Tissue specific regulation of sirtuin and nicotinamide adenine dinucleotide biosynthetic pathways identified in C57Bl/6 mice in response to high-fat feeding

Janice E. Drew, Andrew J. Farquharson, Graham W. Horgan, Lynda M. Williams

PII: S0955-2863(16)30360-6
DOI: doi: 10.1016/j.jnutbio.2016.07.013
Reference: JNB 7618

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 17 December 2015
Revised date: 16 June 2016
Accepted date: 15 July 2016

Please cite this article as: Drew Janice E., Farquharson Andrew J., Horgan Graham W., Williams Lynda M., Tissue specific regulation of sirtuin and nicotinamide adenine dinucleotide biosynthetic pathways identified in C57Bl/6 mice in response to high-fat feeding, *The Journal of Nutritional Biochemistry* (2016), doi: 10.1016/j.jnutbio.2016.07.013

Title: Tissue specific regulation of sirtuin and nicotinamide adenine dinucleotide biosynthetic pathways identified in C57Bl/6 mice in response to high-fat feeding

Short running title: Obesity, glucose intolerance and sirtuins

Janice E. Drew[1*], Andrew J. Farquharson[1], Graham W. Horgan[2], Lynda M. Williams[1]

[1]Rowett Institute of Nutrition and Health, University of Aberdeen, [2]Biomathematics and Statistics Scotland, Aberdeen, AB21 9SB, Scotland.

*Corresponding author:

Janice E. Drew,

Rowett Institute of Nutrition and Health,

University of Aberdeen,

ABERDEEN,

AB25 2ZD,

Scotland

Tel: +44 (0)1224 438775

Fax: +44 (0)1224 716629

Email: j.drew@abdn.ac.uk

Abbreviations: ABCA1, ATP-Binding Cassette, Sub-Family A (ABC1), Member 1, CD38, Cyclic ADP-Ribose Hydrolase 1, HDL, high density lipoprotein, HFD, high fat diet, HIC1, Hypermethylated In Cancer 1, LDL, low density lipoprotein, LFD, low fat diet, KanR, Kanamycin Resistance, NAD, nicotinamide adenine dinucleotide, NADSYN1, NAD Synthetase 1, Nampt, Nicotinamide Phosphoribosyltransferase , NNMT, Nicotinamide N-methyltransferase, Nmnat, Nicotinamide Nucleotide Adenylyltransferase , NMRK, Nicotinamide Riboside Kinase , NAPRT1, Nicotinate

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Phosphoribosyltransferase Domain Containing 1, PARP1, Poly (ADP-Ribose) Polymerase 1, PCA, principal component analysis, PNP, Purine-Nucleoside:Orthophosphate Ribosyltransferase, PSMB6, Proteasome (Prosome, Macropain) Subunit, Beta Type, 61, PPIB, Peptidylprolyl Isomerase B (Cyclophilin B) QPRT Quinolinate Phosphoribosyltransferase, QAPRTase, SIRT, sirtuin, T2D, type 2 diabetes, TDO2, Tryptophan 2,3-Dioxygenase, TNF, tumour necrosis factor, UBE2D2, Ubiquitin-Conjugating Enzyme E2D 2 UBCH5B,

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Abstract

The sirtuin/nicotinamide adenine dinucleotide (NAD) system is implicated in development of type 2 diabetes (T2D) and diet-induced obesity, a major risk factor for T2D. Mechanistic links have not yet been defined. Sirtuin/NAD system gene expression and NAD/NADH levels were measured in liver, white adipose tissue (WAT) and skeletal muscle from mice fed either a low-fat diet (LFD) or high-fat diet (HFD) for 3 days up to 16 weeks. An in-house custom designed multiplex gene expression assay, assessed all 7 mouse sirtuins (*SIRT1-7*) and 16 enzymes involved in conversion of tryptophan, niacin, nicotinamide riboside and metabolic precursors to NAD. Significantly altered transcription was correlated with body weight, fat mass, plasma lipids and hormones. Regulation of the sirtuin/NAD system was associated with early (*SIRT4*, *SIRT7*, *NAPRT1*, *NMNAT2*) and late phases (*NMNAT3*, *NMRK2*, *ABCA1*, *CD38*) of glucose intolerance. *TDO2* and *NNMT* were identified as markers of HFD consumption. Altered regulation of the SIRT/NAD system in response to HFD was prominent in liver compared to WAT or muscle. Multiple components of the sirtuins and NAD biosynthetic enzymes network respond to consumption of dietary fat. Novel molecular targets identified above could direct strategies for dietary/therapeutic interventions to limit metabolic dysfunction and development of T2D.

Key words glucose intolerance; high-fat diet; nicotinamide adenine dinucleotide; mouse; sirtuin

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

1. Introduction

An impaired sirtuin/nicotinamide adenine dinucleotide (NAD) system is linked to development of type 2 diabetes (T2D) [1-3]. Obesity and associated abnormal glucose and lipid metabolism, major risk factors for development of T2D, are also associated with reduced activity of the sirtuin/NAD system [1-3]. The specific components of the sirtuin/NAD system involved in diet-induced obesity and the development of glucose intolerance have not been established, but studies using global and tissue-specific knockout mice indicate the importance of the peripheral tissues liver, white adipose tissue (WAT) and muscle [2].

Previous studies in our lab identified distinct phases in development of glucose intolerance and inflammation in mice consuming a high-fat diet (HFD) in liver, WAT and muscle [4]. The mammalian sirtuins are proposed to be conserved nutritional sensors that operate in a concerted fashion to regulate glucose and lipid homeostasis and inflammatory responses [5-7]. While research has focused on *SIRT1,* all seven mammalian sirtuins (*SIRT1 – 7*) are likely to be involved in a molecular network orchestrating the response to glucose and lipids in a range of tissues [6]. Sirtuin regulation of glucose and lipid induced inflammation may be a mechanistic link in the impact of HFD on metabolic health [5]. Studies in our lab have identified postprandial induction of *SIRT1* gene transcription in parallel with inflammatory responses, with *SIRT1* gene transcription levels negatively associated with TNFα [8]. Sirtuin activity is dependent on the co-factor nicotinamide adenine dinucleotide (NAD). NAD biosynthesis is controlled by a number of enzymes, the dietary intake of tryptophan, niacin and nicotinamide riboside precursors, together with salvage pathways that direct biosynthesis from intermediate molecules [9].

Sirtuins are activated in response to metabolic stressors such HFD, while NAD biosynthesis is compromised at the same time [5,10,11]. This implies the potential for the system to become overwhelmed in response to chronic challenges. This may contribute to the development of

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

metabolic dysfunction. This is supported by studies showing that the sirtuin/NAD system is compromised by HFD, diet-induced obesity and metabolic stress [5,12,13]. Conversely, activation of the sirtuin/NAD system has been shown to improve fasting serum insulin and glucose and insulin sensitivity [3,14].

The liver, white adipose tissue (WAT) and muscle are all involved in glucose and lipid homeostasis and sirtuin regulated processes are known to be important in these tissues [2,15]. Studies in our lab indicated that while HFD can induce marked glucose intolerance within days, increased inflammatory markers in liver, adipose and muscle are initially transitory [4]. However, a second phase of increased glucose intolerance with long-term HFD is associated with marked increased adiposity and increased inflammation [4]. The potential role of the sirtuin/NAD system in the liver, WAT and muscle tissue in response to HFD in this process is not clear. Thus, the interplay between the seven members of the sirtuin gene family and the genes encoding enzymes that regulate the biosynthetic pathways to generate NAD is investigated in this study with a focus on transcriptional regulation to exploit the potential of this system in developing strategies to restore and maintain metabolic health.

2. Materials and Methods

2.1 *Animals*

The present study was conducted using tissue from mice used in our previous study [4]. Animal studies were licensed under the Animal (Scientific Procedures) Act of 1986 with approval from the Rowett Institute of Nutrition and Health's Ethical Review Committee. Briefly, male C57BL/6 mice (n=12 per diet group), 12 weeks of age were fed a HFD (60% of energy from fat) or a LFD diet (10% of energy from fat) (D12492 and D12450B, respectively, Research Diets, NJ, US) *ad libitum.* Sirtuin/NAD system components associated with our previous observations of distinct phases of the development of diet induced obesity and glucose intolerance [4] were examined in mice fed diets for

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

3 or 7 days (acute response) or 1, 4, 8, 12 or 16 weeks (chronic response). Food intakes, body weights, MRI scans (EchoMRI, Houston, TX, USA) and tissue collection (n=8-12) were as reported previously [4]. The mice were housed in a 12 h light/12 h dark cycle. All mice were sacrificed within a 3 hour period beginning at 2.5 hours from the start of the 12 hour light cycle.

2.2 *mSIRTNADplex assay*

Total RNA (liver, WAT, soleus muscle) extraction was described previously [4]. Total RNA (50 ng) was assayed (n=6–8) for sirtuin and NAD biosynthetic enzyme gene expression using our in-house custom designed assay, the mSIRTNADplex. The procedures for design and optimisation of the mSIRTNADplex and the selected gene targets and primer assays are detailed in Supplementary files S1 and S2 respectively.

