns denotes a non-significant difference (t-test).

4.3.3.2 Cardiac function

Cardiac haemodynamic function was assessed invasively by LV catheterisation at baseline and under β -adrenergic stimulation and have been compared to littermate controls. The results of the two groups of creatine-rescue mice have been juxtaposed to the creatine-naïve mice previously described in 4.3.2 (Figure 4-15).

After one week of supplementation, there was a trend towards normalisation of LVESP from 82 ± 8 to 91 ± 4 mm Hg ($p = \text{ns}$), although this remained significantly different from WT. However, there was no marked improvement in HR, in contractility (dP/dt_{max}) or in relaxation (dP/dt_{min}) compared to naïve animals. It is worth noting the high standard deviation in LVEDP measurements and the variability amongst the three WT control groups. This is in part a consequence of the zero pressure drifting during the experiment, for which the cannula used in these experiments is particularly prone, due to its very small size and fragility. Compensation for this drift is made by subtracting the baseline pressure measured at the end of the experiment; however this is an imperfection as the same correction is applied to the entire experiment. Furthermore, baseline drift gets worse as the cannula ages and these groups were studied sequentially.

A similar pattern was observed after 7 weeks of creatine diet, with LVESP further increased to complete normalisation (101 ± 7 versus 101 ± 6 mmHg for the WT group, $p = \text{ns}$), and only marginal improvement in HR, while dP/dt_{max} and dP/dt_{min} remained significantly different to WT controls.

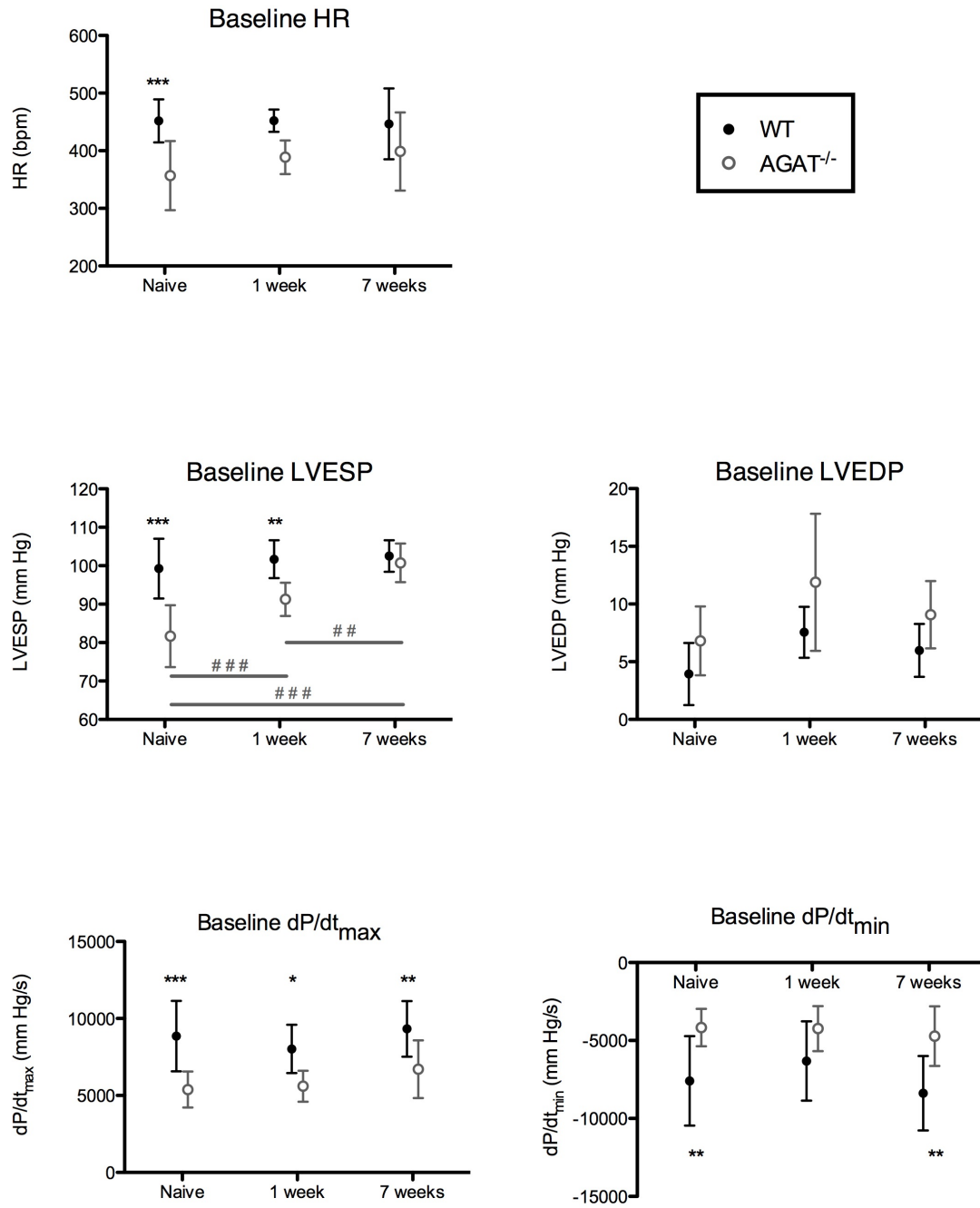


Figure 4-15: Baseline haemodynamic parameters in AGAT^{-/-} Cr-naïve, fed Cr for 1 week and fed creatine for 7 weeks with their respective WT controls. * denotes $p < 0.05$ vs WT, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. # denotes $p < 0.05$ vs same phenotype with different creatine treatment, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. Statistical analysis: two-way ANOVA with Bonferroni's correction. Data are expressed as Mean $\pm$ SD.

All groups (WT, AGAT^{-/-} Cr-naïve, AGAT^{-/-} fed Cr for 1 and 7 weeks) responded in a similar way to β -adrenergic stimulation for HR with a progressive increase from low to high dose dobutamine (Figure 4-16). As expected there was a robust increase in $dP/dt_{\max}$ and $|dP/dt_{\min}|$ in wild-type mice. However, there was barely any contractile reserve in the one-week rescue group with only partial improvement at 7 weeks. In both, maximal contractility remained significantly impaired compared to WT controls.

In summary, only LVESP was rescued by Cr treatment with partial recovery at one week and full normalisation by 7 weeks.

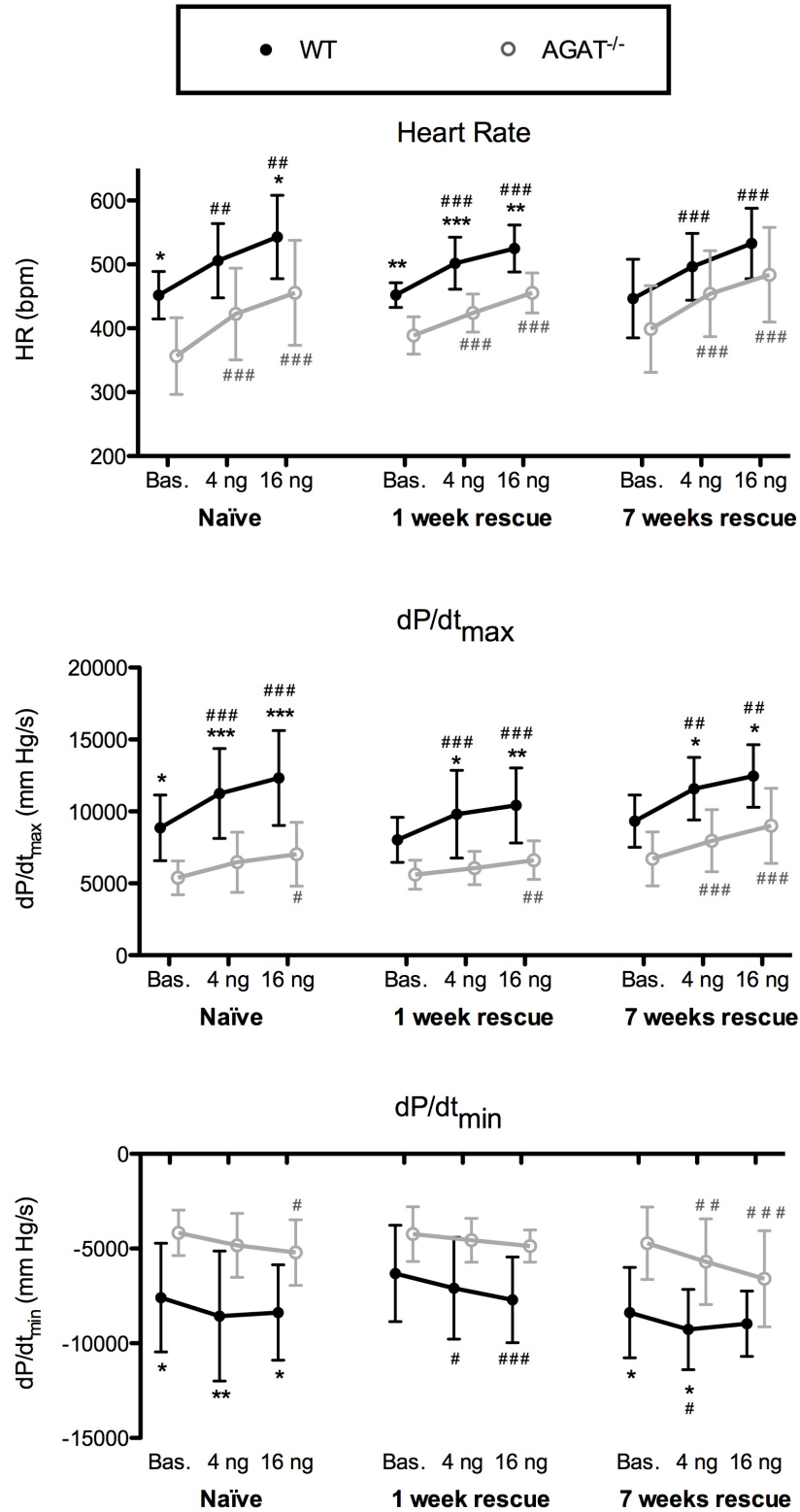


Figure 4-16: Response to dobutamine stimulation in AGAT^{-/-} Cr-naïve, Cr-fed for 1 week and Cr-fed for 7 weeks with their respective WT controls. Bas. stands for baseline; 4 ng and 16 ng for infusion with 4 and 16 ng/gBW/min of dobutamine respectively. * denotes $p < 0.05$ vs WT at the same dobutamine dose, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. # denotes $p < 0.05$ vs baseline, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$. Statistical analysis (two-way ANOVA with repeated measures and Bonferroni's correction) was performed independently for each of the creatine treatment group. Data are expressed as Mean $\pm$ SD.

4.3.3.3 Body composition changes

As the sex ratio between groups was not homogeneous and due to a marked sexual dimorphism for body composition, we randomly selected mice to get matched groups between AGAT^{-/-} and wild-type for each experiment (1 and 7 weeks supplementation).

The data show that after only one week of creatine supplementation, the BW of the AGAT^{-/-} had already increased by 15 % ($p < 0.001$). This change was even more striking since in the meantime, the WT lost 5 % of their initial BW ($p < 0.01$) probably due to reduced food consumption in the first few days after the change in diet. The observed decrease in fat but not lean mass in the WT was in favour of this hypothesis. The rise in BW in the AGAT^{-/-} mice was mostly attributable to an increase in lean mass by 28 % ($p < 0.0001$) (Figure 4-17).

In the group of AGAT^{-/-} fed creatine for 7 weeks, BW raised significantly from 14 ± 2 g to 21 ± 3 g ($p < 0.001$). Both an increase in fat and lean mass by 58 % and 57 % respectively contribute to this dynamic change in body condition. However, the AGAT^{-/-} BW was still 36 % lower than in WT mice ($p < 0.0001$) with lower fat and lean contents. In contrast, after only one week of Cr supplementation, the size of all thoraco-abdominal organs – lungs excepted – was not significantly different from WT mice (Table 4-10).

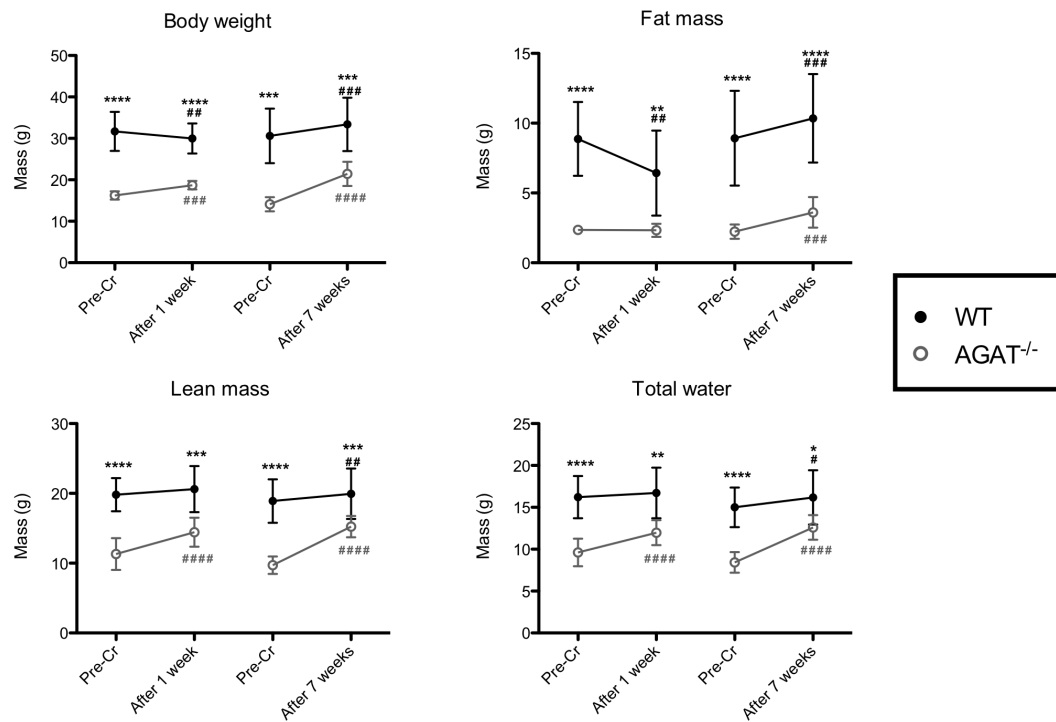


Figure 4-17: Evolution of body composition parameters following creatine supplementation. Two groups of mice were used: $n=4M$ & $3F$ for the 1 week supplementation experiment and $n=2M$ & $4F$ for the 7 weeks supplementation. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ for WT vs AGAT^{-/-} at the same time-point (2-way ANOVA Bonferonni's correction). ## denotes $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ vs baseline data of same genotype (2-way ANOVA with repeated measures, Bonferonni's correction). NB: The 1 week and 7 weeks supplementation groups were analysed separately. Data are given as mean $\pm$ SD.

	WT Cr-fed 1 week (4M, 3F)	AGAT ^{-/-} Cr-fed 1 week (4M, 3F)	WT Cr-fed 7 weeks (2M, 4F)	AGAT ^{-/-} Cr-fed 7 weeks (2M, 4F)
Body weight (g)	30 ± 3	19 ± 3 ****	33 ± 6	21 ± 3 **
Whole heart mass (mg)	129 ± 23	123 ± 19	130 ± 28	124 ± 21
LV mass (mg)	90 ± 16	88 ± 13	90 ± 17	86 ± 14
RV mass (mg)	25 ± 4	22 ± 5	26 ± 9	23 ± 5
Lung weight (mg)	146 ± 12	129 ± 12 *	146 ± 16	126 ± 11 *
Liver weight (mg)	1312 ± 254	1197 ± 166	1413 ± 341	1075 ± 202
Kidney weight (mg)	305 ± 59	283 ± 51	330 ± 98	283 ± 82
Tibial length (mm)	18.4 ± 0.5	17.8 ± 0.5 *	18.5 ± 0.4	17.6 ± 0.2 **
LV mass / BW (x 10 ³)	3.0 ± 0.4	4.5 ± 0.2 ****	2.7 ± 0.2	4.0 ± 0.3 ****
LV mass / tibial length (x 10 ³)	4.9 ± 0.8	5.0 ± 0.6 *	4.9 ± 0.8	4.9 ± 0.7 **

Table 4-10: Post-mortem organ weights in AGAT^{-/-} and WT fed Cr for 1 or 7 weeks. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ (t-test). Data are expressed as Mean ± SD.

4.4 Discussion

This study characterized a new mouse model of creatine deficiency: the AGAT^{-/-} mouse. Lack of creatine induces a decrease in systolic pressure and an impairment of contractile reserve without signs of cardiac remodelling. It is associated with marked changes in body

composition. Moreover, it was established that contrary to myocardial Cr loss, which is a very slow process, Cr uptake in the AGAT^{-/-} mice is rapid but only partially reverses cardiac and body composition abnormalities.

4.4.1 Myocardial creatine efflux

Degradation of Cr and PCr to creatinine is a spontaneous, non-enzymatic and irreversible process. Creatinine is formed at a constant rate, diffusing into the blood before being filtered and excreted by the kidneys¹⁵. Using ¹H-MRS in a longitudinal study for the first time, the progressive creatine depletion of the myocardium was quantified in a small group of AGAT^{-/-} mice. These adult mice, fed creatine since birth, had WT myocardial creatine levels at the beginning of the study. Following dietary creatine withdrawal, myocardial creatine levels decreased at a measured rate of ~2.7 % per day. Despite having been calculated on a limited amount of animals and over a limited number of time points only (due to legal/ethical and technical considerations), this rate of breakdown is in accordance with a total body conversion rate of about 1.7 % per day¹⁵.

This rate of conversion is slow and after 3 months of Cr withdrawal, we could still detect more than 25 % of WT levels by HPLC. This point warrants further discussion. It questions the sensitivity of ¹H-MRS. Indeed, at this time point, ¹H-MRS was unable to detect Cr even after summation of the three spectra acquired for each mouse, which should theoretically increase the SNR by 1.7 fold. We would anticipate that for such a high remaining level of Cr, a resonance would still be detectable, unless part of this pool is MR invisible as already discussed in Chapter 3. Moreover, from the rate constant of daily myocardial creatine loss, we would expect about 10 % remaining at this time point. We have also shown that HPLC

gave false positive in Cr-naïve mice and is not an accurate technique for quantifying very low creatine values. Taking into account all these considerations, it therefore seems likely that this value of 25 % Cr remaining is an overestimate.

No change in cardiac function was detected by cine-MRI over the course of Cr withdrawal. At the final time-point, a thorough invasive haemodynamic examination of the cardiac function with inotropic stimulation did not reveal any impairment in systolic or diastolic functions. The level of creatine remaining is broadly comparable to previous experiments using β -GPA, and is in agreement that there is little functional consequence of reducing creatine in the short-term^{183, 199, 245}. For example, Shoubridge et al.¹⁹⁹ showed in *ex vivo* rat hearts that cardiac function decreased only when total creatine was reduced to 20 % of controls. However, our experiment goes further and suggests that previous findings were not simply due to β -GPA participating in the CK reaction.

4.4.2 Cardiac function and adaptations

Creatine-naïve mice were shown to have systolic dysfunction with a decrease in LVSP and in contractility ($dP/dt_{\max}$). Relaxation was impaired too with a decrease in the maximum rate of pressure decline ($|dP/dt_{\min}|$), an increase in tau and in diastolic wall stress. These changes concern principally the isovolumetric phases of cardiac cycle, which explains why no change in MR parameters was detected. Moreover, the extent of the inotropic response to dobutamine was attenuated in the AGAT^{-/-} mice, underscoring a decrease in contractile reserve. An increase in cardiac index (CO/BW) has been shown, driven by the strong decrease in body weight. However, due to the important changes in body composition and

especially fat content, the validity of this parameter is questionable as the metabolic demand of fat tissue is very low.

The lower heart rate observed in anaesthetised mice (cine-MRI and haemodynamics) was not seen in conscious mice, which suggests this is not an intrinsic difference. A deficit in thermoregulation, as has previously been described in M/mt-CK^{-/-} mice²⁴⁶, could result in changes secondary to hypothermia, but it is unlikely due to the use of supplementary heating during these experiments. More likely is that AGAT^{-/-} mice are more sensitive to the cardiac depressant effects of general anaesthesia. It is probable that extremely low fat levels induced changes in the pharmacokinetics of isoflurane and affected the ability of AGAT^{-/-} to buffer anaesthetic levels. However, this anaesthesia-hypersensitivity renders the interpretation of the haemodynamic phenotype open to question. Is the phenotype due to an enhanced isoflurane-induced cardiodepression or is it an intrinsic phenotype of chronic creatine depletion? Similarities to GAMT phenotype, which had normal HR under general anaesthesia, suggest the latter, but we cannot rule out an exaggeration of the effect by general anaesthesia. Conscious LV haemodynamic characterisation would ideally be required. However, due to the frailty of AGAT^{-/-} mice, this would be extremely difficult.

