Hepatocyte Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) is Decreased by High-Fat Diet and Regulates Metabolism in Mice

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Keywords

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ABBREVIATION LIST

T2D	- Type 2 diabetes mellitus
ARNT	- Aryl hydrocarbon Receptor Nuclear Translocator
LARNT	- Liver-specific ARNT-knockout
ATP	- Adenosine triphosphate
HFD	- High Fat Diet
FC	- Floxed control
AhR	- Aryl hydrocarbon Receptor
HIF1-α	- Hypoxia-Inducible Factor-1-α
HGP	- Hepatic glucose production
HIF2- α	- Hypoxia-Inducible Factor-2-α
GTT	- Glucose tolerance test

ITT - Insulin tolerance test

PCT - Pyruvate challenge test.

TG -Triglyceride

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ABSTRACT

Objective: The liver plays a key role in whole-body metabolism, and liver dysfunction is a major component of type 2 diabetes (T2D) and the metabolic syndrome. Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) plays a role in β-cell function and is decreased in the liver of patients with T2D. This study examined the effects of long-term hepatocyte ARNT deletion on liver metabolism and metabolic homeostasis.

Research Design and Methods: Hepatocyte-specific *ARNT*-knockout (LARNT) mice were created using the Cre-LoxP system.

Results: LARNT mice had significantly increased fasting glucose, impaired glucose tolerance, increased glucose production after pyruvate challenge and markedly lower hepatic ATP than floxed littermate controls. *G6Pase*, *ChREBP*, *Fas* and *Scd-1* mRNAs were increased. Post-prandial triacylglycerides were increased in LARNT animals. High fat diet (HFD) significantly reduced hepatic ARNT in floxed controls (FC) and ameliorated differences in the metabolic phenotype between LARNT and control mice.

Conclusions: Taken together, these results demonstrate that ARNT regulates hepatic ATP content, lipid handling and hepatic glucose production (HGP). Decreased ARNT may play a role in the inappropriate HGP seen in T2D. The decrease in ARNT with HFD in controls may contribute to the development of glucose intolerance in susceptible individuals. Hepatic ARNT therefore, may be a therapeutic target to improve post-prandial hypertriglyceridemia and glucose homeostasis in T2D.

In recent decades there have been dramatic increases in the prevalence of type 2 diabetes mellitus (T2D) and the metabolic syndrome, which have paralleled the increased rates of obesity (1, 2). In healthy individuals, the liver prevents hypoglycemia by hepatic glucose production (HGP) from glycogenolysis and gluconeogenesis. The liver also plays a key role in regulating serum lipids (3). T2D is associated with inappropriate HGP when glucose is normal or even raised, and impaired insulin-mediated suppression of HGP (4, 5). Inappropriate HGP can result in elevated fasting glucose, a key feature of T2D. HGP is higher in diabetic patients even after controlling for higher circulating glucagon (6). T2D is associated with dyslipidemia, characterized by increased serum TG (triacylglycerides) and low HDL cholesterol, often with pronounced post-prandial hypertriglyceridemia (3, 7, 8). Dyslipidemia contributes to the increased risk of cardiovascular complications in people with diabetes or the metabolic syndrome.

Studies in animal models show that liver dysfunction can be sufficient to lead to T2D and components of the metabolic syndrome (5, 9, 10). The elucidation of the mechanisms leading to alterations of glucose and lipid metabolism in the liver is thus of importance to understanding the pathogenesis of T2D and the metabolic syndrome.

Aryl hydrocarbon Receptor Nuclear Translocator (ARNT) is a transcription factor. It heterodimerises with other bHLH/PAS family members including Hypoxia-Inducible Factor-1-α (HIF-1α), Hypoxia-Inducible Factor-2-α (HIF-2α) and Aryl hydrocarbon Receptor (AhR) to form active transcription complexes which regulate genes involved in hypoxic-responses, cell survival, proliferation, glycolysis, angiogenesis and response to xenobiotics (11-14). The endogenous ligand for AhR remains highly contentious; reports have suggested bile acids and cAMP and a recent paper suggests breakdown products from cruciferous vegetables (15-17). Interestingly, acute

increases in the level of liver HIF-1α and HIF-2α via Adenoviral-Cre mediated deletion of *von Hipplel-Lindau* (Vhl) factor in the liver leads to fatty liver together with decreased serum glucose levels (18). Both AhR activation and deletion lead to hepatosteatosis (19-21). It has also recently been reported that liver *HIF-1α* deletion leads to impaired glucose tolerance on high fat/sucrose diet (22).

We have previously reported that there is decreased expression of *ARNT* in isolated pancreatic islets and in the liver of patients with T2D (11, 23), and that short-term adenovirus-induced hepatic *ARNT* deletion caused increased HGP and impaired glucose tolerance (23).

To investigate the effects of long-term *ARNT* deletion in the liver, liver-specific *ARNT*-knockout (LARNT) mice were created using the Cre-LoxP system with Cre-recombinase driven by the albumin promoter. LARNT mice had decreased hepatic ATP, increased fasting glucose, increased HGP and worsened glucose tolerance. Liver triglyceride content was equivalent in fed but decreased in fasting LARNT animals. Post-prandially, serum triglycerides were significantly higher in LARNT mice. High fat diet (HFD) feeding of control mice decreased ARNT. This reduction in ARNT on a HFD resulted in a lessening of the differences between floxed control (FC) and LARNT animals.

EXPERIMENTAL PROCEDURES

Animal studies

All animals received humane care according to the criteria outlined in the "Australian code of practice for the care and use of animals for scientific purposes". Floxed ARNT mice were created as previously described (11, 24) and crossed with Albumin-Cre mice (a kind gift from David James, obtained from Jackson laboratories, Boston) to produce LARNT and floxed-control offspring. All mice were on an inbred C57Bl/6 background.

