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# **Delivery by Caesarean section, rather than vaginal delivery, promotes hepatic steatosis in piglets**

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# **Abstract:**

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There has been a marked increase in the number of babies born by elective caesarean section (CS). Following CS, the lack of normal stimuli that occur at birth alters the thermogenic response but any effects on hepatic metabolism had not been identified. We compared the effect of delivery on hepatic metabolism in piglets, born either by CS or vaginal delivery (VD) and fed by total parenteral nutrition, by measuring lipid metabolism and enzyme activity coupled with metabolomic and genomic approaches. Hepatic lipid in the CS piglets 7 days post-partum was in excess of 5mg/g of liver consistent with hepatic steatosis whereas in the VD piglets the amount of lipid was markedly lower (3mg/g) and below the threshold for a diagnosis of steatosis. Metabolomic analysis indicated that CS resulted in higher hepatic glycerol and lower glycerol phosphate-dehydrogenase activity suggesting that CS causes an decrease in hepatic gluconeogenesis from glycerol. CS also resulted in altered cholesterol handling and gene expression despite the same dietary intake 7 days post-partum. Furthermore the CS piglets had lower expression of interferon responsive genes, but higher expression of markers of immature hepatocytes. The data suggest that VD promotes normal liver maturation and hepatic metabolism, thereby reducing the accumulation of hepatic lipid.

## **Introduction:**

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The number of babies born by elective Caesarean section (CS) has increased dramatically in the past 25 years, exceeding 25 % of births in the UK in 2007 [1] and 30 % in Brazil [2]. Despite the increasing rate of CS deliveries little is known about the health implications for the offspring. Recently, epidemiological research has indicated that CS is a risk factor for type 1 diabetes [3], asthma [4-6] and elevated body mass index [7] in later life, when compared to vaginally delivered (VD) children. Whilst CS is known to result in significant changes in both the hormonal milieu and the consequent plasma metabolite profile immediately post partum [8], little is known about any long term effects on metabolism due to programming in the perinatal period.

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Marked changes in metabolism have to occur at birth to allow the newborn to adapt to extra-uterine life. Pre-partum, fetal blood glucose levels are regulated maternally; fetal glucose supply is continuous and insulin operates as a growth factor. Following birth, glucose supply is discontinuous; and the neonate must regulate its own blood glucose concentration via the insulin/glucagon axis. Consequently, at birth there is a marked decrease in circulating plasma insulin whilst both plasma glucagon concentration and the number of glucagon receptors increase dramatically [9, 10]. These changes result in a low insulin/glucagon ratio which promotes glycogenolysis and gluconeogenesis to maintain plasma glucose concentrations.

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The processes that reduce insulin secretion and promote glucagon production are thought to be transient hypoglycemia following cessation of umbilical blood flow, the surge in stress hormones accompanying VD (predominantly adrenaline and cortisol) and the onset of enteral feeding [11]. During elective CS at least one of the main triggers for altered insulin function is absent and CS has been shown to modify the thermogenic response of both humans [12] and sheep [13]. These observations raise the question of whether CS results in altered glucose metabolism after birth, particularly in the liver.

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Long term abnormalities in glucose regulation can have serious consequences and insulin resistance is linked to the development of obesity, non-alcoholic fatty liver disease (NAFLD) and the metabolic syndrome [14] with its resultant cardiovascular disease all of which are significant and growing problems worldwide [15] in both children and adults [16]. Whilst increased energy intake and a more sedentary lifestyle contribute to insulin resistance, other

factors, particularly perinatal programming events (e.g. disproportionate growth *in utero*) are also involved [17]. Insulin resistance is marked by enhanced net liver glucose production and decreased tissue glucose uptake resulting in elevated blood glucose levels and is associated with obesity and accumulation of hepatic lipid [18, 19].

Neonatal NAFLD is associated with total parenteral nutrition (TPN) [20] and we have previously shown that the neonatal piglet is a useful model to study perinatal factors influencing the development of hepatic lipid accumulation [21]. Given the changes in metabolism that occur at birth we used this model to test the hypothesis that CS delivery would promote the accumulation of hepatic steatosis in response to feeding by TPN. We therefore compared the metabolic and transcriptomic response of TPN fed piglets that were born either by CS or VD.

# Methods:

## Ethical Approval:

Experimental protocols were carried out as defined by the regulations of the Animals (Scientific Procedures) Act 1986, followed local ethical standards, and were approved by the Home Office, UK.

## Experimental design:

Four sows nulliparous (25% Meishan, 12.5% Duroc and 62.5% Large White X Landrace) were artificially inseminated three times in a 36 hour period with pooled semen from multiple boars. This method maximises the genetic variation of the piglets without recourse to large numbers of sows entering the study and allows accurate estimation of the date of parturition. As previously described [21], piglets from two sows were delivered by CS at 112 days post fecundation (dpf). Two sows delivered piglets vaginally (VD) at term (mean 114 dpf). Piglets were selected to balance experimental groups for sex, body weight at birth and maternal influences. All piglets received a 10 ml bolus feed of sow's milk prior to surgical implantation of bilateral jugular vein catheters, approximately 3 hours post partum. Three cm of catheter (0.96 mm OD, 0.58 mm ID; Portex, UK) was inserted into the jugular vein, under isoflurane 0.5-2% (v/v; Schering-Plough Animal Health, UK) anaesthesia, and tunnelled subcutaneously to the back of the neck where it was stiched into the *trapezius* muscle on the shoulder and exteriorised. Piglets were administered analgesia (0.05 ml·kg<sup>-1</sup> tolafenamic acid 4 % (w:v); Tolfine, Vetoquinol, UK) and housed in individual open top cages.

Five CS (three from one sow and two from the other) and four VD (two from each sow) piglets were parenterally fed (PN) with TPN solution (1690 J·mL<sup>-1</sup>; 99.34 mg·mL<sup>-1</sup> glucose; 17.89 mg·mL<sup>-1</sup> amino acids; Portsmouth Hospital NHS Trust, Portsmouth, UK), plus Intralipid 20 %<sup>®</sup> (8.36 MJ·L<sup>-1</sup>; Fresenius Kabi Ltd., UK) administered as previously described [21] for 7 days (final rates TPN solution 5.8 mL·h<sup>-1</sup>kg<sup>-1</sup>; Intralipid 1.0 mL·h<sup>-1</sup>kg<sup>-1</sup>).

Some of the piglets from the same litters (6 CS and 6 VD) were humanely killed using pentobarbitone sodium (Euthatal, Rhone Merieux, UK) at birth; their livers were immediately excised, frozen in liquid nitrogen and stored at -80 °C to provide baseline data. At 7 days the piglets fed parenterally were humanely killed and samples of liver, muscle and plasma were taken for subsequent analysis as described [21].

