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1 **TITLE**

2 **Investigating the effects of an Oral Fructose Challenge on Hepatic ATP Reserves in**

3 **Healthy Volunteers: A ^{31}P MRS Study**

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14 **DEPARTMENT AND INSTITUTION OF STUDY**

15 All work was conducted at the Sir Peter Mansfield Imaging Centre in the University of

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31 **LIST OF ABBREVIATIONS**

32 NAFLD – Non-Alcoholic Fatty Liver Disease

33 NASH – Non-Alcoholic Steatohepatitis

34 ATP – Adenosine Triphosphate

35 MRS – Magnetic Resonance Spectroscopy

36 Pi – Inorganic Phosphate

37 PDE – Phosphodiesters

38 PME – Phosphomonoesters

39 IV – Intravenous

40 ISIS – Image Selective *In vivo* Spectroscopy

41 NOE – Nuclear Overhouser Effect

42 SD – Standard Deviation

43 ADP – Adenosine Diphosphate

44 AMP – Adenosine Monophosphate

45 AMPK – AMP-activated protein kinase

46 UTP – Uridine Triphosphate

47

48 **KEYWORDS**

49   ATP; hepatic ATP; fructose; fructose infusion; oral challenge; NAFLD; 31P; MRS

50

59   **AUTHORS CONTRIBUTIONS**

60   Study conception and design: SB; MS; LM; GA; PM; PG;

61   Acquisition of data: SB; MS

62   Analysis and interpretation of data: SB; MS; GA; IM; LM; PG

63   Drafting of manuscript: SB

64   Critical revision: SB; MS; EC; KH; LM; IM; GA; PM; PG

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ABSTRACT

Background: Impaired homeostasis of hepatic ATP has been associated with NAFLD. An intravenous fructose infusion has been shown to be an effective challenge to monitor the depletion and subsequent recovery of hepatic ATP reserves using ^{31}P MRS.

Aims: The purpose of this study was to evaluate the effects of an oral rather than intravenous fructose challenge on hepatic ATP reserves in healthy subjects.

Methods: Self-reported healthy males were recruited. Following an overnight fast, baseline liver glycogen and lipid levels were measured using Magnetic Resonance Spectroscopy (MRS). Immediately after consuming a 500ml 75g fructose drink (1275 kJ) subjects were scanned continuously for 90 minutes to acquire dynamic ^{31}P MRS measurements of liver ATP reserves.

Results: A significant effect on ATP reserves was observed across the time course ($P <$ 0.05). Mean ATP levels reached a minimum at 50 minutes which was markedly lower than baseline ($80 \pm 17\%$ baseline, $P < 0.05$). Subsequently, mean values tended to rise but did not reach statistical significance above minimum. The time to minimum ATP levels across subjects was negatively correlated with BMI (R^2=0.74, $P < 0.005$). Rates of ATP recovery were not significantly correlated with BMI or liver fat levels, but were negatively correlated with baseline glycogen levels (R^2=0.7, P<0.05).

Conclusions: Depletion of ATP reserves can be measured non-invasively following an oral fructose challenge using ^{31}P MRS. BMI is the best predictor of postprandial ATP homeostasis following fructose consumption.

4

INTRODUCTION

Both NAFLD and non-alcoholic steatohepatitis (NASH) have been associated with impaired homeostasis of hepatic adenosine triphosphate (ATP) levels [1] and baseline hepatic ATP reserves have been shown to be more depleted in obese subjects [2, 3]. It is widely accepted that the inhibition of AMP-activate protein kinase (AMPK) which stimulates ATP synthesis is an important part of liver lipid accumulation [4, 5] and it has also been suggested that an inability to maintain ATP levels may prime hepatocytes to become vulnerable to injury by reactive oxygen species.

Hepatic ATP reserves can be monitored noninvasively using ^{31}P magnetic resonance spectroscopy (MRS) [6]. Early animal studies used this method to monitor ATP following fructose injections and suggested its potential use as a diagnostic method for studying liver disease [7]. A number of more recent studies have used these techniques to measure ATP homeostasis following an intravenous (IV) fructose load [2, 8, 9]. Fructose infusion causes the depletion of hepatic ATP levels due to a lack of phosphorylation feedback which results in continued phosphorylation activating AMP deaminase and uric acid production (supplementary material) [10]. During these studies, subjects undergo continuous ^{31}P MRS immediately following a fructose bolus injection to measure minimum ATP levels and subsequent rates of replenishment.

