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# Myocardial Substrate Changes in Advanced Ischaemic and Advanced Dilated Human Heart Failure

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There has been renewed interest in cardiac metabolism at least partly driven by the dramatic improvements in heart failure outcomes in recent sodium-glucose cotransporter 2 (SGLT2) inhibitor trials (1). It has been proposed that these agents provide “thrifty” substrates that the failing heart can leverage for more efficient energy supply; however, this process is poorly understood. Most prior reports have used circulating metabolites or lipids to infer conclusions regarding human cardiac substrate use (2). However, it is unclear if these perturbations, remote from the heart, adequately reflect actual myocardial substrate metabolism. Therefore, we investigated changes across multiple metabolic pathways in human left ventricular myocardial tissue from 26 end-stage HF hearts: ischaemic cardiomyopathy (ICM) (n=14) and non-ischaemic dilated cardiomyopathy (DCM) (n=12), compared to matched healthy donor hearts (n=17).

Two targeted platforms were used to measure 210 known metabolites on our platforms at Sydney Mass Spectrometry: hydrophilic interaction chromatography (HILIC) – tandem mass spectrometry with positive electrospray ionisation, and HILIC chromatography / amide stationary phase – tandem mass spectrometry and negative electrospray ionisation (3). These methods incorporate a broad range of metabolites including amino acids, acylcarnitines, ketone bodies, energetics, and central carbon metabolism. These methods are highly reproducible, with an average coefficient of variation of 3.85% across all metabolites (4). Nevertheless, two independent mass spectrometry runs were performed for each method, which were combined and analysed. We focussed on the 40 metabolites reported in the Table that were present in all tissue samples in both independent LC-MS runs, and that passed internal quality control assessment.

Therefore, we did not adjust for multiple comparison. Data were analyzed by Wilcoxon rank-sum test using SPSS (SPSS 20. Chicago, IL, USA). Metabolites and proteins with  $P < 0.05$  were considered statistically significant.

Mean age in each group were as follows (mean $\pm$ SD): age ICM 59 $\pm$ 8, DCM 47 $\pm$ 12, donor 45 $\pm$ 15 years. Left ventricular HF samples (ICM and DCM) were procured from hearts of NYHA class III-IV patients with left ventricular ejection fraction (LVEF)  $< 35\%$  at the time of heart transplantation. All patients regardless of the aetiology of heart failure received guideline-directed medical therapy pre-transplant, including angiotensin converting enzyme inhibitor or angiotensin II receptor blocker, beta blocker and spironolactone. No patients were on mechanical assist devices at time of transplantation.

Age-matched donor hearts were procured but not used for heart transplantation (primarily for logistical reasons); they did not have any known cardiovascular risk factors and all hearts underwent formal pathology examination to ensure they were histologically normal – donor hearts did not show histological pathology. Both donor and recipient hearts were procured after preceding cardioplegia. All hearts (donor and heart failure) were immediately processed and stored in liquid nitrogen according to published protocols (5) (Sydney Heart Bank HREC # 2016/923). Fibrotic tissue was avoided when processing samples for metabolomics. Subsequent analysis showed the intra-group variability in metabolite levels to be low, suggesting that myocardial tissue composition was not a significant issue influencing metabolite results. Samples were crushed in Liquid Nitrogen to powder, and exactly 50 mg tissue used for each sample.

We found significant changes in ICM and DCM myocardium in many pathways. Uridine and its metabolites 2-deoxycytidine, cytidine, and cytosine were significantly increased in *both* ICM and DCM. Uridine is reported as being pathological in heart failure: *in vivo* administration of uridine triphosphate induced myocardial fibrosis, and *in vitro* uridine diphosphate initiated fibrosis upon mechanical stretch via its P2Y<sub>6</sub> receptor (6).

Branched-chain amino acids (BCAAs) including valine, leucine, and isoleucine were significantly elevated in both ICM and DCM. Mechanical stress upregulates catabolism of BCAAs, causing cardiac dysfunction either through toxic intermediates -- branched-chain  $\pm$ -keto-acids -- or through direct activation of the protein kinase mTOR (7).

Methionine, homocysteine, and derivatives were also significantly changed. The homocysteine pathway has been widely implicated in cardiovascular disease, particularly in the promotion of atherosclerosis via endothelial dysfunction, LDL oxidation, and inflammation. However, this is the first report, to our knowledge, that demonstrates decreased homocysteine in HF hearts.