2.3 *NAD/NADH assay*

NAD and NADH levels were measured using a commercially available fluorimetric assay kit (Abcam, Cambridge, UK) and a Spectra Max Gemini XS fluorimeter (Molecular Diagnostics) (fluorescence excitation 540nm/emission 590nm). NAD and NADH were extracted from tissue lysates (n=5–8) using the solutions suppled with the kit. NAD and NADH were specifically recognised and detected using an enzyme cycling reaction. Background fluorescence was subtracted from tissue lysate NAD, NADH and total NAD/NADH was then assessed using the standard curves generated with the NAD and NADH standard stock solutions supplied with the kit.

2.4 ELISA

Protein homogenates were prepared by bead-grinding ~20mg tissue with PreCellys 24 (Bertin Technologies, UK) in 400ul of phosphate buffer (pH 7.4). Homogenates were centrifuged at 5000 x g and aliquots of supernatants were frozen at -70 C until analysis. Protein concentration was estimated using Bradford Reagent (B6916 Sigma, UK) and serum bovine albumin standards with

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

absorbance measured at 595 nm. ELISA kits were used to assay SIRT4 (Wuhan EIAab Science Co.,Ltd., antibodies-online GmbH, Germany) and NMNAT3 (MyBioSource, San Diego, USA) in the prepared homogenates according to the manufacturer's instructions using standards supplied with the ELISA kits and a Microquant KC4 (Biotek Instruments Inc.) to measure absorbance at 450nm.

2.5 *Plasma hormones and lipids*

Plasma hormones, cytokines (insulin, leptin, IL-1β, IL-6, TNFα) and lipids (HDL and LDL cholesterol, triacylglycerol and non-esterified fatty acids) were assessed in the same mice (n=6–8) and reported previously [4].

2.6 *Glucose tolerance*

Intra-peritoneal glucose tolerance tests (IPGTTs) were performed on a separate group of mice (n=6-8), as reported previously [4].

2.7 *Statistical Analysis*

Principal Component Analysis (PCA) was performed using SIMCA-P+ 12.0 software (MKS Instruments UK Ltd, Cheshire) on normalised gene expression data. Two-way analysis of variance (ANOVA) was performed on normalised gene expression and protein data with diet and time as factors, using Gen Stat®13th Edition (VSN International, Ltd., Hemel Hempstead, UK). A post-hoc Bonferroni or Tukey's correction for multiple comparisons of time points within an ANOVA was applied (significance level 0.05) to gene expression and protein data respectively. ANOVA was conducted on a log scale if data was skewed. Pearson correlations were calculated using Genstat. Pairwise comparisons of tissue NAD and NADH levels were conducted using Student's ttest (significance level 0.05).

3. Results

3.1 *Acute and chronic responses to HFD*

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Phenotypic data for the mice were reported previously [4] and are summarised here in the Graphical Abstract accompanying this manuscript. Briefly, body weight and adiposity of HFD mice increased significantly after 3 days of HFD and increased linearly with time when compared to LFD mice [4]. The acute response within 3 days of HFD and glucose intolerance assessed by intraperitoneal glucose tolerance tests (IPGTT) (50% increase in area under the curve, AUC) was attributed to increased caloric intake and fat content of the diet respectively. Glucose intolerance then improved to 30% after 1 week, remaining steady up to 12 weeks. After 12 weeks a further increase in glucose intolerance (60% AUC) was observed concomitant with WAT and muscle inflammation (chronic response) characterised by increased expression of inflammatory gene markers (see summary Graphical Abstract).

3.2 ***Transcriptional responses of SIRT/NAD system to HFD in liver, WAT and muscle***

The mSIRTNADplex gene expression profiles were assayed in liver, WAT and muscle from mice fed a HFD for 3 or 7 days (acute response) and for 1, 4, 8, 12 or 16 weeks (chronic response) to determine transcriptional responses of specific components of the sirtuin/NAD system involved in diet-induced obesity and the development of glucose intolerance. Gene expression data analysed using GeNorm (http://medgen.ugent.be/genorm/) identified *UBE2D2* as the most stable reference gene and all data was normalised to *UBE2D2.*

PCA of mSIRTNADplex gene expression profiles revealed distinctly different sirtuin/NAD system gene expression profiles associated with each tissue (Supplementary file S3 Figure S1). This was characterised by high levels of *QPRT* and *TDO2* in liver, *HIC1* and *PARP*1 in WAT and muscle and *NMRK2* in muscle. Tissue specific responses to HFD and differing effects associated with the duration of HFD were apparent from cluster patterns of mSIRTNADplex data from HFD compared to LFD mice in all three tissues, liver (Figure 1A-B), WAT (Figure 1C-D) and muscle (Figure 1E).

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

This prompted further interrogation of significant changes in gene expression. ANOVA confirmed tissue specific and temporal regulation of the sirtuin/NAD system in C57Bl/6 mice in response to a HFD with diet and time a main effect for expression of several genes (Supplementary File S4 Figure S2). In liver this comprised 15 genes influenced by diet as a main effect. *SIRT2* (p=0.017), *PPIB* (p<0.001), *SIRT1* (p<0.001), *SIRT7* (p=0.004), *PARP1* (p=0.022), *ABCA1* (p=0.047) and *NMNAT1* (p=0.011) were up-regulated in the acute phase (3 and 7 days) (Supplementary File S4 Figure S2A), but not during the chronic phase (1 - 16 weeks) (Supplementary File S4 Figure S2B). Conversely, *NMNAT3* (p<0.001) was up-regulated by HFD at 8 and 16 weeks (Supplementary File S4 Figure S2B). *HIC1* (p<0.021), *SIRT4* (p<0.001), *NAPRT1* (p<0.001), *NMNAT2* (p<0.033), *NAMPT* (p<0.018), *TDO2* (p<0.001) and *NNMT* (p<0.001) were up-regulated in HFD mice at all time points (Supplementary File S4 Figure S2). Notably, not all seven mammalian sirtuins transcripts are regulated in response to HFD (Supplementary File S4 Figure S2). Time was also a factor influencing gene expression, with *SIRT1* (p=0.002), *NADSYN1* (p=0.017), *QPRT* (p=0.002), *SIRT4* (p<0.001), *TDO2* (p<0.001) and *NNMT* (p<0.001) transcripts down-regulated in the liver of both LFD and HFD mice during the latter stages of the experiment (Supplementary File S4 Figure S2B).

In WAT HFD was indicated as a main effect for 7 gene targets. *PNP*, *NMNAT2* and *NMNT* were up-regulated. *PNP* (p=0.033) showed an early response to HFD at 3 and 7 days (Supplementary File S5 Figure S3A), while *NMNAT2* (p≤0.009) and *NMNT* (p≤0.004) were elevated in response to HFD throughout the period tested (Supplementary File S5 Figure S3). *SIRT5*, *SIRT6*, *SIRT4* and *NMNAT3* were down-regulated in response to HFD (Supplementary File S5 Figure S3). *SIRT5* (p=0.043) showed an early response to HFD at 3 and 7 days (Supplementary File S5 Figure S3A). While *SIRT4* (p=0.012) and *SIRT6* (p=0.009) were down-regulated in response to HFD within the period 1 to 16 and 1 to 12 weeks respectively (Supplementary File S5 Figure S3B). *NMNAT3* was down-regulated in response to HFD (Supplementary File S5 Figure S3) at 3 and 7 days (p=0.006) and within the period 1 to 16 weeks (p=0.17). Seventeen genes were changed in both LFD and HFD in response to time. *SIRT2* (p<0.001),

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

PPIB (p<0.001), *PSMB6* (p=0.021), *SIRT1* (p=0.01), *SIRT5* (p=0.034), *NADSYN1* (p=0.003), *PNP* (p<0.001), *NRMK1* (p=0.023), *SIRT4* (p<0.001) and *NMRK2* (p=0.002) were all down-regulated over time (Supplementary File S5 Figure S3B). *SIRT3* (p=0.028) and *SIRT7* (p=0.013) reached the lowest levels around week 12 (Supplementary File S5 Figure S3B). *ABCA1* (p=0.001), *NAMPT* (p=0.037) and *NMNAT3* (p=0.027) revealed variable expression with a tendency to reduce with time (Supplementary File S5 Figure S3B).

In muscle HFD was indicated as a main effect for thirteen gene targets. Significant increases in *HIC1* (p=0.007), *PSMB6* (p=0.009), *SIRT5* (p<0.001), *SIRT3* (p<0.001), *NAPRT1* (p=0.022) *NMRK1* (p=0.026), *NMRK2* (p<0.001), *CD38* (p<0.001), *NMNAT1* (p<0.001) and *NMNAT3* (p=0.002) were shown as a main effect of HFD (Supplementary File S6 Figure S4). Conversely, *SIRT7* (p=0.003), *ABCA1* (p<0.001) and *NAMPT* (p=0.007) decreased in response to HFD. Down-regulation of *PPIB* (p<0.001) and *PSMB6* (p<0.001) and up-regulation of *SIRT1* (p=0.001) were observed in the mice as main effects of time (Supplementary File S6 Figure S4).

