Impaired In Vivo Mitochondrial Krebs Cycle Activity After Myocardial Infarction Assessed Using Hyperpolarized Magnetic Resonance Spectroscopy

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Background—Myocardial infarction (MI) is one of the leading causes of heart failure. An increasing body of evidence links alterations in cardiac metabolism and mitochondrial function with the progression of heart disease. The aim of this work was to, therefore, follow the in vivo myocardial metabolic alterations caused by MI, thereby allowing a greater understanding of the interplay between metabolic and functional abnormalities.

Methods and Results—Using hyperpolarized carbon-13 (13C)-magnetic resonance spectroscopy, in vivo alterations in mitochondrial metabolism were assessed for 22 weeks after surgically induced MI with reperfusion in female Wister rats. One week after MI, there were no detectable alterations in in vivo cardiac mitochondrial metabolism over the range of ejection fractions observed (from 28% to 84%). At 6 weeks after MI, in vivo mitochondrial Krebs cycle activity was impaired, with decreased 13C-label flux into citrate, glutamate, and acetylcarnitine, which correlated with the degree of cardiac dysfunction. These changes were independent of alterations in pyruvate dehydrogenase flux. By 22 weeks, alterations were also seen in pyruvate dehydrogenase flux, which decreased at lower ejection fractions. These results were confirmed using in vitro analysis of enzyme activities and metabolomic profiles of key intermediates.

Conclusions—The in vivo decrease in Krebs cycle activity in the 6-week post-MI heart may represent an early maladaptive phase in the metabolic alterations after MI in which reductions in Krebs cycle activity precede a reduction in pyruvate dehydrogenase flux. Changes in mitochondrial metabolism in heart disease are progressive and proportional to the degree of cardiac impairment. (Circ Cardiovasc Imaging. 2014;7:895-904.)

Key Words: citric acid cycle ▪ heart ▪ magnetic resonance spectroscopy ▪ metabolism ▪ myocardial infarction

Myocardial infarction (MI) is the leading cause of heart failure in the developed world and results in high levels of mortality and morbidity in patients.1 MI is caused by a partial or complete obstruction of a coronary artery, resulting in significant reductions in coronary blood flow and in ischemia.2 MI injury results in loss of heart tissue, causing the heart to remodel structurally and metabolically. However, this remodeled state is unsustainable, and the heart eventually fails, where the myocardial wall thins, the left ventricular (LV) cavity dilates, and cardiac output decreases.2 After a MI, the progression into heart failure can be characterized by profound changes in myocardial energy metabolism.13

Clinical Perspective on p 904

Fatty acid oxidation contributes 60% to 70% of the energy required for the resting heart, with glucose and lactate making up the majority of the remaining needs.1,2,4,5 The tightly regulated reciprocal relationship between glucose oxidation through pyruvate dehydrogenase (PDH) and fatty acid oxidation breaks down after an MI.5–7 This is accompanied by alterations in both Krebs cycle enzyme activities and electron chain complexes and contributes to a generalized reduction in the capacity for ATP production.8–12 In vitro experiments of MI have revealed that there are alterations in the electron transport chain as early as 2 weeks after MI,8 and that alterations in cardiac mitochondrial metabolism continue out to 6 months. However, the exact nature and timing of in vivo mitochondrial dysfunction after an MI are unclear, and information about this would provide a greater understanding of the development of metabolic abnormalities after an MI. The use of hyperpolarized 13C-magnetic resonance spectroscopy (MRS)13 allows the study of in vivo mitochondrial metabolism by measuring the flux of 13C-labeled

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895
pyruvate through PDH and into the Krebs cycle. Unlike positron emission tomography, which uses substrates such as 18Fluorodeoxyglucose as a marker of glucose uptake, or in vivo enzyme assays, hyperpolarized 13C-MRS provides a noninvasive, real-time measurement of metabolic flux. The technique allows animals to be scanned serially, offering the potential to track the temporal metabolic changes associated with MI and heart failure progression in vivo.

The aims of this study were to assess in vivo metabolic alterations in mitochondrial function that occur after an MI. Rats were scanned using both hyperpolarized [1-13C] and [2-13C] pyruvate at 1, 6, and 22 weeks after MI. Cardiac function was monitored at each time point using echocardiography. Ejection fraction (EF) was used to correlate in vivo metabolic data with the severity of cardiac functional impairment. Ex vivo metabolomics and biochemical analysis of enzyme activities were used to support the in vivo data at the 22-week time point.

Methods

Detailed Methods are presented in the Data Supplement. Fifteen female Wistar rats (200–250 g; 8–9 weeks; Harlan, United Kingdom) were housed on a 12:12-hour light/dark cycle in animal facilities at the University of Oxford. All animal studies were performed between 7 am and 1 pm with the animals in the fed state. All investigations conformed to Home Office Guidance on the Operations of the Animals (Scientific Procedures) Act 1986, to institutional guidelines and were approved by the University of Oxford Animal Ethics Review Committee.

MI Surgery

Surgical procedures were performed to ligate the left anterior descending coronary artery as previously described. Eleven anaesthetized female Wistar rats underwent 50 minutes of ischemia followed by reperfusion (2%–3% isoflurane in oxygen). Four sham-operated animals underwent the same procedure but without ligation of the left anterior descending.

Echocardiography

Animals were lightly anaesthetized with isoflurane (1.5% in 2 L/min oxygen) and 2-dimensional-echocardiography was performed as described. Measurements of end-diastolic and end-systolic areas and diameter were used to determine EFs (end-diastolic area = end-systolic area/end-diastolic area) at 1, 6, and 22 weeks after MI.

MRI Measurements of Cardiac Function

At 22 weeks after MI, cardiac structure and function were also assessed using CINE MRI as previously described. Briefly, anaesthetized animals (1.5%–2% isoflurane in 2 L/min oxygen) were positioned in an 11.7 T vertical bore MR scanner (Magnet Scientific, Oxon, United Kingdom) interfaced to a Bruker Avance console (Bruker Medical, Ettlingen, Germany) and a shielded gradient system. A 52-mm birdcage transmit/receive radiofrequency coil was used to obtain MRI (Rapid Biomedical, Rimpar, Germany). Sequences were ECG-triggered, and 24 to 31 frames were collected per cardiac cycle. For each heart, the LV mass, EF, stroke volume, scar size, and cardiac output were derived using the free-hand drawing function in ImageJ (National Institutes of Health). The infarct size was determined by measuring the akinetic region in the image.