The effects of fructose consumption on liver lipids [11] and NASH [12] have been considered in the literature, but little research has investigated the immediate ATP response to an oral fructose challenge. The present study investigated postprandial changes to hepatic ATP reserves following an oral fructose intake.

MATERIAL AND METHODS

Subjects

All subjects were self-reported healthy non-obese males with sedentary lifestyles and no known metabolic disorders. All subjects consumed the oral challenge in the time required (5 minutes) and complied well with the lifestyle restrictions and scanning requirements. The mean age for all subjects was 24 ± 4 years and BMI was 25 ± 3 kg/m^2.

Study Design

Ethical Permission

Ethical permission was obtained from the local Medical School Research Ethics Committee and subjects provided written informed consent before participation.

Subjects

At the time of this investigation there were no published data on ^{31}P MRS ATP following an oral fructose challenge which could be used to estimate the power of the study. We therefore chose a sample size for this first exploratory study based on data reported in infusion studies [13, n=8].

Prior to study days subjects were asked to refrain from alcohol for 24hr. On the morning of the study subjects arrived at the test centre between 7:30am and 8:00am having fasted overnight.

On arrival, natural abundance ^{13}C MR spectra were acquired from the liver to determine baseline hepatic glycogen levels, and localized ^{1}H MR spectra were acquired to determine baseline hepatic lipid levels. Subjects were then asked to consume a 500ml drink of 75g fructose solution (1275 kJ) within 5 minutes. Immediately following consumption, subjects

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136   were placed in the scanner and ^{31}P MR spectra were acquired continuously for 90 minutes to

137   assess dynamic changes in ATP and related phosphate metabolites. During the 90 minutes of

138   scanning, subjects were asked to breathe regularly and remain as still as possible and were

139   allowed to listen to the radio or music.

140   **Data Acquisition**

141   All measurements were performed on a Philips Achieva 3T system (Philips, Best, The

142   Netherlands) using the built-in ^{1}H transmit / receive body coil for scout images and voxel

143   placement.

144   *ATP*

145   Dynamic changes in phosphate metabolites were measured using localized ^{31}P MRS. A ^{31}P

146   surface coil (Philips, Best, The Netherlands) was placed on the abdomen over the liver. Scout

147   ^{1}H images were obtained and used for voxel placement in the right lobe of the liver (60 x 60

148   x 60 mm^3 voxel size). ^{31}P spectra were obtained continuously for 90 minutes using a

149   respiratory triggered ISIS sequence with Nuclear Overhouser Effect (NOE) enhancement and

150   proton decoupling (3 kHz bandwidth, 2048 samples, 5000 ms repetition time) as described

151   previously [14, 15]. The voxel for β-ATP was positioned against the abdominal wall with the

152   chemical shift of all other metabolites directed away from the wall to minimize signal leakage

153   from the abdominal muscle (confirmed by a lack of spectral PCr peak) and maximise signal

154   for β-ATP.

155   *Hepatic Lipids*

156   Baseline lipid levels were measured using the integrated ^{1}H body coil. Scout images were

157   obtained and used for voxel placement (30 x 30 x 30 mm^3 voxel size). ^{1}H spectra were

158   obtained using a respiratory triggered, water suppressed STEAM sequence (2 kHz

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159   bandwidth, 1024 samples, 13 ms echo time, 5000 ms repetition time, 40 averages). Two

160   spectra were collected without water suppression for correction to absolute lipid fat fractions

161   as described previously [15].

162   *Glycogen*

163   Baseline glycogen levels were measured using unlocalized ^{13}C MRS. A surface coil with

164   integrated quadrature proton decoupling (PulseTeq, Surrey, UK) was placed on the abdomen

165   over the liver. Scout ^{1}H images were used to determine correct placement. ^{13}C spectra were

166   obtained using a $\pi/2$ pulse-acquire sequence with an adiabatic half passage pulse shape to

167   minimise the effects of B_1 field inhomogeneity within the volume of interest, along with

168   narrow band proton decoupling (7 kHz bandwidth, 512 samples, 2150 ms repetition time, 576

169   averages, ~20 minutes total acquisition time) as previously described [16].