More cautious statistical analysis (application of a post hoc Bonferroni correction) was applied to discriminate the most robust gene regulation responses to HFD in liver, WAT and muscle (summarised in Graphical Abstract). In liver significant increases in *SIRT4* (p<0.001) and *SIRT7* were apparent at 3 days of HFD (Figure 2A). *SIRT4*, *PPIB* and *NAPRT1* were significantly up-regulated in response to HFD at 7 days (p<0.001) (Figure 2A). *TDO2* and *NNMT* expression was higher in HFD mice at all but week 8 (p<0.001) (Figure 2B**)**. In contrast *NMNAT3* (p<0.001) only became significantly elevated following 8 weeks of HFD, remaining significantly elevated at 16 weeks (Figures 2B). *ABCA1* was the only transcript down-regulated with HFD, at 8(p<0.001) and 12 (p<0.004) weeks in muscle (Figure 2D) and liver respectively (Figure 2B). Robust up-regulation in response to HFD was also observed for *NMNAT2* (p<0.003) in WAT at 7 days of HFD (Figure 2C) and *NMRK2* (p<0.001) at 1 week and *CD38* (p<0.001) at 16 weeks in muscle (Figure 2D). In theory it would be expected that

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

significantly altered gene expression between LFD and HFD mice should be the same in the 7 day (acute phase experiment) and 1week (chronic phase experiment) groups. However, while this is not always the case it was noted that differences in gene expression are indeed similar, but fail to reach significance. This is readily explained by reduced statistical power for some genes and the use of Bonferroni adjusted p-values making the requirement for significance more stringent, unavoidably pushing up type II error rates.

3.3 *Correlated SIRT/NAD gene expression*

Several mSIRTNADplex genes exhibited correlated regulation with tissue type or HFD (Supplementary File S7 Table S2). *SIRT4*, *SIRT7* and *NAPRT1* gene expression is highly positively correlated in the liver (Figures 3A, 3B, 3C). In muscle *NMRK2* and *CD38* are significantly positively correlated (Figure 3D). Notably, co-regulated SIRT/NAD gene expression was often dependent on consumption of LFD or HFD. Positively correlated gene expression of *NMNAT3* with *SIRT 4*, *SIRT7*, *NAPRT1* and *NNMT* in liver was lost when mice were fed HFD (Table 1). Conversely, TDO2 positively correlated gene expression with *SIRT7*, *NAPRT1, SIRT 4* and *ABCA1* in liver was only apparent when mice were fed HFD (Table 1). NMNAT3 and TDO2 were negatively correlated in liver only when mice were fed HFD (Table 1). *ABCA1* also exhibited aberrant regulation with diet, being negatively correlated with *NMNAT3* and positively correlated *NAPRT1* only when fed HFD. Negatively correlated gene expression of *ABCA1* and *SIRT7* was only observed in liver of LFD mice (Table 1).

3.4 *Tissue specific SIRT/NAD gene regulation correlates with metabolic markers and HFD*

The genes exhibiting highly significantly altered expression in the liver, WAT and muscle in response to HFD demonstrate significant correlations with a number of metabolic markers (Supplementary File S8 Table S3). Correlation analysis demonstrates the reasons why *PPIB*, initially selected as a potential reference gene, proved unsuitable as a reference gene in this study. *PPIB* expression was negatively correlated with variables such as body weight (-0.43, $p<0.001$), fat mass (-0.45, $p<0.001$),

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

plasma HDL (-0.38, p=0.001), LDL (-0.35, p=0.003), leptin (-0.54, p=0.001) and insulin (-0.49, p=0.002) (Supplementary File S8 Table S3). Liver *NMNAT3* transcripts were observed to be significantly correlated with body weight (0.61, p<0.001), plasma HDL (0.70, p<0.001) and leptin (0.77, p=0.008) (Supplementary File S8 Table S3). *ABCA1* transcripts in liver exhibited a negative correlation with body weight (-0.37, p<0.001) and fat mass (-0.39, p<0.001) (Supplementary File S8 Table S3). *NMNAT2* expression in WAT was significantly positively associated with body weight (0.30, p=0.004), fat mass (0.30, p=0.004), plasma LDL (0.25, p=0.037), HDL (0.34, p=0.004), leptin (0.37, p=0.028), insulin (0.40, p=0.017) and basal glucose (0.31, p=0.024) (Supplementary File S8 Table S3).

However, other SIRT/NAD system genes demonstrate significant correlation with metabolic parameters dependent on dietary fat consumed, providing further evidence of deregulation of the SIRT/NAD system in response to HFD (Table 2). Deregulated transcriptional regulation in liver in response to HFD was demonstrated by the loss of significant positive correlated expression of *SIRT7* with LDL (LFD 0.53, p=0.001, HFD 0.29, p=0.077), *NAPRT1* with body weight (0.44, p=0.002, HFD -0.05, p=0.74), fat mass (0.49, p=0.001, HFD -0.05, p=0.749), plasma HDL (0.35, p=0.04, HFD -0.08, p=0.65) and leptin (0.67, p=0.001, HFD -0.24, p=0.345) and NMNAT3 with plasma and glucose (0.51, p=0.007, HFD -0.02, p=0.907) (Table 2). Conversely, several SIRT/NAD gene transcripts were significantly negatively associated with metabolic markers when mice were fed a HFD. These included liver *SIRT7* with leptin (LFD 0.05, p=0.83, HFD -0.51, p=0.032), *ABCA1* with plasma LDL (LFD 0.01, p=0.966, HFD -0.34, p=0.042), HDL (LFD -0.02, p=0.259, HFD -0.62, p<0.001), TG (LFD 0.11, p=0.529, HFD -0.42, p=0.01) and insulin (LFD -0.27, p=0.286, HFD-0.51, p=0.031), *TDO2* with plasma LDL (LFD -0.17, p=0.35, HFD -0.43, p=0.008), HDL (LFD 0.07, p=0.7, HFD -0.51, p=0.002), TG (LFD 0.05, p=0.78, HFD -0.54, p=0.001), leptin (LFD -0.40, p=0.35, HFD-0.65, p=0.003) and insulin (LFD -0.08, p=0.763, HFD-0.55, p=0.018) and NNMT with HDL (LFD 0.13, p=0.466, HFD -0.58, p<0.001) (Table 2). NMNAT2 in WAT was positively correlated with insulin when mice were fed a HFD (LFD -0.06, p=0.802, HFD 0.51, p=0.038) (Table 2). *NMRK2* expression in muscle was significantly

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

negatively associated with body weight (LFD -0.05, p=0.812, HFD -0.53, p=0.003), fat mass (LFD 0.18, p=0.334, HFD 0.53, p=0.003), plasma HDL (LFD -0.28, p=0.226, HFD -0.54, p=0.01) and basal glucose (LFD -0.20, p=0.536, HFD -0.61, p=0.037) in HFD mice only (Table 2). *ABCA1* expression in muscle was significantly negatively associated with body weight (LFD -0.42, p=0.021, HFD -0.01, p=0.964), fat mass (LFD -0.40, p=0.028, HFD -0.10, p=0.62) in LFD mice only (Table 2).

3.5 *Tissue NAD/NADH levels altered with HFD*

No significant changes in either total NAD+NADH, or NAD:NADH ratios were observed in comparisons of liver (n=5-8) from LFD or HFD mice at 3 days, 1 week or 16 weeks (data not shown). No significant changes in either total NAD+NADH were observed in comparisons of WAT from mice fed LFD or HFD at 3 days (Figure 4A), but HFD mice at 7 days had significantly increased total NAD+NADH (p=0.002) levels, a consequence of increased NAD (p=0.008) (Figure 4B). The NAD:NADH ratio was also significantly higher in WAT (n=5-8) from HFD mice at 3 (p=0.017) and 7 days (p=0.004) (Figure 4C). No significant changes in total NAD+NADH, or NAD:NADH ratios were observed in comparisons of muscle (n=3-6) from LFD or HFD mice at 1 week (Figure 4D). However, at 8 (p=0.069) (Figure 4E) and 16 weeks (p=0.0056) (Figure 4F) HFD mice had a reduction in total NAD+NADH. This indicated a reduction in NADH levels that was significant at 16 weeks (p=0.0096) (Figure 4F). An increased, but non-significant NAD:NADH ratio was also observed in muscle at 1 (p=0.08), 8 (p=0.16) and 16 (p=0.14) weeks of HFD (Figure 4G).

3.6 *SIRT/NAD system transcriptional responses and development of glucose intolerance and inflammation*

IPGTT revealed two phases in development of glucose intolerance in C57Bl/6 mice consuming a HFD [4] (and summarised Graphical Abstract). The first phase occurred within 3 days and was associated with an acute phase response [4] (Graphical Abstract). This was followed by an improvement in glucose tolerance then a stable period with no significant difference between total AUC at the

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

individual time points of 1, 4, 8 and 12 weeks [4] (Graphical Abstract). The second phase was characterised by a 60% increase in AUC together with increased inflammation in the WAT and muscle [4] (Graphical Abstract). The acute phase response was characterised by elevated plasma levels of the inflammatory cytokine IL6 and the acute phase proteins, alpha1-antichymotrypsin (aACT), haptoglobin and serum amyloid A (SAA) and increased transcription of *SERPIN3AN* and *SAA* in liver during the acute phase response [4]. This study demonstrates altered regulation of specific sirtuin/NAD genes in the liver (Supplementary File S4 Figure 2A) in association with the acute response. The most robust changes (Figure 2A) are up-regulation of *SIRT4* and *SIRT7* and the enzyme *NAPRT1* with NAD levels remaining stable. Conversely, NAD levels increase in WAT (Figure 4B) in association with *NMNAT2* up-regulation (Figure 2C).