Physiological testing

Blood samples and physiological test samples were collected after an overnight fast (16 hours). For glucose tolerance tests (GTT) and insulin tolerance tests (ITT), mice were fasted then glucose (2g/kg body weight) or insulin (0.25U/kg) was given by intraperitoneal injection (IP). A tail nick was made and glucose levels were measured at the time-points shown using an Optium glucometer (Abbot Diabetes Care, Doncaster, Australia). For the pyruvate challenge test (PCT), mice were fasted and 2g/kg of pyruvate dissolved in phosphate-buffered saline was given intra-peritoneally (IP).

Mice were sacrificed after an overnight fast at least 1 week after the last physiological test. Livers were divided for formalin fixation or snap-freezing in liquid nitrogen for gene expression and lipid studies.

Gene expression analysis

Liver was homogenized in RLT buffer (Qiagen, USA). RNA was isolated and cDNA was synthesized as previously described (25, 26). Real-time PCR was performed

using specific primers and Sybr Green PCR master mix (Applied Biosystems, Melbourne Australia), and amplification was performed in an ABI7900 light-cycler. Results were corrected to the housekeeping gene TATA-box binding-protein (TBP) which did not differ between groups (data not shown). Primers are shown in Table 1.

Table 1.

mRNA	Fwd primer	Reverse Primer
Tbp	atgatgactgcagcaaatcg	tatcactcctgccacaccag
Arnt	tctccctcccagatgatgac	caatgttgtgtcgggagatg
Glut-1	acctatggccaaggacacac	ctggtctcaggcaaggaaag
Glut-2	catgctgagctctgctgaag	acagtccaacggatccactc
Gk	gagatggatgtggtggcaat	accagctccacattctgcat
Chrebp	gcatcctcatccgaccttta	gatgcttgtggaagtgctga
Pepck	ctaacttggccatgatgaacc	cttcactgaggtgccaggag
F16bp	gaccctgccatcaatgagta	gttggcggggtataaaaaga
G6pase	tcggagactggttcaacctc	acaggtgacagggaactgct
Fas	gaggacactcaagtggctga	gtgaggttgctgtcgtctgt
Scd-1	cctgcggatcttccttatca	gtcggcgtgtgtttctgag
Hmgs	gccgtgaactgggtcgaa	gcatatatagcaatgtctcctgcaa
Hmgr	caaagtttgccctcagttca	gtgccaactccaatcacaag
Pgc1a	gtcaacagcaaaagccacaa	tctggggtcagaggaagaga
Ppara	tggcgtacgacaagtgtgat	gtttgcaaagcctgggatag
Pparg	gaataccaaagtgcgatcaaagta	ccaaacctgatggcattgtgagac
Srebp1-c	gagccatggattgcacattt	ctcaggagagttggcacctg
Cpt-1	cttccatgactcggctcttc	agcttgaacctctgctctgc
Ir	taccgcattgagctgcaggc	aagacaaagatgaggggtcc

Irs-1	tcccaaacagaaggaggatg	cattccgaggagagcttttg
Irs-2	gtagttcaggtcgcctctgc	ttgggaccaccactcctaag
Akt2	cctgaggcttttctcaaacg	cctcagtctcagcctcatcc

Liver histology

Liver was dissected from floxed-control and LARNT mice and the left lobe from each mouse was fixed in 10% buffered formalin. Tissue was paraffin-embedded and 5μm sections were stained with hematoxylin and eosin (H&E) or Sirius Red according to standard protocols.

Insulin Assay

Insulin was measured using the Crystal Chem (Chicago, USA) ELISA kit as per the manufacturer's instructions.

Triglyceride (TG) Assays

Liver was homogenized (30-40mg per mouse) and used to determine total liver TG content which was expressed as μg/mg of liver. Serum and liver TG content was assayed using the Roche TG kit (GPO-PAP, Mannheim, Germany).

Western blot

Western blot for ARNT was performed as described previously (21). Alpha-tubulin was used as a loading control (Abcam, San Francisco, CA). Blots were quantitated by densitometry using ImageJ to calculate ARNT protein level relative to α-tubulin level.

Lipid oxidation

Primary hepatocytes were isolated as previously described (27) and cultured in 25cm^2 flasks. They were washed and incubated in Kreb's buffer plus 0.25% fatty acid free BSA (Sigma-Aldrich, St. Louis, MO) with 6mM glucose, 0.125mM palmitate and 0.25μCi/ml of [1-^{14}C]-palmitic acid (GE Healthcare, Port Washington, NY). Filter paper soaked in 5% KOH was suspended over the cells and the flasks sealed shut. Cells were incubated at 37°C with 5% CO_2 for 24 hrs and the reaction stopped with 500μl of 40% perchloric acid. Filter paper radioactivity was counted in 5ml Microscint-20 (Perkin Elmer, Waltham, Massachusetts, USA) using an LS 6500 Scintillation Counter (Beckman Coulter, Brea, CA, USA) and results were corrected for total protein.

High fat diet

Mice were placed on Hugo's high fat diet (HFD) as previously described (28). Mice were placed on the HFD for 20 weeks then sacrificed and samples collected as described. HFD mice were fasted for 6 hours before ITT otherwise assays were as described above.

Serum triglycerides

Serum was taken 6 hours after re-feeding high-fat fed mice which had been fasted overnight.

ATP assay

Intracellular ATP content was measured as previously reported (26).