The interpretation of traditional indices of macroscopic hypertrophy such as LV mass / BW and LV mass / tibial length ratios was hampered by changes in body weight and tibial length in the AGAT^{-/-} mice. Consequently, we looked at changes at the microscopic and molecular level. There was no difference in myocyte cross-section area. Study of the expression of the hypertrophic gene program showed an increase in the expression of ANP, a decrease in α -SA whereas β -MHC and BNP were unchanged. The decrease in α -SA may indicate cellular atrophy, also suggested by the decrease in LV mass in dietary creatine withdrawn mice. In the latter, a decrease in cellular water content cannot be excluded and this may also

be reflected in the rapid restoration of heart weight upon creatine feeding. The inconsistent pattern of expression of the members of the “hypertrophic gene program” highlights the non-specificity of ANP²⁴⁷⁻²⁵⁰. As suggested by Dorn et al.²⁴⁴, the *sine qua-non* condition to conclude to the presence of cardiac hypertrophy should remain an increased cardiomyocyte size, independently of macroscopic heart changes or variation in gene expression. Therefore, we can conclude that AGAT^{-/-} hearts are not hypertrophied.

In mice models and patients with myocardial energy depletion, cardiac hypertrophy is a frequently observed adaptative mechanism²⁵¹⁻²⁵³. According to the law of Laplace, hypertrophy reduces wall stress and consequently diminishes ATP demand and utilisation. That this is not the case here or in other models of CK shuttle disruption as in GAMT^{-/-86} or in mt-CK^{-/-214}, suggests that these hearts adapt in a different way. Finally, we cannot exclude that cardiac function or remodeling would deteriorate further with ageing although this seems unlikely as no change in cardiac function or LV dimensions were observed between young and old GAMT^{-/-} mice²²⁵.

In mice with alterations to the components of the CK shuttle, morphological and metabolic adaptations of the mitochondria have been described in skeletal muscle and heart. Increases in mitochondrial volume and cytoarchitectural rearrangements occurred^{215, 216, 218}. Moreover, as the CK system is part of an intracellular phosphotransfer network coupling energy production in mitochondria and energy utilization at myofibrils, a redistribution of energy fluxes to alternative pathways such as adenylate kinase (AK) and/or glycolytic enzymes (GAPDH, PK) have been found in CK-deficient mice^{217, 254} and also in models of heart failure²¹⁷. Surprisingly, our experiments did not elicit changes in citrate synthase, a marker of mitochondrial volume, neither in adenylate kinase. Finally, we showed that *in vitro* CK activity is independent of the presence of creatine. The reciprocal is true as creatine levels

were normal in CK-deficient mice²¹². This would suggest that there is no cross-regulation of the different components of the CK shuttle.

Following creatine supplementation, myocardial creatine levels increased extremely quickly within a week. This is concordant with a 2.1 fold in CrT gene expression in creatine-naïve mice. This agrees with observations on the *GAMT*^{-/-} model, where creatine uptake was also increased by 7 fold²⁵⁵ and confirms a strong feedback mechanism by creatine on the CrT. Creatine supplementation resulted in normalisation of LVESP only, with all other parameters of cardiac function remaining significantly different from WT even after 7 weeks. This seven weeks time point was chosen as this duration was sufficient to reverse all structural and functional consequences of aortic banding²²⁸. The difference in the completeness and/or rapidity of reversal in these two models could be due to the fact that in the aortic banding model adaptations occurred at adulthood, whereas in the *AGAT*^{-/-} the adaptations to low creatine levels might come from the developmental phases. Therefore, part of these adaptations may not be reversible or may take longer. Finally, we cannot exclude an alternative function of the *AGAT* gene unrelated to Cr biosynthesis, which could affect cardiac function.

It is important to emphasize that simply correcting creatine levels was insufficient to fully rescue cardiac phenotype. This implies that energy buffering by PCr has less influence on phenotype than the long-term adaptative consequences. Therefore, to reverse the functional changes observed in HF, correcting creatine levels may not necessarily be sufficient.

4.4.3 Creatine deficiency affects body composition

We showed that creatine-free AGAT^{-/-} mice had a very low body weight due to a combination of skeletal growth retardation, reduction of lean mass, and also a particularly striking decrease in body fat content. These findings confirm the unpublished observation from our collaborators on this model²⁵⁶. A similar phenotype (reduction in body weight and fat content) has been described in M/mt-CK^{-/-} mice²⁴⁶, which could suggest a role of CK system as a whole and not a specific role of creatine *per se*. However, the exact mechanism of the body composition changes is unknown²⁵⁶. Moreover, our experiments on AGAT^{-/-} creatine-withdrawal mice suggests that there is a creatine level / threshold of about 20 % of remaining creatine, below which body composition changes occur.

As discussed above, these changes in body composition have proved to be a major limitation to this model and in understanding the precise role of Cr in the heart. On top of the likely effect on anaesthesia sensibility, the knock-on effect on substrate availability/utilization by the heart is unknown but may be profound.

4.4.4 Contribution of AGAT^{-/-} model to understanding the role of Cr in cardiac function

It is of interest to compare the findings in the AGAT^{-/-} mice with the phenotype of GAMT-deficient mice, which also lack creatine but accumulates its precursor – guanidinoacetate.

A summary of the general and cardiac phenotype of both models is shown in Table 4-11. Due to slight differences in haemodynamic protocols, all parameters are not available for both models.

	GAMT^{-/-}	AGAT^{-/-}
Body weight	↓ by 28 %	↓ by 51 %
Tibial length	↓ by 5 %	↓ by 5 %
LV weight	↓ by 19 %	↓ by 27 %
Myocyte cross sectional area	⇒	⇒
Markers of cardiac hypertrophy	ANP ⇒ BNP n.d. β-MHC ⇒ α-SA n.d.	ANP ↑ BNP ⇒ β-MHC ⇒ α-SA ↓
HR (conscious)	⇒	⇒
HR (under anaesthesia)	⇒	↓
Baseline LVSP	↓	↓
Baseline LVEDP	⇒	Trend to ↑
Baseline dP/dt _{max}	⇒	↓
Baseline dP/dt _{min}	⇒	↓
τ	⇒	↑
PRSW	⇒	n.d.
dP/dt _{max} response to dobutamine stimulation	⇒	↓
PRSW response to dobutamine stimulation	↓	n.d.

Table 4-11: Comparison AGAT^{-/-} vs GAMT^{-/-}. N.d.: not determined. PRSW: Preload recruitable stroke work.

This table shows that AGAT^{-/-} phenotype is very similar but more pronounced than GAMT^{-/-}. These data give a better understanding of the GAMT^{-/-} model and suggests that the GAMT^{-/-} phenotype was not due to a toxic effect of guanidinoacetate but that the presence of GA blunted the phenotype by participating in the CK reaction.

Contrary to the CK knockout models, the interest of the AGAT^{-/-} is that it can give insight into all the biological functions of Cr, without limiting it to its role in high energy phosphotransfer. Indeed, we cannot exclude that the phenotype observed in AGAT^{-/-} is partially due to the osmotic effect of Cr. In mice lacking taurine – a key osmolyte in the cell²⁵⁷ – a general and cardiac phenotype, which reproduces in part the one observed in GAMT^{-/-} or AGAT^{-/-} has been described. These mice have a lower body weight despite normal food intake, a moderate decrease in EF observed only in old animals, changes in myofiber and mitochondrial ultrastructure (myofibrillar fragmentation, mitochondrial swelling) and function (reduction of complex II activity) and an impaired exercise capacity²⁵⁸. There are similarities between Cr and taurine in terms of chemical structure, intracellular concentration, and also cellular uptake²⁵⁸, therefore an osmotic stress in AGAT^{-/-} mice cannot be excluded. In this context it is interesting to note that taurine levels were increased in AGAT^{-/-}, which could represent compensation for loss of the osmolyte effect of creatine. Indeed, an osmotic effect could explain the normalisation of organ weights within one week of creatine supplementation.

4.4.5 Future directions

In order to investigate the importance of creatine in maintaining cardiac energy supply, *ex vivo* or ideally *in vivo* ³¹P-MRS would be of particular interest. It would allow us to follow

ATP levels in stress conditions such as inotropic stimulation or ischaemia/reperfusion. Moreover, energy handling in the cells of AGAT^{-/-} mice will be studied further by looking at potential adaptations at the level of mitochondrial respiration.

The role of creatine as an osmolyte also needs to be assessed. This can be done by looking at the expression of osmosensitive genes such as hsp or S100²⁵⁸, or in cell culture by correlating cell volume with creatine loading.

Future work will focus on investigating the response of AGAT^{-/-} hearts to ischaemia/reperfusion injury. We anticipate an increased susceptibility of these hearts to this extreme situation of energy/demand supply mismatch and this, for two main reasons. Firstly, these hearts do not have the energetic reserve provided by PCr and this lack of energy may exacerbate the ischemic injury as seen in the GAMT^{-/-} model. Secondly, Cr has been shown *in vitro* and *in vivo* to have an antioxidant effect²⁵⁹⁻²⁶¹. Consequently, we can hypothesize that creatine could protect against the burst of oxidative stress, which occurs following reperfusion. Moreover, a neuroprotective effect of creatine against brain ischaemia but also in neurodegenerative disorders – probably through its antioxidant effect – have been described^{262, 263}.

Chapter 5

CARDIAC SUPPLEMENTATION WITH RIBOSE AND CREATINE AS A THERAPEUTIC STRATEGY IN CHRONIC HEART FAILURE

5.1 Introduction

As previously discussed, a depletion of the total creatine pool in heart failure has been observed both in patients^{29, 50, 51} and animal models^{16, 30-33, 36, 37, 264} independent of the aetiology. It is associated with a decrease in PCr/ATP ratio, which has been shown to correlate to cardiac function in a mouse model of cardiac hypertrophy²⁶⁵ and to mortality in patients with dilated cardiomyopathy⁵⁰. Therefore, increasing creatine and PCr pools has been proposed by us and by other laboratories as a strategy for treatment of heart failure^{32, 56, 87}.

However, simply feeding excess creatine has proved to be inefficient at increasing myocardial creatine levels, due to a down-regulation of the creatine transporter (CrT)^{266, 267}. This limitation has been bypassed by the generation of transgenic mice, which constitutively over-express the CrT in the heart: the CrT-model⁸⁷.

5.1.1 Creatine transporter-overexpressing mice model (CrT-OE)

A mouse model over-expressing CrT specifically in the myocardium has been created by our group⁸⁷. Two independent lines were generated: the Tg46 line with moderately elevated creatine levels (mean: 100 ± 18 nmol/mg protein; range: 68 to 118 vs WT mean: 66 ± 6 ; range 58 to 74) and the Tg55 line with a wider range of expression (mean: 133 ± 52 ; range 65 to 240). Mice with very high creatine levels developed cardiac hypertrophy, ventricular dilatation and cardiac dysfunction resulting in congestive heart failure. The intensity of systolic and diastolic dysfunction was proportional to the myocardial creatine content.

³¹P-MRS showed an increase in PCr content in the transgenic mouse, which was nonetheless proportionally smaller than the increase in total creatine. This would suggest that the CrT-OE failed to keep the creatine pool adequately phosphorylated especially when the increase in creatine is extreme (up to four fold). This resulted in an increase in free ADP levels and a reduction in free energy available from ATP hydrolysis. Finally, a decrease in enolase – a glycolytic enzyme – content and activity, leading to a decrease in glycolytic capacity was found in the CrT-OE hearts, which could, at least partially, explain the development of cardiac hypertrophy and heart failure, as decrease in enolase content have been reported in the diabetic heart, models of cardiomyopathy or in cardiac hypertrophy²⁶⁸. Despite this, it was concluded from these studies that a moderate increase in Cr levels, for which PCr/Cr ratio remains normal and hence does not induce heart failure, could still prove to be beneficial in maintaining normal Cr and PCr levels in pathological conditions.

5.1.2 Effects of moderate elevation of myocardial creatine levels in a model of congestive heart failure

To address this question, our laboratory conducted a study, in which we investigated the effect of a moderate elevation of creatine in a model of congestive heart failure (CHF) secondary to permanent coronary artery ligation. CrT-OE mice were pre-selected according to their myocardial creatine levels (up to 2-fold increase) using ¹H-MRS, which was performed by the author of this thesis. Following myocardial infarction, creatine levels decreased both in the WT and CrT-OE groups but remained supra-normal in the transgenic group. However, both groups showed similar LV hypertrophy and cardiac dysfunction six

weeks after infarction. This suggested that a moderate increase in myocardial creatine was neither detrimental nor beneficial in this model of CHF (manuscript in preparation). Interestingly, while these experiments were being carried out, a computational model predicted the same result⁵². This model takes into consideration cardiac tissue oxygen transport from capillaries and myocyte energy metabolism, the cell being subdivided into cytoplasmic and mitochondrial compartments. Mitochondrial energy metabolism simulation incorporates tricarboxylic acid cycle fluxes, mitochondrial oxidative phosphorylation fluxes, and substrate and cation transport fluxes. 53 biochemical concentration variables (oxygen concentration, mitochondrial membrane potential, substrates concentrations for e.g.) are integrated (Figure 5-1)²⁶⁹.

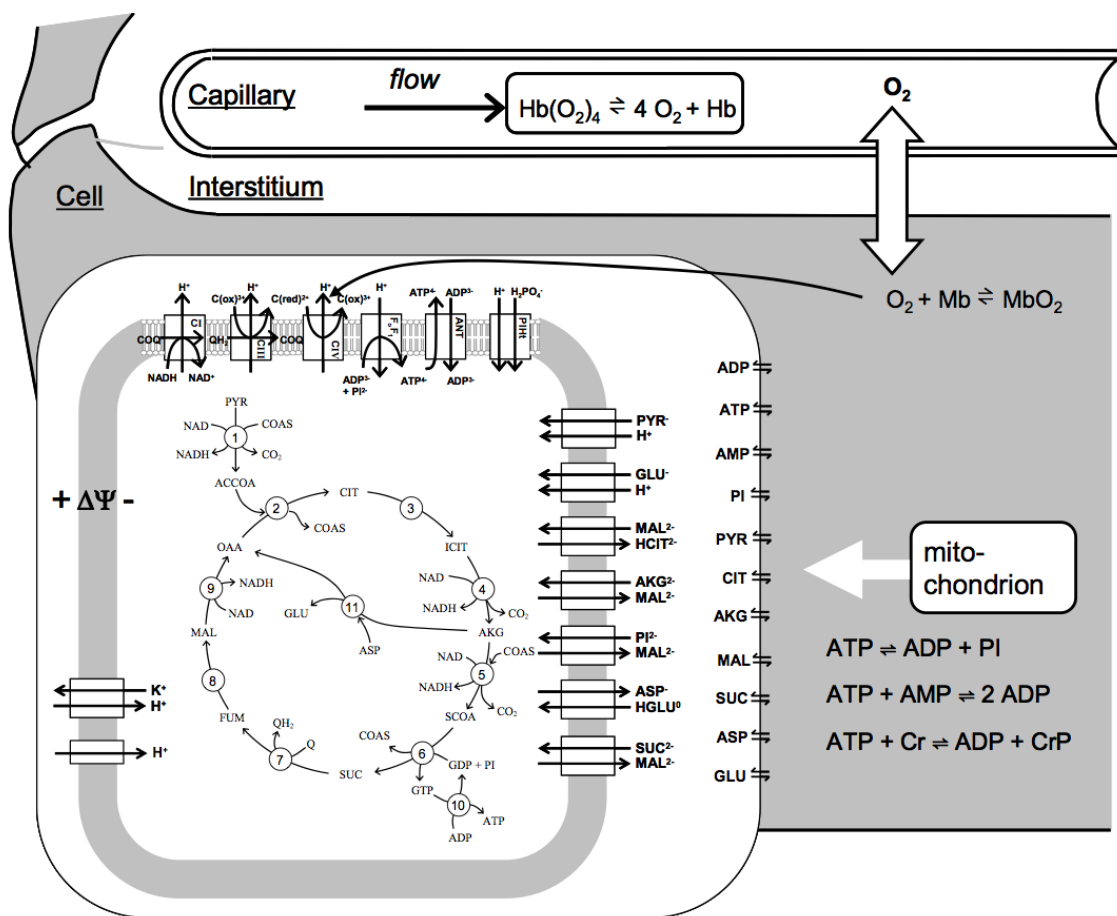


Figure 5-1: Diagram of model used to simulate cardiac tissue oxygen transport and energy metabolism. The simulation integrate the following components: tricarboxylic acid cycle reactions, mitochondrial oxidative phosphorylation fluxes, and substrate and cation transport fluxes. This figure is reproduced from Wu et al.²⁶⁹.

Using this model, they simulated the value of ΔG_{ATP} as a function of total creatine, total adenine nucleotide (TAN) and total exchangeable phosphate (TEP) concentrations (TEP is comprised of ATP, ADP, PCr, Pi and GTP). The modelisation results implied that total creatine, TEP and TAN pools are well balanced with respect to each other even in the dysfunctional heart. Therefore, they anticipated that a beneficial therapy would depend on elevating all three pools simultaneously (Figure 5-2). This is an interesting and testable hypothesis if only there was a method to achieve it. Our CrT-OE model increase both total Cr and PCr. Augmentation of the TAN pool would preserve ATP and ADP. Combined, these would contribute to the TEP. This gave rise to the hypothesis that augmenting the TAN pool in CrT-OE mice would be beneficial in HF.

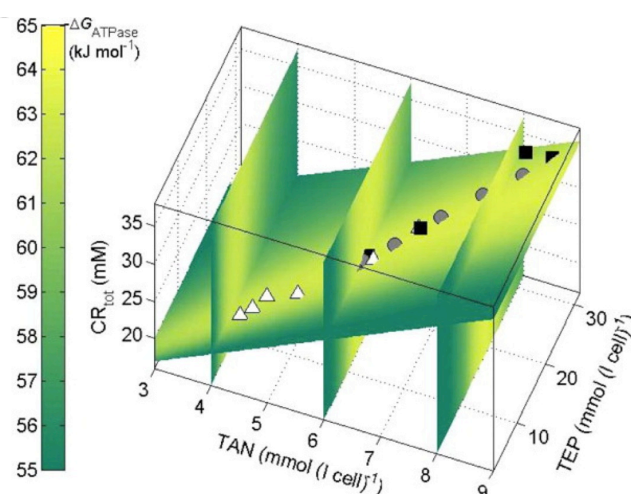


Figure 5-2: Model-prediction of ΔG_{ATP} as a function of total Cr (CR_{tot}), TAN and TEP pools. This figure is reproduced from Wu et al.⁵². Energy available from ATP hydrolysis is maximised only when all three interchangeable pools are elevated.

5.1.3 Adenine nucleotide synthesis

Following events of intense energy requirements, total adenine nucleotide pool (TAN = ATP + ADP + AMP) can be depleted through the diffusion of unphosphorylated adenine and inosine across the muscle sarcolemmal membrane^{20, 270}. It is through this mechanism that TAN pool is depleted in HF (see 1.1.4 for further details). These purines are then unavailable for resynthesis of adenine nucleotides through the “salvage pathway”²⁷¹. Thus, repletion of the adenine nucleotide pool necessitates *de novo* synthesis.

The oxidative phosphate pathway generates the precursors necessary for nucleotide biosynthesis. Glucose-6-P is converted following several enzymatic steps in Ribose-5-P, which is then transformed in 5-phosphoribosyl-pyrophosphate (PRPP). PRPP is the molecule on which purine nucleotides are built, leading to the formation of inosine monophosphate (IMP) that, in turn, can lead to the synthesis of AMP²⁷²⁻²⁷⁴ (Figure 5-3).