The second phase of glucose intolerance developing between 12 and 16 weeks [4] (Graphical Abstract), is preceded by significant up-regulation of liver *NMNAT3* at 8 weeks, which is maintained at 16 weeks and a down-regulation of *ABCA1* at 12 weeks (Figures 2B). In WAT the chronic phase and associated inflammation (12 and 16 weeks) [4] does not appear to be associated with specific sirtuin/NAD system genes. Chronic phase elevations in muscle *IL1β*, *IL6* and *TNFα* and lipid [4] is preceded by up-regulation of *NRMK2* at 1 week, down-regulation of *ABCA1* at 8 weeks and up-regulation of *CD38* by 16 weeks of HFD (Figures 2D) and is associated with decreased muscle NAD levels (Figure 4F).

3.7 SIRT4 and NMNAT3 protein levels in liver

Transcriptional regulation of the SIRT/NAD system to consumption of HFD appears to be more responsive in the liver compared to WAT or muscle. Consequently, the levels of two significantly altered transcripts, *SIRT4* and *NMNAT3* were assessed in liver. Similarly to gene transcription the levels of SIRT4 were reduced significantly over time in the acute experiment (p=0.039) (Figure 5A). Whereas a significant time.diet interaction (p=0.015) (Figure 5A) was observed in the chronic

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

experiment. This was largely attributed to an increased level of SIRT4 at 16 weeks in HFD mice (Figure 5A). In contrast to gene expression NMNAT3 protein levels were significantly decreased in response to HFD in the acute experiment (p=012) (Figure 5B), while there was a trend to decreased NMNAT3 levels at 1 and 8 weeks with levels being equal by 16 weeks (Figure 5B).

4. Discussion

Studies have reported that the sirtuin/nicotinamide adenine dinucleotide (NAD) system is impaired in individuals with type 2 diabetes (T2D) and the associated risk factors, obesity, abnormal glucose and lipid metabolism [1-3]. The sirtuin/NAD system is complex and regulated at both transcriptional, post-translational and enzyme activity level. This study, focused on transcriptional regulation, set out to identify specific components of the sirtuin/NAD system involved in diet-induced obesity and the development of glucose intolerance using a previously validated mouse model that demonstrated two phases of development of glucose intolerance associated with diet induced obesity in liver, WAT and muscle [4]. Phased responses, with altered sirtuin/NAD gene transcription coincident with the acute and chronic responses to HFD were identified. In liver transcriptional responses of *SIRT4*, *SIRT7* and *NAPRT1* are prominent during the acute response, indicating an important role in SIRT/NAD system responses to HFD induced stress in liver. However, there is a lack of concomitant increases in SIRT4 protein levels until 16 weeks of HFD consumption when mice become glucose intolerant. The transcriptional response may be induced in an attempt to maintain SIRT4 levels that appear to initially decrease with consumption of HFD. *SIRT4* gene expression is no longer significantly elevated when SIRT4 protein levels are significantly elevated (Figure 5A). SIRT4 is a negative regulator of oxidative metabolism linked with suppression of enhanced SIRT1 and SIRT3 oxidative capacity [16,17] and is elevated in genetically diabetic mouse models [16]. Notably, changes in expression of *SIRT4* and *SIRT7* in response to HFD are positively correlated with *NAPRT1* (Figure 3). NAPRT1, an important salvage pathway enzyme catalysing conversion of nicotinic acid to β-nicotinic acid mononucleotide (β-NAMN), can alleviate responses to oxidative stress in liver [18]. Loss of NAPRT1

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

regulation with prolonged consumption of HFD is potentially important in contributing to the resulting loss of metabolic homeostasis. Penke *et al*., [19] report increased liver NAD levels in mice consuming high fat diet concomitant with increased NAMPT gene and protein expression. Increased expression of enzymes synthesising NAD, *NAMPT* (salvage pathway synthesis from nicotinamide), (*NAPRT1* (salvage pathway synthesis from nicotinic acid) and *TDO2* (*de novo* synthesis from tryptophan) were apparent in this study, but were not associated with increased NAD levels in liver. It is probable in our study that elevation of *NAMPT, NAPRT1* and *TDO2* contribute to a compensatory response to increased demand for NAD in liver of HFD mice and maintenance of liver NAD:NADH ratios. The study by Penke *et al*., [19] supports this conclusion. Supplementation with NAD precursors could provide added protection to the liver via this route [19]. Both *TDO2* and *QPRT*, highly expressed in liver compared to WAT and muscle, reflect the capacity of the liver to convert tryptophan to NAD. The positive correlation of *NAPRT1* and *TDO2* is lost during the development of obesity, perhaps indicating that increased demand for NAD is limited with increased duration of HFD. Indeed the levels of *TDO2* and *QPRT* tend to decrease from 1 – 16 weeks of HFD (Supplementary File S4 Figure S2). Thus it is likely that NAD/NADH ratios in liver will be adversely affected with continued HFD beyond the 16 week period tested in this study.

The second phase of glucose intolerance in response to HFD, occurring after 12 weeks [4], is preceded by a second phase of liver sirtuin/NAD system gene regulation. The salvage pathway enzyme, *NMNAT3*, is significantly elevated after 8 weeks of HFD and remains so for the remainder of the study. NMNAT3 is proposed to be a mitochondrial salvage pathway enzyme [20] generating NAD from β-nicotinic acid mononucleotide (β-NAMN) and β-nicotinamide mononucleotide (β-NMN). This was disputed with failure to detect NMNAT3 in the mitochondrial proteome [21] and speculation surrounding *NMNAT3* transcript variants [22]. Transcriptional elevation of *NMNAT3* and *TDO2* is not associated with increased levels of NAD in liver and is possibly a response to increased NAD demand and a loss of NMNAT3 protein. Increased *NMNAT3* transcription may be a response to falling levels

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

of NMNAT3 in liver of HFD mice (Figure 5B). The second phase of glucose intolerance is also characterised by significantly down-regulated *ABCA1* at 16 weeks of HFD. Hepatic ABCA1 is a rate-limiting enzyme in HDL biogenesis [23] reported to improve B-cell function and glucose tolerance [24]. Notably, ABCA1 down-regulation in liver is preceded by down-regulation in muscle and elevated plasma HDL and LDL cholesterol and triglycerides. These events may mark the prelude to loss of metabolic homeostasis and development of glucose intolerance in the chronic phase.

NNMT, elevated in HFD mice has been linked to development of obesity, diabetes and metabolic syndrome [25-27]. *NNMT* expression was not correlated with increasing fat mass in HFD mice, but is elevated when mice consume HFD. Research on *NNMT* has largely focused on its association with carcinogenesis [28,29]. Likewise *SIRT7* has also been implicated in carcinogenesis [30, 31]. The up-regulation of these transcripts in response to HFD may have implications for links between obesity and increased risk of hepatic cancer [32]. More recently NNMT has also been linked to metabolic benefits in liver with it's product N^1-methylnicotinamide reported to decrease serum and liver cholesterol [33]. However, although marked increases in *NNMT* expression was consistently observed in mice fed a HFD this did not prevent elevated serum cholesterol, but it is possible that up-regulation of the NNMT gene is an indication of liver responses to attempt to maintain homeostatic control of lipid homeostasis in response to consumption of a HFD.

Transcriptional regulation of the sirtuin/NAD system was less pronounced in WAT compared to liver. This is not surprising as previous analysis indicated gains in adiposity and inflammation are not seen in WAT during the acute response in the liver in response to HFD [4]. However, prolonged HFD resulted in increases in adiposity and inflammation in WAT between 12 and 16 weeks. This is preceded by an elevation of *NMNAT2* following 7 days on HFD, associated with increased levels of NAD and NAD:NADH ratios at 3 and 7 days of HFD. NMNAT enzymes generate NAD from the precursors, β-nicotinic acid mononucleotide (β-NAMN) and β-nicotinamide mononucleotide (β-

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

NMN). This may indicate an adaptation to changes in WAT in response to increasing adiposity. There was a strong correlation of *NMNAT2* with plasma insulin. Thus despite a lack of major changes in the sirtuin/NAD system in WAT, *NMNAT2* transcripts appear to be associated with inflammation in WAT and glucose intolerance.