## Laboratory Analysis:

Total liver lipid content, together with fatty acid profiles of the liver triacylglycerol (TAG) and total liver lipid, were determined as previously described [21]. Hepatic TAG phospholipid, cholesterol, non-esterified fatty acids (NEFA) and cholesterol esters were analysed as previously described [22]. Enzyme activity was determined using previously described assays for glycerol phosphate dehydrogenase [23], phosphoenolpyruvate carboxykinase (PEPCK) [24], succinate dehydrogenase [25], malate dehydrogenase [26]. Hepatic glycerol was measured according to the method of [27] utilising the Free Glycerol

reagent (Sigma Aldrich, UK). Plasma hormones and metabolites were determined using commercially available kits, as per the manufacturer's instructions: insulin – Mercodia Porcine Insulin ELISA (Diagenics Ltd, UK.), glucagon – Linco Research Glucagon RIA Kit (Millipore, UK), triiodothyronine (T<sub>3</sub>) - MP Biomedicals T<sub>3</sub> RIA kit (MP Biomedicals, UK), haptoglobin – Phase Range™ Haptoglobin Kit (Biognosis Ltd., UK), glucose – Infinity Glucose Oxidase Reagent (Thermo, UK.) and plasma cholesterol - Total Cholesterol kit (Thermo, UK.).

#### *Metabolomic Analysis:*

High resolution <sup>1</sup>H NMR spectra were acquired from aqueous liver metabolites and blood plasma on a DRX Bruker Avance II+ spectrometer as previously described (Atherton *et al.*, 2006).

Spectra were processed in ACD 1D NMR Manager (v11; Advanced Chemistry Development Inc.), zero-filled to twice the number of points and multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz. The spectra were Fourier transformed, phased, baseline corrected and referenced to the TSP (trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid) peak (at 0.00 ppm). The spectra were divided into 224 buckets each of 0.04 ppm between 0.2 and 9.4 ppm (excluding the region of the water resonance (4.65 - 4.89 ppm)) and the spectral area of each bucket was integrated. To correct for any variation in sample volumes the integral for each bucket was normalized to the total integral for the spectrum. The compounds detected in the spectra were identified by comparison with entries in the Human Metabolome Database ([www.hmdb.ca](http://www.hmdb.ca);[28]) and spiking known standards into samples. To quantify individual components the peaks were identified and integrated manually.

#### *Gene Expression Analysis:*

Gene expression analysis was carried out using GeneChip® Porcine Genome Array (Affymetrix UK Ltd., UK). RNA was extracted from liver using the Qiagen RNeasy® kit (Qiagen, UK), and run on a bioanalyser (Agilent 2100 BioAnalyzer) to ensure a RNA integrity number (RIN value) above 8.0. Labeling and hybridization of the RNA was carried out by the MRC CSC/IC Microarray Centre (Hammersmith Hospital, London, UK) using the Affymetrix GeneChip® platform. The samples were stained using streptavidin conjugated to R-Phycoerythrin (SAPE). A biotinylated anti-streptavidin antibody was additionally used to amplify the signal. The arrays were scanned on an Affymetrix 3000 7G scanner.

The expression data was analyzed using Rosetta Resolver 7.1 (Rosetta Biosoftware, USA). The genome array data was analyzed to obtain a signature set of genes which showed a fold change between the two modes of delivery of greater than 2 and were significantly different between the treatments ( $p < 0.01$ ) as determined by a two way analysis of variance (ANOVA) followed by Benjamini-Hochberg (FDR) multiple test correction. The genes were identified by BLAST search of the accession number of the probe against the nr and est databases

To confirm the gene array findings, quantitative real time-polymerase chain reaction (qRT-PCR) was performed. RNA was extracted as described above and first strand cDNA generated using Qiagen Omniscript® Reverse Transcription kit. DNA Primers were designed from the *Sus. Scrofa* gene sequences obtained from NCBI ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) over intron-exon boundaries to prevent amplification of genomic DNA (See Supplementary Table I). The qRT-PCR analysis was carried out as described [29] in duplicate on each cDNA sample using RPLPO (acidic ribosomal phosphoprotein P0) as a reference; the annealing temperature was optimized for each individual pair of primers (See Supplementary Table I).

#### *Statistical Analysis:*



Statistical analysis was carried out in Minitab 14 (Minitab Ltd., UK). Data are presented as means  $\pm$  standard error of mean. Data were shown to be normally distributed, (using an  $F$ -test;  $p < 0.05$ ) and ANOVA general linear model was used to test for statistical differences.

## Results:

### *Growth and phenotype*

The birth weight of piglets entered into this study were similar, regardless of whether they were born by CS or on average 2 days later by VD (CS  $1447 \pm 82$ , VD  $1643 \pm 238$ g). Likewise, there were no significant differences in final body weight (CS  $1539 \pm 116$ , VD  $2005 \pm 267$ g) or growth velocities (CS  $62 \pm 11$ , VD  $65 \pm 9$ ; g·day<sup>-1</sup>) of CS and VD piglets.

Gut weight both as an absolute weight and expressed as a percentage of total body weight was significantly ( $p = 0.034$ ) lower in CS piglets compared to VD animals (CS  $3.29 \pm 0.33$ , VD  $4.86 \pm 0.46$ ; % of total body weight). However, there was no difference in the relative or absolute liver weight based on mode of delivery (CS  $3.29 \pm 0.28$ , VD  $3.29 \pm 0.02$ ; % of total body weight).

### *Glucose Homeostasis:*

Immediately after birth, plasma glucose concentration was lower ( $p = 0.001$ ) in the CS piglets compared to the VD animals (CS  $1.92 \pm 0.17$ , VD  $3.13 \pm 0.22$ ; mmol·L<sup>-1</sup>). The higher plasma glucose concentration in the VD animals was accompanied by elevated ( $p < 0.001$ ) plasma glucagon concentration (CS  $62.13 \pm 3.59$ , VD  $124.4 \pm 18.91$ ; pg·mL<sup>-1</sup>), but there was no difference in plasma insulin concentration between the CS and VD piglets (CS  $9.88 \pm 1.10$ , VD  $8.65 \pm 0.90$ ; pg·mL<sup>-1</sup>). The glucagon : insulin ratio of the piglets was lower ( $p = 0.024$ ) in the CS piglets at birth than the VD piglets (CS  $6.69 \pm 0.37$ , VD  $10.08 \pm 0.90$ ).