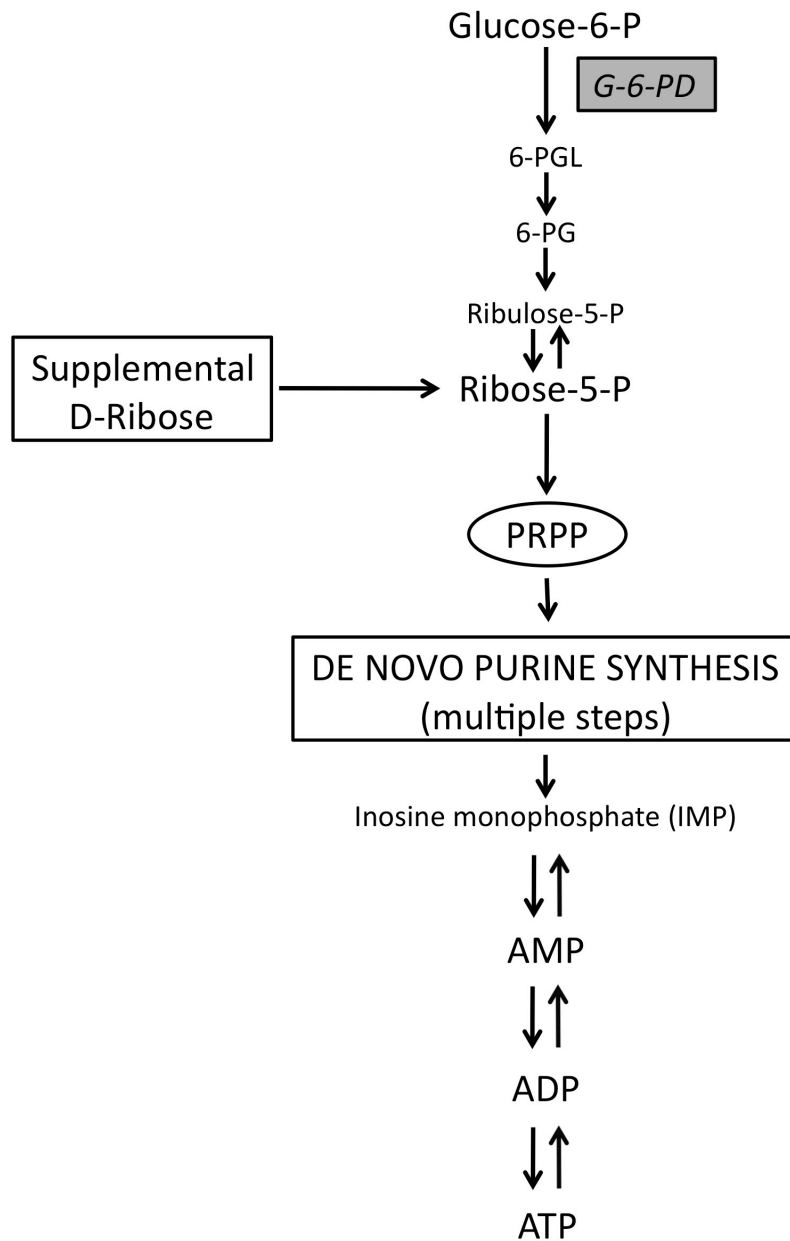


Figure 5-3: Adenine nucleotide de novo synthesis. D-ribose-5-phosphate is pyrophosphorylated to form 5-phospho-D-ribose 1-pyrophosphate (PRPP) leading to adenine nucleotide biosynthesis. Glucose-6-phosphate dehydrogenase (G-6-PD) is the rate-limiting enzyme. 6-PGL: 6-phosphogluconolactone. 6-PG: 6-phosphogluconate (adapted from Pauly and Pepine²⁷² and Zimmer²⁷¹).

In the heart, this process is very slow²⁷⁵ due to the limited availability of the precursor PRPP^{276, 277}. This is the consequence of a low cardiac glucose-6-phosphate dehydrogenase (G-6-PD) activity^{278, 279}, the first and rate-limiting enzyme of the pentose phosphate pathway

(see Figure 5-3). Moreover, despite an increase in *de novo* biosynthesis observed in multiple models of myocardial stress such as β -isoproterenol stimulation^{277, 280}, surgically-induced cardiac hypertrophy^{275, 280}, acute ischaemia²⁷⁵ or permanent coronary artery ligation²⁸¹, this is insufficient to compensate for the loss in total adenine nucleotide (TAN) pool^{275, 277}. Therefore two strategies have been proposed to enhance the pool of available PRPP in the heart: stimulation of the cardiac activity of the rate limiting enzyme G-6-PD or bypassing this step by direct administration of ribose²⁷⁴.

5.1.4 Use of ribose to enhance adenine nucleotide biosynthesis

Administration of D-ribose to bypass the rate-limiting step of the pentose phosphate pathway has been shown to further enhance adenine nucleotide (AN) biosynthesis in multiple models of high-energy demand such as isoproterenol stimulation in the rat^{277, 282, 283} or guinea-pig heart²⁷⁹ or following ischaemia/reperfusion (I/R) insult in the *in vivo* rat²⁸⁴ or dog heart²⁸⁵. In most studies, this attenuated the decrease in ATP pool or accelerated its recovery^{282-284, 286-288}. For example, following I/R in the rat heart, ribose administration restored the depleted ATP pool within 12 hours compared to more than 72 hours in the control group²⁸⁴.

This was shown, as well, in a more chronic setting (over 9 days) following permanent coronary artery ligation, where ribose accelerated the recovery of ATP levels in the rat heart²⁸⁶. However, despite an increase in AN synthesis by 4 to 6 fold, the ATP pool remained unchanged following I/R in dogs after 3 hours of reperfusion²⁸⁵. This would suggest that the rate of AN synthesis in response to ribose might still be insufficient in acute models. This is corroborated by the fact that, in acute settings, these changes at metabolite level translated in functional improvement in some studies^{283, 289} but not all²⁸⁷. More recently, Gonzalez et al.²⁹⁰

suggested that administration of ribose could reduce infarct size in an acute (6 hours) model of permanent coronary artery ligation in the rat. However, the results of this study should be considered with caution as after 6 hours of permanent occlusion, the area of necrosis is surprisingly small compared to what has previously been shown in the literature^{291, 292}, which could be explained by methodological concerns regarding the quantification of infarct size.

Studies in chronic models of heart failure are more limited. In a model of permanent coronary artery ligation, ribose moderately improved diastolic – but not systolic – function two and four days after myocardial infarction²⁸⁶. However, the main limitation of this study is that the groups were not matched for infarct sizes, and it is well established that the size of the infarct obtained following coronary artery ligation is highly variable, even when the suture is placed in a consistent location in genetically identical animals²⁹³. Finally, in a model of monocrotalin-induced right ventricular hypertrophy, the combined oral administration of ribose and folic acid reduced fibrosis and partially improved diastolic function after 4 weeks²⁸⁸.

A few clinical studies suggest that administration of ribose over a few days or weeks, in patients with heart failure, could result in small improvement in cardiac function and/or quality of life²⁹⁴⁻²⁹⁸. However, most of these clinical studies were only presented as “letter-to-the-editor”, in which the available information was very limited and/or showed significant methodological concern (e.g. no control group, comparison with historical data) or were performed on a very small number of patients.

Overall, this literature review shows an effect of ribose on TAN pool in acute settings and over periods up to 9 days. In most of these studies, ribose administration was parenteral and prior to the cardiac injury, which allowed for the evaluation of a preventive but not a true therapeutic effect of ribose. Studies on chronic models of HF with an already existing cardiac dysfunction are lacking.

5.1.5 Aims

The aims of this study were:

- first, to measure the palatability and bioavailability of ribose during oral dosing in drinking water and to assess its consequences on the cardiac function of healthy mice (preliminary study);
- secondly, to assess whether ribose administration can prevent the expected decrease in TAN pool in infarcted mice;
- and finally to test the hypothesis that a concomitant increase in myocardial creatine and ribose levels will have a beneficial effect on cardiac function in chronically infarcted mice.

5.2 Preliminary studies

5.2.1 Material and Methods

5.2.1.1 Changes in TAN pool in the failing mouse heart

C57BL/6J female adult mice underwent coronary artery ligation (see 5.3.1.3) or sham surgery (n = 7 in each group). 4 weeks following surgery, mice were culled, their heart

harvested, the scar removed and total adenine nucleotide pool was measured by HPLC as previously described (see 2.4).

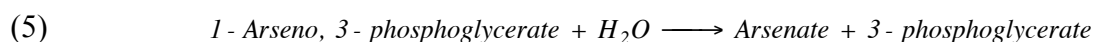
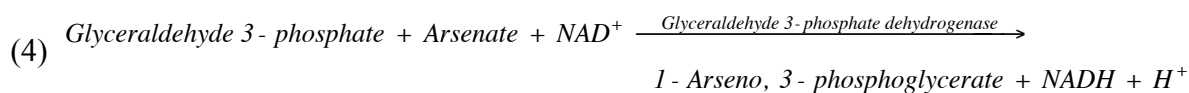
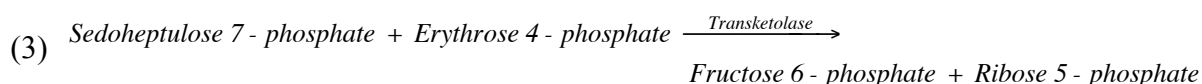
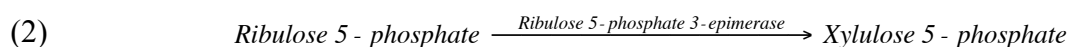
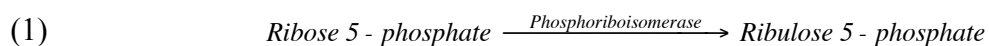
5.2.1.2 Effect of ribose feeding in wild-type mice

Water and food consumption were monitored in a cage of 5 adult, female C57BL/6J mice every two to three days before and following addition of 10 % (w/w) ribose (Sigma Aldrich, UK) in water. Body weight was recorded in parallel.

After 7 weeks of ribose feeding, cardiac function was measured by haemodynamics. Mice were then culled and heart harvested and freeze-clamped for biochemical analysis.

Quantification of Ribose-5-Phosphate in the heart

In vivo ribose is phosphorylated to ribose-1-P and ribose-5-P, which are readily interconverted and can be measured. However, measuring basal sugar phosphate concentrations is difficult because the concentrations are very low (in the nmol/g of wet tissue range). In addition, distinguishing between different sugar phosphates is complicated by the similarities in weight and structure²⁹⁹. Different methods have been described to measure ribose-5-P including a spectrophotometric method and more recently a liquid chromatography - tandem mass spectrometry method^{299, 300}. We chose the spectroscopic method described below, as it is to our knowledge the only method which has been used on heart tissue (rat)³⁰¹. This method is an amalgamation of the methods published by Casazza and Veech³⁰² and by Racker³⁰³. The method is based on the following enzyme sequence³⁰²:



In brief, ribose-5-phosphate is converted to ribulose-5-phosphate and the latter is epimerized to xylulose-5-phosphate. Xylulose-5-phosphate is then determined using transketolase and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The increase in NADH concentration, measured by the change in absorbance at 340 nm is proportional to the amount of ribose-5-phosphate³⁰³.

Tissue was pulverized into fine powder, wet weight recorded (average 80.4 ± 0.005 mg, n=9) and mixed with 1 mL of ice-cold 10 % tricarboxylic acid. Extracts were mixed thoroughly and incubated on ice for 5 min. Tissue debris was sedimented by centrifuging at 13500 rpm for 5 min at 4 °C. Resulting supernatant was neutralized with 1 M NaHCO₃. The assay mixture was prepared containing 162 mM imidazole pH 7.6, 30 mM NAD, 18 mM MgCl₂, 15 mM sodium arsenate, 0.25 mM erythrose 4-phosphate and 0.03 % thiamine pyrophosphate. 0.5 mL of deproteinized and neutralized heart tissue extract and 32 units

GAPDH were mixed with 0.25 mL of assay mixture in a cuvette and incubated for 3 min at 25°C. In the reference cuvette, tissue extract was replaced with corresponding volume of water. The first reaction was initiated by addition of 1.25 U of transketolase and absorbance (A_1) of NADH followed at 340 nm, 25 °C for 10 min. A_1 originates from the presence of glyceraldehyde-3-phosphate and xylulose-5-phosphate in the extract. After the first reaction reached completion, 5 units of ribulose-5-phosphate 3-epimerase and 10 units of phosphoriboisomerase were added to the cuvette. The absorbance (A_2) was recorded at 340 nm, 25 °C for 10 min.

ΔA was calculated from $A_2 - A_1$. The calculated assay values were corrected for dilution during deproteinization to obtain concentration of ribose-5-phosphate in the specimen. The ribose-5-phosphate content of the ventricular tissue was expressed as nmol/g wet tissue weight.

5.2.2 Results

5.2.2.1 Changes in TAN pool in the failing mouse heart

A decrease in TAN pool has been shown in dog³³ and rat²⁸⁸ models of heart failure. However, it has never been demonstrated in the mouse heart. Therefore, it was important to assess whether this decrease was measurable in the chronically infarcted mouse heart. It was found that TAN pool decreased by 14 % in infarcted mice compared to sham operated mice ($p = 0.02$, $n = 7$). This suggests that the alteration in the TAN pool is similar to that observed

in human and larger animals, and represents a suitable model to assess whether ribose supplementation can be used to maintain the TAN pool.

5.2.2.2 Choice of ribose dose

In order to decide which dose of ribose to use, we did a literature review of the posologies previously used in cardiac studies. They are shown in Table 5.1.

Reference	Species	Dose	Duration	Route	Dose in g/kg/day
Zimmer et al. ^{277, 279}	Guinea pig or rat	100 mg/kg	Single dose	i/v	0.1 (single injection)
Zimmer and collaborators ^{282, 283, 304}	Rat	200 mg/kg/h	24 h	i/v	4.8
Zimmer and Ibel ²⁸⁴	Rat	200 mg/kg/h	72 h	i/v	4.8
Gonzalez et al. ²⁹⁰	Rat	200 mg/kg/h	30 h	i/v	4.8
Sawada et al. ²⁹⁶	Human	180 mg/kg/h	4.5 h	i/v	4.3
Pliml et al. ²⁹⁴	Human	15 g <i>q.i.d.</i>	3 days	<i>per os</i>	0.8
Zimmer et al. ³⁰⁴	Rat	200 mg/kg/h	Up to 9 d	i/v	4.8
Omran et al. ²⁹⁷	Human	5 g <i>t.i.d.</i>	3 weeks	<i>per os</i>	0.2
Czarnecki and Hinek ³⁰⁵	Rat	200 mg/kg, 5 times a week	5 weeks	IP	~0.2
Lamberts et al. ²⁸⁸	Rat	150 mg/kg daily (NB: administered with folic acid)	6 weeks	<i>per os</i>	0.15
Maccarther et al. ²⁹⁸	Human	5 g <i>t.i.d.</i>	8 wks	<i>per os</i>	0.2

Table 5-1: Literature review of the posologies of ribose used in cardiac studies. For calculation of the dose in g/kg/day, a standard human being was considered to weigh 75 kg.

We decided to administer ribose in the drinking water, which is the simplest and most ethical route. It does not require animal handling, reducing associated stress, which could be problematic in infarcted animals.

Prior to ribose administration, water intake was measured every other day in a cage of five C57BL/6J mice over a period of 10 days. The average daily water consumption was 3.7 g per mouse. Consequently, considering the doses employed in the literature, it was decided to use a concentration of 10 g of ribose per litre of drinking water, which corresponds for an average 25 g mouse to a daily intake of 1.5 g/kg of ribose.

5.2.2.3 Ribose consumption and absorption

The effects of administration of ribose in drinking water on water and food consumption were monitored. Ribose-containing water was changed twice a week mostly to avoid bacterial contamination as half-life of ribose in solution was shown to be 30 days at 25 °C³⁰⁶. Water and food intake were measured every two to three days from 10 days before to 50 days after adjunction of ribose to water. No change was observed in food or water consumption (Figure 5-4 and Table 5-2, $p = \text{ns}$ both for water and food consumption, t-test). Similarly, body weight was stable over the length of the experiment (Figure 5-4 and Table 5-2).

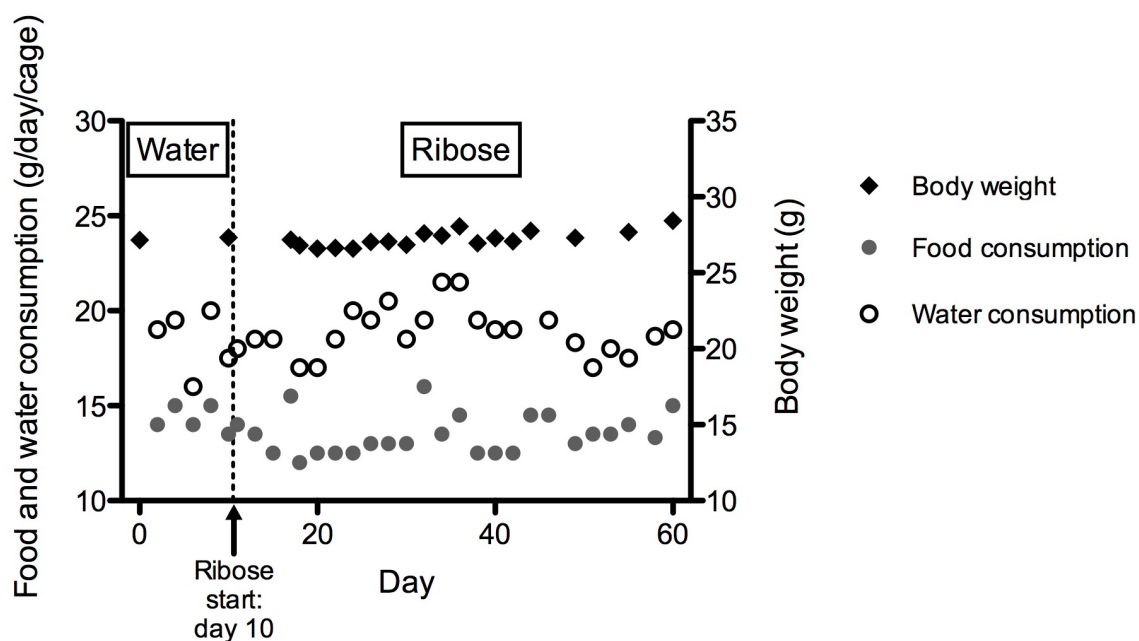


Figure 5-4: Water and food-intake prior and following administration of ribose in water. Changes in body weight are also shown.

	Prior to ribose administration	Following ribose administration
Water consumption (g/cage)	18.4 ± 1.6	18.9 ± 1.2
Food consumption (g/cage)	14.3 ± 0.7	13.5 ± 1.0
Mouse average weight (g)	27.2 ± 0.1	27.2 ± 0.5

Table 5-2: Average daily water/food consumption and body weight prior (5 measurements) and after (25 measurements) ribose administration. Water and food consumption are given for the whole cage of 5 mice. Data are expressed as mean ± SD.

In order to confirm that dietary ribose is taken up by the heart, we measured myocardial ribose-5-P concentrations. Ribose-5-P levels were increased by 2-fold in the ribose-fed group compared to a matched control group receiving pure water ($p = 0.004$, t-test) (Figure 5-5).

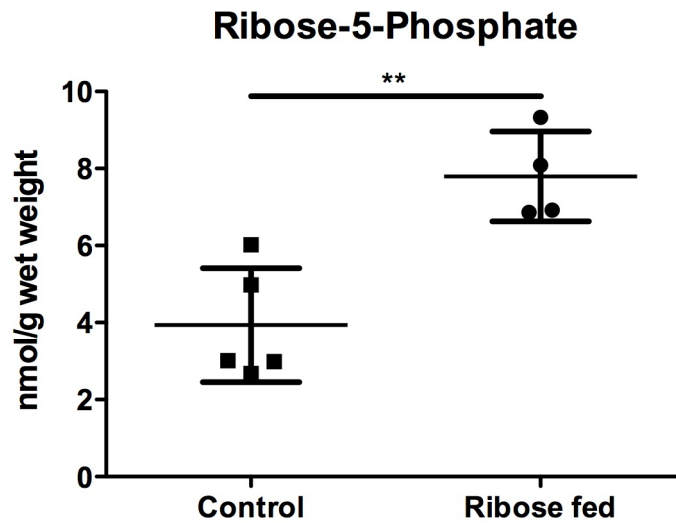


Figure 5-5: Myocardial ribose-5-P levels following ribose feeding. The control group consisted in mice receiving pure water. ** denotes $p < 0.01$ (t-test).

5.2.2.4 Effect of ribose on cardiac function in healthy mice

Ribose dosing had no discernable effect on cardiac function in healthy mice, as determined by LV haemodynamics at baseline and following inotropic stimulation by dobutamine. No difference was found for any of the parameters between the ribose-fed mice and a matched control group (Figure 5-6).