Previous studies indicated that muscle tissue was not associated with the acute response to HFD [4]. The second phase of glucose intolerance in HFD mice revealed up-regulation of *NMRK2* (1 week) and *CD38* (16 weeks) and down-regulation of *ABCA1* at 8 weeks. NMRK2 converts nicotinamide riboside to the NAD precursor, β-NMN [34]. *NMRK2* expression is markedly higher in muscle compared to liver and WAT. NMRK2 increases NAD and insulin sensitivity [35]. High expression levels and robust responses to HFD provide evidence that NMRK2 is important in regulating NAD in muscle. Elevated *NMRK2* is not maintained becoming negatively correlated with increased body weight and fat mass, plasma HDL and basal glucose (see Table 2). The onset of increased glucose intolerance between 12 and 16 weeks is coincident with elevated CD38 (Figure 2D). CD38, a major NADase in tissues, is necessary for diet-induced obesity linked to sirtuins [36] and is supported by the significant positive correlations of muscle *CD38* gene regulation with body weight and fat mass, plasma HDL and leptin (Table S3) and accumulation of lipid in muscle [4]. CD38 knockout mice fed a HFD have increased NAD levels, sirtuin activity and reduced fat droplets in muscle [36]. Notably, CD38 is regulated at the transcriptional level by IL6 [37] which is elevated in muscle of HFD mice at 12 and 16 weeks [4]. The reduced levels of *NMRK2* with duration of HFD and the up-regulation of *CD38* at 16 weeks, may contribute to the observed reduction in total NAD/NADH levels at this time. Analogous to the changes seen in WAT there was a trend towards increased NAD:NADH ratios in muscle in HFD mice. Christensen *et al*., [38] propose that increased NAD:NADH ratios are associated with altered energy metabolism and metabolic phenotype indicating reduced glycolysis and increased fatty acid biosynthesis. This may contribute to eventual loss of insulin sensitivity observed with long term high fat feeding.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

The present study demonstrates a level of complexity in the regulation of the sirtuin/NAD system that has not previously been reported. This study identifies novel tissue specific transcriptional activation of key sirtuin/NAD system components in response to high fat diet, development of obesity and glucose intolerance and potential regulatory factors and transcriptional linkages involved. The regulation of rate-limiting enzymes of NAD biosynthesis appears to be important in the adaptive compensatory response in liver during the acute phase to prevent the negative impact of high fat diet and maintain glucose homeostasis and insulin sensitivity. Indeed, the novel finding of transcriptional activation of *NAPRT1* supports previous reports of the importance of this enzyme in maintaining NAD levels in liver [40]. While activation of *NMNAT3* implies a compensatory response specifically aimed at maintaining NMNAT3 protein and subsequently NAD pools in mitochondria. Ultimately, prolonged high fat feeding overwhelms the capacity to maintain homeostatic control concomitant with loss of insulin sensitivity in WAT and muscle. Notably, the activation of *NMRK2*, an important transcriptionally regulated salvage pathway enzyme in skeletal muscle [39] is not sustained with prolonged high fat feeding. ABCA1 was identified as an important component marking prelude to loss of metabolic homeostasis and development of glucose intolerance. In summary this study has shown that multiple sirtuins that act as control points linked to NAD biosynthetic enzymes regulate metabolic signals, implicating several new sirtuin/NAD molecular targets for targeted intervention to protect against metabolic damage induced by diet-induced obesity.

Role of the funding sources

The funding sources had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Acknowledgements

We thank the Genomics Unit, Analytical Services and Amanda Morris, Christine Grant, Gail Hempseed, Rowett Institute of Nutrition and Health, University of Aberdeen for technical support. This work was supported by the Scottish Government's Rural and Environment Science and Analytical Services Division.

J.E.D and L.M.W. designed the study; J.E.D, A.J.F, G.W.H .and L.M.W. researched and interpreted data; J.E.D. wrote the manuscript; J.E.D, A.J.F, G.W.H .and L.M.W. discussed/edited the manuscript; all authors critically revised the manuscript for important intellectual content and gave approval of the final version.

None of the authors declares a conflict of interest.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

References

[1] Haigis, M.C., Sinclair, D.A. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol. 2010; 5: 253-95.

[2] Dong, X.C. Sirtuin biology and relevance to diabetes treatment. Diabetes Manag (Lond). 2012; 2: 243-57.

[3] Huynh, F.K., Hershberger, K.A., Hirschey, M.D. Targeting sirtuins for the treatment of diabetes. Diabetes Manag. (Lond). 2013; 3: 245-57.

[4] Williams, L.W., Campbell, F.M., Drew, J.E., Koch, C., Hoggard, N.M., Rees, W.D. et al. The development of diet-induced obesity and glucose intolerance in C57Bl/6 mice on a high-fat diet consists of distinct phases. PLoS ONE 2014; 9: e106159.

[5] Pfluger, P.T., Herranz, D., Velasco-Miguel, S., Serrano, M., Tschöp, M.H. Sirt1 protects against high-fat diet-induced metabolic damage. Proc Natl Acad Sci USA. 2008; 105: 9793-8.

[6] Houtkooper, R.H., Pirinen, E., Auwerx, J. Sirtuins as regulators of metabolism and healthspan. Nat. Rev. Mol Cell Biol. 2012; 13: 225-38.

[7] Caron, A.Z., He, X., Mottawea, W., Seifert, E.L., Jardine, K., Dewar-Darch, D., et al. The SIRT1 deacetylase protects mice against the symptoms of metabolic syndrome. FASEB J. 2014; 28: 1306-16.

[8] Drew, J.E., Farquharson, A.J., Horgan, G.W., Duthie, S.J., Duthie, G.G. Stratification of study subjects detected by gene expression profiling reveals differences in postprandial cell defence system responses. Molec Nutr Food Res. 2014; 58: 2066–79.

[9] Bogan, K.L., Brenner, C. Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD+ precursor vitamins in human nutrition. Annu Rev Nutr. 2008; 28: 115-30.

[10] Kendrick, A.A., Choudhury, M., Rahman, S.M., McCurdy, C.E., Friederich, M., Van Hove, J.L., et al. Fatty liver is associated with reduced SIRT3 activity and mitochondrial protein hyperacetylation. Biochem J. 2011; 433: 505-14.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

[11]Ishikawa, S., Li, G., Takemitsu, H., Fujiwara, M., Mori, N., Yamamoto, I., et al. Change in mRNA expression of sirtuin 1 and sirtuin 3 in cats fed on high fat diet. BMC Veterinary Res. 2013; 9: 187.

[12]de Kreutzenberg, S.V., Ceolotto, G., Papparella, I., Bortoluzzi, A., Semplicini, A., Dalla Man, C., et al. Down-regulation of the longevity-associated protein sirtuin 1 in insulin resistance and metabolic syndrome: potential biochemical mechanisms. Diabetes 2010; 59: 1006-15.

[13]Hirschey, M.D., Shimazu, T., Jing, E., Grueter, C.A., Collins, A.M., Aouizerat, B., et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Mol Cell. 2011; 44: 177-90.

[14]Yoshino, J., Mills, K.F., Yoon, M.J., Imai, S. Nicotinamide mononucleotide, a key NAD(+) intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. Cell Metab. 2011; 14: 528-36.

[15]Chang, H.C., Guarente, L. SIRT1 and other sirtuins in metabolism. Trends Endocrinol Metab. 2014; 25: 138-45.

[16]Nasrin, N., Wu, X., Fortier, E., Feng, Y., Bare, O.C., Chen, S., et al. SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells. J Biol Chem. 2010; 285: 31995-2002.

[17]Laurent, G., de Boer, V.C., Finley, L.W., Sweeney, M., Lu, H., Schug, T.T.,et al. SIRT4 represses peroxisome proliferator-activated receptor α activity to suppress hepatic fat oxidation. Mol Cell Biol .2013; 33: 4552-61.

[18]Hara, N., Yamada, K., Shibata, T., Osago, H., Hashimoto, T., Tsuchiya, M., et al. Elevation of cellular NAD levels by nicotinic acid and involvement of nicotinic acid phosphoribosyltransferase in human cells. J Biol Chem. 2007; 282: 24574-82.

[19]Penke M., Larsen P.S., Schuster, S., Dall, M., Jensen, B.A., Gorski, T., et al. Hepatic NAD salvage pathway is enhanced in mice on a high-fat diet. Mol Cell Endocrinol. 2015; 412: 65-72.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

[20]Berger, F., Lau, C., Dahlmann, M., Ziegler, M. Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. J Biol Chem 2005; 280: 36334–41.

[21]Rhee, H.W., Zou, P., Udeshi, N.D., Martell, J.D., Mootha, V.K., Carr, S.A., et al. Proteomic mapping of mitochondria in living cells via spatially restricted enzymatic tagging. Science 2013; 339: 1328–31.

[22]Felici, R., Lapucci, A., Ramazzotti, M., Chiarugi, A. Insight into molecular and functional properties of NMNAT3 reveals new hints of NAD homeostasis within human mitochondria. PLoS One 2013; 8: e76938.

[23]Oram, J.F., Lawn, R.M. ABCA1. The gatekeeper for eliminating excess tissue cholesterol. J Lipid Res 2001; 42: 1173-9.

[24]de Haan, W., Karasinska, J.M., Ruddle, P., Hayden, M.R. Hepatic ABCA1 expression improves β-cell function and glucose tolerance. Diabetes 2014; 63: 4076-82.

[25]Kraus, D., Yang, Q., Kong, D., Banks, A.S., Zhang, L., Rodgers, J.T., et al. Nicotinamide N-methyltransferase knockdown protects against diet-induced obesity. Nature. 2014; 508: 258-62.

[26]Kannt, A., Pfenninger, A., Teichert, L., Tönjes, A., Dietrich, A., Schön, M.R., et al. Association of nicotinamide-N-methyltransferase mRNA expression in human adipose tissue and the plasma concentration of its product, 1-methylnicotinamide, with insulin resistance. Diabetologia. 2015; 58: 799-808.

[27]Giuliante, R., Sartini, D., Bacchetti, T., Rocchetti, R., Klöting, I., Polidori, C., et al. Potential involvement of nicotinamide n-methyltransferase in the pathogenesis of metabolic syndrome. Metab Syndr Relat Disord 2015; 13: 165-70.