Hyperpolarized 13C-MRS Protocol

At 1, 6, and 22 weeks after MI, animals were scanned with both [1-13]Cpyruvate and [2-13C]pyruvate (Sigma-Aldrich, Gillingham, United Kingdom), as previously described. Briefly, anaesthetized animals (1.5%–2% isoflurane in 2 L/min oxygen) were positioned in a 7T horizontal bore MR scanner (Varian Inc, Varnton, United Kingdom), and signal from the heart was localized using a custom-built 1H/13C butterfly coil. Correct positioning was confirmed with an axial proton fast low-angle shot image, and an ECG-gated shim was used to reduce the proton line width to ±120 Hz. One milliliter of either hyperpolarized [1-13C]- or [2-13C]pyruvate was injected >10 s through a tail vein cannula (dose of 0.32 mmol/kg). Sixty individual, ECG-gated, 13C-MR pulse-acquire cardiac spectra were acquired after injection (TR, 1 s; excitation flip angle, 5°; sweep width, 13,593 Hz; acquired points, 2048; frequency centered on the 13C pyruvate resonance, total acquisition time, 60 s). The [1-13C]pyruvate and [2-13C]pyruvate infusions were conducted ±1 hour apart in all animals, and the sequence of infusions was randomized.

MRS Data Analysis

Cardiac 13C-MRS spectra were analyzed as previously reported. Briefly, for [1-13C]pyruvate MRS data, the peak areas of metabolites at each time point were quantified using a kinetic model. The acquired [2-13C]pyruvate spectra were summed for the first 30 s after the appearance of [2-13C]pyruvate, to increase the sensitivity of measurements because of a low signal/noise ratio. The peak areas of metabolites were quantified in the summed spectra.

Tissue Collection

One day after the 22-week hyperpolarized scans, animals were anaesthetized using an overdose of isoflurane. The hearts were removed and placed in ice-cold PBS (Sigma-Aldrich). The hearts were immediately blotted, the scar excised and viable tissue weighed before being freeze-clamped, and stored at −80°C for subsequent biochemical analysis.

Tissue Homogenization and Enzyme Activity Assays

Tissue was homogenized using the following method for all assays unless stated otherwise. Frozen powered cardiac tissue (15 mg) was homogenized in 1 mL of ice-cold homogenization buffer using a polytron homogenizer (Kinematic, Luzern, Switzerland) for 30 s. To each sample, 10 μL of triton X-100 was added before being briefly vortexed and incubated on ice for 10 minutes. Samples were centrifuged (375g for 10 minutes at 4°C), and used for assays as described below.

The activity of citrate synthase (CS) was determined spectrophotometrically in a reaction that monitored the increase in absorbance at 412 nm because of the conversion of 5,5′-dithiobis(2-nitrobenzoic acid) to 2-nitro-5-thiobenzoate. The activity of carnitine acetyltransferase was determined spectrophotometrically in a coupled reaction monitoring the production of nicotinamide adenine dinucleotide using previously developed methods. The activity of isocitrate dehydrogenase and aconitase was determined spectrophotometrically.

The activity of PDH was measured using a spectrophotometric assay, as previously described. The cardiac tissue used for the PDH activity assay was homogenized in different buffers depending on whether the active or total activity was analyzed. The reaction monitored the increase in absorbance at 340 nm because of the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) to 2-nitro-5-thiobenzoate.

The following metabolites were assessed: glutamate, citrate, malate, and creatine. Data were expressed as relative to the control peak of TSP.

NMR Metabolomic Analysis

Metabolites were extracted using methanol–chloroform–water, and the aqueous extract was analyzed as previously described using both gas chromatography-mass spectrometry and 1H-NMR spectroscopy. The following metabolites were assessed: glutamate, citrate, malate, and creatine.

Acylcarnitine-Free Carnitine Assay

Cardiac tissue (50 mg) was pulverized and extracted using methanol/chloroform/water. Butylated extracts were injected (10 μL) into a Quattro Premire XE Triple Quadrupolar Mass Spectrometer (Waters Ltd, Elstree, United Kingdom) coupled with an electrospray ionization source in positive ion mode. The acylcarnitines were analyzed by multiple reaction monitoring in a positive ion mode. Samples were introduced by direct infusion. Data were processed using the Neolynx software package (Waters Ltd).
Statistics
All data underwent a Kolmogorov–Smirnov normality test with the Dallal–Wilkinson–Lillie for corrected P value. All results were expressed as correlations against the assessed EF, across the shams and infarcted animals. Data that were normally distributed underwent a Pearson linear regression test; all others underwent a Spearman Rank. Statistical significance was defined as $P \leq 0.05$. In addition, a group analysis (Data Supplement) was undertaken to compare the hearts with an EF>65% (normal function n=6) with those with an EF<50% (impaired function n=5). A 2-tailed Student t test was performed to assess statistical differences between the groups in the group analysis. All statistical analysis was undertaken using Prism 6 (GraphPad Software, San Diego, CA). All graphs show a trendline of linear regression with the 95% confidence levels indicated by the displayed bounds.

Results
Alterations in Cardiac Structure and Function After Myocardial Function
Echocardiography showed that 1 week after surgery, animals that had undergone MI surgery displayed a range of EFs (from 28% to 81%) when compared with sham animals (72%–84%). Cardiac function was also assessed by CINE MRI at 22 weeks after surgery to allow a more in-depth analysis of the structural and functional remodeling in these animals. EFs measured after MI using CINE MRI and echocardiography at 22 weeks were tightly correlated and validated the use of echo at each time point (Figure IA in the Data Supplement). The use of echo was necessary to reduce the length of time the animals were under anesthesia because echo provides a quick, but reliable measurement of cardiac function. EF at 1 and 22 weeks significantly correlated, indicating that cardiac function did not alter over the time of the experiment (Figure IB in the Data Supplement). Scar size, measured as the thinned and akinetic regions of the myocardium, negatively correlated with EF (determined using CINE MRI), ranging from 4% to 37% of the LV (Figure 1). End-diastolic volume also negatively correlated with EF, whereas cardiac output and stroke volume correlated positively with EF. The dilation of the LV and decrease in cardiac output were consistent with functional abnormalities associated with the progression into heart failure. Average viable mass was not significantly different over the range of EFs, consistent with compensated hypertrophy of the remote myocardium (Figure II in the Data Supplement).