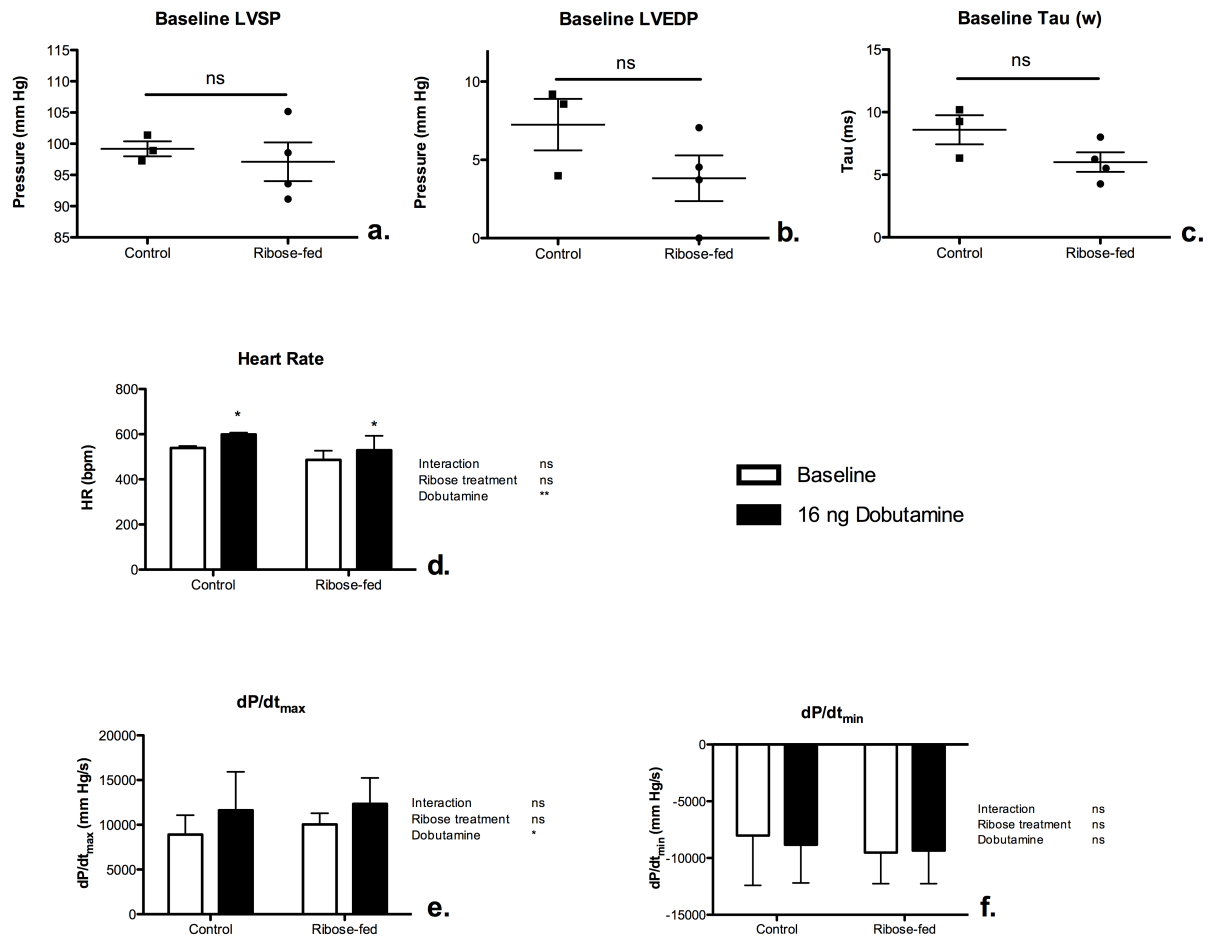


Figure 5-6: Haemodynamics parameters of healthy WT mice fed ribose. Baseline LVSP (panel a.), LVEDP (b.), tau (c.) and effect of dobutamine (16 ng/gBW/min) on HR (d.), dP/dt_{max} (e.), dP/dt_{min} (f.) are shown. The control group consisted of an identical group of mice receiving pure water. Only 3 mice in the control and 4 in the ribose-fed group are available due to technical issues during haemodynamic surgery. * denotes $p < 0.05$, ** $p < 0.01$. A t-test was used to compare the two groups at baseline. A 2 way-ANOVA with Bonferroni post-hoc test was used for the effect of dobutamine on cardiac function.

5.2.2.5 Influence of scar tissue on accuracy of creatine measurements

In order to assess myocardial ATP levels, harvesting and freezing of the heart needs to be as prompt as possible. Removal of the scar tissue would delay the process. As the scar tissue is very thin compared to remote myocardium and mostly composed of collagen^{307, 308}, we anticipated that it would not contribute to the creatine and TAN pool. However, as HPLC

data are normalized to the amount of protein, keeping the scar tissue could underestimate the concentrations of the metabolite of interest due to the presence of protein in the scar in the form of collagen. We used a Lowry assay for protein determination, which reacts with tyrosine or phenylalanine. Hence, proteins deficient or poor in these aminoacids such as collagen do not contribute much to the change in coloration^{115, 309, 310}. Consequently, we anticipated that the presence of scar tissue in our samples would not bias the measurement of creatine and high-energy phosphates in the remote myocardium.

To verify this hypothesis, three C57BL/6J female mice were infarcted. Two weeks later when the scar had reached full maturation³¹¹, the mice were culled and the hearts harvested. Scar tissue was separated from the remote left ventricular myocardium. The remote myocardium was then split in two halves, to one of which the scar was added. Creatine was then measured by HPLC for each heart both in the remote myocardium and in the LV/scar blend. No systematic difference was observed and variation was within the normal limits for this technique (Table 5-3). Therefore, we concluded that keeping scar tissue (i.e. analysing the whole ventricle) would not bias metabolite measurements in the remote myocardium, but will allow us to minimise harvesting time.

	LV	LV + scar	Variation (%)
Mouse 1	47.3	45.7	-3.5
Mouse 2	48.4	48.9	+1.1
Mouse 3	47.2	44.1	-6.6

Table 5-3: Creatine concentration (in nmol/mg prot) in the remote myocardium and a mixture of remote myocardium and scar in the infarcted hearts of three mice. Measurements were done by HPLC.

5.3 Effects of a combination of increased ribose and creatine levels in the infarcted heart

5.3.1 Material and methods

5.3.1.1 Description of the groups

All mice used in this study were female as, following coronary artery ligation (CAL), a higher proportion of males die from cardiac rupture²⁹³. The study comprised four groups:

- Group **S** consisted of **WT** mice, which underwent **sham** surgery and were not fed ribose;
- Group **I** consisted of **WT** mice, which underwent myocardial infarction (**MI**) surgery and were not given ribose in drinking water;
- Group **R** consisted of **WT** mice, which underwent **MI** surgery and were given **ribose** in drinking water;
- Group **C** consisted of **CrT-OE** mice, which underwent **MI** surgery and were fed **ribose** in drinking water.

All mice were aged between 3 and 7 months at the time of surgery. WT mice for groups S, I & R consisted in a random mixture of wild-type littermates of CrT-OE and age-matched C57BL/6J obtained from a commercial breeder (Harlan, UK). As CrT-OE are generated by crossing, at each generation, a heterozygous male with a C57BL/6J female (Harlan, UK), the

genotype of WT littermates of CrT-OE and commercially available C57BL/6J mice was considered to be identical.

5.3.1.2 Protocol

CrT-OE mice underwent ^1H -MRS examination (as detailed in Chapter 3) for pre-selection of those with moderately elevated creatine levels ranging from 88 to 140 nmol/mg prot. The lower limit was set as twice the standard deviation above the previously reported WT mean¹⁶. The upper limit was based on the previous findings that mice with creatine levels >150 nmol/mg protein developed clear LV dysfunction and hypertrophy while mice below this level did not⁸⁷. A lower cut-off of 140 nmol/mg protein was adopted to allow for measurement errors. ^1H -MRS examination was performed at least 1 week prior to surgery to ensure complete recovery from general anaesthesia.

WT and CrT-OE mice with creatine levels within the chosen range underwent CAL surgery to induce chronic myocardial infarction. Ribose was added to drinking water in the relevant groups 24 hours following surgery. Three to 4 weeks following surgery, mice received an echocardiogram under light general anaesthesia to exclude those with very small or no infarcts. Seven and a half weeks after surgery, all mice underwent ^{31}P -MRS to measure ATP and PCr levels *in vivo* (full analysis of data is not available yet and consequently we will not present the results in this thesis). A few days later, they underwent cine-MRI to measure infarct size and assess cardiac function. Finally, 4 to 5 days later, to allow for recovery, cardiac function was assessed invasively by haemodynamics at baseline and following maximal stimulation with 16 ng/gBW/min dobutamine. Hearts were then harvested, quickly rinsed in ice-cold heparinised saline before freeze-clamping in liquid nitrogen of both

ventricles and scar. LV samples were then kept in liquid nitrogen until analysis by HPLC, which was performed maximum 48 hours after harvesting.

Mice with infarct size below 25 % of LV circumference were excluded as these animals do not develop the hallmarks of heart failure²⁹³. At the end of the study, some mice were excluded in order to retrospectively match all infarcted groups and obtain the same range of infarct sizes. This is essential to allow inter-group comparison of LV function and remodeling parameters, which are highly dependent on the extent of myocardial injury.

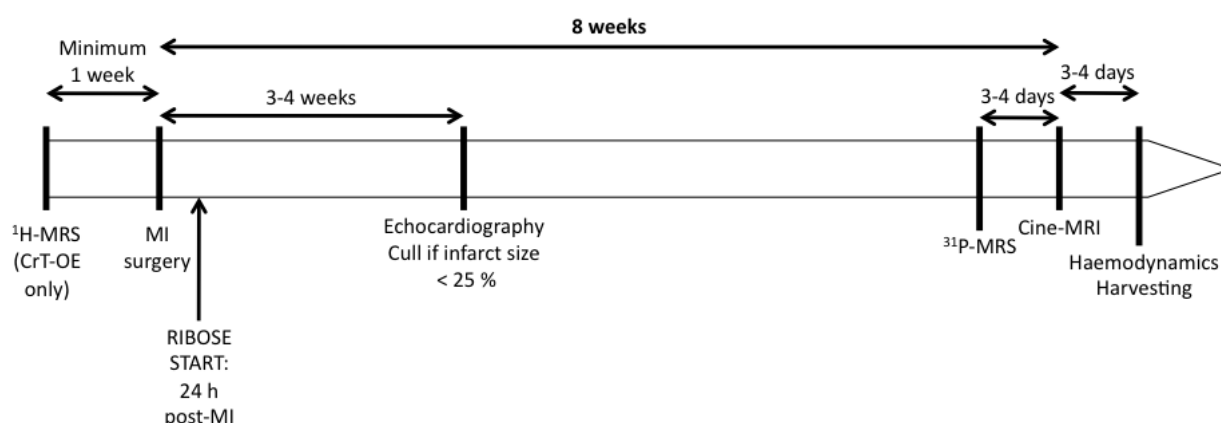


Figure 5-7: Time-line of study.

5.3.1.3 Coronary artery ligation (CAL)

Permanent coronary artery ligation was performed as previously described in details by Lygate³¹¹. In brief, following induction of anaesthesia with isoflurane (ISF), mice received 0.27 mg of buprenorphine subcutaneously (Vetergesic, Alstoe Animal Health, UK). They were intubated and ventilated with a tidal volume of 250 μ L and respiratory rate of 150/min (Hugo-Sachs MiniVent type 845, Harvard Apparatus, UK). Anaesthesia was maintained

using 2 % ISF in 100 % O₂. A left thoracotomy was performed in the fourth intercostal space; the left lung was deflated using a small piece of gauze soaked in saline. The pericardium was then removed and an intramyocardial ligature placed 1-2 mm below the atrioventricular groove using a 6-0 polyethylene suture (Ethicon, UK). Lungs were then reinflated before the thorax was closed. Sham mice underwent the same protocol with the exception of the ligation of the coronary artery.

5.3.1.4 Results analysis

Cine-MRI and haemodynamic data were analysed blinded to treatment group. Statistical analysis was performed using one-way ANOVA with Bonferroni's post-hoc test, or two-way ANOVA for analysis of the cardiac response to dobutamine stimulation. Differences were considered significant when $p < 0.05$.

5.3.2 Results

5.3.2.1 ¹H-MRS in CrT-OE mice

LV creatine levels were measured by ¹H-MRS in 28 female Tg46 mice (Figure 5-8). The distribution of creatine levels is shown in Figure 5-8. 24 mice were within the range of interest (88-140 nmol/mg prot) and underwent myocardial infarction.

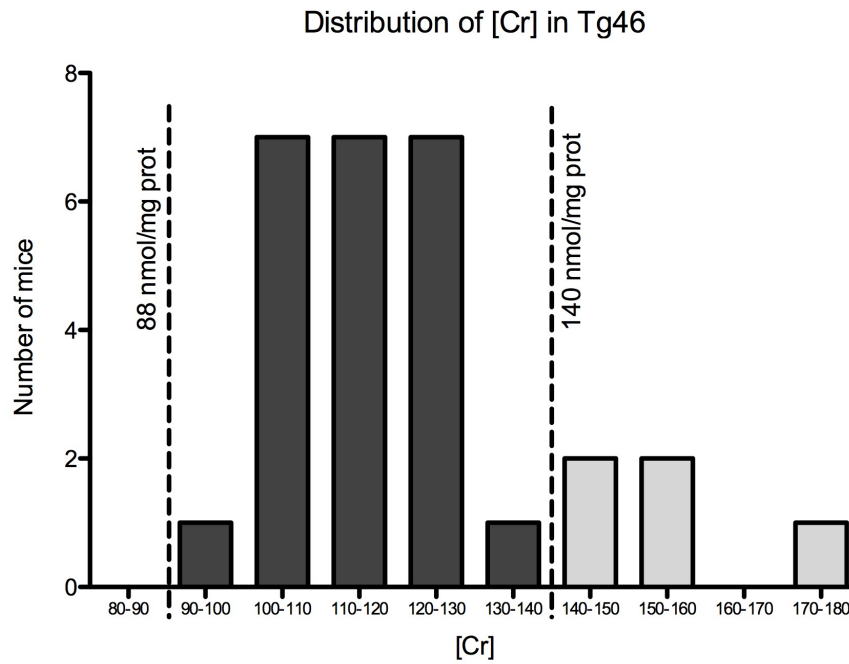


Figure 5-8: Distribution of myocardial creatine levels measured by ^1H -MRS in CrT-OE. The range of interest lies between the two dotted vertical bars.

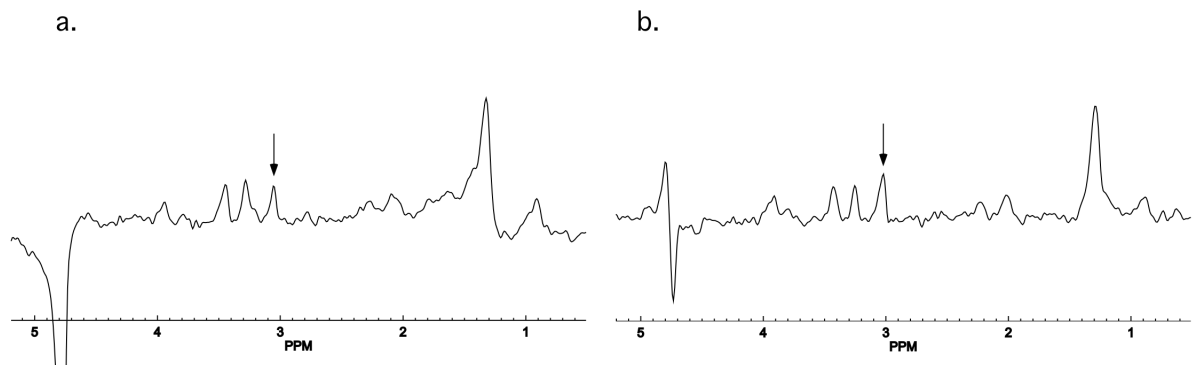


Figure 5-9: Two representative myocardial ^1H -MRS spectra acquired in Tg46 mice. The arrow shows the creatine resonance. Fitting of the spectra gave an estimated value for creatine concentration of 90 nmol/mg protein (a.) and 142 nmol/mg protein (b.) respectively.

5.3.2.2 Outcome of mice entering the study

The fate of the mice entering the study is summarized in Table 5-4. There was no difference in peri-surgical mortality (during or within 24 hours of surgery) between WT and Tg46 (χ^2 -test, $p = ns$). Similarly, there was no difference in long-term mortality (after 24 hours) between the four study groups (χ^2 -test, $p = ns$).

	WT			CrT-OE
Number of mice entering study for ^1H -MRS scan	N/A			28
- excluded as outwith target [Cr] range	N/A			4
Number of mice undergoing infarct surgery	83			24
- peri-surgical death (within the first 24 hours)	14			4
	Sham, no ribose Group S	MI, no ribose Group I	MI, ribose Group R	MI, ribose Group C
Number of mice at start of ribose feeding	20	28	22	20
- died 24 hours to 8 weeks (peri-MR death excluded)	0	0	1	3
- peri-MR death	2	1	2	1
- excluded due to small infarcts	0	7	12	9
- died during haemodynamics	2	5	2	1
Number of mice completing all aspects of study	16	15	5	6
- excluded to create groups matched for infarct size	0	2	1	0
Number of mice in final analysis	16	13	4	6

Table 5-4: Outcome of mice entering the study.

At the end of the study, mice were retrospectively matched to obtain similar infarct sizes in all infarcted groups (Figure 5-10). Infarct sizes were 39 ± 8 % of total LV in group S, 42 ± 8 in group R and 39 ± 10 in group C.

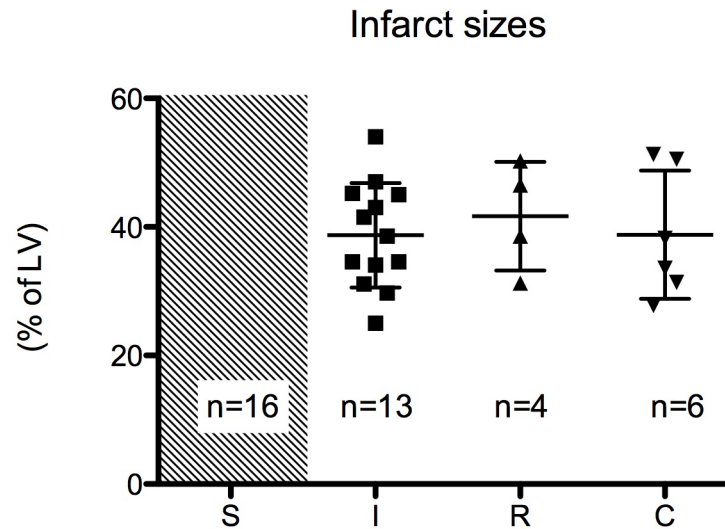


Figure 5-10: Infarct sizes measured by cine-MRI in all groups of infarcted mice.

5.3.2.3 Cardiac function

Cine-MRI

All infarcted groups showed a clear cardiac hypertrophy with a significant elevation in LV mass compared to sham despite the loss of tissue to infarct scar (101 ± 9 mg in group I; 117 ± 3 in group R; 98 ± 10 in group C vs 79 ± 9 in group S; $p < 0.0001$ vs WT for all three infarcted groups) (Figure 5-12). LV mass was higher in group R than in the two other infarcted groups ($p < 0.05$ for group R vs group I and group C). Representative mid-ventricular slices are shown in Figure 5-11.

Cardiac dysfunction was noted in all infarcted groups with an increase in cardiac volumes both in diastole and systole and a decrease in ejection fraction by 60 to 68 % compared to WT ($p < 0.0001$ for all three infarcted groups vs WT). Cardiac output was maintained to similar levels as in non-infarcted mice (14 ± 3 mL/min in group S; 15 ± 2 in group R; and 14 ± 3 in group C vs 16 ± 2 in group S). This suggests that LV dilatation was sufficient to maintain normal stroke volume and cardiac output despite significantly impaired systolic function.