[28]Sartini, D., Muzzonigro, G., Milanese, G., Pozzi, V., Vici, A., Morganti, S., et al. Up-regulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test.Cell Biochem Biophys. 2013; 65: 473-83.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

[29]Zhou, W., Gui, M., Zhu, M., Long, Z., , Huang, L., Zhou, J., et al. Nicotinamide N-methyltransferase is overexpressed in prostate cancer and correlates with prolonged progression-free and overall survival times. Oncol Lett 2014; 8: 1175-80.

[30]Lu, C.T., Hsu, C.M., Lin, P.M., Lai, C.C., Lin, H.C., Yang, C.H., et al. The potential of SIRT6 and SIRT7 as circulating markers for head and neck squamous cell carcinoma. Anticancer Res 2014; 34: 7137-43.

[31]Kiran, S., Anwar, T., Kiran, M., Ramakrishna, G. Sirtuin 7 in cell proliferation, stress and disease: rise of the seventh sirtuin! Cell Signal 2015; 27: 673-82.

[32]Byers, T., Sedjo, R.L. Body fatness as a cause of cancer: epidemiologic clues to biologic mechanisms. Endocr Relat Cancer 2015; 22: R125-34.

[33]Hong S., Moreno-Navarrete, J.M., Wei, X., Kikukawa, Y., Tzameli, I., Prasad, D., Nicotinamide N-methyltransferase regulates hepatic nutrient metabolism through Sirt1 protein stabilization. Nat Med. 2015; 21: 887-94.

[34]Bieganowski, P., Brenner, C. Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD+ in fungi and humans. Cell 2004; 117: 495-502.

[35]Chi, Y., Sauve, A.A. Nicotinamide riboside, a trace nutrient in foods, is a vitamin B3 with effects on energy metabolism and neuroprotection. Curr Opin Clin Nutr Metab Care 2013; 16: 657-61.

[36]Barbosa, M.T., Soares, S.M., Novak, C.M., Sinclair, D., Levine, J.A., Aksoy, P., et al. The enzyme CD38 (a NAD glycohydrolase, EC 3.2.2.5) is necessary for the development of diet-induced obesity. FASEB J. 2007; 21: 3629-39.

[37]Malavasi, F., Deaglio, S., Funaro, A., Ferrero, E., Horenstein, A.L., Ortolan, E., et al. Evolution and function of the ADP ribosyl cyclase/CD38 gene family in physiology and pathology. Physiol Rev. 2008; 88: 841-86.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

[38]Christensen, C.E., Karlsson, M., Winther, J.R., Jensen, P.R. Lerche, M.H. Non-invasive in-cell determination of free cytosolic [NAD+]/[NADH] ratios using hyperpolarized glucose show large variations in metabolic phenotypes. J Biol Chem 2014; 289: 2344-52.

[39]Ruggieri, S., Orsomando, G., Sorci, L., Raffaelli N. Regulation of NAD biosynthetic enzymes modulates NAD-sensing processes to shape mammalian cell physiology under varying biological cues. Biochim Biophys Acta 2015; 1854: 1138-49.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Figures

Graphical Abstract Summary of phenotypic data reported previously [4] and associated SIRT/NAD responses to HFD development of obesity and glucose intolerance.

Figure 1 Principal component analysis (PCA) biplots of *UBE2D2* normalised GeXP mSIRTNADplex data from (A, B) liver, (C,D) WAT and (E) muscle of mice fed either a low (Δ) or high (▲) fat diet. The number adjacent to each symbol represents the duration on the diet (A and C) at 3 and 7 days and (B, D and E) at 1, 4, 8, 12 and 16 weeks. The PCA biplot permits visualisation of inherent clustering patterns of individual tissue samples and associated gene expression levels (denoted by ● and gene symbol). The measurements all fall within the Hotelling T2 95% confidence limit.

Figure 2 mSIRTNADplex gene expression profiles of mice fed either low (LF) or high (HF) fat diets. Mean expression for each gene (n=6-8 $\pm$ SE) are shown for liver (A) 3 and 7 days and (B) 1, 4, 8 12 and 16 weeks, WAT (C) 3 and 7 days and muscle (D) 1, 4, 8 12 and 16 weeks as indicated in the legend associated with each chart. Gene expression was assessed by mSIRTNADplex assay and normalised to internal reference gene *UBE2D2*. Significant ($p < 0.05$) differences in gene expression between LF and HF fed mice at time point tested assessed by ANOVA with Bonferroni correction are indicated (*). Significant gene changes are also summarised in Graphical Abstract. See Supplementary File S2 Table S1 for details of the genes in mSIRTNADplex and Supplementary Files 4, 5 and 6 for mean expression profiles of all genes in the mSIRTNADplex for liver, WAT and muscle respectively.

Figure 3 Correlation of SIRT/NAD system genes and metabolic markers. The experimental mice from both acute (Δ or ▲) and chronic (○ or ●) response experiments are shown. Responses to low (Δ or ○) and high (▲or ●) fat diet at 3 and 7 days (Δ or ▲) and 1, 4, 8, 12 and 16 weeks (○ or ●) are

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

indicated. (A-C) Correlations between liver *SIRT4*, *SIRT7* and *NAPRT1* expression (relative to *UBE2D2*) (n=92). (A) *SIRT4* and *SIRT7*, (B) *NAPRT1* and *SIRT4*, (C) *NAPRT1* and SIRT7. Correlation coefficients of *SIRT4* and *SIRT7* were ρ=0.48, p<0.001 for all, ρ=0.48, p=0.001 for LF and ρ=-0.53, p<0.001 for HF mice, *SIRT4* and *NAPRT1* were ρ=0.66, p<0.001 for all, ρ=0.52, p<0.001 for LF and ρ=-0.53, p<0.001 for HF, and *NAPRT1* and *SIRT7* were ρ=0.39, p<0.001 for all, ρ=0.41, p=0.005 for LF and ρ=-0.34, p=0.022 for HF. (D) Correlations between muscle *CD38* and *NMRK2* expression (relative to *UBE2D2*) in experimental mice (n=60). Correlation coefficients of *CD38* and *NMRK2* were ρ=0.37, p=0.003 for all, ρ=0.11, p=0.55 for LF and ρ=-0.15, p=0.45 for HF mice. (E-F) Correlations between liver *TDO2* (relative to *UBE2D2*) and body weight and fat mass (n=92). (E) *TDO2* and body weight (g), (F) *TDO2* and fat mass (g). Correlation coefficients of *TDO2* with body weight were ρ=-0.10, p=0.33 for all, ρ=-0.40, p=0.007 for LF and ρ=-0.57, p<0.001 for HF mice and with fat mass were ρ=-0.11, p=0.302 for all, ρ=-0.39, p=0.007 for LF and ρ=-0.60, p<0.001 for HF mice. (G) Correlations between liver *NNMT* expression (relative to *UBE2D2*) and plasma HDL (n=70). Correlation coefficients were ρ=0.17, p=0.16 for all, ρ=0.14, p=0.47 for LF and ρ=-0.58, p<0.001 for HF mice respectively.

Figure 4 NAD and NADH assay of WAT and muscle. Total NAD, NADH in response to LFD (□) or HFD (■) in WAT during acute phase (A) 3 and (B) 7 days and in muscle during chronic phase (D) 1 (E) 8 and (F) 16 weeks. The corresponding NAD/NADH ratios in (C) WAT and (G) muscle are shown. Pairwise comparisons of tissue NAD and NADH levels were conducted using Student's ttest (significance level 0.05).

Figure 5 Expression levels of SIRT4 and NMNAT3 in liver of mice fed either low (LF) or high (HF) fat diets. Mean expression of proteins (n=6 $\pm$ SE) are shown for SIRT4 (A) and NMNAT3 (B) the acute phase (3 and 7 days) and chronic phase (1, 8 and 16 weeks) experiments. Significant ($p < 0.05$) differences in protein expression between LF and HF fed mice at time point tested assessed by ANOVA with Tukey's correction are indicated (*).

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Tables

Table 1 Sirtuin/NAD system gene expression in liver correlated with dietary fat.

Table 2 Sirtuin/NAD system genes in liver, WAT and muscle that significantly correlate with dietary fat.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Table 1

Sirtuin/NAD system gene expression in liver correlated with dietary fat

	Diet Group	SIRT7	NAPRT1	SIRT4	*ABCA1*	*NNMT*	*TDO2*
NMNAT3	ALL	**0.21**	**0.25**	**0.44**	**-0.40**	**0.34**	-0.03
	LF	**0.32**	**0.48**	**0.70**	-0.07	**0.40**	-0.03
	HF	0.07	-0.21	-0.05	**-0.68**	-0.10	**-0.58**
TDO2	ALL	**0.27**	**0.56**	**0.64**	**0.51**	-	-
	LF	0.06	0.12	0.11	0.31	-	-
	HF	**0.32**	**0.53**	**0.58**	**0.73**	-	-
ABCA1	ALL	0.16	**0.25**	-	-	-	-
	LF	**0.33**	0.03	-	-	-	-
	HF	0.04	**0.41**	-	-	-	-

ALL = all experimental mice, LF = low and HF = high fat fed mice. **Bold font** and **underline** indicates $p < 0.05$. Negative correlation indicated by negative value.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Table 2 Sirtuin/NAD system genes in liver, WAT and muscle that significantly correlate with dietary fat.