RESULTS

Fasting glucose levels in LARNT mice

Fasting glucose levels were normal in floxed controls, and 42% higher in LARNT mice ($p<0.001$, **Figure 1A**).

Glucose tolerance was mildly impaired in LARNT mice

LARNT mice had mildly but significantly worse glucose tolerance than FC, shown in **Figure 1B**. Area under the curve of glucose tolerance was increased by 19% (P=0.026), **Figure 1C).**

LARNT mice had increased serum glucose following pyruvate challenge.

To examine the effect of hepatic *ARNT* deletion on HGP, PCTs were performed. Fasting glucose levels were higher, and glucose levels were significantly greater after pyruvate loading in LARNT mice (**Figure 1D**, p=0.005 by ANOVA for repeated measures). Because the fasting glucose was greater, the results are also presented as the AUC of the *increase* from baseline fasting glucose. This was 22% higher in LARNT mice following pyruvate challenge (**Figure 1E,** p=0.021). There were no differences in body weight **(Figure 1F)**.

LARNT mice had no significant change in whole-body insulin sensitivity

Fasting serum insulin did not differ between LARNT and floxed control mice (**Figure 2A**). To assess whole-body insulin sensitivity, ITTs were performed. Results are presented as percentage fall from baseline. LARNT and control mice had similar whole-body insulin sensitivity (**Figure 2B**).

ARNT deletion altered hepatic gene expression

Arnt mRNA was decreased to 20.4±1.4% of FC levels ($p<0.00001$) by real-time-PCR (**Figure 2C**). Expression of glucose transporters 1 (*Glut1*) and 2 (*Glut2*) were decreased and expression of the transcription factor Carbohydrate responsive element binding protein (*Chrebp)* was increased **(Figure 2C).** For the rate limiting steps in gluconeogenesis, there was a trend to increased Phosphoenolpyruvate carboxykinase (*Pepck)* expression (~20%, $p=0.07$), and Glucose 6-phosphatase *(G6Pase)* was increased by ~60% (**Figure 2D**). There were also significant decreases in mRNA for Insulin receptor (*Ir*) and Insulin receptor substrate 2 (*Irs2*), and trends to decreased Insulin receptor substrate 1 (*Irs1*) and *Akt2* (**Figure 2D**). In line with increased *Chrebp* expression LARNT mice had significantly increased levels of lipogenic genes Steroyl Co-A-desaturase 1 (*Scd-1*), HMGCoA-synthase (*Hmgs*) and a trend to increased Fatty-acid synthase (*Fas*) (**Figure 2E**). No changes were present in HMGCoA-reductase (*Hmgr*) (**Figure 2E**) or other lipid regulatory genes (**Figure 2F**).

Hepatic and serum TG were altered

Liver sections were stained with H&E or Sirius red. There were no changes in liver histology as assessed by H&E **(Supplementary Figure A and B).** Sirius red staining did not indicate any significant differences in liver collagen content **(Supplementary figure C and D).** Despite increases in the expression of lipogenic genes, liver TG content was 26% lower in LARNT mice after 16 hour fast **(Figure 3A).** To investigate the cause of the reduced TG content, we assessed rates of lipid oxidation in LARNT hepatocytes. **Figure 3B** shows that lipid oxidation was reduced in isolated

LARNT hepatocytes. ATP concentrations were correspondingly reduced in LARNT livers to 38.9 % of FC levels (P=0.0182) **(Figure 3C).**

We next assessed serum triglycerides. These were not different in fasting mice **(Figure 3D)**, but were significantly higher post-prandially (~40% increase, p=0.0187) **(Figure 3E)**.

HFD feeding decreased the differences between the phenotypes of FC and LARNT mice

To determine whether physiological stress worsened the phenotype, mice were placed on a HFD. At completion, body weights were equivalent **(Figure 4A)**. Surprisingly, there was no longer a significant difference in glucose tolerance **(Figure 4B).** Insulin sensitivity was now also equivalent **(Figure 4C)**. Glucose tolerance deteriorated as expected for both groups, with tendency to larger deterioration in the controls. A small difference persisted in response to pyruvate challenge but the AUC was no longer significantly different **(Figures 4D and E).**

Loss of phenotype corresponded to a loss of ARNT in control mice fed HFD

With our previous report that hepatic *Arnt* is decreased in people with T2D (23), we hypothesized that the loss of phenotype with HFD might be due to decreasing hepatic ARNT in controls. This was confirmed. HFD treatment reduced the level of *Arnt* to 71% of chow fed levels ($p<0.000652$) **(Figure 5A)**. Protein levels were examined by western blotting **(Figure 5B)** and were found by densitometry to be 52% of levels in chow fed mice (p=0.0129) **(Figure 5C).**

HFD feeding also decreased differences in triglyceride handling and ATP

HFD fed LARNT mice had a trend to reduced hepatic TG content after overnight fasting (p= 0.09) **(Figure 6A).** TG were equivalent in HFD LARNT and FC animals after fasting or re-feeding **(Figures 6B and C).**

DISCUSSION

Hepatocyte *ARNT* deletion resulted in impaired glucose tolerance, increased glucose production after pyruvate challenge and increased post-prandial serum TG. These changes are similar to changes seen in people with T2D and metabolic syndrome. Assessment of liver ATP and triglyceride content showed that both were significantly reduced in LARNT mice in fasted animals, however these alterations became non-significant with HFD feeding. HFD caused a reduction in ARNT in control animals, thus reducing the differences in ARNT expression as the LARNT mice were unaffected. This also suggests that the physiological differences were a result of decrease in hepatocytes, rather than other liver cell types.