After 7 days of parenteral feeding, plasma glucose concentrations tended to be lower in CS piglets (CS  $3.80 \pm 0.16$ , VD  $5.13 \pm 0.76$ ; mmol·L<sup>-1</sup>;  $p = 0.178$ ) and whilst there was no difference in plasma glucagon concentration (CS  $105.33 \pm 45.54$ , VD  $153.69 \pm 78.29$ ; pg·mL<sup>-1</sup>), the CS pigs were hyperinsulinaemic compared to the VD animals (CS  $56.79 \pm 11.77$ , VD  $23.55 \pm 9.71$ ; pg·mL<sup>-1</sup>;  $p = 0.037$ ). There was no difference in the glucagon : insulin ratio of the piglets on day 7 (CS  $2.17 \pm 1.03$ , VD  $7.82 \pm 5.65$ ;  $p = 0.212$ ).

### *Thyroid Hormones:*

The plasma concentration of triiodothyronine (T<sub>3</sub>), a potent mediator of metabolism post-partum, was determined immediately after birth. Plasma T<sub>3</sub> (CS  $1.43 \pm 0.09$ , VD  $3.75 \pm 1.78$ ; nmol·L<sup>-1</sup>;  $p = 0.003$ ) was significantly lower in the CS animals. After 7 days there were no differences in plasma concentration of T<sub>3</sub> (CS  $0.63 \pm 0.18$ , VD  $0.84 \pm 0.15$ ; nmol·L<sup>-1</sup>;  $p = 0.417$ ).

### *Hepatic Lipid Metabolism:*

Consistent with a role for mode of delivery in the development of hepatic steatosis in response to TPN, CS piglets accumulated more hepatic lipid, 7 days after birth, than VD piglets (Fig. 1). Indeed the amount of lipid in the liver of CS piglets was 5.2mg/g tissue, above that for a clinical definition of steatosis. In contrast for the VD piglets it was 3.5 mg/g, which is significantly below the clinical definition of steatosis. While in none of the VD piglets was a hepatic lipid in excess of 5mg/g measured. Comparison of the hepatic lipid showed no difference in phospholipid, cholesterol or NEFA, but markedly higher TAG and cholesterol esters in the CS piglets (Table 1). In addition to elevated hepatic cholesterol esters, plasma cholesterol was lower in CS piglets (CS  $1.04 \pm 0.25$  vs. VD  $1.73 \pm 0.32$  mmol·L<sup>-1</sup>;  $p = 0.046$ ), suggesting that mode of delivery affects cholesterol metabolism. In spite of the elevated hepatic TAG in the CS piglets, there was no difference in plasma TAG levels between the piglet groups.

It was possible that differences in hepatic lipid content at birth were responsible for the difference in lipid on day 7. A comparison of the hepatic lipid content of CS and VD piglets within 1 hour of birth showed that the livers of CS piglets contained less lipid than VD piglets at this time point (Fig. 1), consistent with the possible accumulation of fuel stores just prior to delivery. Therefore, over the 7 days of TPN, CS piglets accumulated more hepatic lipid than VD piglets.

Despite being on the same diet, the liver fatty acid profiles were substantially different between CS and VD piglets (See Table 2). The proportion of the different fatty acids present in the livers of the CS piglets was similar to that of the TPN solution but markedly different from that in the livers of VD piglets. These data suggest that the metabolism of lipids in the livers of the CS piglets is relatively passive, whereas there is marked fatty acid modification in the livers of the VD piglets. For example, the proportion of total saturated fatty acids was the same in the diet and the livers of CS piglets but there was a marked increase in the proportion of saturated fatty acids in the livers of the VD piglets (diet; 26.29 g·100g<sup>-1</sup>; CS; 26.98, VD 37.73, g·100g<sup>-1</sup>). Delivery by CS also resulted in a smaller ratio of stearic acid (18:0) to palmitic acid (16:0) (CS 0.58±0.02 vs. VD 1.17±0.06  $p < 0.001$ ). Similarly, the ratio of oleic acid (18:1 (ω9)) to stearic acid (18:0), which has been used previously as an index of Δ9-desaturase activity [30], in CS livers was the same as the composition of the diet (CS 1.90±0.19 vs. Diet 2.00). However, this ratio was significantly lower in the livers of VD animals (0.71±0.11;  $p = 0.003$ ). As hydrogenation of oleic acid is not known to occur *in vivo*, these data suggest reduced elongation of palmitic acid and higher Δ9-desaturase activity in CS piglets.

Analysis of the polyunsaturated fatty acids was also consistent with reduced elongase activity in the CS delivered piglets. For example, the proportion of 20:4ω6 and 22:6ω3 were much lower in the CS piglets, whereas their respective essential precursors (18:2ω6 and 18:3ω3) were significantly higher. Furthermore, the ratios of both 20:4ω6/18:2ω6 and 22:6ω3/18:3ω3 are much lower in CS than in VD piglets (CS 0.14 vs VD 0.50 and CS 0.92 vs VD 4.96, respectively). These findings indicate that in addition to lower elongase activity, the Δ6 and Δ5 desaturases are also less active in the CS piglets.

#### *Liver Metabolomics:*

To determine the effects of delivery mode on carbohydrate and amino acid metabolism, we compared the metabolic profiles of aqueous liver extracts by <sup>1</sup>H NMR spectroscopy. Multivariate statistical analysis by partial least squares-discriminant analysis (PLS-DA) of this data showed clear separation of metabolic profiles between the CS and VD groups (Fig. 2A). Analysis of the loading plots identified succinate, aspartate, oxaloacetate, α-ketoglutarate, glucose and glutamate as contributors to the separation. Independent integration of the peaks corresponding to these metabolites revealed higher (approximately 50%) oxaloacetate, aspartate, α-ketoglutarate, and glutamate and significantly lower glucose and succinate in the livers of CS piglets fed for 7 days by TPN (Figs. 2B and C).

#### *Hepatic Enzyme Activity:*

To determine whether the changes in metabolite levels were accompanied by changes in hepatic enzyme activity we measured the activity of succinate dehydrogenase, malate dehydrogenase and phosphoenolpyruvate carboxykinase. Delivery by CS resulted in lower succinate dehydrogenase activity and higher malate dehydrogenase activity but did not alter PEPCK activity (Fig 3A). The lack of change in PEPCK activity suggests that mode of delivery does not alter the use of oxaloacetate for gluconeogenesis.