170   **MRS analysis**

171   *ATP*

172   ^{31}P spectra were line broadening by 30 Hz and data were averaged over 15 minute windows

173   at 5 minutes intervals across the time-course. The β-ATP peak position was defined in the

174   spectra and peak area calculated across the time course (Figure1). The β-ATP peak provides a

175   way of measuring total ATP because the phosphate signal from ADP overlaps with the α-

176   ATP and γ-ATP peaks. The first time point was taken as a reference to measure changes in

177   ATP and recorded as % of baseline value.

178   Time to reach minimum ATP levels was calculated, and the rate of recovery of absolute ATP

179   was determined using the gradient across the first 4 time points of recovery using linear

180   fitting. For recovery rates, ratios of β-ATP to total phosphorous levels were taken as used in

181   previous studies [3].

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182 *Hepatic Lipids*

183 ^{1}H spectra were zero filled to 1024 datapoints and phase corrected before peak areas were

184 calculated using the AMARES algorithm in jMRUI (Universiteit Leuven, Belgium) [17]

185 (Lorentzian curve fitting of water peak at ~4.8ppm and -$[CH_2]_n$- at ~1.3 ppm). Water

186 suppression was applied during spectral acquisition for better resolution of the fat peak,

187 followed by unsuppressed spectra with identical parameters to determine the water peak area.

188 Peak areas were corrected for T_2 relaxation as determined from previous studies and

189 lipid/water ratios used to determine absolute fat fractions as described by Stephenson *et al*

190 [23].

191 *Glycogen*

192 ^{13}C spectra were zero filled to 4096 datapoints and 100 Hz line broadening was applied

193 before Lorentzian curve fitting using in house software. Integrals of the C1-glycogen peak

194 (100.4 ppm) and of an external reference peak were measured and ratios used to account for

195 varying loading factors. Quantification was achieved by comparing glycogen/reference ratios

196 with a phantom [18].

197 **Statistical Analysis**

198 All results are expressed as means (±SD). A repeated measures ANOVA F-test was used to

199 determine a significant effect across the timecourse, and a means difference T-tests were

200 subsequently used on individual time points to determine significant changes. Significances

201 in correlations were determined using linear regression analysis with Pearson correlation

202 coefficients quoted. In all cases significance was attributed to $P < 0.05$. The statistical

203 package used for analysis was SPSS version 21 for Windows (SPSS, Inc., Chicago, IL).

204

RESULTS

Baseline Hepatic Lipid and Glycogen

The mean baseline liver lipid fat fraction was 4 ± 3 % and correlated significantly with BMI ($R^2 = 0.48$, $P \leq 0.05$) as expected.

The mean baseline hepatic glycogen concentration was 219 ± 81 mmol/l and there were no correlations between individual values and age, BMI or baseline liver lipid levels.

ATP Reserves following Oral Fructose Challenge

Mean postprandial hepatic ATP levels began to decline from 15 minutes after the oral fructose challenge (Figure 2). A statistically significant variation from baseline was found across the time course (One way ANOVA F-test, $P < 0.05$). Mean values continued to decline and were significantly below the first two points at t = 30 minutes ($86 \pm 14\%$, $P < 0.05$), t = 40 minutes (85 ± 16 %. $P < 0.05$) and t = 45 minutes ($84 \pm 14\%$, $P < 0.005$) until reaching minimum at = 50 minutes ($80 \pm 17\%$, $P < 0.05$). There was a trend for values to recover after 50 minutes, but the increase was not statistical significance compared to nadir and levels remained lower than baseline at the end of the study.

No subject showed any recovery of ATP levels during the first 6 time points (until t = 40 mins). The mean AUC across this period (t=0 to t=40 mins) was 232 ± 19 % h and showed a strong negative correlation with BMI ($R^2 = 0.65$, $P < 0.01$).