People with T2D have increased HGP (6). PEPCK and G6Pase are critical enzymes in gluconeogenesis (29, 30) and PEPCK is increased in T2D (31). G6Pase catalyses the final step in gluconeogenesis and is increased in diabetic animals (30, 32). Taken together with the fact that *ARNT* is decreased in human liver from people with T2D (23) the increase in *G6Pase* and trend to increased *Pepck* in LARNT mice are consistent with a mechanistic role for ARNT in the increased HGP of T2D.

It has recently been demonstrated by magnetic resonance spectroscopy that both ATP and flux through ATP (fATP) are reduced in the livers of T2D patients compared to age matched controls (33, 34). Further, liver ATP correlated with hepatic insulin sensitivity even after controlling for hepatocyte lipid content (34). We found that hepatic *ARNT* deletion in LARNT animals resulted in significantly decreased liver ATP and this reduction in ATP occurred alongside increased HGP.

We previously reported an acute model of *ARNT* ablation using adenoviral delivery of Cre-recombinase (23). In common with short-term deletion, long-term loss of *ARNT* also led to alterations in gluconeogenic and lipogenic mRNAs in the liver, increased HGP and worsened glucose tolerance. Short term *ARNT* deletion led to reduced hepatic TG after fasting, similar to what we have shown with long term *ARNT* deletion. Interestingly a decrease in ARNT protein after streptozicin-induced diabetes was also reported (23). We also report here a reduced level of *Arnt* mRNA after HFD, similar to that seen in the liver of type 2 diabetic patients (23). This data is supportive of a role for reduced ARNT in HFD-induced diabetes. We hypothesize that this decrease may be mediated by ChREBP in response to increased glucose, as is the case in pancreatic β-cells (35).

The alterations in lipid handling following *ARNT* deletion are noteworthy in that despite hepatic insulin resistance, these mice showed reduced hepatic lipid content on fasting compared to controls. Lipogenic gene expression was increased in these animals and lipid oxidation was reduced in culture, suggesting that liver TG content should have been elevated. The mechanism for this effect is unclear, but may relate to alterations in lipid import into the liver. With feeding, serum TG increased in LARNT mice, suggesting that increased fatty acid synthesis and reduced lipid oxidation results in increased hepatic triglyceride export. As expected, the lipid handling differences were abrogated on high fat feeding which reduced ARNT levels in control mice.

These results demonstrate that ARNT is an upstream regulator of hepatic glucose and lipid homeostasis. The increased HGP, reduced hepatic ATP and increased fed serum TG in *ARNT* deleted mice together with the reduction in ARNT on a HFD in control

mice suggests that this factor plays an important role in liver metabolism, and in the pathogenesis of obesity induced-T2D and the metabolic syndrome. Taken together, these results suggest that normalizing hepatocyte ARNT levels may be beneficial for the treatment of T2D and the metabolic syndrome.

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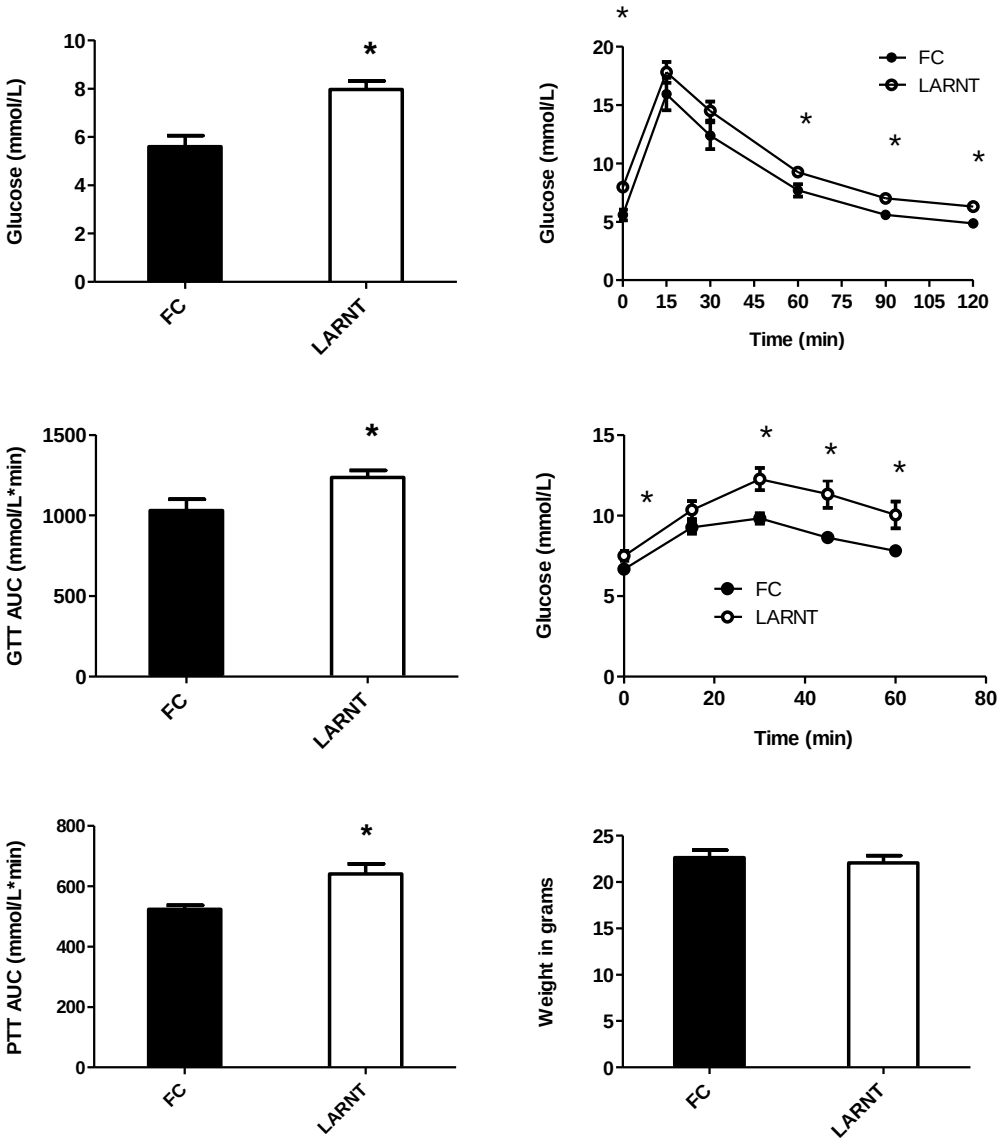