Glycerol derived from glyceride metabolism is an alternative source of carbon for gluconeogenesis, and the lower levels of hepatic lipid in the VD piglets indicates its use for gluconeogenesis in these animals. The carbon for gluconeogenesis from lipids only comes

from glycerol. We measured both glycerol content and glycerolphosphate dehydrogenase activity in liver extracts. Hepatic glycerol content was lower (Fig 3B) and glycerolphosphate dehydrogenase activity was higher in VD piglets (Fig 3A). This data suggests that glycerol in the VD piglets was converted into glucose, whereas in the CS piglets the glycerol was converted into lipid. There is very little adipose tissue in the neonatal piglet making adipose tissue an unlikely source of the glycerol and suggesting that the glycerol is produced by hydrolysis of the TAG provided in the lipid emulsion during TPN.

#### *Liver Gene Expression:*

As the animals received the same diet for the 7 days it seemed likely that the differences in metabolism were underpinned by gene expression patterns that resulted from the mode of delivery. We therefore compared gene expression using RNA microarrays and found that 7 days after delivery 118 genes were expressed at least 2 fold lower (32 were expressed more than 4 fold lower) and 243 genes were expressed at least 2 fold higher in the livers of the CS piglets (46 more than 4 fold higher) (see Table 3 and Supplementary Tables II & III).

Twenty of the genes expressed at lower levels in the CS piglets were associated with inflammation. Of these 14 were readily identifiable as interferon inducible genes and these constituted 3 of the 4 most markedly reduced genes. Furthermore, interferon  $\gamma$  was suppressed (1.9 fold) in the CS animals an observation consistent with previous demonstrations of reduced interferon  $\gamma$  in CS compared to VD piglets [31] and in response to CS delivery compared to normal or assisted delivery in humans [32]. The fourth gene in this group of most highly suppressed genes (approximately 30 fold) encoded the orexigenic peptide galanin-like peptide (GALP). Analysis of a 2Kb fragment of the promoter of this gene using TESS (<http://www.cbil.upenn.edu/tess>) showed that it contained a consensus sequence for the interferon responsive transcription factors IRF1 and IRF2 implying that it too is an interferon responsive gene. Similarly, expression of another orexigenic peptide, angiopoietin-like 4, was lower in CS piglets and promoter analysis showed that it also contains IRF1/2 binding sites. Amongst the changes in inflammatory gene expression we also noted lower expression of markers of the innate immune system, including the p80 NK receptor in the CS piglets. To identify any inflammatory condition in the VD piglets we measured the haptoglobin concentration (a marker of immune response [33]) in the plasma on day 7 but found no significant difference between the groups (CS  $1.21 \pm 0.01$ , VD  $0.95 \pm 0.18$ ; ng/ml).

Twenty genes reduced in the CS piglets were associated with metabolism, of which 5 were involved in lipid metabolism. These genes were Apolipoprotein A1, PID1, lipoprotein lipase H, and StAR-related lipid transfer protein (START; a cholesterol responsive protein involved in cholesterol and lipid trafficking [34]). These observations suggest modified cholesterol handling by the CS piglets and consistent with this, expression of 3-hydroxy-3-methylglutaryl Co-enzyme A synthase determined by qRT-PCR was lower in CS piglets (CS  $9.18 \pm 3.30$ ; VD  $45.87 \pm 8.34$ ;  $p = 0.005$ ).

Of the genes more highly expressed in the CS animals, levels of calbindin and myo-inositol synthetase 1 were 100 and 20 fold higher respectively than in VD animals. Eight genes associated with inflammation including 5 antimicrobial peptides were also more highly expressed in the CS livers. There was higher expression of genes previously described as markers of hepatocyte stem cells (e.g. orosomucoid-1, PEG3, PEG10, NOPE, DUSP 9 MAGED1, MAGED4 and  $\alpha$ -fetoprotein) [35], suggesting that the livers of CS piglets were less differentiated than those of VD piglets. The livers of the CS piglets also showed higher expression of genes associated with lipid accumulation including adipose differentiation related protein (ADRP), hepatic lipase, two acyl CoA synthetases, ACAT2 (Acetyl-Coenzyme A acetyltransferase), sterol C5 desaturase and SDR7.



In addition to the inflammatory and lipid metabolism changes, there were significant differences in the expression of components of the activin signaling pathways. Expression of inhibin B was lower but that of the activin II receptor was higher in the CS animals implying a reduction in activin signaling after normal VD that did not occur or was reduced in response to delivery by CS. Previous studies using inhibin deficient mice have shown that increased activin signaling in the liver results in liver damage [36]. A further marker of liver damage,  $\gamma$ -glutamyl transferase, was also elevated in CS livers.

The array data was confirmed using qRT-PCR to analyze the expression of a subset of the genes which showed significant differences. This analysis showed that changes on the array were supported by changes in the RNA expression, as measured by qRT-PCR except for PID1 (Supplementary Table IV).

## Discussion:

### *Factors Underlying the Hepatic Phenotype:*

This study establishes mode of delivery as an important additional contributor to the development of hepatic steatosis with CS delivery resulting in the amount of hepatic lipid exceeding 5mg/g (the threshold for defining NAFLD in man [37]) compared to 3.5 mg/g in VD piglets. One possible factor that could influence hepatic lipid accumulation is calorific intake and it is known that birth by CS can reduce the suckling reflex and milk intake over the first few days of life [e.g. 38]. However, by using TPN fed piglets it was possible for us to ensure the dietary intake was the same in all animals, precluding differences in dietary intake as a factor influencing our findings. TPN is associated with an increased risk of steatosis in human babies and our data suggest that this response may be much larger in babies born by CS and fed by TPN than in those born vaginally.

In determining the mechanism leading hepatic steatosis in response to CS delivery we found that CS also has a significant impact on metabolism and gene expression that persists beyond the immediate post birth period. Analysis of the fatty acid profiles was consistent with higher  $\Delta 9$  desaturase activity (stearoyl CoA desaturase (SCD-1)) in the CS piglets. SCD-1 has been implicated in the accumulation of lipid in a number of conditions and mice that lack SCD-1 have reduced lipogenesis and are resistant to hepatic steatosis [39]. The lipid analysis also suggested that normal VD was required to increase fatty acid elongase activity and the activity of  $\Delta 5$  and  $\Delta 6$  desaturases. However, the contribution of the failure to increase the activity of these enzymes to the accumulation of hepatic lipid in CS animals remains to be established. The alterations in aqueous metabolites and enzyme activities that occur alongside the reduction in lipid accumulation were consistent with reduced activation of gluconeogenesis from lipid derived sources (i.e. glycerol) in CS delivered piglets. Consequently, in these piglets, glycerol is diverted into hepatic lipid rather than glucose (Fig. 4).