Altered In Vivo Metabolism in the Infarcted Heart
Hyperpolarized $^{13}$C-MRS was used to measure the real-time conversion of [1-$^{13}$C]pyruvate into both [$^{13}$C]bicarbonate and
13CO₂, which directly assesses flux through PDH. At 1 and 6 weeks after induction of MI, there was no significant correlation between EF and PDH flux (Figure 2), despite several animals with severely reduced EFs. This result was supported by the group analysis, which revealed no significant difference between hearts with normal and impaired function (Figure III in the Data Supplement). This would imply that the contribution to energy generation from pyruvate was unaltered ≤6 weeks after infarction, despite observed functional deficits early after MI. However, by 22 weeks after surgery, a significant positive correlation between EF and PDH flux was observed (Figure 2; Figure III in the Data Supplement). Therefore by 22 weeks, the use of pyruvate for acetyl-CoA generation, and subsequent acetyl-CoA processing via the Krebs cycle, was reduced in infarcted hearts proportional to the degree of functional impairment. Incorporation of the hyperpolarized 13C label into [1-13C]lactate and [1-13C]alanine was also evaluated in these experiments. No correlation between EF and 13C-label incorporation from [1-13C]pyruvate into [1-13C]lactate was observed at any time point. At 1 and 6 weeks, 13C-label incorporation into [1-13C]alanine did not correlate with EF, interestingly although at 6 weeks, there was a significant positive correlation with EF. It is possible that these data indicate limited nonoxidative fates of pyruvate after MI surgery (Figure IV in the Data Supplement).

### Altered In Vivo Activity of the Krebs Cycle

To evaluate the effects of MI on Krebs cycle metabolism, the conversion of [2-13C]pyruvate into several downstream mitochondrial products, namely [5-13C]glutamate, [1-13C]citrate, and [1-13C]acetyl carnitine, was also evaluated. Figure 3 shows a representative [2-13C]pyruvate spectra from the 22-week time point of both a sham and an infarcted heart. 13C-label incorporation into glutamate, citrate, and acetyl carnitine showed no significant correlation with EF at 1 week after surgery (Figure 4). However, at 6 weeks after MI, incorporation of the 13C-label into the Krebs cycle (citrate) was reduced in proportion to contractile dysfunction, independent of any changes in PDH flux. This was also accompanied by a positive correlation between EF and 13C-label flux into acetyl carnitine and glutamate. These findings were supported by the group analysis, which showed significant reductions in the levels of 13C incorporation into citrate, glutamate, and acetyl carnitine in the hearts with impaired function (Figure V in the Data Supplement). Such results may reflect several different perturbations in these hearts, namely a reduction in the activity of the enzymes required to synthesize these products, or reductions in the pool sizes of these metabolites, or a combination of both. These changes in the Krebs cycle remained at 22 weeks after MI but were also accompanied by alterations in PDH flux. Therefore, the observed differences at this later time point were accompanied by decreased amounts of 13C-label reaching the Krebs cycle because of decreased PDH flux.

### Altered In Vivo Metabolite Signals Over the Duration of the Study

A 2-way repeated measures ANOVA of the data revealed a general trend (statistically significant for all metabolites except lactate, data not shown) for 13C-label incorporation to increase during the 22-week time frame of the study.
This effect was caused by the ratiometric nature of the kinetic analysis that explored the relative proportion of metabolite generated from the injected $^{13}$C-labeled pyruvate. As the amount of pyruvate injected was held constant across the time course of the study and the amount of viable myocardium in the sensitive region of the radiofrequency coil increased as the animals grew, an increase in the relative metabolite levels was observed.

**Altered Metabolite Pool Sizes in the Infarcted Heart**

In vitro $^1$H-NMR spectroscopy was used to determine whether the decreased $^{13}$C-label incorporation into the Krebs cycle was linked with alterations in the metabolite pool sizes at 22 weeks. The relative pool size of citrate did not significantly correlate with EF, thereby demonstrating that any reduction in the production of citrate must have also been coupled with a reduced use. In contrast to this, a positive correlation was observed between the pool sizes of both glutamate and malate, and EF, indicating depletion of Krebs cycle intermediates (Figure 5; Figure VI in the Data Supplement). Consistent with other studies of contractile dysfunction, a positive correlation was seen between creatine and EF. Decreased creatine highlights an alteration in energy handling within the infarcted heart.

**Alterations in Carnitine Availability in the Infarcted Heart**

Carnitine is the carrier for mitochondrial membrane transport of fatty acyl-CoA and acetyl-CoA and exists as a finite pool within the heart. Direct infusion mass spectrometry was used to measure carnitine levels within the heart. Changes in carnitine availability could lead to alterations in acetylcarnitine production and, therefore, $^{13}$C-label incorporation into $[1^{-13}C]$...
acetylcarnitine. There was a significant negative correlation between the ratio of acylcarnitine:free carnitine and cardiac function (Figure 5; Figure VII in the Data Supplement). Furthermore, the ratio of free carnitine:total carnitine positively correlated with cardiac function. Taken together, these findings suggest that there was a reduction in the relative availability of free carnitine in the infarcted hearts, which may make some contribution to the reduction in [1-13C]acetylcarnitine.

MI Affected the Activity of Krebs Cycle Enzymes

Finally, to understand the observed alterations in in vivo flux through PDH and in the Krebs cycle, changes in key mitochondrial enzyme activities were assessed. Consistent with decreased flux into bicarbonate and CO2, PDH activity positively correlated with EF at 22 weeks (Figure 6; Figure VIII in the Data Supplement).

The positive correlation of 13C-label flux into acetylcarnitine and EF seems to have been driven by multiple factors. Along with altered carnitine availability, the activity of carnitine acetyl-transferase positively correlated with cardiac function, indicating that decreased 13C-label incorporation might be a result of both decreased enzyme activity and decreased free carnitine. Decreased CS activity was also seen to correlate with cardiac function, demonstrating that altered 13C-label flux into citrate was because of changes in CS activity and not increased usage of citrate. Interestingly, aconitase activity did not significantly correlate with EF, suggesting that this enzyme reaction was not a rate-determining step, whereas isocitrate dehydrogenase (ICDH) activity was found to correlate with EF positively. Changes to both CS and ICDH activity mean that flux into glutamate, whether derived from the [2-13C]pyruvate or endogenous substrates, will have been reduced. This would explain the observed reductions in both 13C-label incorporation and glutamate pool size, which were proportional to cardiac function.