Figure 1. LARNT mice compared to FC mice on chow diet. Standard error bars are shown *p<0.05 FC mice are shown in black (bars or circles) and LARNT mice in white (bars or circles) N = 8-13 per group **(A)** Average fasting blood glucose levels in FC and LARNT mice. **(B)** Glucose tolerance test FC and LARNT. **(C)** Area under the curve of glucose tolerance test.. **(D)** Pyruvate challenge tests (PCT) in FC and LARNT KO female mice. **(E)** Area under the curve (AUC) of PCT on female mice. **(F)** Average weight of FC and LARNT mice in grams.

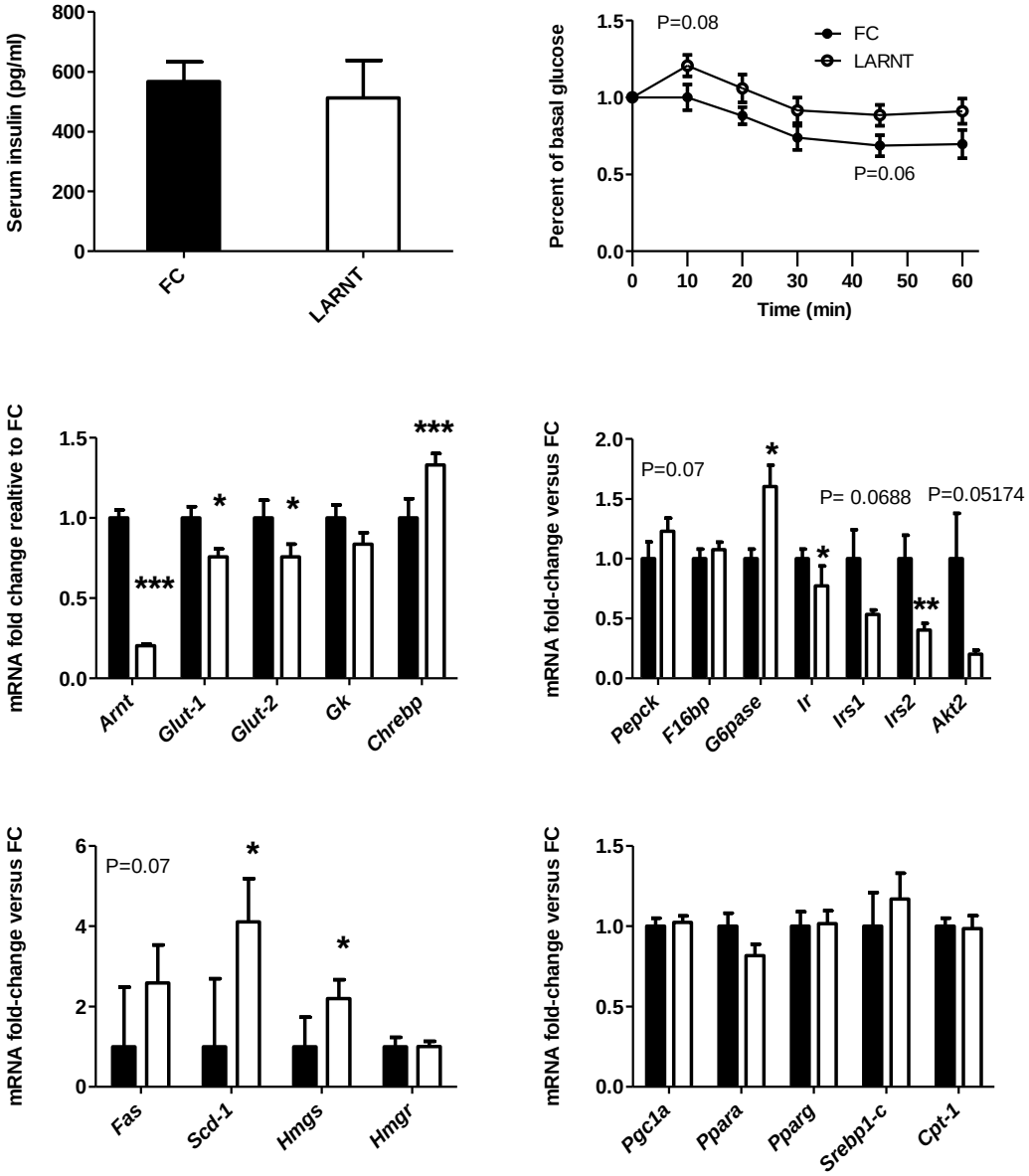


Figure 2. Insulin level, ITT and the effects of liver ARNT deletion on expression of gluconeogenic and lipogenic genes. . FC are shown in black (bars or circles) and LARNT mice in white (bars or circles) Standard error bars are shown *p<0.05. N= 5-6 **(A)** Average fasting serum insulin levels FC and LARNT mice. **(B)** Insulin tolerance test (ITT) of female FC and LARNT mice. Levels are expressed as a percentage of blood glucose level at 0 min. **(C)** Relative mRNA levels of Aryl hydrocarbon nuclear translocator (*Arnt*), Glucose transporter 1 (*Glut-1*), Glucose transporter-2 (*Glut-2*), Glucokinase (*Gk*) and Carbohydrate response element-binding protein (*Chrebp*), N=5-6 **(D)** Relative mRNA levels for Phosphoenolpyruvate