There was increased lipid metabolism in HF myocardium. Short-chain acylcarnitines isovalerylcarnitine, butyrylcarnitine, and 2-methylbutyrylcarnitine were significantly decreased in ICM and DCM compared to donor hearts. These data are consistent with previous reports of increased utilisation of short-chain acylcarnitines in advanced HF and a low myocardial:plasma ratio (2). Ketone bodies were also significantly changed – acetoacetate was significantly decreased in DCM compared to donor, whereas <sup>2</sup>-hydroxybutyrate was significantly increased

in DCM compared to donor hearts. However, there was no consistent change in ketone bodies across both types of HF, which is at odds with reports suggesting the failing heart is dependent on ketone bodies as its major substrate. Interestingly, recent work examining mechanisms underpinning benefits of SGLT2 inhibitor empagliflozin in the hearts of db/db mice reported increased glucose and fatty acid oxidation, but no change in ketone body oxidation (8).

Deoxyribose-5-phosphate (DRP), a pentose phosphate pathway (PPP) intermediate, was significantly elevated in both ICM and DCM, aligned with reported elevation of PPP intermediates post myocardial infarction (MI). Consistent with previous reports of reduced metabolic flexibility and changes in glycolysis in advanced HF, we found glycolytic intermediates glucose-6-phosphate and fructose-6-phosphate to be significantly reduced in ICM myocardium compared to donor hearts. These and other metabolomic changes are summarised in the Figure.

Herein for the first time, we provide a cross-sectional report on human myocardial substrate perturbations common to heart failure of different aetiologies, as compared to matched, healthy donor hearts. The findings in the current study were limited to those with end-stage, as opposed to earlier-stage, heart failure. Indeed, a spectrum of disease severity and associated metabolite changes are likely to exist, and should be more rigorously explored in animal models in relation to time-course and severity of heart failure development. Our work significantly informs recent work using circulating metabolites and previous murine models of HF.

Furthermore, this work implicates many of the metabolic pathways found to be altered by SGLT2 inhibition.

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**Figure Legend**

**A.** Significantly changed metabolites. FC: Fold change.

**B.** Pathway visualisation.



## HILIC Metabolites

Glycine  
Alanine  
Serine  
Proline  
Valine  
Threonine  
Cysteine  
Isoleucine\_Leucine  
Isoleucine  
Leucine  
Aspartate  
Lysine  
Glutamine  
Glutamate  
Methionine  
Histidine  
Glucose  
Phenylalanine  
Arginine  
Tyrosine  
Methionine sulfone (MetSul)  
Methylsulfone (MES)  
Tryptophan  
Asparagine  
Valine-d8  
Phenylalanine-d8  
1-methylhistamine  
2'-deoxyadenosine  
2'-deoxycytidine  
3-hydroxykynurenine (3-HK)  
5-hydroxyindoleacetic acid (5-HIAA)  
Adenosine  
Asymmetric dimethylarginine (ADMA)  
±-keto-γ-(NG,NG-dimethylguanidino)valeric acid (DMGV)  
Anserine  
beta-Alanine  
Betaine  
Butyrlcarnitine  
Cyclic adenosine monophosphate (cAMP)  
Carnosine  
Choline  
Citrulline  
Cotinine  
Creatine  
Cystamine  
Cysteamine  
Cytidine  
Cytosine

Gamma aminobutyric acid (GABA.1)  
GABA.2  
Glycerol  
Histamine  
Kynurenic acid  
Ornithine  
Phosphocholine  
Phosphoethanolamine  
Serotonin  
Spermine  
Taurine  
Thiamine  
Thyroxine  
trans-hydroxyproline (trans-HYP)  
Triiodothyronine  
Trimethylamine N-oxide (TMAO)  
Thymidine  
Uridine  
3-deazadenosine  
3-indolepropionic acid (3-IPA)  
<sup>2</sup>-aminoisobutyric acid (BAIBA)  
Acetylcarnitine  
Acetylcholine  
Carnitine  
L-Homoserine  
N-acetylglutamine  
NG-monomethyl-L-arginine (L-NMMA)  
Purine  
Pyridoxine  
Riboflavin  
Anandamide  
2-Arachidonyl glycerol  
Arachidonic acid  
Aminoadipic acid (AAD)  
Colchicine  
Homocysteine  
Acetoacetic Acid  
Glyoxylic acid  
5-Aminolevulinic Acid  
α-ketoisocaproic acid.1  
α-ketoisocaproic acid.2  
Isovalerylcarnitine.1  
Isovalerylcarnitine.2  
α-keto-β-methylvaleric acid.1  
α-keto-β-methylvaleric acid.2  
2-methylbutyrlcarnitine.1  
2-methylbutyrlcarnitine.2  
2-methylbutyrlcarnitine.3  
±-ketoisovaleric acid (KIV)  
Propylcarnitine