Discussion

An increasing body of evidence links alterations in cardiac metabolism with the progression of heart disease.1 In this study, using hyperpolarized 13C-MRS, an in vivo assessment of mitochondrial metabolism after MI was performed. At 6 weeks after MI, activity in the Krebs cycle positively correlated with cardiac function, indicative of early in vivo mitochondrial defects. Early metabolic alterations in the heart after MI occurred independently of changes in PDH flux, which were not observed until 22 weeks after MI.

Reduced Capacity to Oxidize Acetyl-CoA After MI

One week after MI, there were no detectable alterations in cardiac mitochondrial metabolism over the range of EFs observed. This was an early adaptive phase after infarction, where scar formation and remodeling of the heart were occurring.30–32 Six weeks after an MI, impairment in in vivo mitochondrial Krebs cycle activity was seen to correlate with the degree of cardiac dysfunction. Although the observation of mitochondrial Krebs cycle alteration after an MI is not novel, the fact that these were detected in vivo provides an interesting opportunity to monitor the effects of modified cardiac function on cardiac metabolism. Interestingly, these changes in Krebs cycle activity were independent of alterations in PDH. Thus, in vivo alterations in Krebs cycle activity may define an early maladaptive phase in metabolic derangement that is associated with the progression into heart failure. Reductions in 13C-label incorporation into citrate and acetylcarnitine, which correlated with cardiac function, have highlighted a reduced capacity of the infarcted heart to oxidize acetyl-CoA, despite a normal ability to decarboxylate pyruvate. Uncoupling means acetyl-CoA is produced at the same rate by PDH, but there is a reduction in its oxidation because of a slowdown in Krebs cycle activity. Without information of β-oxidation,
it is unclear whether changes in fatty acid usage lead to less acetyl-CoA from fat, thereby preventing the normal feedback inhibition of PDH. Although altered 13C-label incorporation into glutamate may have been a result of reduced label flux through CS, the reduced 13C-label incorporation into citrate shows that in vivo the initial reactions of the Krebs cycle were defective at this early time point.

The animals were subsequently studied at 22 weeks after MI. CINE MRI analysis showed that, consistent with a 22-week chronically infarcted heart, EF positively correlated with scar size, indicating that LV remodeling was related to the degree of myocardial damage. Measurements of the viable myocardium (both in vitro and in vivo using MRI) indicated no relationship between viable myocardium and EF at 22 weeks (Figure II in the Data Supplement), meaning that any decreases in in vivo 13C-label incorporation were not because of reductions in the mass of viable tissue in the infarcted hearts. At 22 weeks after MI, there was a general breakdown in in vivo mitochondrial function. Although EF remained relatively stable during the course of the study, the deterioration in in vivo mitochondrial function was progressive during the 22-week time frame. Along with the defects in Krebs cycle activity described at 6 weeks after MI, flux through PDH also correlated with cardiac function at the 22-week time point. These alterations highlight defects in pyruvate oxidation at 22 weeks after MI. Our in vivo data support and advance the link between cardiac and mitochondrial function, which has previously been reported in vitro.

13C-label incorporation into citrate demonstrates the flux of pyruvate-derived acetyl-CoA into the Krebs cycle and provides a measure of Krebs cycle activity. Decreased 13C-label incorporation into citrate at 22 weeks was because of multiple factors, including decreased CS activity and reduced acetyl-CoA production (by PDH). In vitro analysis of key metabolic enzymes reveals reduced activity in the failing heart: consistent with in vivo data, pyruvate dehydrogenase, citrate synthase, and carnitine acetyl-transferase activity positively correlated with ejection fraction. Aconitase activity was unaltered with altered ejection fraction, possibly because this enzyme is not a rate-determining step. However, isocitrate dehydrogenase did show reduced activity with reduced cardiac function. Highlighting abnormalities in key enzymes involved in pyruvate oxidation and Krebs cycle flux with reducing ejection fraction.

Figure 6. In vitro analysis of key metabolic enzymes reveals reduced activity in the failing heart: consistent with in vivo data, pyruvate dehydrogenase, citrate synthase, and carnitine acetyl-transferase activity positively correlated with ejection fraction. Aconitase activity was unaltered with altered ejection fraction, possibly because this enzyme is not a rate-determining step. However, isocitrate dehydrogenase did show reduced activity with reduced cardiac function. Highlighting abnormalities in key enzymes involved in pyruvate oxidation and Krebs cycle flux with reducing ejection fraction.

13C-label incorporation into citrate was recently highlighted as an important factor in understanding the development of heart failure.

Interestingly, 13C-label incorporation into citrate did not relate to citrate pool size in this study because the pool size of citrate did not alter during the range of EFs observed. The maintenance of a normal citrate pool size was because of the decreased activity of ICDH, which along with CS, showed a positive correlation with EF.

Label incorporation into glutamate provided information on the activity of the initial reactions of the Krebs cycle. At both 6 and 22 weeks, positive correlations between cardiac function and 13C-label incorporation into glutamate were observed, which were accompanied by a decreased glutamate pool size. The alteration in ICDH activity suggests a decrease in the production of α-ketoglutarate, which may explain both the decreased pool size and the reduced 13C-label incorporation into glutamate. However, it does not preclude a possible contribution from increased α-ketoglutarate efflux in the reduction of 13C incorporation into glutamate. The reduction in myocardial glutamate levels, in addition to reduced malate levels, may also indicate alterations in the malate–aspartate shuttle. In an environment where there is a reduced activity of both CS and ICDH, it is also possible that an increased mitochondrial uptake of glutamate may maintain α-ketoglutarate levels and keep the second span of the Krebs cycle flowing.
At both 6 and 22 weeks, there was a reduced capacity for acetylcarnitine formation, which normally acts as a buffering system for excess acetyl-CoA production, similar to triacylglycerol in β-oxidation.\textsuperscript{14} Reductions in \textsuperscript{13}C-label flux into acetylcarnitine may be a indicative of a reduction in available free carnitine and reduced acetylcarnitine acetyl-transferase enzyme activity.\textsuperscript{43,44} Previous data suggest that carnitine becomes trapped in the acylcarnitine form because of reductions in β-oxidation, therefore, preventing free carnitine from being available to buffer excess acetyl-CoA.\textsuperscript{45–47} We have presented evidence here that this could be the case with a possible reduction in free carnitine in the viable myocardium of low EF animals at 22 weeks. Coupled with this, measurement of the activity of carnitine acetyl-transferase revealed that although reductions in free carnitine may play some role in the reduced acetylcarnitine flux, the enzyme must also carry a defect, which reduces the activity. The breakdown of this auxiliary pathway could explain the reason for carnitine trapping, because of a reduction in free CoA, which is itself trapped in acetyl-CoA. This complicated cycle of trapping of CoA and carnitine eventually leads to reductions in PDH and Krebs cycle activity, and possibly β-oxidation, although this was not measured here.\textsuperscript{48}