carboxylase 1 (*Pepck*), Fructose-1-6-bisphosphatase (*F16bp*), Glucose-6-phosphatase (*G6pase*), Insulin receptor (*Ir*), Insulin receptor substrate 1 (*Irs1*), Insulin receptor substrate 2 (*Irs2*) and *Akt2*,. **(E)** Relative mRNA levels of Fatty acid synthase (*Fas*), Stearoyl-CoA-desaturase (*Scd-1*), HMG-CoA-Synthase (*Hmgs*) and HMG-CoA-Reductase (*Hmgr*). **(F)** Relative mRNA levels of Peroxisome Proliferator-Activated Receptor-Gamma, Coactivator-1 Alpha (*Pgc1a*), Peroxisome Proliferator-Activated Receptor-Alpha (*Ppara*), Peroxisome Proliferator-Activated Receptor-Gamma (*Pparg*), Sterol regulatory element-binding protein (*Srebp1-c*) and Carnitine Palmitoyltransferase 1 (*Cpt-1*).. *p<0.05. **p<0.01, *** p<0.001

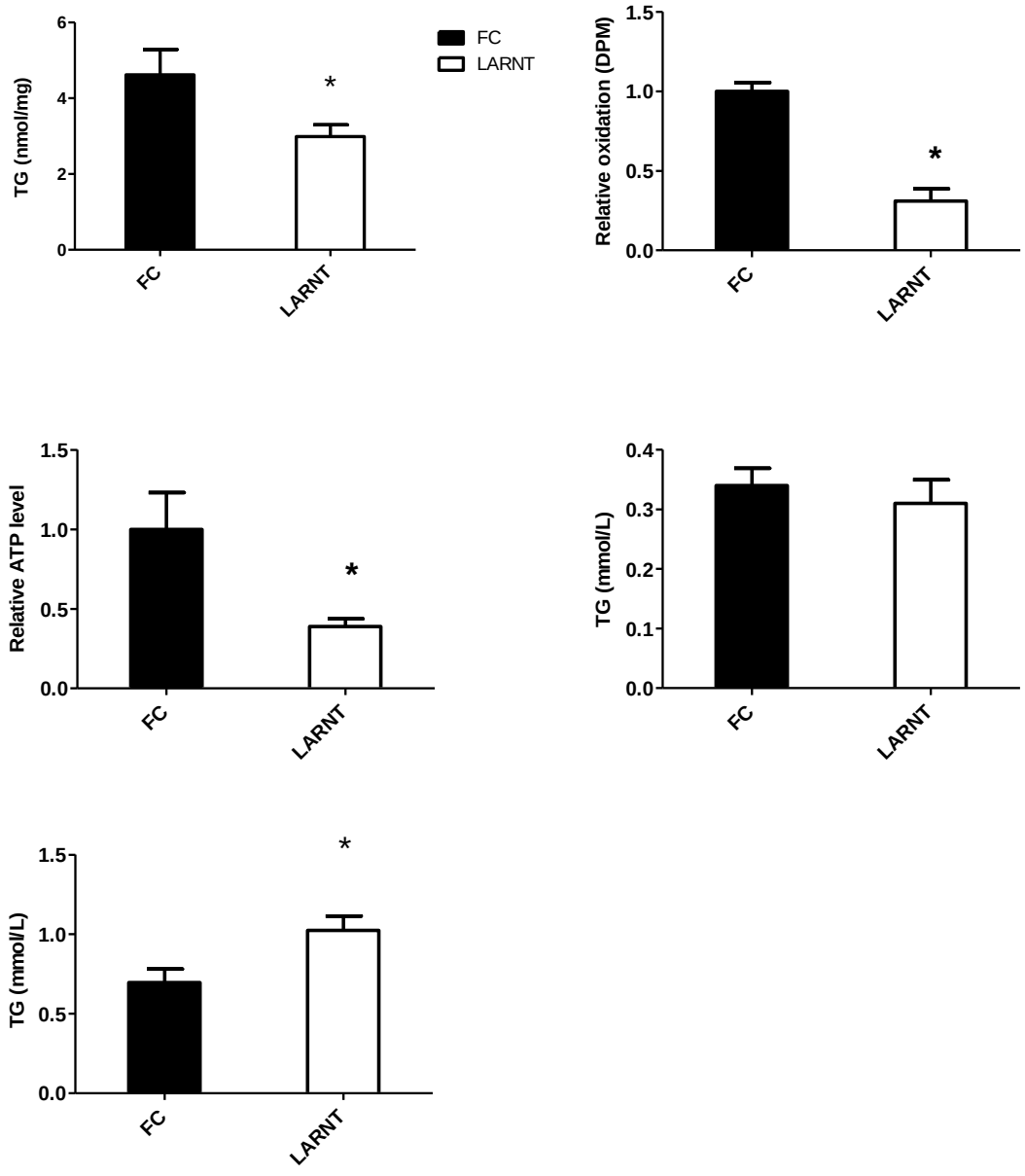


Figure 3. TG handling in LARNT mice compared to FC. Standard error bars shown in all figures. *p<0.05. FC are shown in black bars and LARNT mice in white bars **(A)** Liver TG levels in FC and LARNT mice. Liver TG in FC and LARNT mice are shown the fasted state. **(B)** Lipid oxidation in isolated hepatocytes from FC or LARNT animals. **(C)** Whole liver ATP levels in fasted LARNT compared to FC animals. **(D)** Serum TG's in FC and LARNT animals after a 16hour fast. **(E)** Serum TG in FC and LARNT animals 6 hours after feeding.