Time to minimum ATP

For two subjects the minimum ATP time point was at the end of the scanning period, and as such the final time point was taken as their time to minimum ATP (which may in fact have been after the scan period). A significant negative correlation was found between time to

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227   minimum ATP and BMI ($R^2 = 0.74$, P < 0.005) as shown in Figure 3. No such correlation

228   was observed with age ($R^2 = 0.01$, P = 0.78) or baseline glycogen ($R^2 = 0.003$, P = 0.88) but

229   the correlation approached significance with baseline liver fat ($R^2 = 0.39$, P = 0.07).

230   *Rate of recovery*

231   Figure 4 shows the relationship between rate of recovery and baseline glycogen reserves,

232   which had a strong negative correlation that was statistically significant ($R^2 = 0.71$, P < 0.05).

233   This correlation was not observed with BMI, liver fat, or any other baseline measures.

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DISCUSSION

The underlying physiological hypothesis of this study is that ATP homeostasis, which provides a measure of AMPK activity, acts as a biomarker for NAFLD and NASH. Rather than fructose infusion, this study explored using ^{31}P MRS following an *oral* fructose challenge, which is more physiological, more patient-acceptable and much simpler to administer. The results showed that after oral consumption there is a measurable decline in ATP reserves (β-ATP) followed by a partial recovery. This observation is characteristic of fructose metabolism and can be explained as a result of the immediate rapid phosphorylation of the monosaccharide. Under normal physiological conditions an increased cellular level of adenosine monophosphate (AMP) activates AMPK resulting in the regeneration of ATP, whereas under conditions where AMPK activity is lower (e.g. following fructose consumption) the production of uric acid is favoured over ATP (supplementary material). In addition to this, fructose has been shown to up-regulate Glut5 and Fructokinase [19], and subjects with NAFLD and a higher intake of fructose have been shown to have a greater hepatic mRNA expression of fructokinase [20].

In a small study of 4 subjects Buemann *et al*. tested the effects of an oral dose of 30g D-Fructose and D-Tagatose on hepatic ATP reserves at 1.5T [21] and reported no drop in ATP following D-fructose consumption (however, they did find a drop following D-Tagatose which reached a maximum at 51 minutes). The data from the present study suggests that a greater concentration of fructose and high resolution spectra (3T scanner) may be required to observe significant reductions.

In the present study ATP levels took longer to recover compared to previous infusion studies. This is probably due to the extra stages necessary to transfer fructose to the hepatic tissue, namely gastric emptying and intestinal absorption. Gastric emptying has been shown to be

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259   dependent on meal energy and volume [22], which becomes relevant to the techniques used

260   here when considering the optimum energy content and volume of the fructose challenge to

261   induce sufficient depletion of hepatic ATP. Another confounding factor is the variation in

262   fructose intestinal absorption rates reported in the literature. A previous study showed a high

263   variability in intestinal absorption of fructose in healthy subjects following an oral fructose

264   drink [23]. The amount of fructose used in the present study was sufficient for intestinal

265   absorption and delivery to the liver in all subjects, but this factor should be considered in

266   future experiments, and it may be that lower doses and volumes of fructose will not have the

267   same effect.

268   This study showed a negative correlation between BMI and time to minimum ATP levels.

269   Given that the hepatic ATP response is a combination of depletion and recovery and fructose

270   is known to deplete ATP reserves, these findings suggest that individuals with lower BMI

271   have a more effective hepatic ATP recovery in response to a high fructose challenge. This

272   result may be confounded by changes in gastrointestinal function, but also confirms previous

273   studies that have shown that obese subjects have an impaired efficiency of ATP

274   replenishment [2]. Surprisingly this correlation was not observed with liver fat levels as

275   might be expected. Previous studies have shown that there is an impaired hepatic ATP

276   homeostasis in Type2 diabetes [24] and it has been suggested that this may precede the

277   development of steatosis in these patients [3]. Whether or not there is a causal link between

278   rates of ATP synthesis and metabolic disorders remains to be established. Related to this, it

279   has been suggested that regular consumption of fructose upregulates fructokinase and that

280   this may be a factor in NAFLD development and the high incident rates observed currently

281   [20]. In the present study we did not acquire a full dietary history, but future studies in this

282   area should explore the effects of prior exposure and its relevance to ATP depletion and

283   recovery rates and steatosis.