**Limitations**

As with any study, the limitations of the technique used need to be taken into consideration when interpreting the results. With the use of hyperpolarized pyruvate, there is a requirement for injection of supraphysiological concentrations of the tracer; however, the physiological effect of the pyruvate injection has previously been shown to be minimal.\textsuperscript{16} In this study, sequential injections of hyperpolarized pyruvate (labeled at either the C1 or C2 positions) were given with an interval of 1 hour between injections. Although the order of the injections was randomized to ensure that there was no bias in the acquired data, there is the possibility that any long-term physiological effects of the multiple injections may have contributed to the level of variation observed in the acquired data.

Another limitation is that pyruvate only probes one side of the metabolic process, namely carbohydrate metabolism, and is unable to report directly on fatty acid oxidation. Recent advances in the field of hyperpolarized \textsuperscript{13}C-MRS have allowed the use of butyrate as a metabolic probe for short-chain fatty acid metabolism. The use of this compound may provide more information on fatty acid metabolism in the infarcted heart.\textsuperscript{49}

Although in previous studies using hyperpolarized pyruvate it has been possible to observe increases in the level of anaplerosis through the production of [3,\textsuperscript{13}C]citrate, it was not possible to quantify this peak in this study reliably.\textsuperscript{52} This does not, however, rule out an increased reliance on anaplerosis in the infarcted heart, which has been observed previously in the diseased rat heart.\textsuperscript{50}

Finally, the use of imaging of the metabolism of the infarcted heart would significantly strengthen this study. Although technical developments have been made to enable the imaging of hyperpolarized [1,\textsuperscript{13}C]pyruvate in the isolated rat heart, the ability to perform these studies has not yet been routinely translated into the in vivo rat heart.\textsuperscript{51}

**Conclusions**

In this study, the ability of hyperpolarized \textsuperscript{13}C-MRS to measure in vivo alterations in cellular metabolism at multiple time points has offered new insights into the pathogenesis of heart failure after MI. The technique has shown that there were early maladaptive alterations to in vivo mitochondrial function at 6 weeks after MI, where Krebs cycle activity was reduced, whereas flux through PDH remained normal, possibly indicating an uncoupling of pyruvate oxidation and Krebs cycle activity. At 22 weeks, there were significant decreases in both PDH flux and label incorporation into the Krebs cycle, proportional to the decreased cardiac function. This may imply that alterations in the myocardial energy demand associated with diminished cardiac function lead to progressive alterations in Krebs cycle and PDH activity.

With the completion of the first human trials of hyperpolarized \textsuperscript{13}C-MRS in cancer human applications for the study of in vivo cardiovascular metabolism is on the horizon.\textsuperscript{53} The results from this study provide an interesting insight into the development of in vivo metabolic defects in the in vivo rat heart. With the continued development of this technology,\textsuperscript{53} it is hoped that hyperpolarized \textsuperscript{13}C-MRS will provide a useful diagnostic and prognostic tool in the study of the metabolic alterations in the ischemic and failing human heart.

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**Disclosures**

None.

**References**


37. Dodd et al. Myocardial Infarction Impairs Krebs Cycle Activity 903


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**CLINICAL PERSPECTIVE**

Hyperpolarized ¹³C magnetic resonance spectroscopy provides an unprecedented sensitivity with which to study cardiac metabolism in real-time and in vivo. This article uses a rodent model of myocardial infarction to explore the links between functional and structural alterations in the heart after myocardial infarction and the resulting changes in cardiac metabolism. Six weeks after the induction of myocardial infarction, significant decreases in in vivo Krebs cycle flux were observed, which preceded alterations to pyruvate dehydrogenase flux, which were not observed until 22 weeks. These alterations may serve as valuable diagnostic markers for the decrease of glucose oxidation associated with the development into heart failure.
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**Supplementary Figure 1 – Comparison of Ejection fraction**

A significant correlation was detected between the ejection fraction measured using cine-MRI and echo. B) A comparison between the ejection fraction at week 1 and week 22. A significant correlation was seen between the two different time points.
Supplementary Figure 2 – The viable mass of the heart does not correlate with ejection fraction – A) Following sacrifice and tissue collection, the infarct was excised and the rest of the heart was weighed and compared to the in vivo ejection fraction. B) The left ventricular mass, minus the scar was assessed in vivo by MRI at 22 weeks and compared with the ejection fraction. Both measurements showed that there was no change in either heart mass or LV mass when compared to ejection fraction.
Supplementary Figure 3 – Group analysis of PDH flux – No change is seen in PDH flux until the 22 week time point, where there is a significant reduction in the impaired function group. **p < 0.01. Data is mean with ± SEM.
Supplementary Figure 4 – *In vivo* $^{13}$C label incorporation into lactate and alanine was measured at each time point post-MI – No significant correlation was detected over the range of ejection fractions for lactate but a significant correlation was observed between alanine and ejection fraction at the 6 week time point. (Alanine – 6 weeks and Lactate – 22 weeks were analysed using Spearman Rank Correlation).
Supplementary Figure 5 – Group analysis of *in vivo* $^{13}$C label incorporation into citrate, glutamate and acetylcarnitine was measured at each time point post-MI – By 6 weeks post MI, there was a significant reduction in label incorporation into citrate, glutamate and acetylcarnitine in the impaired function group. This difference was maintained at 22 weeks. * p < 0.05 and *** p < 0.001. Data is mean with ± SEM.
Supplementary Figure 6 – Group analysis of in vitro analysis of metabolite pool sizes at 22 weeks – No difference in citrate pool size was seen between normal and impaired function animals. Glutamate, malate and creatine were all reduced in the impaired hearts. ** p < 0.01. Data is mean with ± SEM.
Supplementary Figure 7 – Group analysis of *in vitro* carnitine availability at 22 weeks – A significant increase in the acylcarnitine to free carnitine ratio is seen in the impaired function group. Coupled with this, there is a significant reduction in the free carnitine to total carnitine ratio. **p < 0.01. Data is mean with ± SEM.
Supplementary Figure 8 – Group analysis of *in vitro* analysis of enzyme activity at 22 weeks – A significant decrease in enzyme activity of PDH, citrate synthase, carnitine acetyl-transferase and isocitrate dehydrogenase is seen in the impaired function group. No difference is seen between groups for the activity of aconitase. * p < 0.05 ** p < 0.01. Data is mean with ± SEM.
**Detailed Methods**