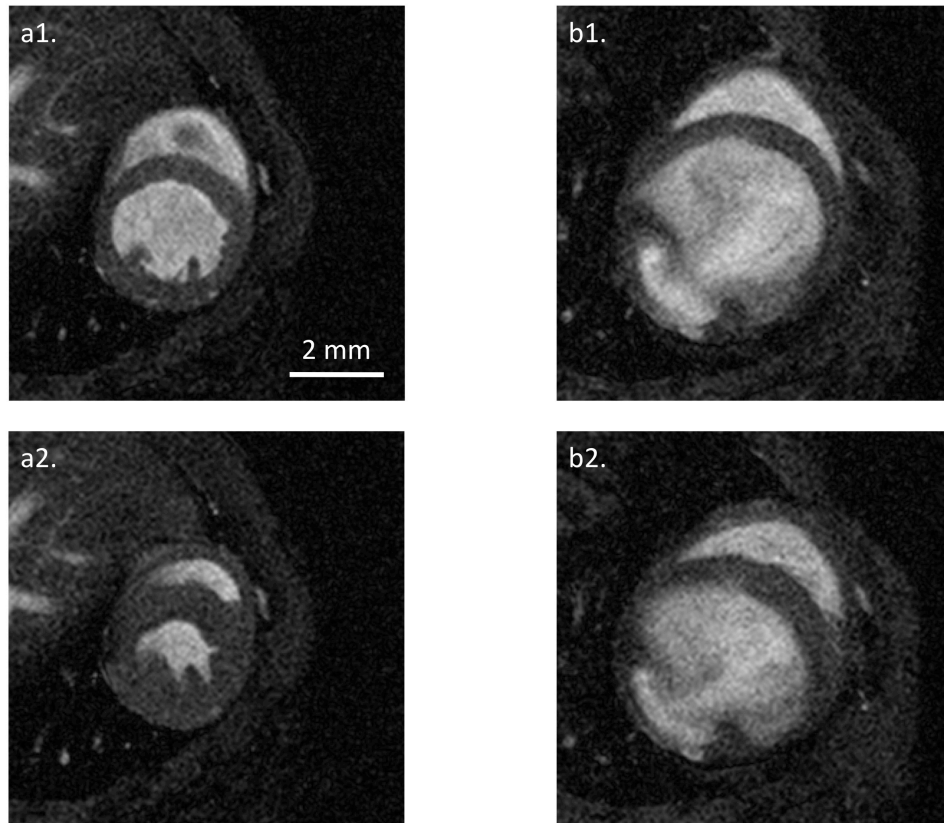


Figure 5-11: Representative mid-ventricular end-diastolic (top row) and end-systolic (bottom row) MR images from a sham mouse (Group S, diastolic frame (a1), systolic frame (a2)) and an infarcted mouse (Group I, diastolic frame (a1), systolic frame (a2)). The ejection fractions were 69% in the sham mouse and 19% in the infarcted mouse (infarct size = 54% of LV). Note the dilatation of the ventricle and the poor contractility of the infarcted heart.

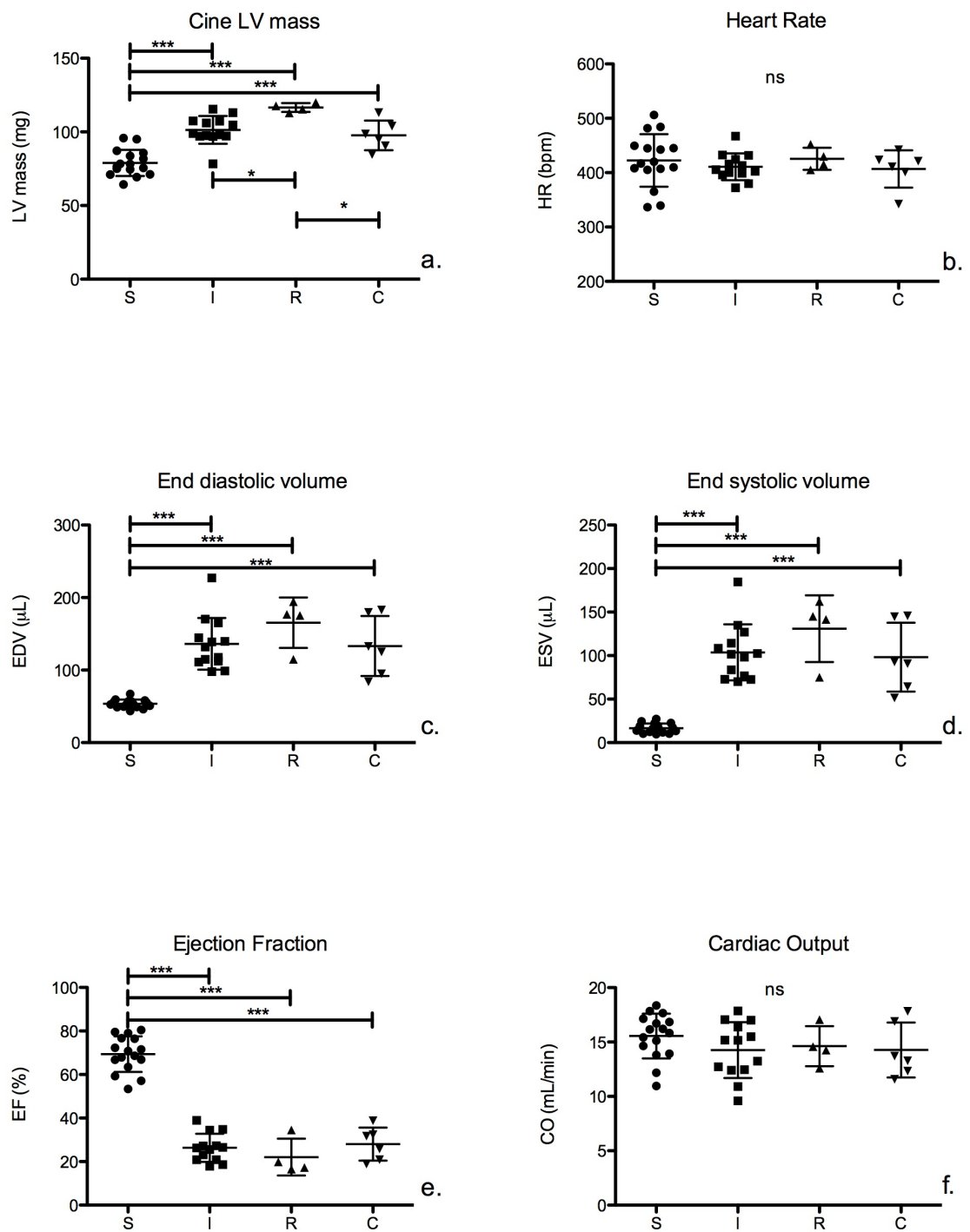


Figure 5-12: Cine-MRI parameters. LV mass (panel a.), heart rate (b.), end-diastolic volume (c.), end-systolic volume (d.), ejection fraction (EF) (e.), cardiac output (f.). * denotes $p < 0.05$, *** $p < 0.001$ and **** $p < 0.0001$ (one-way ANOVA with Bonferroni post-hoc test).

A strong correlation between infarct size and ejection fraction was found irrespective of the genotype or ribose treatment (Figure 5-13). The coefficient of determination (R^2) of the linear regression suggests that 67 % of the variability is due to differences in infarct size.

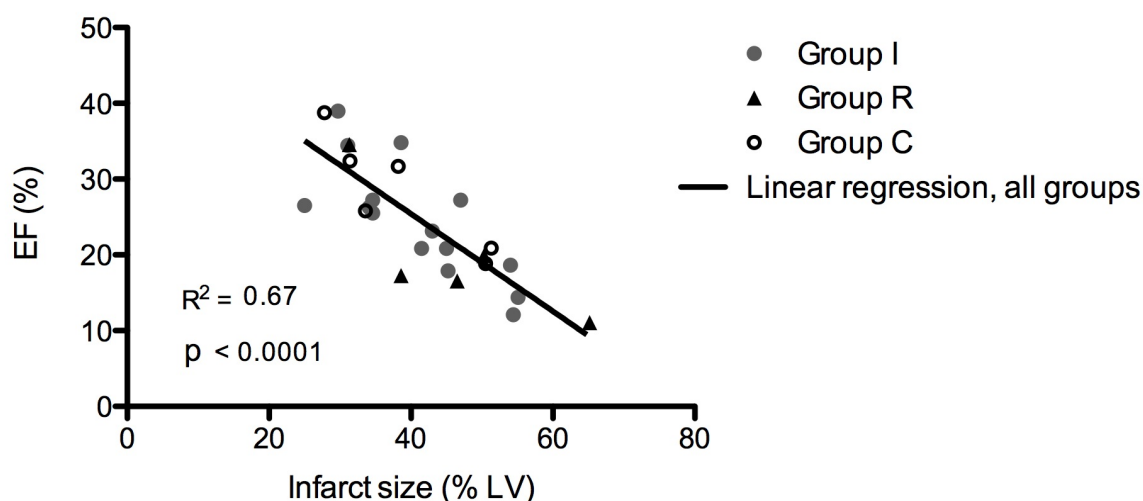


Figure 5-13: Correlation between infarct size and ejection fraction (EF) in all three infarcted groups. No difference between groups was found (linear regression).

Invasive assessment of cardiac function

At baseline, heart rate was comparable between all groups indicating a similar anaesthesia depth. LVSP was lower and LVEDP was elevated in all three infarcted groups (Figure 5-14). A decrease in the inotropic (decrease in $dP/dt_{\max}$) and lusitropic (decrease in $|dP/dt_{\min}|$, increase in tau) properties of the heart was also measured. All these changes are characteristic of the failing heart. However, there was no difference between the three groups of infarcted hearts.

Dobutamine induced an increase in $dP/dt_{\max}$ in all groups. However, the increase was more pronounced in sham than in infarcted groups (increase by 1468 ± 812 in group S vs 708 ± 453 mm Hg in group I ($p < 0.05$); 486 ± 316 in group R ($p = \text{ns}$); 841 ± 815 in group C ($p = \text{ns}$)), indicating an impaired contractile reserve in infarcted hearts. No difference was seen between infarcted groups (Figure 5-15).

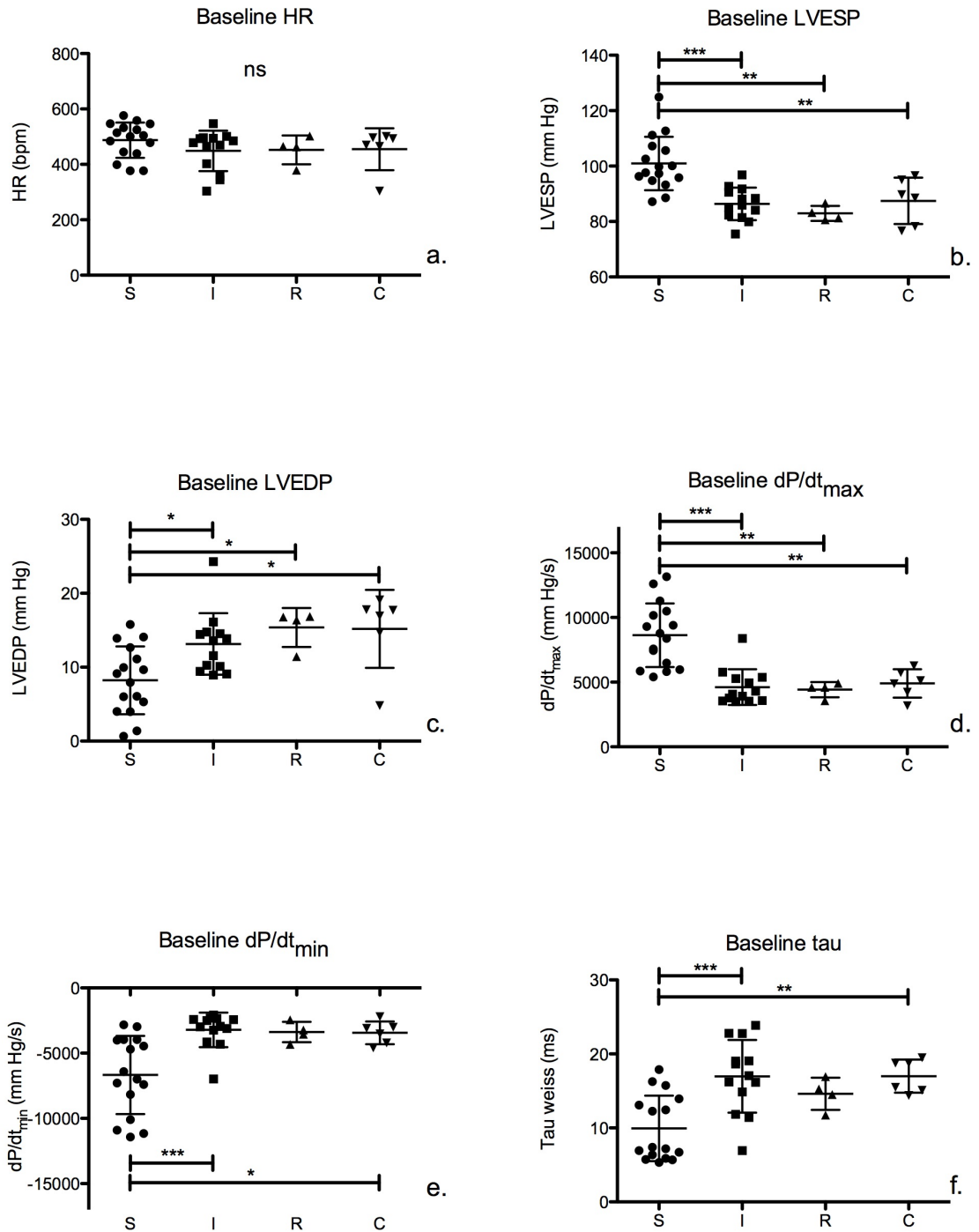


Figure 5-14: Baseline haemodynamics data. Heart rate (HR) (a.), left ventricular systolic pressure (LVSP) (b.), left ventricular end diastolic pressure (LVEDP) (c.), rate of pressure development dP/dt_{max} (d.), rate of pressure decline dP/dt_{min} (e.) and tau (f.) are shown. * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA with Bonferroni post-hoc test). Data are expressed as Mean $\pm$ SD.

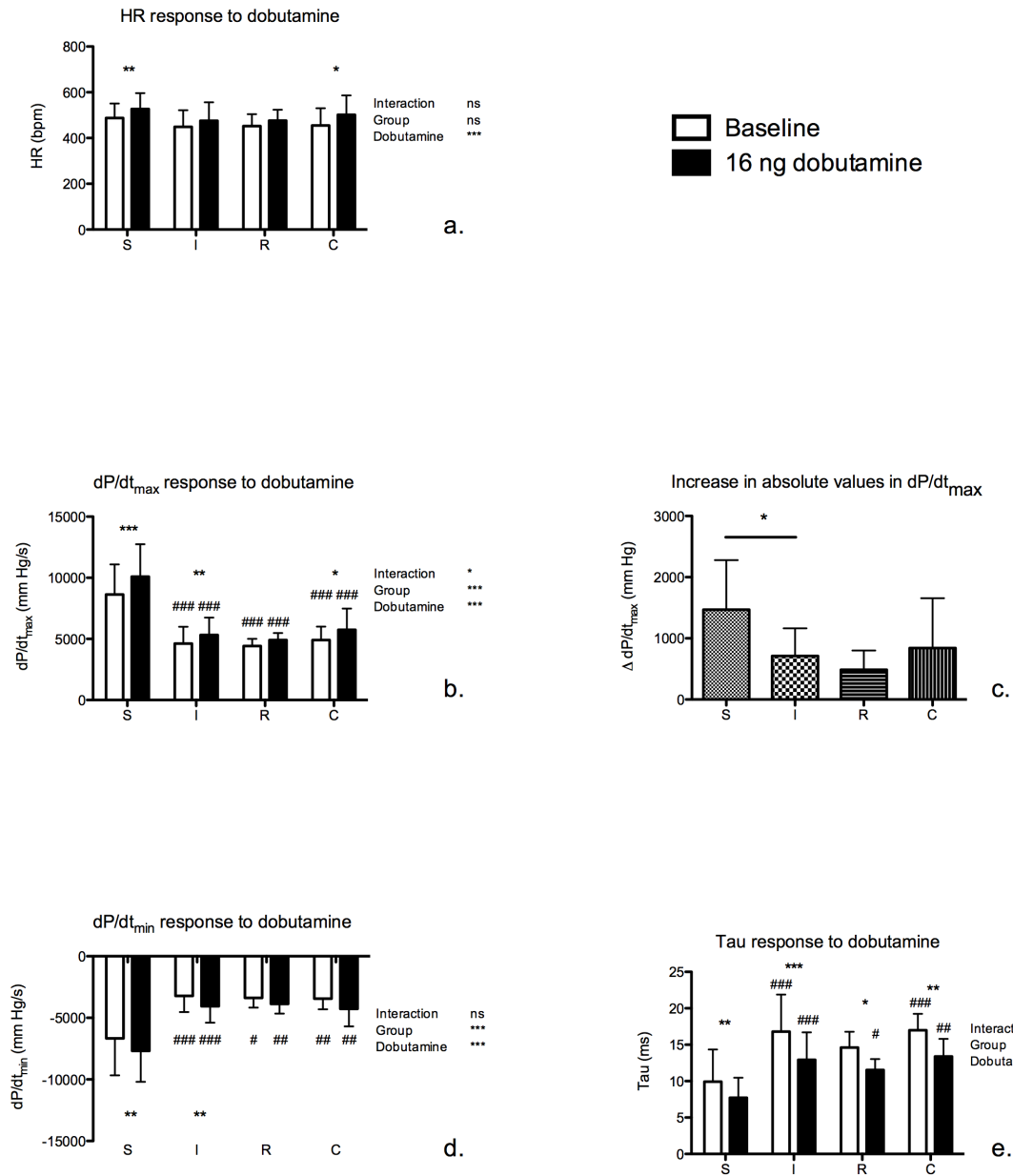


Figure 5-15: Effect of dobutamine (16 ng/gBW/min) on cardiac function. Evolution of HR (a.), dP/dt_{max} (b.), dP/dt_{min} (d.), tau (e.) and the difference between dobutamine-stimulated and baseline dP/dt_{max} (c.) are shown. In panels a., b., d. and e.: * denotes $p < 0.05$ for dobutamine vs baseline, ** $p < 0.01$, *** $p < 0.001$. # denotes $p < 0.05$ vs group S at the same dobutamine dose (2-way ANOVA with Bonferroni post-hoc test). In panel c.: * denotes $p < 0.05$ vs group S (one-way ANOVA with Bonferroni post-hoc test). No difference was found between the three infarcted groups for all parameters. Data are expressed as Mean $\pm$ SD.

There was again a clear correlation between infarct size and LVSP irrespective of the group of infarcted mice (Figure 5-16) with infarct size explaining 46 % of the observed variability in LVSP.

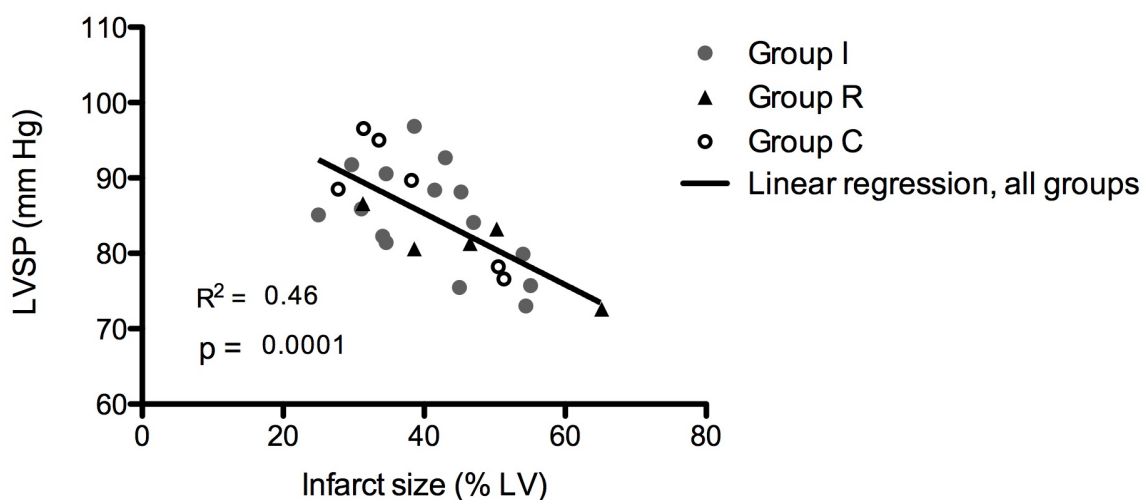


Figure 5-16: Correlation between LVSP and infarct size in all three groups of infarcted mice. No difference between groups was found (linear regression).