The proposed difference in gluconeogenic activity between the CS and VD animals is consistent with the altered insulin : glucagon ratio at birth and the raised plasma insulin concentration seen after 7 days. Other reports in the literature indicate that differential insulin : glucagon ratio between CS and VD neonates may arise as a result of the increase in stress related endocrine changes (increased catecholamine and cortisol production) associated with VD and which are absent during elective CS [8, 9]. However, a normal action of insulin is to activate *de novo* fatty acid synthesis but the similarity between the lipid profiles of the TPN solution and the lipid composition of the hepatic triacylglycerols suggest that the increased lipid is the result of storage of the lipid supplied and not synthesis. Any role therefore for the persistent hyperinsulinaemia in the CS piglets in the development of steatosis is therefore most likely to be due to the suppression of hepatic gluconeogenesis.

The metabolic changes are accompanied by changes in gene expression that are associated with fat accumulation. For example increased hepatic ADRP expression is one of the earliest markers of adipose differentiation [40, 41] and is associated with NAFLD [42]. Furthermore antisense inhibition of ADRP has previously been shown to reduce hepatic steatosis and insulin resistance in ob/ob mice [42]. Increased expression of the ACAT, acyl-Coenzyme A synthase long chain family member 5 and sterol C5 desaturase have also been shown to increase in insulin resistant mice with NAFLD [43] implying that the mechanisms resulting in hepatic lipid accumulation in our CS piglets may be similar to those found in NAFLD and the metabolic syndrome in man.

*Altered immune gene expression:*

In addition to altered metabolism, we also observed marked differences in the expression of genes associated with inflammation and activated by interferon in the CS compared to the VD piglets. A number of studies have shown that parturition itself is associated with a marked increase in inflammatory markers and leukocytes in the myometrium [44]. Furthermore, analysis of the fetal membranes has shown that although there is no increase in leukocyte number there is a marked acute inflammatory gene expression signature [45]. Previous studies have also shown reduced interferon production by white blood cells in humans born by CS [32] and reduced serum interferon levels in piglets 14 days after CS delivery [31]. In this study we show that the inflammatory gene signature is still present 7 days after birth in animals born by VD. However, our data on their own do not determine whether the lower expression of interferon and markers of the innate immune system in the CS piglets are the result of the increased lipid accumulation or a result of the birth process itself. Interestingly, CS has been shown to alter the activity of cell types associated with the innate immune system [46] and results in an increased risk of Type-1 diabetes [3] and asthma [6] also suggesting long term changes in the immune system as a result of CS.

It is possible that the reduced expression of genes associated with the innate immune system is important in the accumulation of lipid. The liver, has a major role in the innate immune system, in particular, the liver contains Kupffer cells (KC, a class of hepatic macrophages), natural killer (NK) cells and NKT cells and recently, it has become evident that the innate immune system may play an important role in the development and progression of NAFLD. In particular, studies using leptin deficient (ob/ob) mice, which develop symptoms of dyslipidaemia, insulin resistance and NAFLD, have demonstrated that KC dysfunction may play a role in the onset of NAFLD [47, 48].

It is not clear whether the changes in the expression of NK cell markers result from increased recruitment or differentiation of these cells. However, NKT cells express adrenergic receptors [49] and in response to catecholamines, the number of NKT cells increases [50]. Thus, lower secreted concentrations of noradrenaline during CS compared to VD [8] may result in lower NKT cell populations.

We also found marked elevation of two orexigenic peptides, GALP and angiopoietin like 4, and the presence of IFR binding sites in the promoters of these genes suggests that they may also be interferon response genes in the liver. This observation implies that the interferon response that occurs at birth may contribute to the stimulation or regulation of appetite. The expression and activity of GALP has previously been analyzed in the brain [51] and the role of the GALP expression that we observe in the liver remains to be established. However, it is possible that this hepatic expression is part of the initial sensing of a requirement to feed orally. As our animals were fed by TPN any effect of this difference in GALP expression was not quantifiable by measuring feed intake. However, consistent with this suggestion a reduced suckling reflex after CS delivery has been noted in humans [38] and rats [52]. It is not known whether normal levels of the peptide are established once the CS baby is fed enterally over an extended period of time or whether VD is required to instigate normal appetite control. There is some evidence to suggest that CS babies are more likely to become

overweight in later life [7], favoring the hypothesis that VD is required for optimal appetite regulation. It is interesting that leptin, like GALP and angiopoietin like 4, appears to have a role in regulating the inflammatory response of the liver [50] strengthening the association between appetite regulation and the innate immune system.

It is possible that the differences in hepatic lipid accumulation, gene expression and metabolism observed between CS and VD animals may be a direct effect of the procedures of CS delivery. Alternatively, the absence of both the hormonal and mechanical stimulation of VD in pre-labour CS delivered infants results in abnormal patterns of post-partum metabolism and physiology, that we observe. It seems to us most likely that the latter suggestion is the case and that the lack of exposure to the increase in stress hormones that accompanies normal vaginal delivery is the primary cause of the observed differences. Consistent with this suggestion stress hormones are known to be markedly lower following CS and these hormones are known to modify metabolism, and the differentiation or recruitment of innate immune cells in a manner consistent with our observations.

#### *Maturation or Mode of Delivery?:*

In the final few days of *in utero* life the fetus responds to changes in circulating concentrations of endogenous hormones, secreted from the maturing fetal endocrine glands, as well as exogenous maternal/placental hormones, which together act to increase fuel deposits and mature some of the organ systems to allow them to respond to the imminent change in environment at birth. It is possible that some of the differences we observe in our model are due to the lack of exposure to these changes in hormones. However, CS delivery was performed just 2 days prior to the expected delivery, as close to the window of natural birth as possible within our experimental system. By this time in the pig, cortisol levels are reported to have approximately doubled and reached a plateau prior to the surge in cortisol that accompanies delivery [53]. It therefore seems unlikely that further maturation events will be initiated before the surge in stress hormones that accompanies birth and that it is this surge in hormones that is responsible for the changes in metabolism and gene expression that we observe. It should be noted that in human pre-labour CS there is a longer period prior to delivery date suggesting that the livers of babies born by pre-labour CS will have experienced even less of the hormonal changes and may therefore be less mature than those of the piglets we studied.