**Myocardial Infarction:**

The left anterior descending (LAD) coronary artery of female Wistar rats (~200g, n =11) was occluded approximately 2 mm from its origin. Rats were anaesthetized with 2.5 % isoflurane in O₂, intubated, and maintained at 80-90 breaths per minute with a tidal volume of 2-3 ml. Thoracotomy was performed in the 4th intercostal space, the pericardium was removed and a 5-0 prolene suture was placed under the LAD. The suture was tied around a small piece of PE tubing, occluding the LAD and the chest closed. Animals were subjected to 3,000 seconds (sec) of coronary artery occlusion, the chest was re-opened and the tubing removed to allow reperfusion. Sham-operated animals underwent the same procedure but without coronary ligation (n = 4).

**Echocardiography**

Animals were lightly anaesthetized with isoflurane and 2D-echocardiography was performed as described. LV short axis images were acquired at the mid-papillary level using a Philips SONOS 5500 system with a 12MHz transducer giving one image every 8.3ms. Three separate acquisitions were made at approximately the same mid-papillary level, and endocardial borders and dimensions were measured, excluding papillary muscles, at end systole and end diastole from three consecutive heart cycles. Images acquired during inspiration were excluded. Ejection fraction (EF = End systolic area/End diastolic area) were calculated from this mid-papillary slice.

**MRI measurements of cardiac function**

At week 22, post hyperpolarized MRS analysis, cardiac function was assessed using MRI as described previously. The animals remained anesthetized with 1.5% to 2.5% isoflurane in O₂ and positioned supine in a purpose-built, temperature- regulated cradle. ECG electrodes were inserted into the
forepaws and a respiration loop was taped across the chest. The cradle was lowered into a vertical-bore 500 MHz, 11.7 T MR system with a Bruker console and a 52-mm birdcage RF coils (Rapid Biomedical, Würzburg, Germany). ECG and respiration trigger levels were adjusted so that acquisitions were triggered at the same point in the cardiac cycle. Long and short-axis scout images were acquired so that true short-axis images could be planned using a segmented, ECG-triggered fast low-angle shot (FLASH) sequence. The RF coil was then tuned and matched, followed by slice selective shimming. Cine-MR images, consisting of 28–35 frames per heart cycle, were acquired in seven to eight contiguous slices in the short-axis orientation covering the entire heart. The imaging parameters were as follows: field of view (FOV) = 51.2 × 51.2 mm, matrix size = 256 × 256, slice thickness = 1.5 mm giving a voxel size 0.015 mm3, echo time (TE)/repetition time (TR) = 1.43/ 4.6 ms, 0.5 ms/17.5° Gaussian RF excitation pulse, and four averages. The total experimental time, including animal preparation, was approximately 50 min per animal. Heart rate remained stable throughout the procedure. End-diastolic (ED) and end-systolic (ES) frames were selected as those with the largest and smallest cavity volumes, respectively. Epicardial and endocardial borders were outlined using the free-hand drawing function of ImageJ (National Institutes of Health, USA). Measurements from all slices were summed to calculate ED volume (EDV), (SV = EDV - ES volume), ejection fraction (EF = SV/EDV) and cardiac output (CO = SV × heart rate).

**Hyperpolarized 13C MR protocol**

Animals received either a [1-13C]- or [2-13C]pyruvate scan, 1 hour apart with the order randomised (Sigma-Aldrich, UK). A home-built 1H/13C butterfly coil (loop diameter, 2 cm) was placed over the rat chest, localizing signal from the heart. Rats were positioned in a 7 T horizontal bore MR scanner interfaced to an Inova console (Varian Medical Systems). Correct positioning was confirmed by the acquisition of an axial proton FLASH image (TE/TR, 1.17/2.33 ms; matrix size, 64 x 64; FOV, 60 x 60 mm; slice thickness, 2.5 mm; excitation flip angle, 15°). An ECG-gated shim was used to reduce the proton linewidth to ~120 Hz. One millilitre of hyperpolarized pyruvate was injected over 10 s into
the anesthetised rat. Sixty individual ECG-gated $^{13}$C MR pulse-acquire cardiac spectra were acquired over 60 sec after injection (TR, 1 s; excitation flip angle, 5°; sweep width, 13,593 Hz; acquired points, 2,048; frequency centred on the C1 pyruvate resonance).

**MR data analysis**

All cardiac $^{13}$C spectra were analysed using the AMARES algorithm in the jMRUI software package (Naressi 2001). Spectra were DC offset-corrected based on the last half of acquired points. The peak areas of in vivo $[1-^{13}\text{C}]$pyruvate, $[1-^{13}\text{C}]$lactate, $[1-^{13}\text{C}]$alanine, $[^{13}\text{C}]$carbon dioxide and $[^{13}\text{C}]$bicarbonate (for $[1-^{13}\text{C}]$pyruvate), $[1-^{13}\text{C}]$acetyl carnitine, $[1-^{13}\text{C}]$citrate and $[5-^{13}\text{C}]$glutamate (for $[2-^{13}\text{C}]$pyruvate) at each time point were quantified and used as input data for a kinetic model. The kinetic model developed for the analysis of hyperpolarized $[1-^{13}\text{C}]$ and $[2-^{13}\text{C}]$pyruvate MRS data is based on a model initially developed by Zierhut et al. and further developed by Atherton et al. Firstly the change in labeled pyruvate signal over the 60 s acquisition time was fit to the integrated labeled pyruvate peak area data using equation [1]:

$$M_{\text{pyr}}(t) = \begin{cases} 
\frac{\text{rate}_{\text{inj}}}{k_{\text{pyr}}} \left(1 - e^{-k_{\text{pyr}}(t-t_{\text{arrival}})}\right) & t_{\text{arrival}} \leq t < t_{\text{end}} \\
M_{\text{pyr}}(t_{\text{end}}) e^{-k_{\text{pyr}}(t-t_{\text{end}})} & t \geq t_{\text{end}}
\end{cases}$$