Post-mortem examination

There was a trend to an increase in lung mass/BW ratio in the infarcted groups characteristic of left-sided congestive heart failure ($p < 0.05$, one-way ANOVA, differences between groups did not reach statistical significance with Bonferroni's correction) (Table 5-5). A moderate decrease in liver and kidney weights was observed in group C; this is most likely due to a slightly lower BW in this group as there was no difference in these organs/body weight ratio. An effect of ribose is unlikely as on the contrary an increase in kidney mass and inconsistent changes in liver mass were shown in rats fed diets containing up to 20 % (w/w) ribose for 13 weeks³¹².

Finally, LV mass at post-mortem was not available as hearts were freeze-clamped immediately after excision for high-energy phosphate measurements. However, it had been assessed by cine-MRI (*vide supra*).

	WT, sham, no ribose (S)	WT, MI, no ribose (I)	WT, MI, ribose-fed (R)	CrT-OE, MI, ribose-fed (C)
Post-mortem				
Body weight (mg)	25 ± 3	24 ± 2	24 ± 2	23 ± 1
Lung weight (mg)	144 ± 13	163 ± 45	192 ± 51	173 ± 49
Liver weight (mg)	1112 ± 115	1018 ± 99	1053 ± 31	933 ± 45**
Kidney weight (mg)	241 ± 25	224 ± 16	231 ± 10	214 ± 12*
Tibial length (mm)	18.2 ± 0.3	18.3 ± 0.2	18.2 ± 0.3	18.2 ± 0.2
Lung / BW (x 10 ³)	5.7 ± 0.9	6.6 ± 1.6	7.9 ± 1.7	7.5 ± 2.2
Liver / BW ratio (x 10 ³)	44 ± 3	43 ± 4	44 ± 4	40 ± 2
Kidney / BW ratio (x 10 ³)	9.5 ± 0.9	9.5 ± 0.9	9.7 ± 1.2	9.2 ± 0.4
Cine-MRI				
LV mass (mg)	79 ± 9	101 ± 9***	117 ± 3*** #	98 ± 10***
LV / BW (x 10 ³)	3.1 ± 0.4	4.3 ± 0.4*****	4.9 ± 0.4*****	4.2 ± 0.4*****

Table 5-5: Weights of the major organs measured at post-mortem. LV mass had been assessed by Cine-MRI only (see text). * denotes $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs group S. # denotes $p < 0.05$ vs groups I and C. One-way ANOVA with Bonferroni post-hoc test was used.

5.3.2.4 Total creatine levels and TAN pool.

As expected in HF, total Cr levels were lower by 18 % in infarcted WT mice than in sham ($p < 0.01$, t-test) (Figure 5-17). A similar reduction in Cr concentration by 16 % was observed in group R ($p < 0.05$, t-test). Infarcted CrT-OE mice maintained significantly

higher Cr levels compared to sham (40 % higher, $p < 0.001$) despite a 10 % decrease in myocardial creatine concentration with development of HF (Figure 5-18).

PCr contributed only to 11 to 17 % of total creatine, when about 2/3 of Cr is normally phosphorylated *in vivo*¹ (Figure 5-17) . This shows the exceptionally high lability of PCr following death, even with rapid dissection and freeze-clamping.

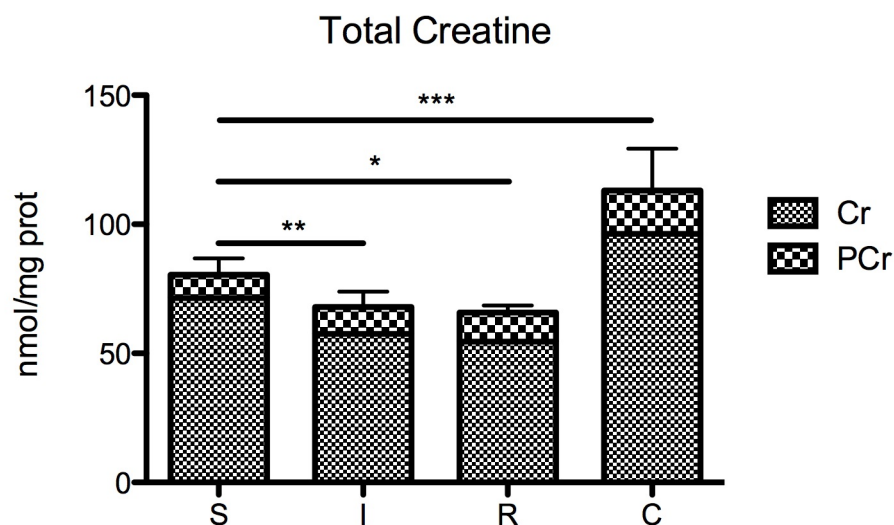


Figure 5-17: Cr and PCr concentrations in LV. The error bars correspond to the SD of total Cr (= Cr + PCr).

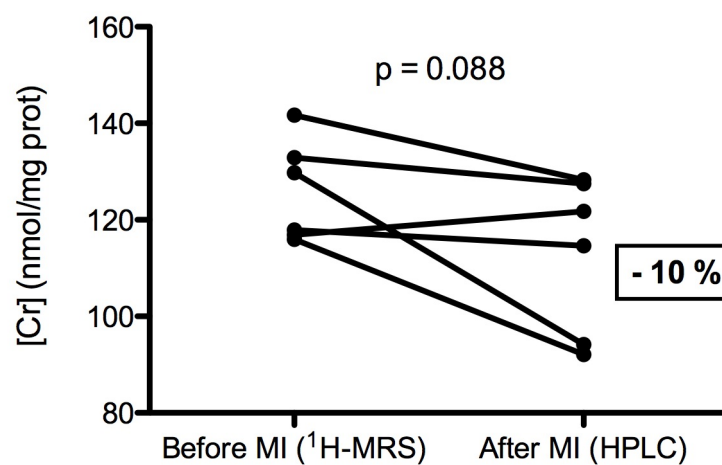


Figure 5-18: Evolution of creatine levels in CrT-OE mice following myocardial infarction. Creatine concentrations were measured by ¹H-MRS prior to MI and HPLC after MI.

As regards to TAN pool, a significant reduction in AMP and ADP levels was measured in all infarcted groups. On the contrary, ATP levels were conserved with even a trend towards higher levels in the two-ribose fed groups compared to the sham group. Therefore, a significantly higher ATP/ADP ratio in the two ribose-fed groups (group R and C) was observed ($p < 0.01$ for group R vs S and $p < 0.05$ for group C vs S) (Table 5-6).

	Sham, no ribose (S)	MI, no ribose (I)	MI, ribose (R)	MI, ribose (C)
TAN (nmol/mg prot)	45 ± 7	40 ± 4	42 ± 3	39 ± 1
ATP (nmol/mg prot)	16 ± 4	17 ± 3	20 ± 4	18 ± 1
ADP (nmol/mg prot)	15 ± 2	12 ± 1**	12 ± 1	12 ± 1**
AMP (nmol/mg prot)	15 ± 3	11 ± 3 *	10 ± 3 *	9 ± 1 ***
ATP/ADP ratio	1.1 ± 0.3	1.4 ± 0.2	1.7 ± 0.5**	1.5 ± 0.1*

Table 5-6: Myocardial adenine nucleotide concentrations in LV. * denotes $p < 0.05$, ** $p < 0.01$ vs group S (one-way ANOVA with Bonferroni post-hoc test).

5.4 Discussion

In this study, we reported the following findings:

- 1) Ribose administered *per os* for seven weeks is palatable and bioavailable resulting in increased myocardial ribose-5-P levels.
- 2) Chronic ribose administration had no effect on function of healthy myocardium.
- 3) There was a trend to a decrease in TAN pool in infarcted hearts, which was not prevented by ribose feeding either in WT or CrT-OE hearts.
- 4) There was no difference in cardiac function between infarcted WT mice with or without ribose and CrT-OE mice supplied with ribose.

5.4.1 Ribose is absorbed *per os* and taken up by the myocardium

We demonstrated that ribose administered in drinking water was well tolerated by mice. It induced an increase in ribose-5-P concentration by 2 fold in the myocardium of WT mice fed ribose. This confirms that ribose is bioavailable when given orally in the mouse and is taken up by the heart. Naito³¹³ and Gross et al.³¹⁴ showed that intestinal absorption is high both in human (> 85 %) and rat. During absorption, ribose is phosphorylated, which is a pre-requisite step for ribose metabolism^{313, 315}. Ribose reaches the blood pool without first passage effect³¹⁴. Cellular uptake is then probably mediated through a glucose transporter³¹⁶. We found an increase in myocardial ribose levels. However, we cannot compare the proportion of this increase to studies from the literature. Indeed, to our knowledge

myocardial ribose concentration has never been measured following ribose supplementation, which is a major weakness of the published literature. Nevertheless, the concentration we found in control hearts was in keeping with the published literature for untreated rat heart³⁰¹.

5.4.2 Ribose supplementation failed to prevent the decrease in TAN pool in the failing heart

Myocardial supplementation with ribose failed to maintain TAN pool in WT and CrT-OE infarcted hearts. Different hypotheses could be advanced to explain what could seem, at first, a surprising result in regard to the literature.

First, despite a two-fold increase, we cannot exclude that the levels of ribose reached in the myocardium were insufficient to substantially increase *de novo* biosynthesis. Moreover, the increase in ribose levels was shown in healthy hearts and could not be confirmed on infarcted hearts as ribose assay requires a high quantity of tissue, which was needed for other biochemical measurements. Therefore, we cannot exclude - even if unlikely - that ribose uptake was impaired in the remote myocardium of the infarcted hearts.

Secondly, in all published studies, ribose was administered prior to the induction of the pathological model. Consequently, this could have brought the myocardium into a more favorable condition to handle the subsequent insult. Moreover, we cannot exclude that, in these studies, part of the effect of ribose in the conservation of TAN pool originates from an enhancement of adenine nucleotide salvage pathway^{304, 317}, even if the stimulating effect of ribose in this pathway has been questioned³¹⁸. Ribose-5-P is the direct precursor of PRPP, which is involved in both *de novo* and salvage pathways of nucleotide synthesis. In our

model, as we wanted to assess the potential therapeutic effect of a simultaneous elevation in ribose and creatine on an already dysfunctional heart, ribose administration was started 24 hours following myocardial infarction. Hence, in these hearts, significant loss of adenine nucleotide may probably already have occurred even in the remote myocardium, which experiences an acute stress response to catecholamines in order to maintain post-infarct cardiac output. Indeed, Zimmer et al.²⁷⁷ showed a reduction of TAN pool after only 5 hours of isoproterenol stimulation. This could readily be tested by measuring TAN pool in remote myocardium a few hours after infarction.

Thirdly, most studies looked at the effect of ribose in the acute setting. Only one study looked at the effect of ribose following permanent coronary artery ligation and only until day 9 post-infarction; they showed a quicker restoration of TAN pool in rat continuously infused ribose intravenously²⁸⁶. On the contrary, another chronic study did not find any effect of ribose feeding in adriamycin-induced cardiomyopathy in rats³⁰⁵. Therefore, we cannot exclude model or species differences.

Finally, we showed that ribose was taken up by the hearts; the increase in ATP/ADP ratio support this. The mechanism of this finding, which should be confirmed in a bigger group size, is unclear. However, a high ATP/ADP and ATP/AMP have been shown to favour both nucleotide *de novo* synthesis and salvage pathways³¹⁹. This could therefore suggest an attempt of these hearts to maintain the TAN pool.

5.4.3 Moderate elevation of myocardial creatine has no effect on cardiac function

In CrT-OE mice, we managed to maintain supraphysiological creatine levels following infarction. However, this had no effect on cardiac function, neither beneficial nor deleterious. This confirms that the range of creatine concentrations prior to infarction was adequately chosen to achieve the objectives of the study. Indeed higher concentrations would have resulted in cardiac dysfunction due to the inability of the heart to maintain adequate phosphorylation of the creatine pool⁸⁷. This finding is in accordance with what we have previously found (see 5.1.2) and shows that within this context, a moderate increase in creatine pool is safe in HF. This is an important finding since we have data to show a protective effect of increased creatine levels in ischaemia / reperfusion for which HF may be a co-morbidity.

5.4.4 Limitations

To date, the main limitation of this study is the small size of the two-groups of ribose-fed mice. We were unlucky to get so few in the ribose-treated groups completing the study; this was due to the learning curve for MI surgery. We are therefore underpowered to conclude that there are no differences between treatment groups. Moreover, this restricts our ability to do a perfect matching for infarct size of all three infarcted groups. The aim of the chronic model of myocardial infarction is not to assess whether an intervention changes infarct size (ischaemia-reperfusion should be used for this) but to determine whether for the same insult,

LV function is modified by the intervention²⁹³. Therefore, a good matching for infarct size is critical as LV remodeling and function is directly proportional to infarct size^{111, 311, 320}. More mice are currently being added to the study to allow for better matching and confirm the above-mentioned findings.

The pilot study for ribose-5-P myocardial concentration was run over the same time-scale as the infarct study. Hence, we do not know how soon after starting ribose supplementation ribose-5-P became elevated, although the acute studies suggest that uptake should be rapid.

Measuring high-energy phosphates *ex vivo* suffers from the high lability of those compounds. This was attenuated by keeping the scar tissue, which decreased harvesting time and by freeze-clamping the ventricles. However, during this time, PCr levels massively dropped and we cannot exclude a decrease in ATP levels too. However, we do not believe that the higher ATP/ADP ratio in ribose-fed groups is due to difference in harvesting times. If anything, harvesting would have taken longer for infarcted groups due to adhesions of the infarct region to the chest wall. Finally, this cannot be an artefactual finding secondary to keeping the scar for HPLC measurements. An eventual bias induced by the scar would result in the same amplitude both for ATP and ADP, hence cancelled by the ratio.

In order to overcome this problem PCr and ATP levels were measured *in vivo* by ³¹P-MRS. All mice included in this study underwent a ³¹P-MRS CSI (chemical shift imaging) examination (Figure 5-19). However, the technique is still at the experimentation stage and data will be analyzed quantitatively in the near future.

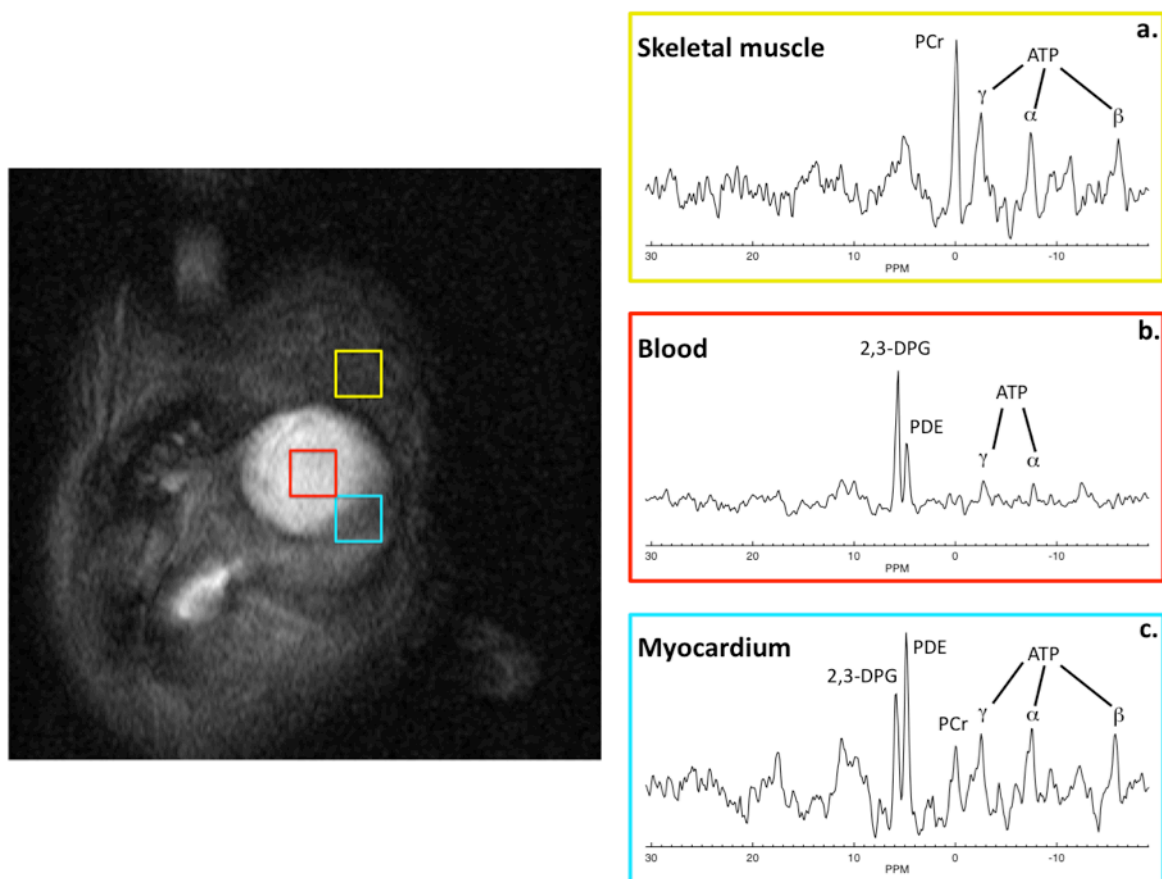


Figure 5-19: ^{31}P -MRS spectra from the skeletal muscle (a.), blood (b.) and myocardium (c.) of an infarcted mouse from group C. The apparently low PCr/ATP ratio in the myocardial spectrum is partially due to blood contamination (as confirmed by the presence of 2,3-DPG and PDE in the spectrum) and to partial saturation due to differences in spin-lattice relaxation times (T_1).

5.4.5 Conclusions and future directions

This study addressed three aims. It demonstrated that ribose administered in drinking water was taken up by the heart but that ribose treatment is not sufficient to maintain normal TAN pool in chronic heart failure. Consequently, the concomitant increase in myocardial creatine and ribose levels was not found to be beneficial in this mouse model of HF. Once confirmed by increasing sample size, this will suggest that other approaches are required in order to simultaneously augment total Cr, TAN and TEP pools. These could include creation of

5'-nucleotidase knockout mice, or control of the expression / activity of G-6-PD, the first and rate-limiting enzyme of the pentose phosphate pathway²⁷¹. Alternatively, *in vitro* studies on isolated cardiomyocytes suggested that the best way to preserve ATP levels during cold cardioplegia is a combination of ribose and adenine associated with either adenosine kinase and deaminase inhibitors³²¹, or with nucleotide transporter inhibitors³²². The applicability of this approach to heart failure remains to be tested. Further studies to test augmentation of HEP in chronic HF are merited.

Chapter 6

SUMMARY AND GENERAL DISCUSSION

The aim of this thesis was to contribute to the understanding of the role of Cr/PCr/CK system on cardiac function. Two approaches were used to evaluate the importance of this phosphotransfer network.

First, we studied the implications of a defect in HEP metabolism by using transgenic mice unable to synthesise creatine (and therefore PCr) due to targeted disruption of the AGAT gene. We studied for the first time the rate of Cr loss from the myocardium using AGAT^{-/-} mice previously fed Cr and subsequently withdrawn from any dietary Cr source. About 3 % of Cr pool was lost every day from passive diffusion through the sarcolemmal membrane. This finding already has practical implications since the Neubauer laboratory has established that elevated creatine is cardio-protective in models of ischaemia/reperfusion injury, and is screening for small molecules that activate the CrT. It suggests that pharmacological dosing to alter CrT activity need only be intermittent, as once creatine is within the target range it will take weeks to return to baseline values.