284 This experiment required 2 hours of scanning on a high field MRI scanner , which may be

285 impractical and costly in a clinical setting. However, it is possible that other related measures

286 may provide a more convenient marker. For example, there was a significant negative

287 correlation between the AUC over the first 6 time points and BMI. These measures can be

288 made over a shorter scan duration. Future studies should consider a wider range of liver fat,

289 as well as NAFLD and NASH patients. In particular, studies that separate BMI from liver fat

290 to determine which of these is a better predictor of ATP homeostasis, although admittedly it

291 would be difficult to recruit for this given the correlation between BMI and liver fat.

292 Baseline glycogen measurements gave a wide range of values, which suggests variability in

293 the timing and content of the previous evening meal across subjects [16]. Whilst this may

294 reveal a potential limitation in the study design, the results showed for the first time a

295 significant negative correlation between rates of ATP synthesis and baseline glycogen levels

296 which may be relevant to patients with glycogen storage disease and other metabolic

297 disorders. Previous studies have shown that a fructose load activates glycogen synthase

298 resulting in increased glycogenesis, and also that fructose-1-phosphate produced during

299 fructose metabolism is a competitive inhibitor of phosphorylase a [25] resulting in a slowed

300 glycogenolysis. These factors result in an increase in glycogen synthesis following fructose

301 consumption. The relationship between glycogen levels and ATP reserves has been explored

302 in a number of publications and correlations between glycogen synthesis and ATP turnover in

303 muscle [26] and between absolute hepatic glycogen levels and total hepatic ATP content

304 during glycogen repletion [27] have been reported. This has been explained as the need for

305 increased uridine triphosphate (UTP) during periods when unidirectional flux of glycogen

306 synthesis is greater than glycogenolysis, which results in greater ATP synthesis. A possible

307 explanation for the negative correlations between rates of ATP synthesis and baseline

308 glycogen levels observed in the present study is that there is a greater demand from hepatic

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309 glycogen in subjects with lower baseline glycogen levels, resulting in an increased rate of

310 glycogen synthesis and indirectly ATP synthesis. Whilst it is beyond the scope of this study

311 to determine this causal link, this study shows that baseline hepatic glycogen levels are an

312 important factor in the ATP response to fructose.

313 The present study has some limitations. Firstly, breath samples were not obtained to estimate

314 levels of intestinal fructose malabsorption [28]. Measuring changes in serum uric acid would

315 also be ideal as hyperuricemia has been associated with an impaired hepatic ATP

316 homeostasis in response to high fructose intake [3]. Future experiments should obtain blood

317 samples to measure this also. Secondly, histological comparisons were not made due to the

318 ethical considerations of liver biopsies in healthy subjects. As such, although all subjects in

319 this study had no known liver health problems this was not confirmed through histological

320 analysis. Other studies should investigate the postprandial ATP effects in patients with

321 NASH in comparison with healthy weight and obese people, as well as individuals with Type

322 2 diabetes. Similarly, all subjects in this study were healthy non-obese male volunteers and it

323 should be acknowledged that the response may be different in women or an obese cohort.

324 Subjects also found 500 ml fluid difficult to consume and the scan time was long and

325 potentially uncomfortable. Future studies should optimize the experimental protocol, in

326 particular the time duration, time resolution and volume or concentration of fructose

327 challenge used.

328 In summary, this study has shown that depletion in hepatic ATP reserves following an oral

329 fructose challenge is observable using ^{31}P MRS in healthy subjects, allowing for a completely

330 non-invasive assessment of ATP synthesis. BMI was negatively correlated with the time to

331 minimum ATP levels and with ATP levels immediately post consumption   indicating an

332 impaired hepatic energy homeostasis in subjects with higher BMI.