In this equation, $M_{\text{pyr}}(t)$ represents the $[1-^{13}\text{C}]$ or $[2-^{13}\text{C}]$pyruvate peak area as a function of time. This equation fits the parameters $k_{\text{pyr}}$, the rate constant for pyruvate signal decay ($\text{s}^{-1}$), $\text{rate}_{\text{inj}}$, the pyruvate arrival rate (a.u. $\text{s}^{-1}$), $t_{\text{arrival}}$, the pyruvate arrival time (s) and $t_{\text{end}}$, the time correlating with the end of the injection (s). These parameters were used in equation [2] along with the dynamic metabolite data to calculate $k_{\text{pyr-\text{X}}}$, the rate of $^{13}$C label incorporation into each metabolite pool from pyruvate ($\text{s}^{-1}$) and
$k_x$, the rate constant for signal decay of each metabolite (s$^{-1}$) which was assumed to consist of metabolite T$_1$ decay and signal loss from the 5° RF flip angle pulses. Where t’ is t - t$_{delay}$, and accounts for delay in the circulation of hyperpolarized pyruvate through the cardiac micro-vascular.

$$M_x(t') = \begin{cases} 
\frac{k_{pyr-x}t_{delay}}{k_{pyr}-k_x} \left(1-e^{-k_x(t'-t'_{arrival})} \right) & t'_{arrival} \leq t'<t'_{end} \\
\frac{M_{pyr}(t'_{end})}{k_{pyr}-k_x} \left(e^{-k_x(t'-t'_{end})} - e^{-k_{pyr}(t'-t'_{end})} \right) + M_x(t'_{end})e^{-k_x(t'-t'_{end})} & t' \geq t'_{end}
\end{cases}$$

**Enzyme Activity analysis**

**Citrate synthase activity assay**

The activity of citrate dehydrogenase was determined spectrophotometrically in a reaction monitoring the increase in absorbance at 412 nm due to the conversion of 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) to 2-nitro-5-thiobenzoate (TNB). To a plastic cuvette, add assay buffer [100 µl; 1 M tris HCl, 1 mmol/L DTNB, neutralized to pH 8.1 with sodium hydroxide and made up to 100 ml in double distilled water], acetyl-CoA [50 µl; Add 1 ml of ice cold dd H$_2$O to 10 mg Coenzyme A hydrate, then add 100 µl 1 M potassium bicarbonate and 2 µl acetic anhydride, mix and incubate on ice for 600 sec. Add a further 2 µl acetic anhydride, mix and incubate for another 600 sec. Dilute with 4.416 ml of dd H$_2$O], 750 µl dd H$_2$O and 50 µl of homogenised sample. The cuvette was incubated in a heat block at 30 °C for 300 sec and used to zero the UV spectrophotometer at 340 nm. 50 µl of 10 mmol/L oxaloacetate was added, mixed and the increase in absorbance recorded at 340 nm and 30 °C for 240 seconds (using an extinction coefficient for NADH of 6270 mmol/L.cm$^{-1}$), using UV probe chart reader software (Shimadzu) and a Shimadzu UV-1700 spectrophotometer. Each supernatant was run in duplicate and the average change in absorbance was used to calculate the activity in (nmol/ min/mg).
**Carnitine acetylcarnitine activity**

The activity of carnitine acetyltransferase was determined spectrophotometrically by the methods of Marquis and Fritz (1965), Pearson *et al* (1974) and Grizard *et al* (1992), in a coupled reaction monitoring the production of NADH.\(^6\)–\(^8\) 10 µl of 30 µmol/L O-Acetyl-L-carnitine hydrochloride (Sigma-Aldrich, UK) was added to assay buffer [840 µl; 100 mmol/L tris base, 10 mmol/L L-malic acid sodium salt, 1.25 mmol/L ethylene glycol tetra-acetic acid (EDTA) dipotassium salt (first three reagents were added to approximately 40 ml of double distilled water and neutralized to pH 7.4 with hydrochloric acid), 0.5 mmol/L \(\beta\)-nicotinamide adenine dinucleotide hydrate (NAD) and 0.125 mmol/L Coenzyme A hydrate, 4 µM rotenone, 35 U citrate synthase (porcine heart) and 275 U malate dehydrogenase (pigeon heart), made up to 50 ml in double distilled water] and incubated for 600 sec at 37 \(^\circ\)C. 150 µl of homogenised sample was added to the assay buffer/acetylcarnitine and incubated for a further 30 seconds at 37 \(^\circ\)C. Absorbance was then recorded at 340 nm and 37 \(^\circ\)C for 100 seconds (using an extinction coefficient for NADH of 6270 mmol/L.cm), using UV probe chart reader software (Shimadzu) and a Shimadzu UV-1700 spectrophotometer. Each supernatant was run in duplicate and the average change in absorbance was used to calculate the activity in (nmol/min/mg).

**Aconitase activity**

The activity of aconitase was determined spectrophotometrically in a reaction monitoring the decrease in absorbance at 240 nm due to the conversion of *cis*-aconitate to isocitric acid.\(^5\) Using quartz cuvettes, 890 µl of 50 mmol/L tris hydrochloride (neutralized to pH 7.5 using sodium hydroxide) was used to zero the UV spectrophotometer at 240 nm. The addition of 10 µl *cis*-aconitate, results in a small increase in absorbance for 60 sec, before stabilizing. 100 µl of homogenised sample was added, mixed and decrease in absorbance recorded at 240 nm and 37 \(^\circ\)C for 100 seconds (using an extinction coefficient for *cis*-aconitate of 3600 mmol/L.cm), using UV probe chart reader software (Shimadzu) and a Shimadzu UV-1700 spectrophotometer. Each supernatant was run in duplicate and the average change in absorbance was used to calculate the activity in (nmol/min/mg).
Isocitrate dehydrogenase activity assay

The activity of isocitrate dehydrogenase was determined spectrophotometrically in a reaction monitoring the increase in absorbance at 340 nm due to the conversion of NAD to NADH. Using plastic cuvettes, 900 µl of assay buffer was used to zero the UV spectrophotometer at 340 nm [Assay buffer: phosphate buffer [33.3 mmol/L dipotassium phosphate, 22.2 mmol/L potassium phosphate were added to approximately 30 ml of double distilled water and neutralized to pH 7 with potassium hydroxide], 2.8 mmol/L isocitrate, 20 mmol/L citrate, 19.7 mmol/L magnesium chloride, 5 mmol/L adenosine diphosphate, 2.2 mmol/L β-nicotinamide adenine dinucleotide hydrate, 0.05 % bovine serum albumin and 0.18 % triton X-100], and made up to 50 ml in double distilled water]. The assay buffer was incubated in a heat block at 30 °C for 120 sec. 100 µl of sample was added, mixed and the increase in absorbance recorded at 340 nm and 37 °C for 60 seconds (using an extinction coefficient for NADH of 6270 mmol/L.cm⁻¹), using UV probe chart reader software (Shimadzu) and a Shimadzu UV-1700 spectrophotometer. Each supernatant was run in duplicate and the average change in absorbance was used to calculate the activity in (nmol/ min/ mg).