#### *The Wider Implications:*

The effects of mode of delivery on metabolism and gene expression that we observe are present 7 days after birth and suggest that VD is required for the liver to develop properly. As alluded to above, whether, given time, the liver will completely mature following CS remains to be established. However, it is possible that the lack of complete hepatic differentiation will lead to a propensity to develop NAFLD, insulin resistance and thereby a tendency to obesity and type 2 diabetes. At the present time there are no reported links between delivery by elective CS and type 2 diabetes, however, there is an association between type 1 diabetes and CS delivery that can be seen in humans [3]. As a number of risk factors associated with type 1 diabetes and type 2 diabetes are the same (e.g. high birth weight, obesity and catch up growth, [54]), it seems likely that CS delivery may also be associated with increased risk of developing type 2 diabetes.

In conclusion, our data indicate that in animals born by CS there is a markedly lower level of glycerolphosphate-dehydrogenase activity that reduces the entry of glycerol into the gluconeogenic pathway as illustrated in Figure 4. The excess glycerol is then re-esterified with the acyl CoA derived from the lipid supplied in the TPN solution facilitating fat storage and hepatic steatosis in the liver. Our data showing an association between mode of delivery and altered hepatic metabolism contributing to hepatic steatosis emphasise that the longer term consequences of Caesarian section on offspring hepatic metabolism now need to be tested.

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# Author Contribution:

In addition to reviewing the final manuscript MJH: experimental design, obtaining and analysis of samples, joint author of first draft of paper; JLG: metabolomic analysis revision and critical review of the manuscript; EH: liver lipid and fatty acid analysis, revision and critical review of the manuscript; CDB: data analysis, drafting the manuscript; LC: experimental design, supervision of the animal study, data analysis, revision and critical review of the manuscript; PRK: experimental design, supervised sample analysis, joint author of first draft of paper.

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# Figure Legends:

**Figure 1: Mode of delivery alters lipid and carbohydrate storage in the liver:** (A) Hepatic lipid content 7 days post-partum, in piglets born by CS (day 112 post-fecundation) or by VD (day 114) and fed by TPN (CS; n = 5, VD; n = 4). (B) Comparison of the hepatic lipid content of animals born by either CS (n = 6) or VD (n = 6) at birth, and after 7 days of parenteral nutrition (CS; n = 5, VD n = 4). All bars represent mean  $\pm$  SEM

**Figure 2: Mode of delivery modifies metabolomic profile 7 days post partum in piglets fed by TPN.** (A) PLS-DA analysis of <sup>1</sup>H NMR spectra of aqueous metabolites from parenterally fed piglets born either by CS ( $\square$ ; CSPN; n = 5) or VD ( $\triangle$ ; VDPN; n = 4). (B) Representative <sup>1</sup>H NMR spectra of aqueous liver extracts from piglets fed by TPN and born either by CS or VD. (C) Relative integral values for the peaks on the <sup>1</sup>H NMR spectra of aqueous liver extracts from piglets fed by TPN and born either by CS or VD. Peaks were individually integrated, lactate - 1.31-1.35 ppm; glutamate 2.34-2.35 ppm; glutamate/glutamine, 2.35-2.37 ppm; succinate, 2.39-2.41 ppm; aspartate, 2.62-2.68 ppm;  $\alpha$ -ketoglutarate, 3.00-3.02 ppm; glucose, 3.24-3.25 ppm. All bars represent mean  $\pm$  SEM, black bars represent CS 7 days post birth, grey bars represent VD 7 days post birth.

**Figure 3: Mode of delivery modifies glucose homeostasis:** (A) Mode of delivery modifies TCA and gluconeogenic enzyme activities in piglets fed by TPN (CS; n = 5, VD; n = 4). (B) Mode of delivery modifies liver glycerol content in the same animals. All bars represent mean  $\pm$  SEM. Black bars represent CS 7 days post birth, grey bars represent VD 7 days post birth

**Figure 4: Proposed mechanism by which CS results in hepatic lipid accumulation:** Following lipolysis of parenteral lipid, fatty acid and glycerol are taken up by the liver in the absence of mature adipose tissue in the CS neonate. Once in the liver, the reduced glycerolphosphate dehydrogenase activity reduced the utilization of glycerol for gluconeogenesis resulting in increased glycerol for triacylglycerol assembly and reduced glucose for export to the plasma. Little of the fatty acid is probably utilised in ketogenesis as piglets (and human neonates) have very limited ketogenic activity [55] and the lower glucagon : insulin ratio in CS piglets also supports the possibility of reduced ketogenic activity. Whilst there was no measurable difference in PEPCK activity in CS vs. VD piglets, the hyperinsulinaemia is likely to reduce gluconeogenesis from PEP, thus the requirement for  $\beta$ -oxidation of NEFA to provide energy for gluconeogenesis is likely to be small. Fatty acid and glycerol entering the liver are esterified to produce TAG which is stored in the liver, rather than being exported, resulting in fatty liver.



**Table 1: Mode of delivery alters the lipid composition of the liver 7 days post-partum.** Piglets were born by CS or VD and fed parenterally (CS; n = 5; VD; n = 4) for 7 days. Hepatic lipid composition was determined by thin layer chromatography followed by densitometry.

Lipid Class	Caesarean Delivered			Vaginally Delivered			<i>p</i> value
Phospholipid	14.32	±	2.97	17.46	±	2.48	0.390
Cholesterol	1.05	±	0.17	1.35	±	0.19	0.233
Non-esterified fatty acid	0.28	±	0.14	0.05	±	0.02	0.142
Triacylglycerol	8.98	±	1.99	0.75	±	0.79	0.007
Cholesterol ester	1.81	±	0.22	0.68	±	0.19	0.003

All values are mean (mg·g liver<sup>-1</sup>) ± SEM.

**Table 2:** Mode of birth significantly alters the fatty acid profile of the liver 7 days post-partum, in piglets fed parenterally with TPN, compared to the fatty acid composition of the diet.

Fatty Acid	Amount of Fatty Acid Present (g/100 g total fatty acids)			<i>p</i> value
	Diet	CS (n = 5)	VD (n = 4)	
14:0	0.72	0.62 ± 0.10	0.28 ± 0.05	0.007
16:0	15.75	16.67 ± 0.53	17.24 ± 0.52	0.207
16:0 (ω7)	2.03	1.90 ± 0.37	1.20 ± 0.40	0.096
17:1	0.16	0.15 ± 0.03	0.00 ± 0.00	0.001
18:0	9.82	9.69 ± 0.77	20.21 ± 0.95	<0.001
18:1 (ω9)	19.65	18.15 ± 0.33	14.17 ± 1.38	0.009
18:2 (ω6)	36.63	36.69 ± 1.39	23.46 ± 0.51	<0.001
18:3 (ω6)	0.58	0.45 ± 0.01	0.35 ± 0.13	0.216
18:3 (ω3)	2.13	2.66 ± 0.25	1.14 ± 0.14	<0.001
20:1 (ω9)	0.39	0.13 ± 0.15	0.00 ± 0.00	0.178
20:2 (ω6)	1.33	1.48 ± 0.13	0.84 ± 0.29	0.02
20:3 (ω6)	0.98	0.93 ± 0.02	0.80 ± 0.10	0.085
20:4 (ω6)	5.05	5.30 ± 0.58	11.63 ± 0.43	<0.001
20:5 (ω3)	0.36	0.32 ± 0.06	0.72 ± 0.08	0.002
22:1 (ω9)	0.20	0.03 ± 0.02	0.03 ± 0.03	0.443
22:4 (ω6)	0.83	0.88 ± 0.16	0.99 ± 0.26	0.340
22:3 (ω3)	0.45	0.45 ± 0.21	0.25 ± 0.13	0.196
22:5 (ω3)	0.34	0.43 ± 0.17	0.00 ± 0.00	0.012
22:6 (ω3)	1.73	2.46 ± 0.19	5.66 ± 0.37	<0.001