The AGAT^{-/-} mouse is a unique model and the study reported in this thesis represents the first description of its cardiac phenotype. Despite a strong reduction in myocardial Cr levels of ~70-90 %, no change in cardiac function was observed. In contrast, mice fully creatine-deprived showed impairment in contraction and relaxation properties of the heart and a reduced contractile reserve. Taken together, these results suggest that a small amount of creatine may be sufficient to maintain cardiac function under basal and stimulated conditions. This agrees with previous work in β -GPA-fed animals, but represents the first time that the confounding effects of alternative guanidino substrates (either participating in the CK reaction or having direct toxic effects) can be excluded. Similarly, it is notable that the AGAT^{-/-} phenotype was more pronounced than in GAMT^{-/-} mice, which do not have Cr either, but accumulate the precursor guanidinoacetate. This suggests that despite a slower

reaction rate, the contribution of GA to HEP flux was sufficient to maintain baseline function. We can deduce from all these data that only a very limited proportion of the activity of the CK system is required to drive energy requirements at basal workloads, i.e. that there is considerable functional redundancy within phosphotransfer circuits in the heart. Rescue of the AGAT^{-/-} phenotype by dietary creatine, represents a quicker and cleaner experiment than has previously been possible using cessation of β -GPA feeding, and provided unique insights. Replenishment of the myocardial creatine pool was shown to be rapid (< 7 days), but was insufficient to fully restore cardiac function even after 7 weeks. This suggests that the observed haemodynamic phenotype is not a thermodynamic consequence of altered high-energy phosphates *per se*, e.g. via impaired ΔG_{ATP} , since this would be expected to resolve immediately upon restoration of creatine. Rather the phenotype appears to be a consequence of the heart adapting to chronic creatine deficiency in this case by slightly reducing the pressure generating capacity, which will be energy sparing. In contrast to observations reported in other models of impaired cardiac energetics, the adaptive mechanisms did not involve hypertrophy.

The main limitation of the AGAT^{-/-} model in understanding the role of Cr in HEP metabolism was the presence of body composition changes, which complicated the interpretation of cine-MRI & invasive haemodynamic function, where an increased susceptibility to general anaesthesia could not be entirely excluded. The low body weight in AGAT^{-/-} mice mirrors that observed in other related models (e.g. GAMT^{-/-} and CK knockouts), but is much more severe. This study established a threshold value for creatine of ~20% of starting levels, below which major weight loss was precipitous. The mechanism for this is not obvious as these mice continue to feed normally, but is worthy of further study.

The difficulties of interpretation due to adaptations or unrelated side-effects are common to all loss-of-function models of HEP metabolism described to date (see 4.1), and demonstrates that no single knockout model is uniquely informative, but that patterns emerge from careful comparisons between models.

A second, more direct approach was taken to evaluate therapeutic potential of increased HEP in an appropriate disease models. The use of ribose as an enhancer of AN biosynthesis in the injured myocardium is an old idea, with multiple studies performed in the 1980's showing a beneficial effect of ribose administration to restore the depleted TAN pool in models of acute injury; which translated to improved functional recovery (see 5.1.4). The study reported here built on these, and is original in multiple aspects, not least because it examined longer time-points than previously reported, in order to assess the effects of ribose on chronic heart failure rather than recovery from injury. It is also the first study to investigate the effects of oral ribose administration in the mouse. This required pilot studies to determine the palatability of ribose administered in drinking water, and to confirm bioavailability by measuring myocardial ribose-5-P levels in the heart. Remarkably, this had always been assumed in previous studies, whereas this study showed that exogenous ribose could elicit a 2-fold increase in the heart. Importantly this did not result in any change in the haemodynamic parameters of the healthy heart suggesting the innocuousness of the treatment. Before commencing the main study it was also necessary to demonstrate that TAN levels are reduced in the failing murine heart following coronary artery ligation, as this had never been measured before. This confirms the adequacy of this model to test the potential benefit of ribose administration in restoring the TAN pool.

One of the major findings is that, despite an increase in ribose myocardial levels, ribose administration did not maintain TAN levels. This is the first robust test of ribose as a

potential therapeutic agent in chronic HF, since previous studies have not controlled for obvious confounders such as matching for infarct sizes. The data presented here therefore suggest that ribose on its own is not beneficial in chronic HF.

On the strength of *in silico* modeling studies it was hypothesized that a concomitant increase in myocardial total Cr and TAN pools would augment HEP metabolism, and thereby improve post-infarction remodeling and function. While we managed to create favorable conditions for testing our hypothesis, with an increase in both Cr and ribose levels, this did not result in improvement of LV haemodynamic or morphological parameters. It can be concluded that the combination of creatine and ribose are not beneficial to the failing heart, but nor are they deleterious, which may be important for future potential therapies. Indeed, since TAN pool was unaffected by ribose treatment, the hypothesis that increasing Cr, TAN and TEP pools will be beneficial remains to be fully tested, and alternative approaches to maintain TAN levels in combination with total Cr should be investigated.

Both of the above studies required a technique to non-invasively assess cardiac creatine levels, for which ^1H -MRS is the only available methodology. Consequently, ^1H -MRS was validated in phantoms and in the *in vivo* mouse heart and extensively applied to quantify a wide-range of myocardial creatine levels. This allowed changes in Cr levels to be followed longitudinally in AGAT^{-/-} mice, but also to pre-select mice according to their creatine levels for the ribose study. Indeed, due to the variability of creatine levels in the CrT-OE mice, pre-selection for suitable myocardial Cr concentration was necessary; therefore this study would not have been possible without ^1H -MRS and is the first of its kind in the world.

The application of non-invasive MRI also made an essential contribution to these studies. Without this technique, measurement of infarct sizes would have required histology, a destructive method, making impossible biochemical analyses on these hearts.

MRI-determined LV mass made post-mortem weighing of LV unnecessary, reducing the time taken to remove and freeze the tissue, and thereby improving the accuracy of myocardial HEP quantification. Furthermore, LV mass assessed longitudinally by MRI, was able to identify the progressive loss of LV weight as creatine levels fell in AGAT^{-/-} mice.

The non-invasive nature and versatility of these MR technologies are revolutionizing cardiac phenotyping of genetically-modified mice, and justify continuing investment in MR technique development. By multiplying the number of functional or metabolic parameters assessed, ideally within a single anaesthesia, this will make new types of study possible, improve study designs, and help reduce the number of animals used in research.

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APPENDICES

Appendix 1:

Related publications and conferences presentations

Journal papers

1. Lygate C.A., Bohl S., ten Hove M., Faller K.M.E, Ostrowski F., Zervou S., Medway D.J., Aksentijevic D., Sebag-Montefiore L., Wallis J., Clarke K., Watkins H., Schneider J.E. Neubauer S. Creatine protects against myocardial ischaemia/reperfusion injury. *Submitted*.
2. Faller K.M.E *et al*. Manuscript in preparation describing the cardiac phenotype of AGAT knockout mice and their rescue by dietary creatine.
3. Faller K.M.E *et al*. Manuscript in preparation describing the combined approach of elevated creatine and TAN pool in chronic heart failure.

Conference presentations

1. Faller K.M.E., Lygate C.A., Sebag-Montefiore L.M., Medway D., Isbrandt D., Neubauer S., Schneider J.E. ^1H -MRS for longitudinal quantification of myocardial creatine concentration in AGAT-KO mice. 26th Scientific Meeting of the European Society of Magnetic Resonance in Medicine and Biology. October 2009, Antalya (Turkey).
2. Faller K.M.E., Lygate C.A., Neubauer S., Schneider J.E.S. Assessment of cardiac creatine visibility by ^1H -MRS. 16th Annual Meeting of the British Chapter of the International Society for Magnetic Resonance in Medicine. September 2010, Nottingham (UK).
3. Faller K.M.E., Lygate C.A., Medway D., Schneider J.E., Neubauer S. Myocardial creatine metabolism – Insights from transgenic models. British Heart Foundation Center of Research Excellence – Mid-term site visit. March 2011, Oxford (UK).
4. Faller K.M.E., Lygate C.A., Neubauer S., Schneider J.E.S. ^1H -MRS can be used to investigate creatine metabolism in multiple organs within a single examination in the mouse. 19th Annual Meeting of the International Society for Magnetic Resonance in Medicine. May 2011, Montreal (Canada).

¹H-MRS for longitudinal quantification of myocardial creatine concentration in AGAT-KO mice
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Purpose/Introduction

Proton Magnetic Resonance Spectroscopy (¹H-MRS) is a non-invasive method for investigation of cardiac metabolism, enabling longitudinal examinations. We validated the use of ¹H-MRS for measurement of myocardial creatine *in vivo*, and applied this to L-Arginine:Glycine amidinotransferase deficient mice (AGAT-KO). With AGAT being the first enzyme in the *de novo* biosynthesis of creatine, KO mice are dependent on dietary intake of creatine. We aimed to determine the time course of myocardial creatine depletion following dietary creatine withdrawal.

Subjects/Methods

Experiments were conducted on a Varian 9.4T/210mm bore Magnex magnet with a Varian Direct Drive console (Varian Inc.) using a 33mm quadrature birdcage coil (Rapid Biomedical). Water-suppressed (256 averages) and unsuppressed (16 averages) spectra were acquired during diastole from a 2μl septal voxel using a cardiac/respiratory-gated PRESS sequence (TE=8ms/TR~2s) [1]. Automatic volume selective shimming was performed over a 4mm³ spherical volume around the voxel centre [2]. Creatine and water peak amplitudes were quantified using AMARES [3].

¹H-MRS was validated against HPLC (gold standard) for the absolute quantification of myocardial creatine. Six spectra (three with and three without water-suppression) were acquired in wild-type (n=3) and creatine-transporter (n=7) over-expressing mice [4], covering a wide range of myocardial creatine concentrations.

Similarly, six adult AGAT-KO mice were examined at baseline and repeatedly over three months following dietary creatine withdrawal. Hearts were subjected to HPLC 83 days after creatine withdrawal.

Results

An excellent correlation (r=0.99) between creatine/water ratio (¹H-MRS) and total creatine concentration (HPLC) was observed (Fig.1). The calibration curve was used to quantify myocardial creatine concentration in the AGAT-KO mice.

In the AGAT-KO mice, following dietary creatine withdrawal, myocardial creatine concentration decreased gradually throughout the time course of the study (Fig.2). By day 83, no creatine resonance was observed (Fig.3). However, when assessed by HPLC, the residual creatine concentration was 20±3.9 nmol/mg protein.

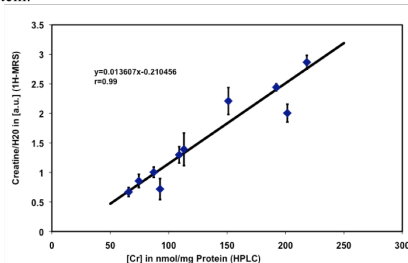


Fig.1: Correlation between creatine/water ratio assessed by ¹H-MRS and creatine concentration measured by HPLC.

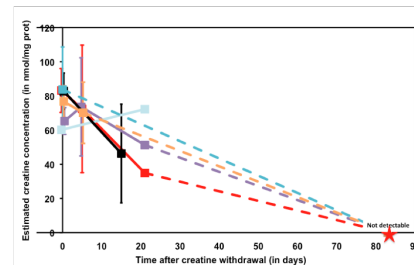


Fig.3: Evolution of the estimated myocardial creatine concentration after creatine withdrawal in AGAT-KO mice. The standard deviation is shown when available.

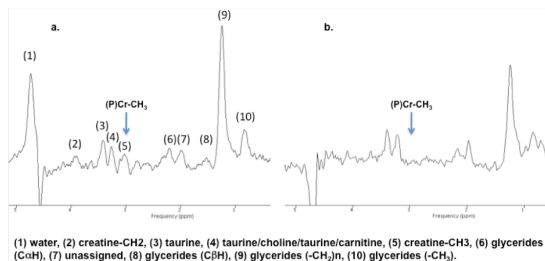


Fig.2: Two representative spectra from the same mouse acquired before (2.a) and 83 days (2.b.) after creatine withdrawal

Discussion/Conclusion

¹H-MRS enabled quantification of the myocardial creatine concentration *in vivo* and monitoring of its temporal evolution. In two mice, the measured creatine concentration increased between subsequent time points, possibly due to fitting errors or technical issues. Furthermore, ¹H-MRS was insufficiently sensitive to detect extremely low creatine concentrations (<20nmol/ mg protein), which is in agreement with the calibration curve. Despite these limitations, ¹H-MRS was suitable for quantifying cardiac creatine *in vivo* over a wide range of physiological and pathophysiological concentrations, making it a valuable tool to study cardiac energetics in mice.

Acknowledgements

This work was funded by research grants from the British Heart Foundation (BHF).

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Is the entire myocardial creatine pool MR-visible? An ^1H -MRS-study in mouse hearts

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Introduction: ^1H -MRS is the only available method to assess myocardial creatine levels non-invasively. This assumes that the entire pool of creatine can readily be detected. However, our previous experiments suggest a reduced MR-visibility of creatine in the heart of rats [1] and mice [2], a phenomenon already described in different organs for other metabolites such as lactate [3]. The aim of this abstract is to investigate the MR-visibility of creatine in murine hearts by applying ^1H -MRS *in vivo*, *ex vivo* in intact myocardium and *ex vivo* in a myocardial solution.

Methods: All experiments were conducted on a 9.4T/210mm bore Magnex magnet with a Varian Direct Drive console (Varian Inc.) using 33 mm and 13 mm quadrature birdcage coils (Rapid Biomedical).

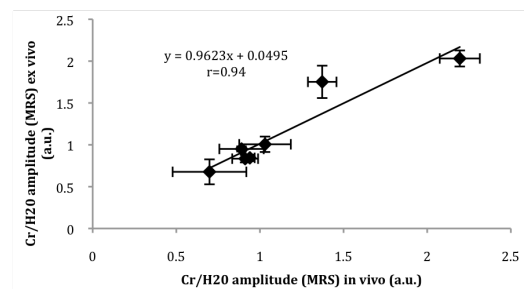
^1H -MRS was performed on hearts of three wild-type and four creatine-transporter over-expressor mice under three different conditions using a PRESS sequence (TE/TR=8/2000ms).

1. *In vivo* MRS: Water-suppressed (256 averages) and unsuppressed (16 averages) spectra were acquired during diastole from a 2 μl septal voxel as reported previously [4].
2. *Ex vivo* MRS on intact myocardium: mice were sacrificed at the end of the *in vivo* experiment, the hearts were excised, the right ventricular wall removed, the left ventricle (LV) rinsed in saline and split in two. One part of the LV was embedded in formalin and four water-suppressed (64 averages) and four unsuppressed (4 averages) spectra were acquired within 30 mins post excision from a 2 μl voxel.
3. MRS on a myocardial solution: The remaining part of the LV was prepared with a standard method [5] used for HPLC measurement of creatine. HPLC was performed on an aliquot of this solution and the remaining solution was subjected to ^1H -MRS. Spectra were acquired from an 8 μl voxel (3 suppressed spectra (NA=6000), 3 water spectra (NA=16)). HPLC and MRS results were then standardized against the amount of proteins (Lowry assay [6]).

Creatine and water peak amplitudes from all spectra were quantified using AMARES [7]. The water peak of the non-suppressed spectra was used as internal reference.

Results: Fig. 1 shows creatine concentrations assessed by ^1H -MRS *in vivo* vs *ex vivo*. Correlation analysis yielded a slope of 0.96 and a y-offset of 0.050 ($r=0.94$).

Fig. 1: Comparison of the amplitude of creatine peak assessed by MRS *in vivo* and *ex vivo* in intact myocardium.



Figures 2a-c show the creatine levels assessed by MRS *in vivo*, *ex vivo* and in myocardial solution versus creatine concentration measured by HPLC. While *in vivo* and *ex vivo* correlations showed a y-offset of 15 and 8.9, respectively, the y-offset for the myocardial solution is close to 0. The correlations are $r=0.85$ (*in vivo*), $r=0.96$ (*ex vivo*) and $r=0.99$ (myocardial solution), respectively.

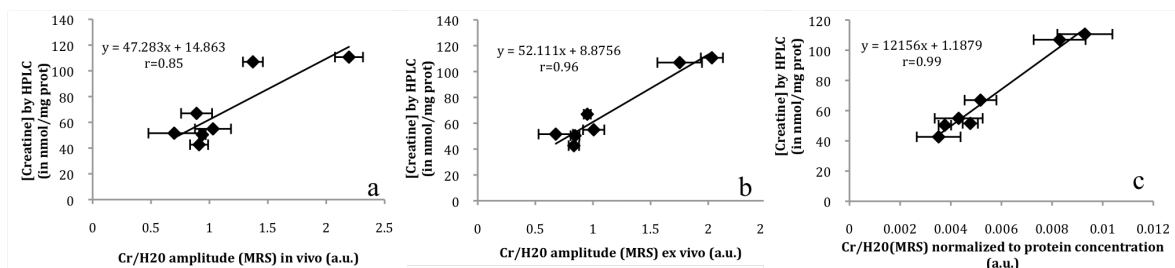


Fig 2: Correlation between creatine amplitude assessed by MRS *in vivo* (a), *ex vivo* (b), and in myocardial solution versus creatine concentration assessed by HPLC.

Conclusions: The correlation plots in Fig 2a & b (*in vivo* & *ex vivo* in intact myocardium) between MRS and HPLC hint towards a decreased MR-visibility of creatine in tissue indicated by the offset correlation analysis. On the contrary, the constant factor found for the correlation in the solution state (Fig. 2c) is very small, suggesting that the preparation of myocardial solution rendered the whole creatine pool measurable by MRS. This decrease in creatine visibility could suggest a compartmentalization of creatine in intact myocardium as first suggested by Lee *et al.* [8]. Work is in progress to add more samples.

Acknowledgements: This work was funded by the British Heart Foundation (BS/06/001 and FS/07/065)

References: [1] Schneider JE *et al.* *Magn Reson Med.* 2000;43(4):497-502; [2] Faller K *et al.* ESMRMB 2009 Congress. [3] Kotitschke K *et al.* *NMR Biomed.* 1994;7(8):349-355; [4] Schneider JE *et al.* *Magn Reson Med.* 2004;52(5):1029-1035; [5] Neubauer S *et al.* *J Mol Cel Cardiol.* 1989;21(11):1163-1178; [6] Lowry OH *et al.* *J Biol Chem.* 1951;193(1): 265-275; [7] Vanhamme L *et al.* (1997) *J.Magn.Reson.*;129(1):35-43. [8] Lee YC *et al.* *Proc Natl Acad Sci U S A* 1961;47:1510-1515.

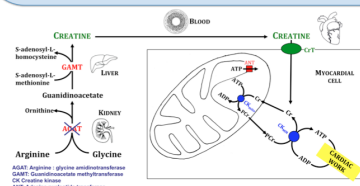
Introduction

The failing heart is characterized by changes in high-energy phosphate metabolism. For example, a decrease in total creatine (Cr), phosphocreatine (PCr), and Creatine Kinase (CK), which together play a key role in cellular energy transport and buffering.

The aim of this ongoing study is to determine whether reduced myocardial creatine levels *per se* can directly impair cardiac function.

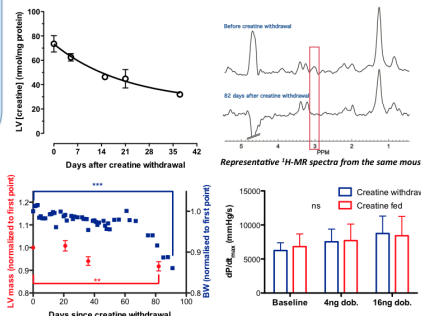
Our group has previously demonstrated reduced inotropic reserve in the GAMT-KO mouse model, which cannot synthesise creatine but accumulates its precursor, guanidinoacetate (GA). However, this model has two limitations:
i) GA can participate in the CK reaction albeit at a very low rate (~1% of Cr)
ii) Phenotype could be due to toxic levels of GA (inhibition of Na⁺/K⁺ ATPase).

A newly available model, the AGAT-KO mouse, which can not make GA and is reliant on dietary creatine, will improve our understanding of creatine metabolism in the heart.



Loss of creatine from the heart is very slow

6 AGAT-KO mice were fed creatine from birth. At adulthood (~20 weeks), dietary creatine was withdrawn



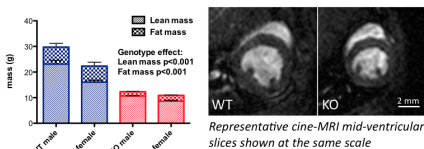
For the first time, the kinetics of myocardial creatine elimination has been established *in vivo* using ¹H-MRS.
- Creatine levels decrease by ~1-2% a day and no creatine was visible by ¹H-MRS after 82 days.
- However, residual creatine of 20 nmol/mg protein was detectable by HPLC.
- Creatine withdrawal resulted in loss of LV mass (by MRI), and beyond 65 days a precipitous drop in body weight.
- Welfare considerations limits the utility of this approach at a time when ~25% of myocardial creatine remains.
- No change in cardiac function was observed by cine-MRI or haemodynamics.
- Potential adaptations could have developed during this slow creatine "wash-out".