PDH Activity

The activity of the active and total fractions of PDH (PDHa and PDHt) were determined spectrophotometrically by the method of Seymour and Chatham. The PDH assay required the preparation of cardiac tissue with one of two homogenisation buffers for either PDHa or PDHt measurement. PDHa was assessed when PDH was extracted under conditions in which both PDP and PDK were inhibited [25 mmol/L N-2-hydroxyethylpiperazine-N’-2- ethanesulfonic acid (HEPES), 25 mmol/L KH₂PO₄, 25 mmol/L KF, 1 mmol/L DCA, 3 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L adenosine diphosphate (ADP), 1 mmol/L dithiothreitol, 0.05 mmol/L leupeptin, 1% Triton X-100; pH 7.2]. PDHt was assessed under conditions in which PDP was stimulated by Mg²⁺ and PDK was inhibited by DCA and ADP (75 mmol/L HEPES, 5 mmol/L DCA, 5 mmol/L MgCl₂, 1 mmol/L ADP, 1 mmol/L dithiothreitol, 0.05 mmol/L leupeptin, 1% Triton X-100; pH 7.2). Frozen cardiac
tissue was powdered and 0.2 g was homogenised in 1 mL of the appropriate homogenisation buffer using a polytron (30 s). The sample was snap frozen in liquid nitrogen, thawed and rehomogenised three times. The sample was centrifuged (1000 x g, 420 sec) and the supernatant was removed for analysis. Assay buffer [950 mL; 50 mmol/L HEPES, 1 mmol/L MgCl₂, 0.08 mmol/L ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid, 1 mmol/L dithiothreitol, 4 mmol/L rotenone, 1.7 mmol/L nicotinamide adenine dinucleotide (NAD), 0.1 mmol/L coenzyme A, 0.2 mmol/L thiamine pyrophosphate HCl, 16.7 mmol/L lactate; pH 7.2] was incubated with 2 mL lactate dehydrogenase (LDH) at 30 °C for 300 sec. PDH activity was determined by adding and mixing a 25 mL aliquot of either PDHa or PDHt extract with the assay buffer and immediately following the reaction at 340 nm using the kinetic program on a spectrophotometer (120 sec for both PDHa and PDHt samples). The rate of NADH production over the first 30 sec was used to determine the activity in units of mmol/ min/ g wet weight.

**NMR metabolomic analysis**

Metabolites were extracted from heart tissue using methanol/chloroform/water. Frozen tissue (~100 mg) was placed in methanol-chloroform (2:1, 600 µl) and homogenised. Samples were then sonicated for 5 min before chloroform-water (1:1) was added (200 µl of each). Samples were centrifuged (13,500 rpm, 20 min), and the aqueous layer was pipetted off and dried overnight in an evacuated centrifuge (Eppendorf, Hamburg, Germany).

The dried extracts were rehydrated in 600 µl of D₂O and buffered in 0.24 M sodium phosphate (pH 7.0) containing 1 mM sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP; Cambridge Isotope Laboratories, Andover, MA) as an internal standard. The samples were analysed using an Avance II+ spectrometer operating at 500 MHz for the ¹H frequency (Bruker) equipped with a 5 mm Broadband TXI Automatic Tuning and Matching (ATMA) probe. Spectra were collected using a solvent suppression pulse sequence based on a 1D- nuclear Overhauser effect spectroscopy pulse sequence to saturate the residual [¹H] water proton signal (relaxation delay = 2 s, τ₁ = 3 µs, mixing time
= 150 ms, solvent pre-saturation applied during the relaxation time and the mixing time). One hundred twenty-eight transients were collected into 16 K data points over a spectral width of 12 ppm at 37 °C. NMR spectra were processed using an ACD SpecManager 1D NMR processor (version 8; ACD, Toronto, Canada). Spectra were Fourier transformed after multiplication by a line broadening of 1 Hz and referenced to TSP at 0.0 ppm. Spectra were phased and baseline corrected manually. Each spectrum was integrated using 0.04 ppm integral regions between 0.5–4.5 and 4.7–9.5 ppm. Each 0.04 ppm region was treated as an independent variable during analysis. To account for any difference in concentration between samples, each spectral region was normalised to total integral area. Resonances in the NMR spectra were assigned with reference to the literature or through the analysis of standard compounds.

**Acylcarnitine : free carnitine assay**

Cardiac tissue (50 mg) was pulverised and extracted using methanol/chloroform/water as described above. The dried organic and aqueous fractions were butylated with hydrogen chloride-1-butanol (50 µl, 3 M) at 60°C for 15 min, dried under nitrogen and reconstituted in 200 µl acetonitrile containing 8 isotopically-labelled carnitine derivatives of known concentration as an internal standard (Cambridge Isotope Laboratories Ltd., USA). Samples were injected (10 µl) into a Quattro Premiere XE Triple Quadrupolar Mass Spectrometer (Waters Ltd, UK) coupled with an ESI source in positive ion mode. The source temperature was 110°C and the capillary voltage used was 3.5 kV. The acylcarnitines were analysed by multiple reaction monitoring (MRM) in positive ion mode. The cone voltage was 35 V, collision energy was 15 eV and the MRM transitions used incorporated a common loss of m/z = +85 corresponding to loss of the carnitine head group. Samples were introduced by direct infusion (analysis time = 2 min, flow rate = 10 µl/min, increased linearly by 100 µl/min during the final min, mobile phase = 1:1 acetonitrile: isopropanol + 0.2% formic acid). Data were processed using the Neolynx software package (Waters Ltd, UK).
References