			DIET GROUP		
			ALL	**LFD**	**HFD**
	TISSUE	**GENE**			
Body weight	Liver	*NAPRT1*	**0.26**	**0.44**	-0.05
	Muscle	*NMRK2*	0.06	-0.05	**-0.53**
		ABCA1	-0.33	**-0.42**	-0.01
Fat mass	Liver	*NAPRT1*	0.26	**0.49**	-0.05
		NMNAT3	**0.65**	0.28	**0.65**
	Muscle	*NMRK2*	0.14	0.18	**-0.53**
		ABCA1	**-0.38**	**-0.53**	-0.10
Plasma LDL	Liver	*SIRT7*	**0.40**	**0.53**	0.29
		ABCA1	-0.20	-0.01	**-0.34**
		TDO2	**-0.20**	-0.17	**-0.43**
Plasma HDL	Liver	*NAPRT1*	**0.34**	**0.35**	-0.08
		ABCA1	**-0.37**	-0.20	**-0.62**
		TDO2	0.11	0.07	**-0.51**
		NNMT	0.17	0.17	**-0.58**
	Muscle	*NMRK2*	0.15	-0.28	**-0.54**
Plasma TG	Liver	*ABCA1*	-0.19	0.11	**-0.42**
		TDO2	**-0.38**	0.05	**-0.54**
HDL/LDL ratio	Liver	*NAPRT1*	-0.17	**-0.46**	0.20
		ABCA1	**0.26**	0.09	**0.43**
		TDO2	**0.24**	0.18	**0.58**
Plasma leptin	Liver	*SIRT7*	-0.32	0.05	**-0.51**
		NAPRT1	0.09	**0.67**	-0.24
		TDO2	-0.15	-0.40	**-0.65**
Plasma insulin	Liver	*ABCA1*	-0.28	-0.27	**-0.51**
		NMNAT3	**0.59**	-0.28	**0.66**
		TDO2	-0.15	-0.08	**-0.55**
	WAT	*NMNAT2*	**0.40**	-0.06	**0.51**
Basal glucose	Liver	*NMNAT3*	**0.32**	**0.51**	-0.02
	Muscle	*NMRK2*	0.09	0.20	**-0.61**

ALL = all experimental mice, LF = low and HF = high fat fed mice. **Bold font** and **underline** indicates $p < 0.05$. Negative correlation indicated by negative value.