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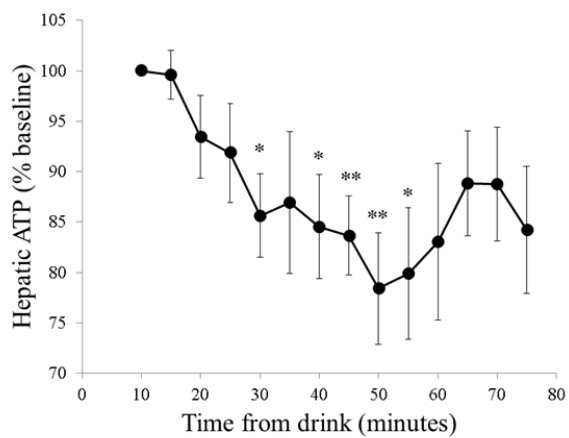426
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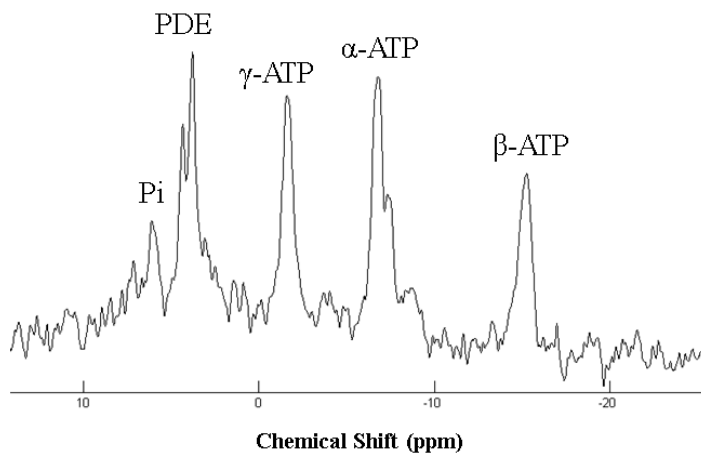
Figure 1. ^{31}P Magnetic Resonance Spectrum from one subjects showing signal peaks from ATP (β-ATP, α-ATP and γ-ATP), phosphodiesters (PDE) and inorganic phosphate (Pi).
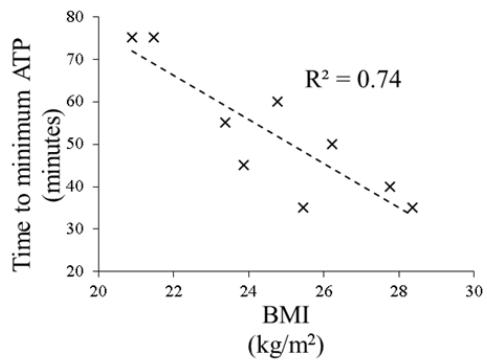
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Figure 2. Changes in Hepatic ATP (β-ATP peak) from baseline in response to a 75g oral fructose challenge measured using ^{31}P MRS (n=9). * P < 0.05, ** P < 0.01
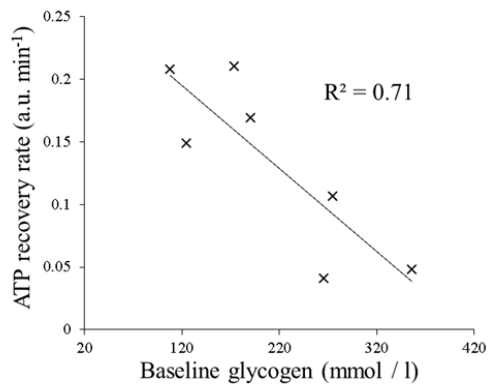
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Figure 3. Correlation between time to minimum ATP (β-ATP peak) and BMI (P <0.005).

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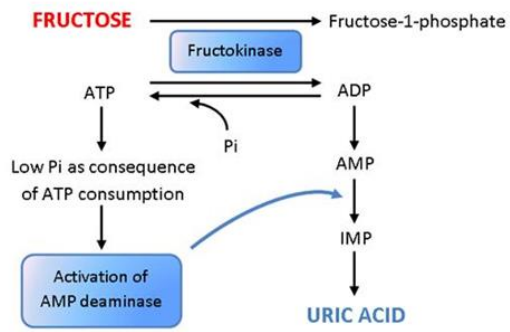

Figure 4. Correlation between rate of ATP recovery (β-ATP peak) and baseline glycogen levels measured using ^{31}P MRS (P < 0.05). Recovery rate is measured as the gradient of [β-ATP signal/total phosphorous signal] across the first four points of recovery

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Supplementary Figure. Fructose metabolism showing ATP depletion and Uric Acid production

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