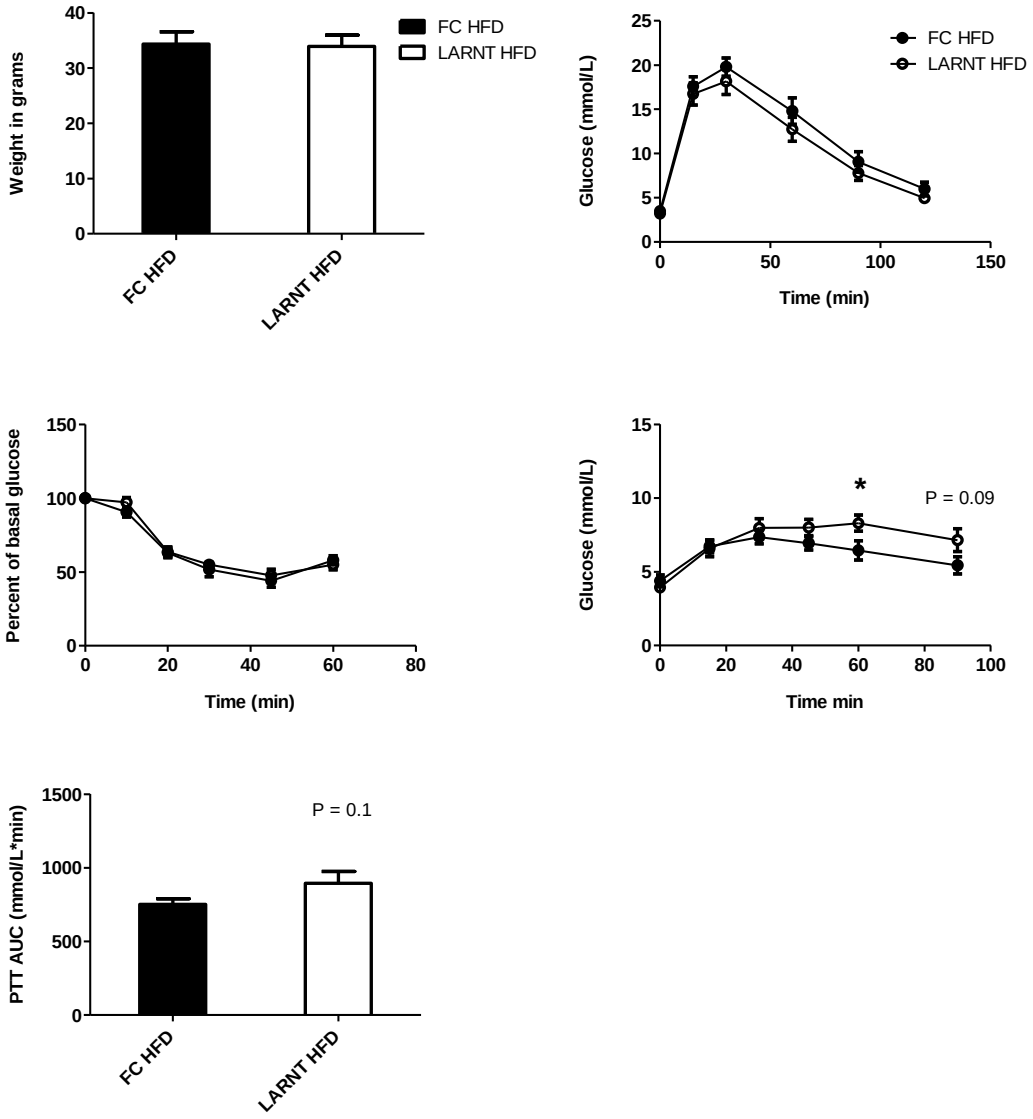


Figure 4. LARNT compared to FC mice on HFD. FC are shown in black bars/ circles and LARNT mice in white bars/circles. Standard error bars are shown. * p <0.05. **(A)** Weight did not differ in FC and LARNT mice fed HFD. **(B)** GTT after HFD. **(C)** ITT of FC and LARNT mice after HFD. Levels are expressed as a percentage of baseline blood glucose. **(D)** PCT in FC and LARNT KO female mice after HFD. **(E)** Area under the curve (AUC) of PTT. *p<0.05.