All values are mean ± SEM.

**Table 3;** Genes which are significantly ( $p < 0.01$ ) differentially expressed between the livers of CS and VD piglets fed parenterally for 7 days post-partum.

Accession Number	Primary Sequence Name	Our assignment	Fold Change
<b>Down-regulated by Caesarean delivery compared to vaginal delivery</b>			
<b>Metabolism</b>			
CO953054	Ssc.27557.1.S1_at	PID1	8.10
BX915625	Ssc.21663.1.A1_at	Transcribed locus LIPG (endothelial lipase)	6.22
CF176316	Ssc.17934.1.S1_at	Steroidogenic acute regulatory protein (StAR)	2.71
BQ604621	Ssc.6240.1.A1_at	Apolipoprotein A1	2.28
<b>Immune Response</b>			
NM_213825.1	Ssc.4875.1.S1_at	Galanin Like peptide (GALP)	27.16
NM_214061.1	Ssc.221.1.S1_at	Interferon-inducible protein p78	17.47
BI403807	Ssc.12825.1.A1_at	NK P80NK rec	8.79
AW313822	Ssc.2641.1.S1_at	ISG ligase UbcH8	8.23
CK465051	Ssc.12504.1.A1_at	ISG12a	6.91
BI119523	Ssc.30752.2.A1_at	Interferon induced with tetratricopeptide repeats	6.41
NM_213826.1	Ssc.336.1.S1_at	ISG15 specific protease	5.48
CF789161	Ssc.21162.1.S1_s_at	Interferon regulatory factor 7	3.29
CO953941	Ssc.29329.1.A1_at	Programmed death ligand 1 (activated by interferon)	2.68
AY293733.1	Ssc.4093.1.A1_at	Interferon-gamma	2.06
BI183736	Ssc.8980.1.A1_at	Angiopoietin-like 4	2.58
<b>Up-regulated by Caesarean delivery compared to vaginal delivery</b>			
<b>Ion transport</b>			
L13068.1	Ssc.429.1.A1_at	Calbindin D-9k	100.00
<b>Metabolism</b>			
CN157463	Ssc.15723.1.S1_at	Myo-inositol 1-phosphate synthase A1	13.35
BI403313	Ssc.12695.1.S1_at	Acyl-CoA synthetase long-chain family member 4	3.98
BQ604949	Ssc.21999.1.S1_a_at	Aldehyde dehydrogenase 3 family, member B1	3.96
BQ604008	Ssc.4426.1.S1_at	Acetyl-Coenzyme A acetyltransferase 2 (ACAT2)	3.55
AU058728	Ssc.6441.2.S1_at	SDR family member 7 (DHRS7)	2.94
BX675010	Ssc.19105.1.S1_at	Lipase, hepatic (LIPC)	2.79
BF710977	Ssc.7534.1.A1_at	Gamma-glutamyltranspeptidase	2.69
AY550037.1	Ssc.6323.1.S1_at	Adipose differentiation-related protein (ARDP) 1	2.19
CK450664	Ssc.10695.2.S1_at	Acyl-CoA synthetase short-chain family member 3 (ACSS3)	2.01
<b>Antimicrobial</b>			
CK464499	Ssc.20938.1.S1_at	Azurocidin 1 p	10.84
NM_214450.1	Ssc.27609.1.S1_at	PR39 protegrin family peptide	8.37
M81327.1	Ssc.13769.1.S1_at	Lactotransferrin	5.01
L24745.1	Ssc.420.2.S1_x_at	Homologue of proline/arginine rich antibacterial peptides	4.92
NM_213863.1	Ssc.420.1.S1_a_at	Antimicrobial peptide precursor (PG-2)	4.86
NM_213863.1	Ssc.420.1.S1_x_at	Antimicrobial peptide precursor (PG-2)	4.40
L39641.1	Ssc.420.4.S1_a_at	Antibacterial peptide precursor (PMAP37)	4.19

Cell cycle and signaling			
AF324155.1	Ssc.3831.1.S1_at	Corticosteroid binding globulin	4.55
Markers of fetal hepatocytes and paternally expressed genes			
CN162607	Ssc.13476.1.A1_at	Paternally expressed (PEG) 10	7.04
CK458546	Ssc.25001.1.A1_at	Dual specificity phosphatase (DUSP) 9	7.04
CK456429	Ssc.2397.1.A1_at	Neighbor of Punc E (NOPE) 11	5.54
CN165575	Ssc.3014.1.S1_at	Melanoma antigen family D (MAGED), 4	3.48
NM_001001860.1	Ssc.22086.1.A1_at	MAGED, 1	3.44
CK453813	Ssc.22623.2.S1_at	PEG 3	2.99
NM_214317.1	Ssc.7131.1.A1_at	Alpha fetoprotein	2.98
BF711624	Ssc.7669.1.A1_at	Orosomucoid 2 agp-2	2.47
BQ604762	Ssc.2112.1.S1_at	Putative DUSP4	2.29



Figure 1.

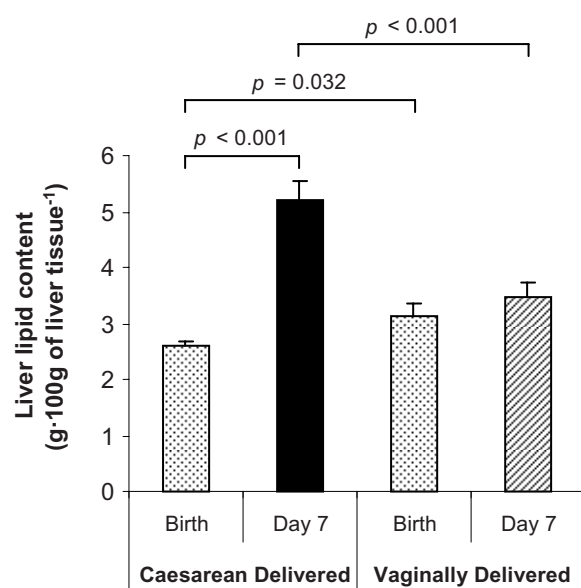


Figure 2.