## Amide Metabolites

2-hydroxy-2-methylbutyrate (2H2MB)  
2-hydroxyglutarate (2-HG)  
2-isopropylmalate (2-IPM)  
2-ketohexanoate (2-KH)  
2-methylacetoacetate (2-MAA)  
2-oxobutanoate (2-OB)  
3-methyl-2-oxobutyrate (3M2OB)  
2-oxodipate (2-OD)  
3-hydroxy-3-methylglutaryl coenzyme A  
3-hydroxybutyrate (3-HB)  
3-hydroxykynurenine (3-HK)  
3-phosphoglycerate (3-PG)  
4-hydroxyphenylacetate (4-HPA)  
5-hydroxyindoleacetate (5-HIAA)  
Acetylphosphate (ACP)  
Aconitate  
ADP.1  
ADP.2  
Allantoin  
2-aminoadipate (AAD)  
Ascorbate  
ATP.1  
ATP.2  
Cholesteryl sulfate (CholSO<sub>4</sub>)  
Cyclic adenosine monophosphate (cAMP)  
Cholate  
Citraconate  
Deoxycholate (DCA)  
Deoxyribose-phosphate (DRP)  
Fructose 1,6-bisphosphate (Fruc-1,6-bP)  
Fructose 6-phosphate  
oxidized glutathione (GSSG)  
reduced glutathione (GSSH)  
Glyceraldehyde  
Guanidoacetate (GAA)  
Homocysteate (HCA)  
Homogentisate  
Hydroxyisocaproate (HICA)  
Isocitrate  
Kynurenate  
Kynurenine  
Malate  
Fumarate  
Malonyl-coenzyme A  
Mevalonate (MEV)  
N-Acetyl-L-Alanine  
Nicotinate

p-aminobenzoate (p-AB)  
Phenyllactic  
Phenylpyruvate  
Phosphoenolpyruvate (PEP)  
Pyroglutamate  
Pyrophosphate  
Pyruvate  
Saccharopine  
S-adenosyl-L-homocysteine  
Taurine  
Taurodeoxycholate (TDCA)  
Thiamine pyrophosphate (TPP)  
Thymidine  
Uracil  
Uridine  
Xanthosine  
Xanthurenate (XAN)  
Acetyl-coenzyme A  
Adenosine monophosphate (AMP.1)  
Adenosine monophosphate (AMP.2)  
Anthranilate (AA)  
Arginosuccinate  
Biotin  
Erythrose 4-phosphate (E4P.1)  
E4P.2  
Glyceraldehyde 3-phosphate (G3P)  
Glutarylcarbitine (C5-DC)  
Inosine  
Malonate  
N-carbomyl-aspartate  
Niacinamide  
Oxaloacetate  
Phytonadione  
Prephenate  
Quercetin  
Riboflavin 5'-monophosphate (R5'MP.1)  
R5'MP.2  
Tropisetron  
UDP-N-acetyl-glucosamine  
Ureidopropionate  
Uridine 5'-diphosphate (U5'dP)  
Phenylalanine-d8  
Thymine-d4  
Glucose 6-phosphate  
Succinate  
Lactate  
Camphorsulfonate (CSA)  
NADH  
Glucose  
Nicotinamide Adenine Dinucleotide (NAD)

Reduced nicotinamide adenine dinucleotide phosphate (NADPH)

Nicotinamide adenine dinucleotide phosphate (NADP.1)