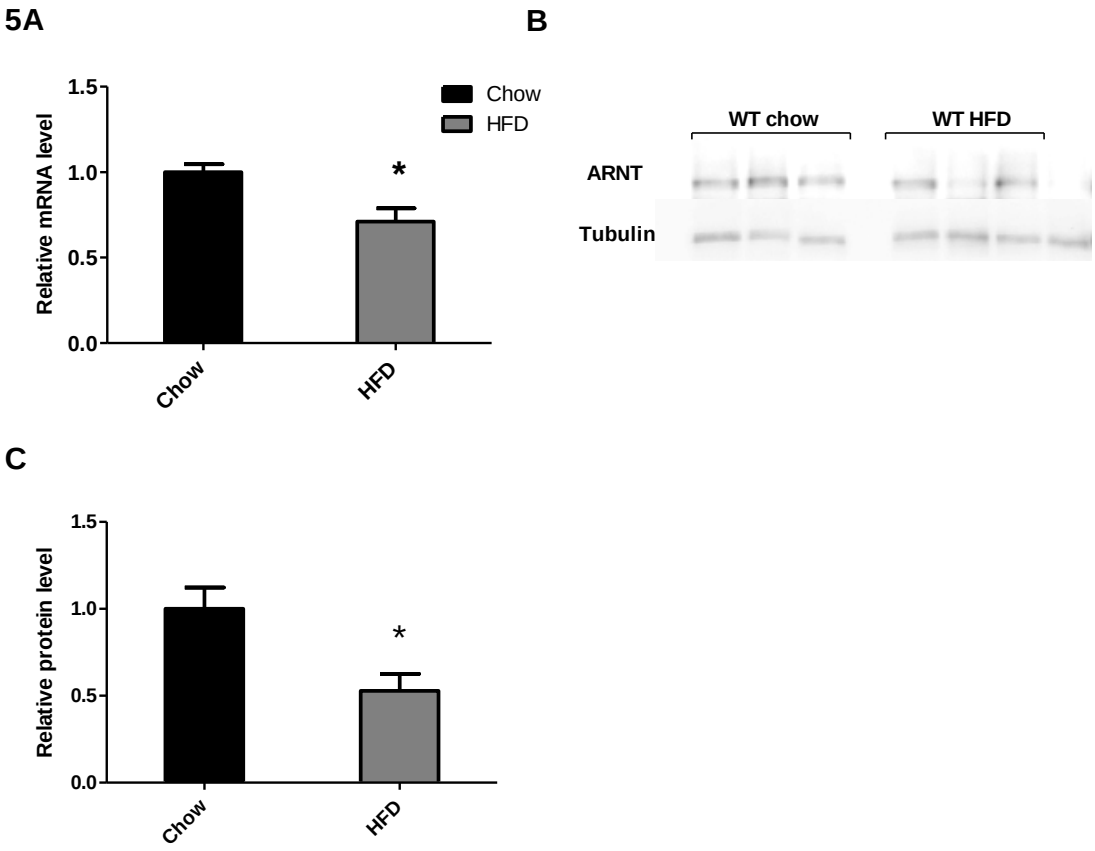
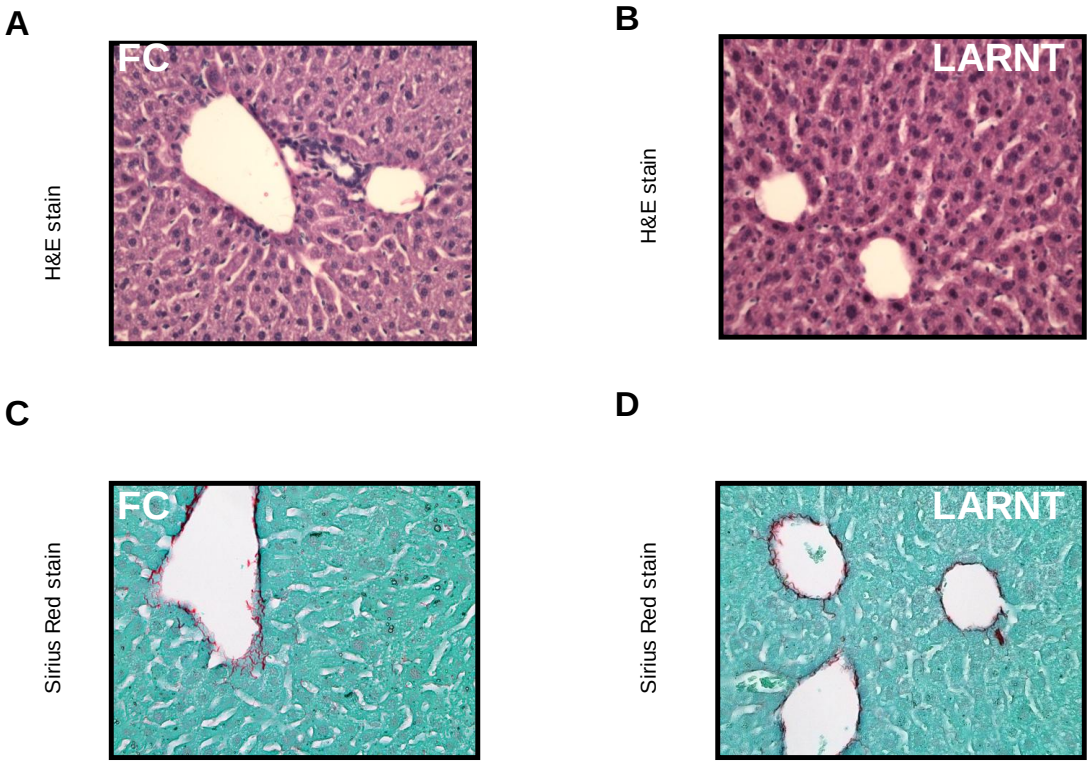


Figure 5. Changes in ARNT expression after HFD. Standard error bars are shown *p<0.05 **(A)** ARNT mRNA in FC animals fed chow (black) or HFD (grey). **(B)** Representative western blot for ARNT in WT chow fed compared to HFD fed animals. α-tubulin shown as loading control. **(C)** Densitometry of ARNT western blot. ARNT levels were normalized to tubulin expression. Relative levels shown.



Supplementary Figure 1 Haemotoxylin and Eosin staining of liver sections from FC **(A)** and LARNT **(B)** mice. Sirius Red staining from FC **(C)** and LARNT **(D)** mice.