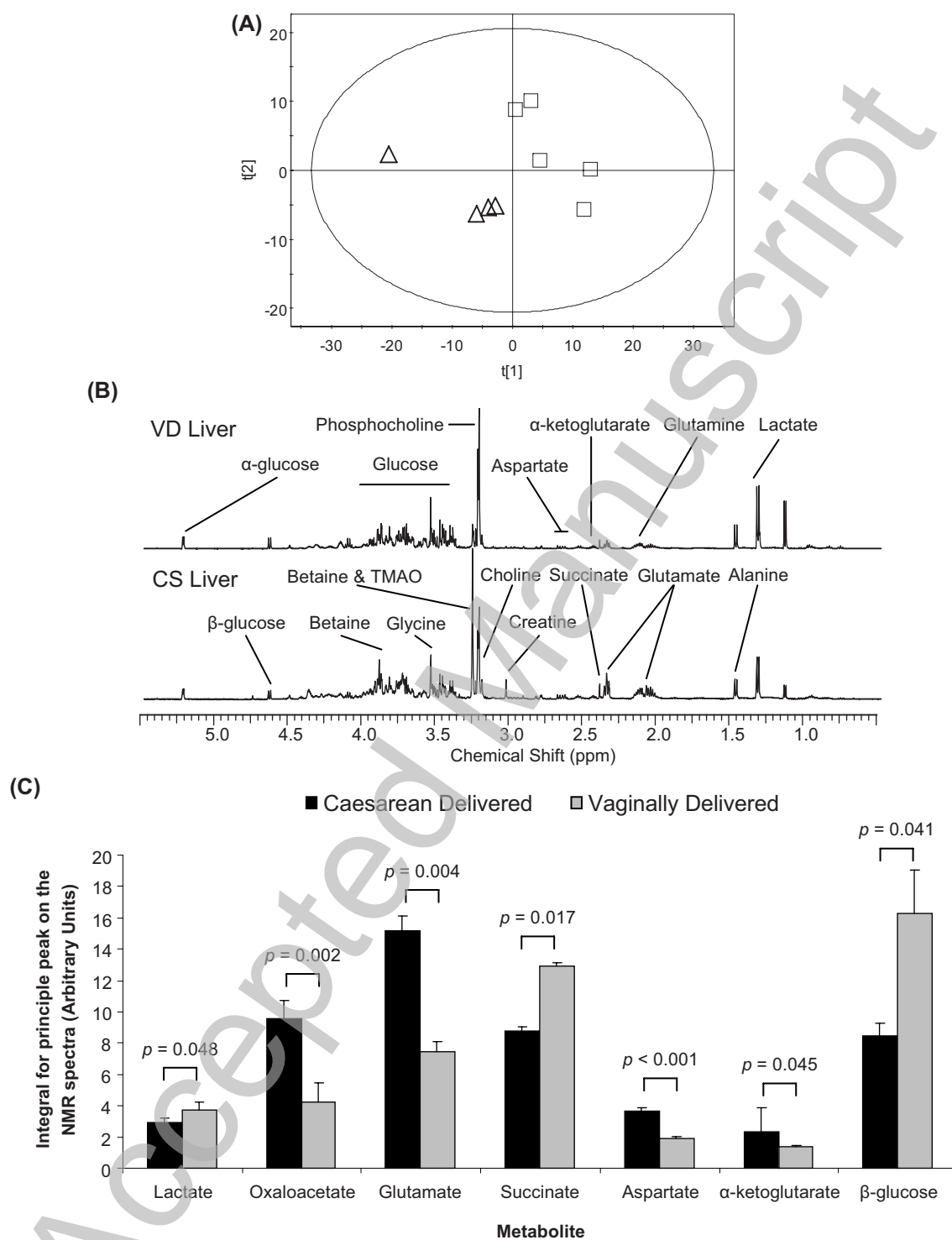


Figure 3.

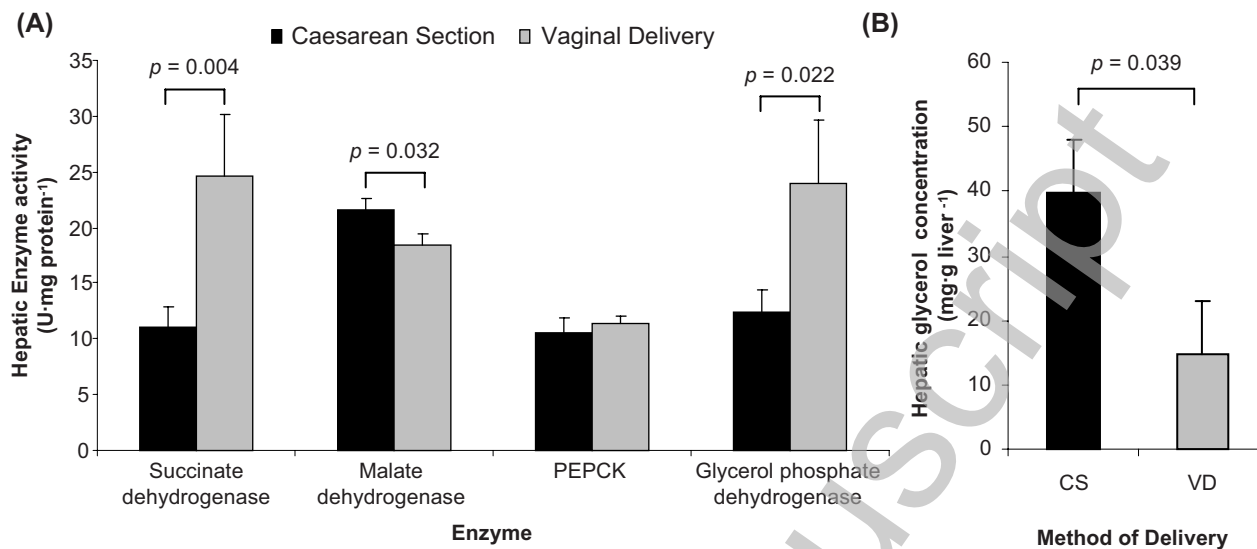


Figure 4.