Total body creatine deficiency results in LV dysfunction

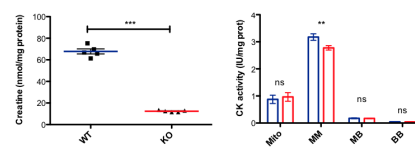
AGAT-KO and littermate controls (~20 weeks old) fed creatine-free diet from birth (creatine-naïve)

Anatomical phenotyping

• Creatine naïve mice both leaner and smaller
• Lower LV weight but LV/BW increased → cardiac hypertrophy?

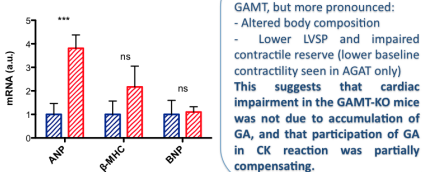
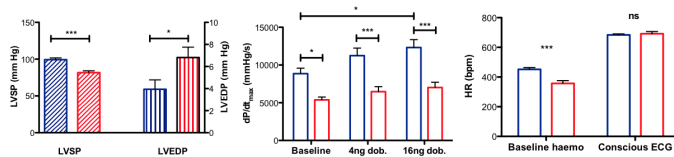


Biochemical alterations



Cardiac function (LV haemodynamics)

• Impaired contractility in the AGAT-KO mice
- Reduced contractile reserve

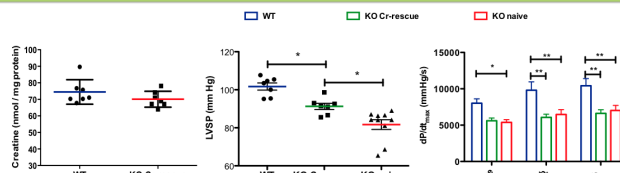


AGAT-KO phenotype is similar to GAMT, but more pronounced:
- Altered body composition
- Lower LVSP and impaired contractile reserve (lower baseline contractility seen in AGAT only)
This suggests that cardiac impairment in the GAMT-KO mice was not due to accumulation of GA, and that participation of GA in CK reaction was partially compensating.

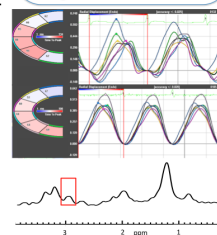
Can *in vivo* phenotype be rescued by creatine feeding? (work in progress)

Creatine-naïve AGAT-KO and littermate controls fed creatine in diet for 7 days

- Creatine accumulation is rapid: 7 days of creatine feeding in previously creatine naïve mice restores myocardial levels.
- This results in partial improvement of LVSP but not in contractility (dP/dt_{max}).
- Can not rule out long-term (mal-) adaptations to low creatine, which are not reversed within 7 days.
- Further experiments are ongoing in mice fed creatine over 6 weeks.



New high-frequency ultrasound partially funded by the CRE will allow more detailed analysis of LV function than previously possible. Example below shows dyssynchronous myocardial displacement in a KO mouse, that resolves after 7 days of creatine feeding.



Future work

• The presence of creatine in AGAT-KO creatine-naïve mice (shown by HPLC) needs to be confirmed by another technique (mass spectroscopy).
• Very low body fat in KO mice could cause changes in circulating adipokines and adipose derived-hormones, which might contribute to the observed phenotype. We intend to study whether variations in their levels correlate with cardiac function.
• The absence of cardiac hypertrophy will be confirmed microscopically by measuring myocyte cross-sectional area.
• Adenylate kinase activity will be measured to assess its potential adaptive role in energy handling in the AGAT-KO myocardium.

Acknowledgements

This work was funded by BHF grant RG/10/002/28187; K. Faller is funded by a BHF 4-year PhD studentship (FS/07/065); The Visualsonics 2100 Ultrasound system was partially funded by the CRE.

¹H-MRS can be used to investigate creatine metabolism in multiple organs within a single examination in the mouse

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Introduction

Creatine is essential in energy transfer and buffering in organs with high and fluctuating energy demands such as brain, heart and skeletal muscle. Whereas a lack of creatine induces neurological, cardiac and muscular defects [1,2], creatine supplementation protects against ischemic and oxidative insults [3]. Therapies targeting creatine metabolism of these organs require a technique to monitor non-invasively creatine levels; ¹H-MRS is to date the only available method. Furthermore, organ-specific differences in creatine metabolism [4] necessitate the investigation / measurement of creatine concentrations in multiple organs, ideally within a single recovery MRS examination to reduce the number of animals and to increase statistical power. Therefore, the aim of this study was to evaluate the feasibility and reproducibility of a ¹H-MRS technique using a simple set-up, which does not require time-consuming mouse or coil repositioning.

Material and Methods

All experiments were conducted on a 9.4T/210mm bore Magnex magnet with a Varian Direct Drive console using a 33 mm quadrature birdcage coil (Rapid Biomedical). The same PRESS sequence was used to scan the heart, the thalamus and the tibialis anterior (TE/TR=8/2000 ms). Mice were anaesthetized with Isoflurane and cardiac and respiratory parameters recorded throughout the experiment.

1. Reproducibility experiments

- Cardiac MRS: Three water-suppressed (256 averages) and three unsuppressed (16 averages) spectra were acquired interleaved during diastole from a 2 µl septal voxel using a cardiac/respiratory-gated PRESS sequence in 33 wild-type mice and 53 transgenic mice with elevated cardiac levels, as previously described [5].
- Brain MRS: Three water-suppressed (256 averages) and three unsuppressed (16 averages) spectra were acquired from a 8 µl voxel localised in the thalamus of the same wild-type mouse on five different days. No gating was used.
- Skeletal muscle MRS: Three water-suppressed (256 averages) and three unsuppressed (16 averages) spectra were acquired from a 4.5 µl voxel localised in the tibialis anterior of the same wild-type mouse on five different days. No gating was used.

2. Follow-up of creatine concentration in multiple organs

A wild-type mouse was scanned on two following days. Three scans were acquired from the cardiac septum, one from the thalamus and one from the tibialis anterior. Between each organ, the mouse was repositioned in the scanner by simply pushing the whole cradle inside the volume coil, repositioning the voxel and reshimming using an automatic method [6].

3. Data analysis

Creatine and water peak amplitudes from all spectra were quantified using AMARES [7]. The water peak of the non-suppressed spectra was used as an internal reference.

Results

1. Reproducibility experiments

Representative spectra from all three organs are shown in Figure 1. The average intra-day Coefficient of Variation (CV) of the creatine cardiac measurements was of 16.9%. The average intra-day CV of the creatine thalamic measurements was of 6.6% and the inter-day of 4.1%. In the tibialis anterior, the intra-day and inter-day CV were of 5.8% and 2.8% respectively.

These results confirm the reproducibility and repeatability of the method. However, due to a higher intra-day variability of the method in the heart, three cardiac spectra were acquired in the multi-organ examination, whereas single spectra were acquired from the brain and the skeletal muscle.

2. Follow-up of creatine concentration in multiple organs

The whole acquisition of spectra from the heart, the brain and the skeletal muscle was performed within a single anaesthesia and lasted roughly 2.5 hours, including preparation time. During this period the physiological parameters were stable (HR=418±13, RR=28±12). The variations in the creatine measurements between the two consecutive days were of 9% for the heart, 2.5% for the brain and 6.7% for the skeletal muscle.

Conclusions / Discussion

We have shown that creatine metabolism can be assessed in three different organs using a simple set-up without the need for RF coil repositioning as would be required for surface coils. Very few studies in rodents have investigated the reproducibility of MRS. However, our inter-day CV is similar to the one from a study by Öz et al. [8] in the mouse brain with a CV for creatine measurement less than 5%. Therefore, this technique allows us to accurately follow creatine levels *in vivo* in multiple organs. An extended study in mice with different pathophysiological conditions is ongoing.

References: [1] Stöckler S. et al., *Am. J. Hum. Genet.* 1996; 58(5):914-922; [2] ten Hove M. et al. *Circulation* 2005; 111(19):2477-2485; [3] Sullivan P.G. et al., *Ann. Neurol.* 2000; 48(5):723-729; [4] Ipsioglu O.S. et al., *Life Sci.* 2001; 69(15): 1805-1815; [5] Schneider JE, et al. *Magn Reson Med.* 2004;52(5):1029-1035; [6] Schneider et al. *Proc.Intl.Soc.Mag.Reson.Med.* 2009;17:1782. [7] Vanhamme et al. *J.Magn.Reson* 1997.;129(1):35-43; [8] Öz G. et al. *J. Neurosci.* 2010; 30(10): 3831-3838.

Acknowledgements: This work was funded by the British Heart Foundation (BS/06/001 and FS/07/065)

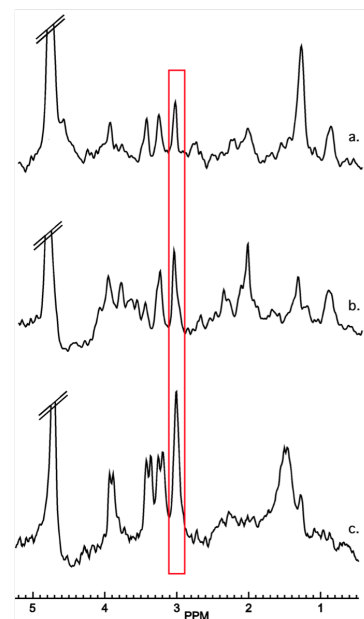


Figure 1: Representative spectra from the myocardium (a), the thalamus (b) and the anterior tibialis (c). The creatine peak is highlighted in red.

Appendix 2:

Composition of the rodent diets used

1. Standard chow (Harlan Teklad Global 16 % Protein Rodent diet irradiated (Ref 2916))

Harlan Laboratories

2016

Teklad Global 16% Protein Rodent Diet

Product Description- 2016 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support growth and maintenance. 2016 does not contain alfalfa or soybean meal, thus minimizing the occurrence of natural phytoestrogens. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. **Also available certified (2016C) and irradiated (2916). For autoclavable diet, refer to 2016S (Sterilizable).**

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, wheat middlings, corn gluten meal, calcium carbonate, dicalcium phosphate, soybean oil, brewers dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, magnesium oxide, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B₁₂ supplement, folic acid, biotin, vitamin D₃ supplement, cobalt carbonate.

Standard Product Form: **Pellet**

Macronutrients		
Crude Protein	%	16.4
Fat (ether extract) ^a	%	4.0
Carbohydrate (available) ^b	%	48.5
Crude Fiber	%	3.3
Neutral Detergent Fiber ^c	%	15.2
Ash	%	4.9
Energy Density ^d	kcal/g (kJ/g)	3.0 (12.6)
Calories from Protein	%	22
Calories from Fat	%	12
Calories from Carbohydrate	%	66
Minerals		
Calcium	%	1.0
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.2
Potassium	%	0.6
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	70
Manganese	mg/kg	100
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.0
Glutamic Acid	%	3.3
Alanine	%	0.9
Glycine	%	0.7
Threonine	%	0.6
Proline	%	1.5
Serine	%	0.8
Leucine	%	1.9
Isoleucine	%	0.7
Valine	%	0.8
Phenylalanine	%	0.9
Tyrosine	%	0.5
Methionine	%	0.3
Cystine	%	0.3
Lysine	%	0.8
Histidine	%	0.4
Arginine	%	0.8
Tryptophan	%	0.2

Vitamins		
Vitamin A ^{e, f}	IU/g	15.0
Vitamin D ₃ ^{e, g}	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K ₃ (menadione)	mg/kg	50
Vitamin B ₁ (thiamin)	mg/kg	17
Vitamin B ₂ (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	75
Vitamin B ₆ (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B ₁₂ (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1030
Fatty Acids		
C16:0 Palmitic	%	0.5
C18:0 Stearic	%	0.1
C18:1ω9 Oleic	%	0.7
C18:2ω6 Linoleic	%	2.0
C18:3ω3 Linolenic	%	0.1
Total Saturated	%	0.6
Total Monounsaturated	%	0.7
Total Polyunsaturated	%	2.1
Other		
Cholesterol	mg/kg	--

^a Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

^b Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

^c Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

^d Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

^e Indicates added amount but does not account for contribution from other ingredients.

^f 1 IU vitamin A = 0.3 µg retinol

^g 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.

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2. Diet creatine-free used for AGAT^{-/-} mice (Harlan Teklad Global 19 % Protein Rodent diet irradiated (Ref 2919))

Harlan Laboratories

2019

Teklad Global 19% Protein Extruded Rodent Diet

Product Description- 2019 is a fixed formula, non-autoclavable diet manufactured with high quality ingredients and designed to support gestation, lactation, and growth of rodents. It is particularly useful for poorly breeding strains of rodents and genetically engineered stocks and strains. 2019 does not contain alfalfa or soybean meal, minimizing the occurrence of natural phytoestrogens, which can be beneficial for reproductive toxicology. Typical isoflavone concentrations (daidzein + genistein aglycone equivalents) range from non-detectable to 20 mg/kg. Exclusion of alfalfa reduces chlorophyll, improving optical imaging clarity. Absence of animal protein and fish meal minimizes the presence of nitrosamines. The extruded form ensures that despite its high fat content, it remains firm with minimal wastage. **Also available certified (2019C) and irradiated (2919). For autoclavable diet, refer to 2019S (Sterilizable).**

Macronutrients		
Crude Protein	%	19.0
Fat (acid hydrolysis) ^a	%	9.0
Carbohydrate (available) ^b	%	44.9
Crude Fiber	%	2.6
Neutral Detergent Fiber ^c	%	12.1
Ash	%	5.0
Energy Density ^d	kcal/g (kJ/g)	3.3 (13.8)
Calories from Protein	%	23
Calories from Fat	%	22
Calories from Carbohydrate	%	55
Minerals		
Calcium	%	0.9
Phosphorus	%	0.7
Non-Phytate Phosphorus	%	0.4
Sodium	%	0.1
Potassium	%	0.4
Chloride	%	0.4
Magnesium	%	0.2
Zinc	mg/kg	60
Manganese	mg/kg	80
Copper	mg/kg	15
Iodine	mg/kg	6
Iron	mg/kg	200
Selenium	mg/kg	0.23
Amino Acids		
Aspartic Acid	%	1.1
Glutamic Acid	%	3.5
Alanine	%	1.2
Glycine	%	0.7
Threonine	%	0.6
Proline	%	1.8
Serine	%	0.9
Leucine	%	2.3
Isoleucine	%	0.8
Valine	%	0.9
Phenylalanine	%	1.1
Tyrosine	%	0.5
Methionine	%	0.5
Cystine	%	0.3
Lysine	%	0.9
Histidine	%	0.4
Arginine	%	0.8
Tryptophan	%	0.2

Ingredients (in descending order of inclusion)- Ground wheat, ground corn, corn gluten meal, wheat middlings, soybean oil, calcium carbonate, dicalcium phosphate, brewers dried yeast, L-lysine, iodized salt, magnesium oxide, choline chloride, DL-methionine, calcium propionate, L-tryptophan, vitamin E acetate, menadione sodium bisulfite complex (source of vitamin K activity), manganous oxide, ferrous sulfate, zinc oxide, niacin, calcium pantothenate, copper sulfate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B₁₂ supplement, folic acid, biotin, vitamin D₃ supplement, cobalt carbonate.

Standard Product Form: **Extruded**

Vitamins		
Vitamin A ^{e, f}	IU/g	15.0
Vitamin D ₃ ^{e, g}	IU/g	1.5
Vitamin E	IU/kg	110
Vitamin K ₃ (menadione)	mg/kg	50
Vitamin B ₁ (thiamin)	mg/kg	17
Vitamin B ₂ (riboflavin)	mg/kg	15
Niacin (nicotinic acid)	mg/kg	75
Vitamin B ₆ (pyridoxine)	mg/kg	18
Pantothenic Acid	mg/kg	33
Vitamin B ₁₂ (cyanocobalamin)	mg/kg	0.08
Biotin	mg/kg	0.40
Folate	mg/kg	4
Choline	mg/kg	1200
Fatty Acids		
C16:0 Palmitic	%	0.9
C18:0 Stearic	%	0.2
C18:1ω9 Oleic	%	1.7
C18:2ω6 Linoleic	%	3.9
C18:3ω3 Linolenic	%	0.4
Total Saturated	%	1.2
Total Monounsaturated	%	1.7
Total Polyunsaturated	%	4.4
Other		
Cholesterol	mg/kg	--

^a Ether extract is used to measure fat in pelleted diets, while an acid hydrolysis method is required to recover fat in extruded diets. Compared to ether extract, the fat value for acid hydrolysis will be approximately 1% point higher.

^b Carbohydrate (available) is calculated by subtracting neutral detergent fiber from total carbohydrates.

^c Neutral detergent fiber is an estimate of insoluble fiber, including cellulose, hemicellulose, and lignin. Crude fiber methodology underestimates total fiber.

^d Energy density is a calculated estimate of *metabolizable energy* based on the Atwater factors assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

^e Indicates added amount but does not account for contribution from other ingredients.

^f 1 IU vitamin A = 0.3 µg retinol

^g 1 IU vitamin D = 25 ng cholecalciferol

For nutrients not listed, insufficient data is available to quantify.

Nutrient data represent the best information available, calculated from published values and direct analytical testing of raw materials and finished product. Nutrient values may vary due to the natural variations in the ingredients, analysis, and effects of processing.

Teklad Diets are designed and manufactured for research purposes only.



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3. Diet enriched with 5 mg/kg creatine monohydrate used for creatine-rescue AGAT^{-/-} mice (Ssniff R/M-H maintenance diet).

ssniff® R/M-H

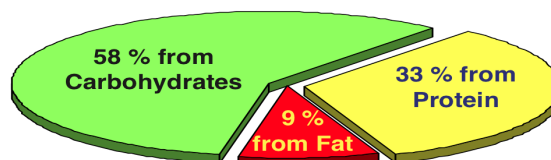
Complete feed for rats and mice - maintenance



Description

That feed is designed for rats and mice in maintenance metabolism. The diet is also suitable for long lasting experiments (e.g. in the field of toxicology), because of its well-balanced nutrient concentrations and moderate energy density as well as the very low nitrosamine contents.

Crude Nutrients	[%]	Energy	[MJ/kg]
Dry matter	87.7	Gross Energy (GE)	16.3
Crude protein (N x 6.25)	19.0	Metabolizable Energy (ME) *	12.8
Crude fat	3.3		
Crude fibre	4.9		
Crude ash	6.4		
N free extracts	54.1		
Starch	36.5		
Sugar	4.7		



Minerals	[%]	Amino acids	[%]	Vitamins	per kg
Calcium	1.00	Lysine	1.00	Vitamin A	15,000 IU
Phosphorus	0.70	Methionine	0.30	Vitamin D ₃	1,000 IU
Sodium	0.24	Met+Cys	0.65	Vitamin E	110 mg
Magnesium	0.22	Threonine	0.68	Vitamin K (as menadione)	5 mg
Potassium	0.91	Tryptophan	0.25	Thiamin (B ₁)	18 mg
		Arginine	1.14	Riboflavin (B ₂)	23 mg
		Histidine	0.44	Pyridoxine (B ₆)	21 mg
		Valine	0.88	Cobalamin (B ₁₂)	100 µg
		Isoleucine	0.76	Nicotinic acid	135 mg
		Leucine	1.30	Pantothenic acid	43 mg
		Phenylalanine	0.85	Folic acid	7 mg
		Phe+Tyr	1.43	Biotin	525 µg
		Glycine	0.80	Choline-Chloride	2,990 mg
		Glutamic acid	3.90	Inositol	100 mg
		Aspartic acid	1.61		
		Proline	1.25		
		Alanine	0.79		
		Serine	0.89		

Fatty acids

	[%]
C 14:0	0.01
C 16:0	0.47
C 16:1	0.01
C 18:0	0.08
C 18:1	0.62
C 18:2	1.80
C 18:3	0.23
C 20:0	0.01
C 20:1	0.02
C 20:5	—
C 22:6	—

Feed composition

descending order of feedingstuffs (FMV)

Grain and grain by-products, oil seed products, minerals, vegetable oils, vitamins, trace elements.

* ME calculated according to the pig formula, Annex 4 of the German feed regulation

Main products

V1530	Meal, single ground
V1534	10 mm pellets
V1535	15 mm pellets

Production and sale

ssniff Spezialdiäten GmbH
Phone: +49-(0)2921-9658-0
Fax: +49-(0)2921-9658-40
E-Mail: mail@ssniff.de
www.ssniff.de

Energy density [MJ ME/kg] and protein/energy ratio [g CP/MJ ME]