NADP.2

Citrate-D4

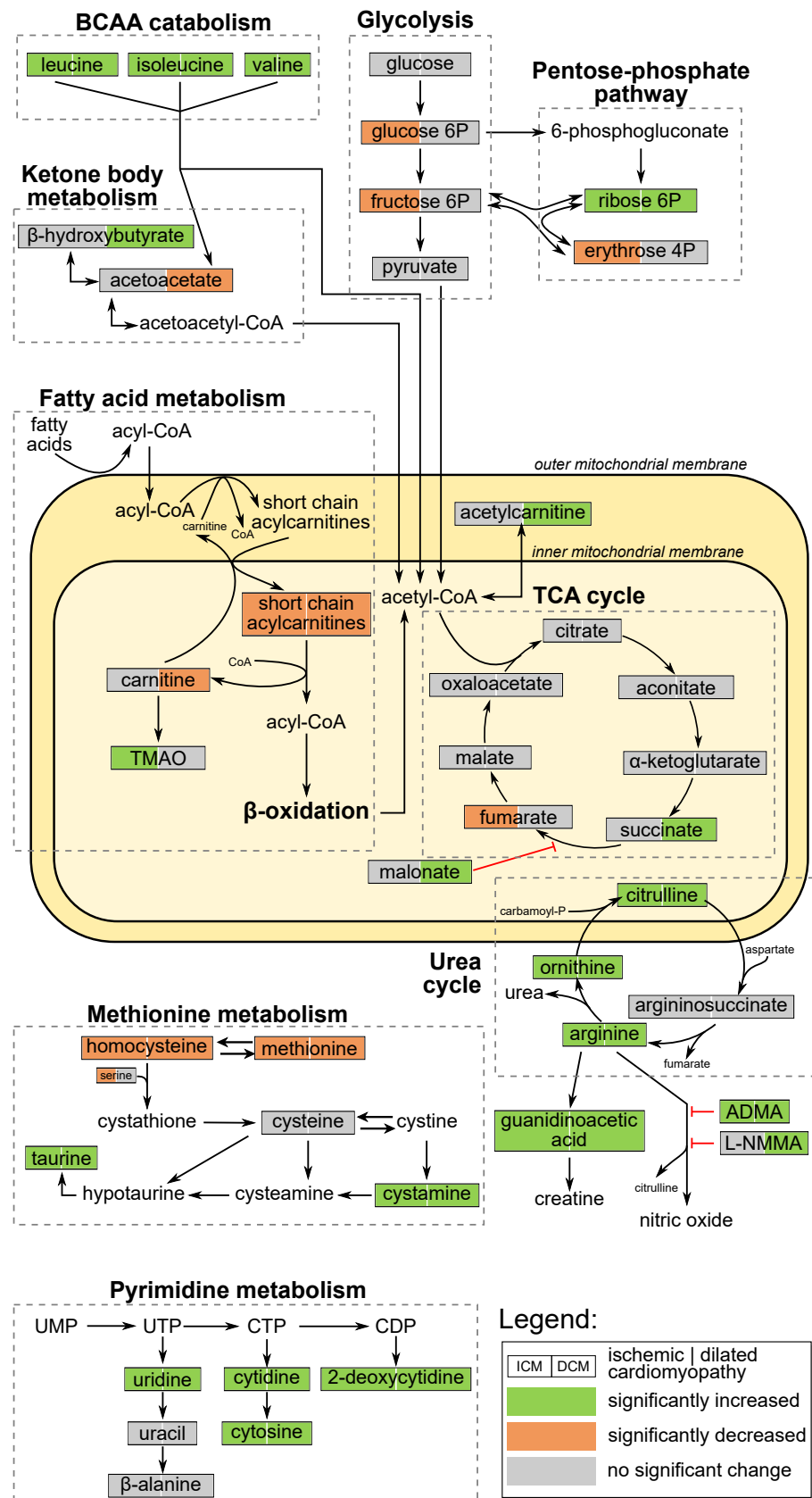
Citrate

3-hydroxyanthranilate (3-HAA)