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

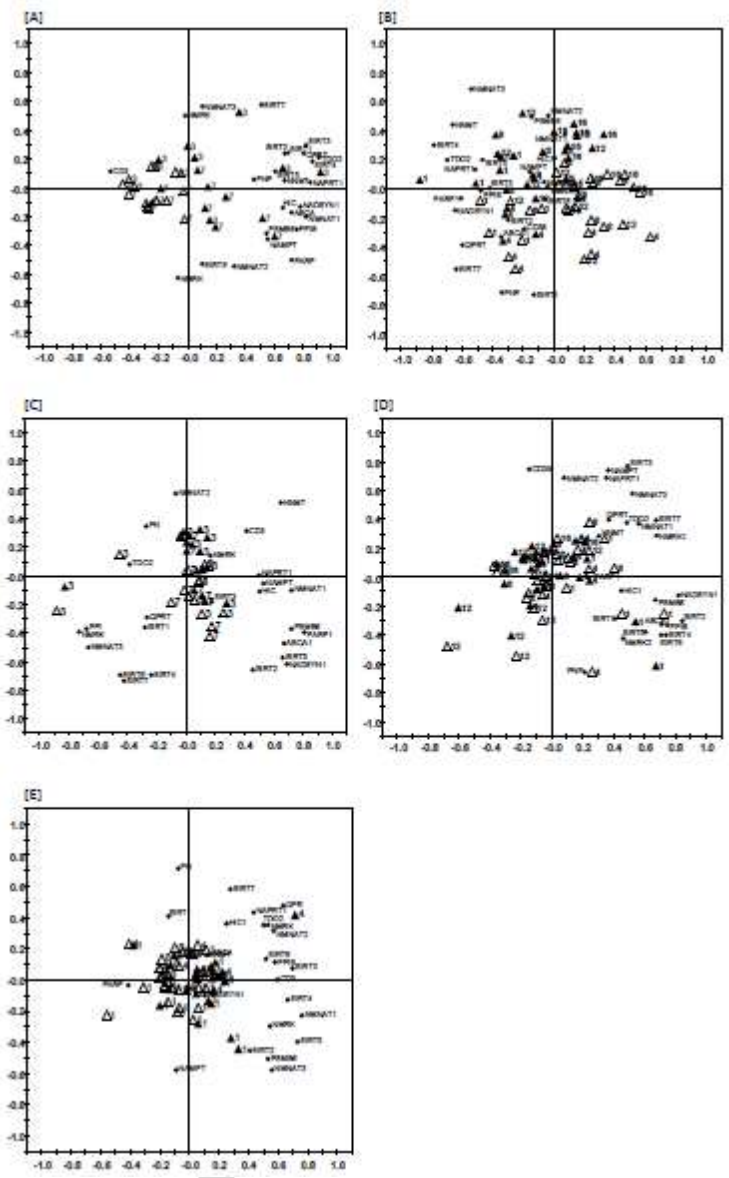


Figure 1

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

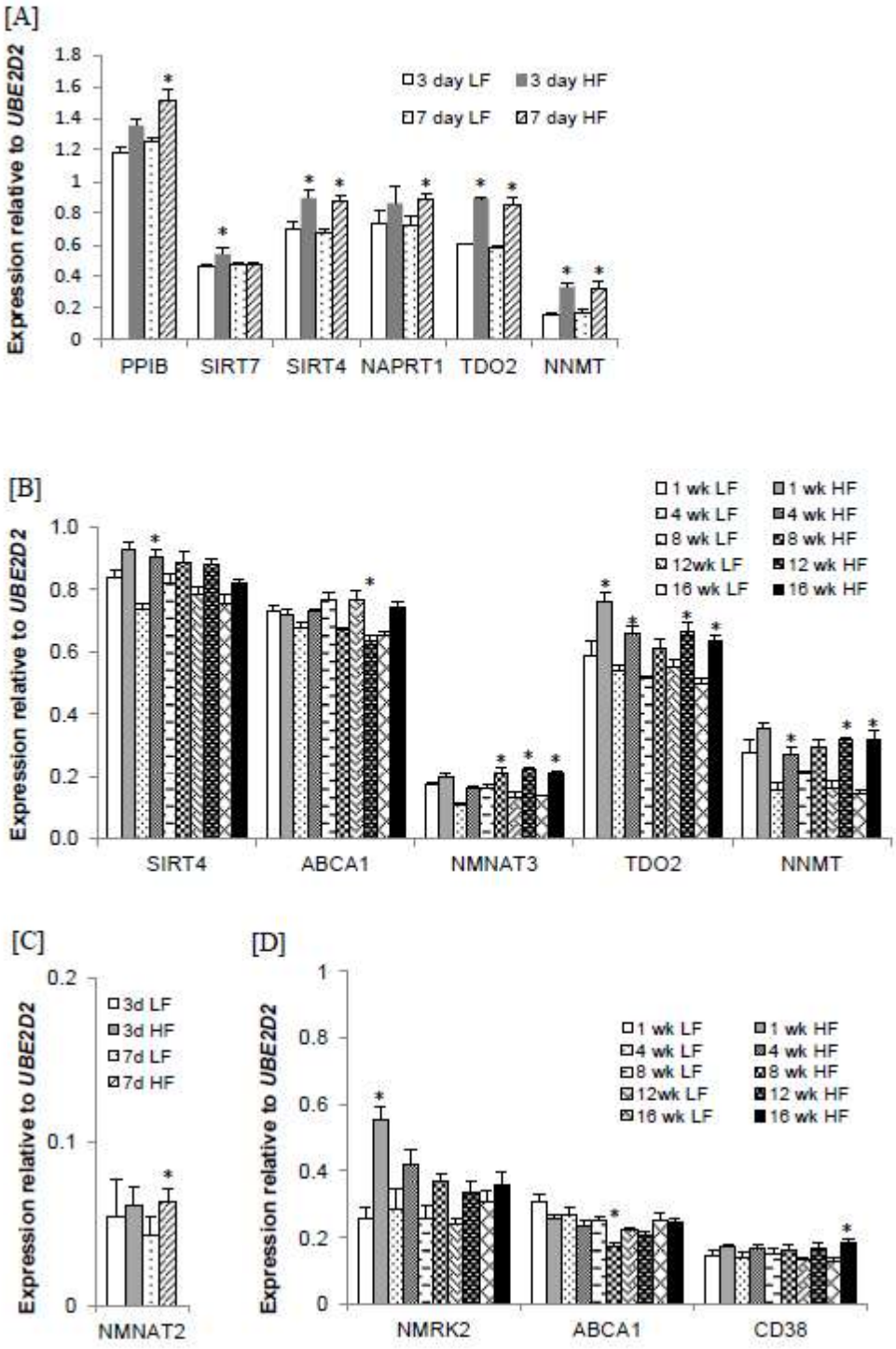


Figure 2

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

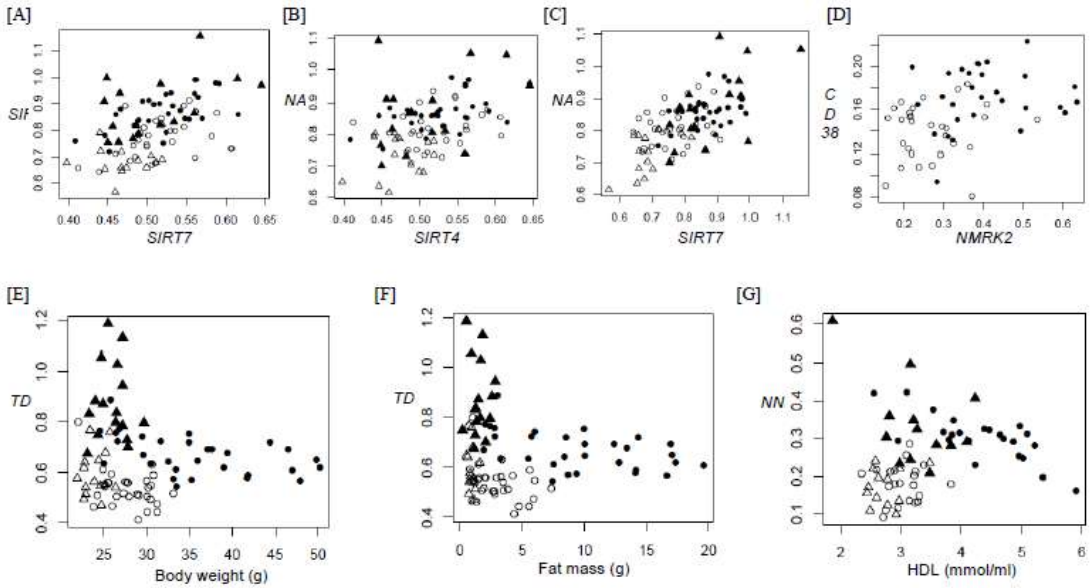


Figure 3

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

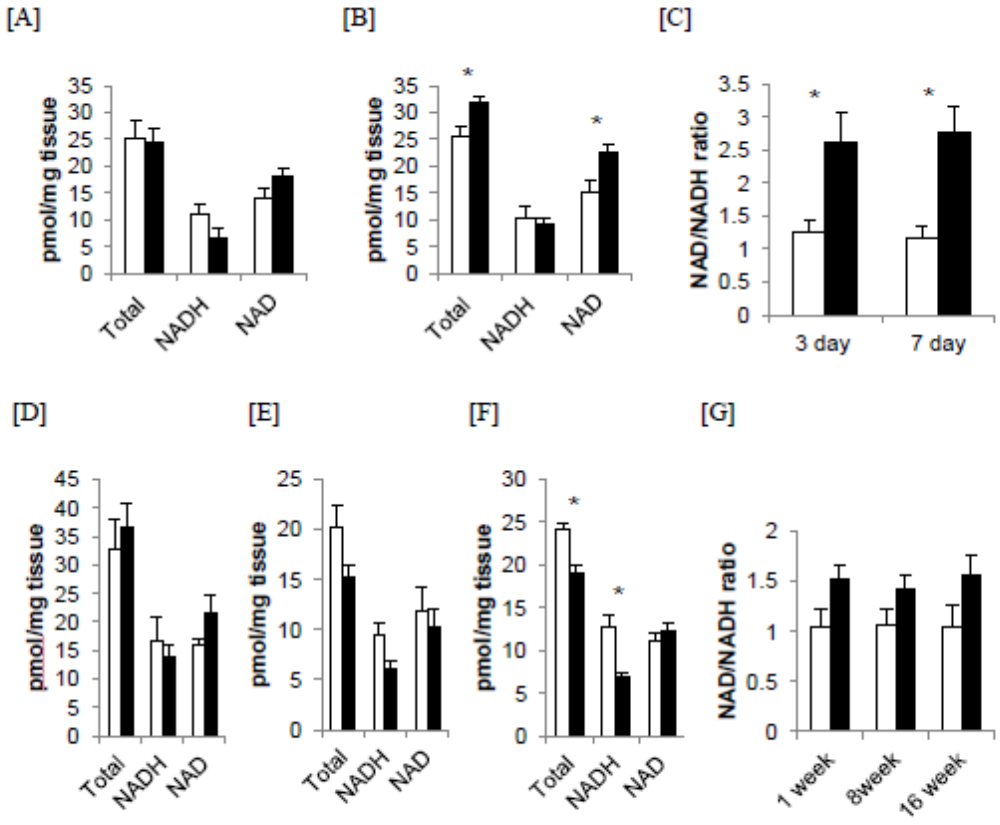
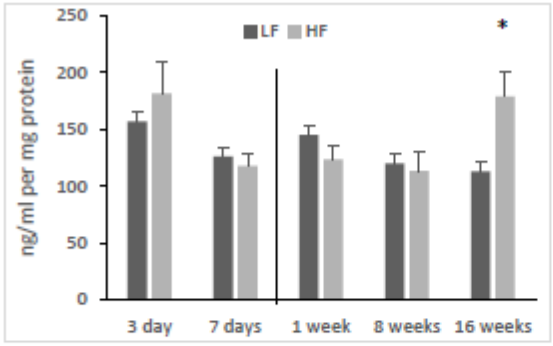


Figure 4

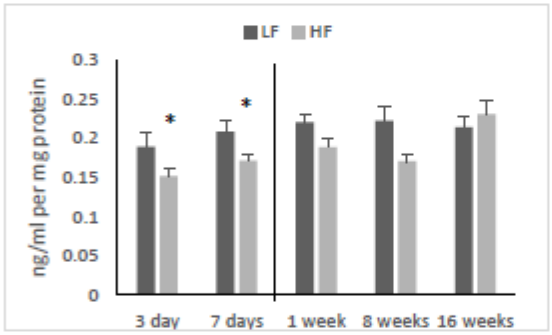
Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.

Figure 5

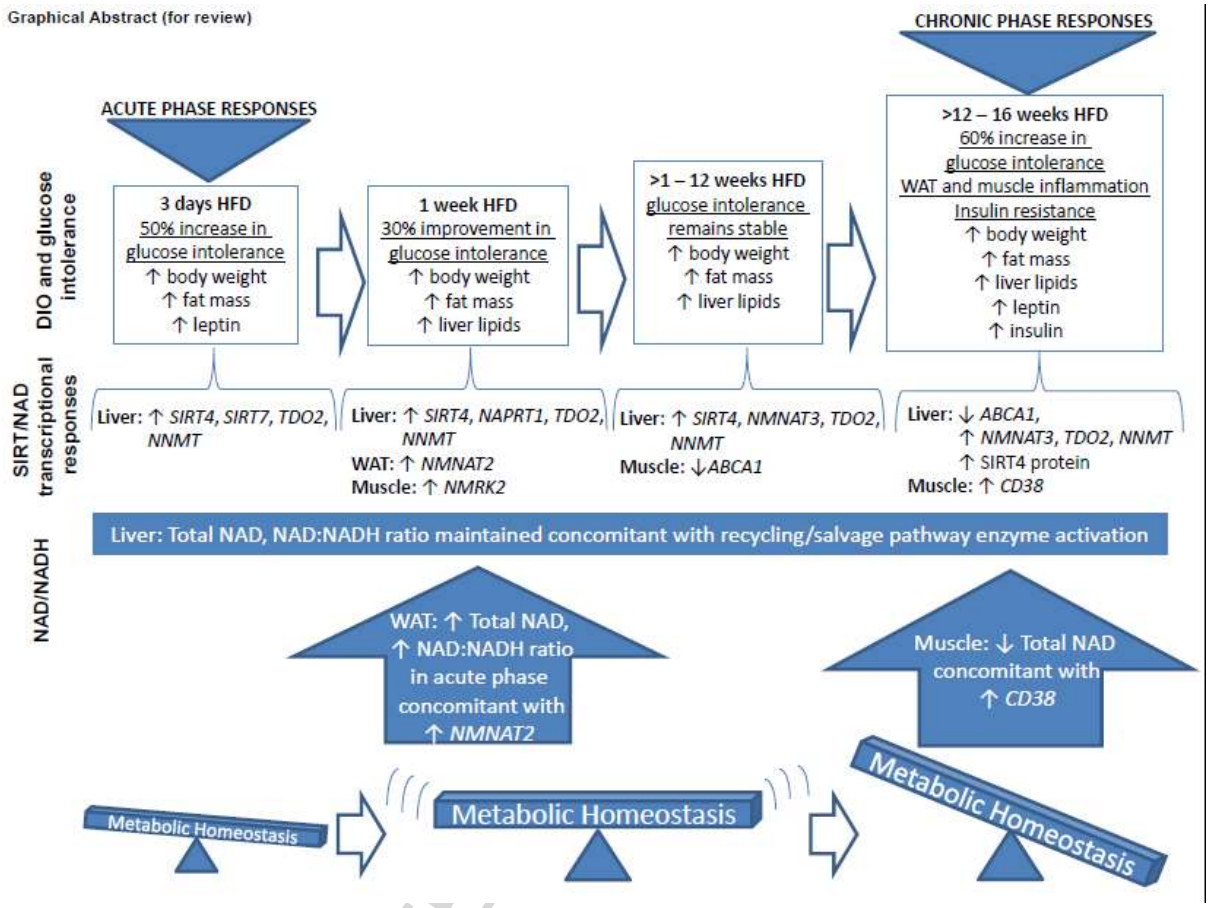
[A]



[B]



Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.



Graphical Abstract

Funding: The Scottish Government's Rural and Environment Science and Analytical Services Division.