A

| Pathway                           | Metabolite               | ICM vs. Control |          | DCM vs. Control |          |
|-----------------------------------|--------------------------|-----------------|----------|-----------------|----------|
|                                   |                          | FC              | P Value  | FC              | P Value  |
| Pyrimidines                       | Uridine                  | 2.76            | 1.00E-06 | 2.72            | 7.30E-07 |
|                                   | 2-deoxycytidine          | 4.2             | 1.20E-05 | 3.4             | 1.62E-04 |
|                                   | Cytosine                 | 3.21            | 1.20E-05 | 2.79            | 2.60E-04 |
|                                   | Cytidine                 | 4.87            | 1.90E-05 | 4.24            | 2.60E-04 |
|                                   | N-Carbamoyl aspartate    | 3.99            | 1.00E-03 | 2.75            | 7.00E-03 |
| Methionine Metabolism             | Homocysteine             | 0.77            | 8.00E-03 | 0.86            | 2.40E-02 |
|                                   | Taurine                  | 1.73            | 5.00E-03 | 1.97            | 1.40E-05 |
|                                   | Methionine               | 0.66            | 1.00E-03 | 0.69            | 7.00E-03 |
|                                   | Cystamine                | 1.94            | 1.30E-02 | 2.66            | 2.04E-04 |
| BCAA                              | Valine                   | 1.65            | 3.00E-03 | 1.63            | 6.00E-03 |
|                                   | Leucine                  | 1.74            | 3.00E-03 | 1.82            | 4.00E-03 |
|                                   | Isoleucine               | 1.48            | 5.00E-02 | 1.75            | 7.00E-03 |
| Nitric Oxide Metabolism           | Arginine                 | 1.47            | 2.00E-03 | 1.46            | 2.70E-02 |
|                                   | ADMA                     | 1.44            | 2.00E-03 | 1.49            | 6.00E-03 |
|                                   | Citrulline               | 2.14            | 2.60E-02 | 2.1             | 1.20E-02 |
|                                   | L-NMMA                   | 1.35            | 1.00E-01 | 1.46            | 4.80E-02 |
| Urea Cycle                        | Ornithine                | 2.23            | 1.56E-04 | 1.51            | 6.00E-03 |
|                                   | Guanidinoacetic acid     | 1.38            | 2.60E-02 | 1.51            | 1.00E-03 |
| Free Carnitines                   | Carnitine                | 0.88            | 2.10E-01 | 0.75            | 8.00E-03 |
| Short Chain Acylcarnitines        | Acetylcarnitine          | 1.17            | 8.40E-02 | 1.21            | 4.30E-02 |
|                                   | Isovalerylcarnitine      | 0.64            | 3.20E-02 | 0.47            | 4.00E-03 |
|                                   | 2-methylbutyrylcarnitine | 0.45            | 6.00E-03 | 0.37            | 2.00E-03 |
|                                   | Butyrylcarnitine         | 0.83            | 1.00E-03 | 0.91            | 6.00E-03 |
|                                   | Glutaryl carnitine       | 0.61            | 2.20E-02 | 0.68            | 7.89E-02 |
| Ketone Bodies                     | Acetoacetate             | 0.85            | 5.60E-02 | 0.78            | 1.80E-02 |
|                                   | 3-hydroxybutyrate        | 1.06            | 4.70E-01 | 1.57            | 2.20E-02 |
| TCA                               | Succinate                | 1.27            | 1.90E-01 | 1.32            | 3.40E-02 |
|                                   | Fumarate                 | 0.75            | 4.70E-02 | 0.82            | 1.70E-01 |
|                                   | Malonate                 | 1.05            | 5.50E-01 | 1.44            | 2.70E-02 |
| Glycolysis (intermed and related) | Glucose-6-Phosphate      | 0.43            | 3.50E-02 | 0.61            | 2.40E-01 |
|                                   | Fructose-6-Phosphate     | 0.41            | 3.50E-02 | 0.57            | 1.80E-01 |
|                                   | Serine                   | 0.6             | 9.00E-03 | 1.03            | 1.00E+00 |
| Pentose Phosphate Pathway         | Deoxyribose-5-phosphate  | 1.88            | 1.00E-03 | 1.71            | 4.00E-03 |
|                                   | Erythrose 4-phosphate    | 0.47            | 4.60E-02 | 0.51            | 1.30E-01 |
| Energetic                         | 5-Aminolevulinic         | 0.78            | 4.95E-04 | 0.74            | 8.00E-06 |
| Second Messenger                  | cAMP                     | 0.63            | 8.00E-03 | 0.72            | 1.80E-01 |
| Tryptophan Metabolites            | Serotonin                | 2.21            | 3.00E-03 | 1.71            | 5.90E-02 |
|                                   | Tryptophan               | 1.63            | 8.00E-03 | 1.83            | 2.00E-03 |
| Hormone                           | Thyroxine                | 1.53            | 2.00E-03 | 1.38            | 2.10E-01 |
| Amine oxide (microbiome)          | TMAO                     | 2.56            | 5.00E-03 | 2.06            | 3.50E-01 |

B



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