Intermittent calorie restriction largely counteracts the adverse health effects of a moderate-fat diet in aging C57BL/6J mice

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Abstract

Scope

Calorie restriction (CR) has been shown to extend life- and health-span in model species. For most humans, a life-long CR diet is too arduous to adhere to. The aim of this study was to explore whether weekly intermittent CR can 1) provide long-term beneficial effects and 2) counteract diet-induced obesity in male aging mice.

Methods and results

In this study we have exposed C57Bl/6J mice for 24 months to an intermittent (INT) diet, alternating weekly between CR of a control diet and *ad libitum* moderate-fat (MF) feeding. This weekly intermittent CR significantly counteracted the adverse effects of the MF diet on mortality, body weight and liver health markers in male 24-month-old mice. Hepatic gene expression profiles of INT-

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exposed animals appeared much more comparable to CR than to MF-exposed mice. At 12 months of age, a subgroup of MF-exposed mice was transferred to the INT diet. Gene expression profiles in the liver of the 24-month-old diet switch mice were highly similar to the INT-exposed mice. However, a small subset of genes was consistently changed by the MF diet during the first phase of life.

Conclusion

Weekly intermittent CR largely, but not completely, reversed adverse effects caused by a MF diet.

Calorie restriction (CR) has been shown to extend life- and health-span in model species. However, for most humans, a life-long CR diet is too arduous to adhere to. In this study we explored the caused by life-long intermittent CR in the liver. Our results revealed that weekly intermittent CR largely, but not completely, reversed the adverse effects caused by a MF diet at old age.

Key words

Aging, moderate-fat diet, intermittent calorie restriction, transcriptomics, obesity, liver

Abbreviations

MA: microarray, IPA: Ingenuity Pathway Analysis, MA: microarray, MF: moderate fat, C: control, CR: calorie restriction

1 Introduction

The beneficial health effects of a calorie restricted (CR) diet, avoiding malnutrition, can be appreciated from two perspectives. First, in our obesogenic society, where about half of the population is overweight or obese [1], maintaining a reduced energy intake is the best nutritional strategy to achieve and maintain weight loss [2, 3]. Since obesity causes a wide range of serious chronic diseases, the negative energy balance induced by a CR diet will result in weight loss and induce, concomitantly, health promoting effects. Secondly, CR has been acknowledged as the most successful approach to increase longevity in a wide range of species [4]. Apart from life-span, reduced food intake also increases health-span. Here, the health-promoting effect is not achieved by counteracting obesity-related disorders, but by ameliorating a wide range of aging-related diseases [5-7]. It is worth mentioning, however, that these obesity- and age-related disorders fundamentally overlap.

In order to achieve longevity, the application of a CR diet requires life-long adherence to a very strict dietary regimen. Severe food restriction is very arduous and too difficult to practice and to sustain for most individuals. Importantly, long-term exposure to a CR diet might also cause

substantial side effects like amenorrhoea, osteoporosis, decreased fertility and libido, impaired wound healing and increased susceptibility to infections [8-11]. Different variants of intermittent energy restriction (CR for intermittent periods of time) have been reported to have health-promoting effects. These beneficial health effects include improvements of body composition [12-14], skin wound healing [9], blood pressure and cardiovascular health markers [15, 16], neurological health and cognitive performance [17-19] and retarded tumour growth/formation [20-23]. By applying repetitive cycles of fasting/CR and regular eating, the negative side effects of CR are thought to be circumvented. Moreover, an intermittent CR regimen is more feasible to maintain. Importantly, increasing evidence points out that the beneficial health effects of (intermittent) CR are not solely caused by reduced body weight [24, 25]. Timing and limitation of meal frequency affect the circadian rhythm and might induce a repetitive challenge that most likely will contribute to the health promoting effects [26-29]. We recently reported that an intermittent energy restriction (INT) diet maintains metabolic health and reverses the adverse effects of the moderate-fat (MF) diet, when provided for 10 months to 9-week-old male C57BL/6J mice [30].

One of the most frequently affected organs in obese individuals is the liver, which is recognized to be the most important metabolic organ and supporting nearly every other organ in the body. Obesity induces a spectrum of abnormalities in the liver called non-alcoholic fatty liver disease (NAFLD), which is currently the most common chronic liver disease in developed countries. NAFLD is seen in 20-40% of the general adult population, but incidence in severe obese adult individuals is much higher (70-90%) [31]. The mildest form of NAFLD is simple hepatic steatosis (HS) and is characterized by intrahepatic accumulation of lipids alone. In around 47% of the severely obese adults this benign hepatic lipid accumulation evolves into non-alcoholic steatohepatitis (NASH) characterized by inflammatory infiltration of the liver and low-level fibrosis [32]. Between 10 to 29% of the individuals with NASH develop advanced fibrosis, cirrhosis and ultimately, hepatocellular carcinoma (HCC) [33, 34]. This progressive disease development is characterized by increasing severity and predisposition to mortality. It has been reported that both the prevalence of NAFLD as well as progression into more severe forms of NAFLD in the general population increase with age [35-37]. The early stages of NAFLD are considered to feature a benign, non-progressive and reversible disease [31]. Management of HS and NASH is mainly focussed on treatment of obesity by introducing lifestyle modifications including increased exercise and decreased calorie intake. Although there is strong evidence that the early stages of NAFLD caused by an obesogenic diet are reversible, it is currently not clear whether all detrimental effects are completely restored after long-term exposure to such a diet.

In this study we explored the effects of intermittent calorie restriction (INT) regimen on NAFLD development during aging by applying the same strict and robust form of the INT diet in male C57BL/6J mice, as previously reported [30]. By alternating weekly between a 40E% calorie restricted control diet and an *ad libitum* moderate-fat (MF) diet, the mice were challenged to adapt to differences in 1) energy intake, 2) macronutrient composition of the diet and 3) continues food exposure versus a one-portion-a-day feeding pattern. The effects of this exceptionally challenging diet on overall body health in the period between middle-aged (12 months) and old (24 months) C57BL/6J mice were explored. In addition, we examined the biochemical, morphological and molecular effects this diet caused in the liver of the 24-month-old mice. The obesity-counteracting effects of this diet were identified by introducing a diet switch in life-long MF exposed mice at the age of 12 months to the INT diet. In 24-month-old mice we examined into what extent the adverse health effects caused by the MF diet, are reversible by applying the INT diet on whole-body as well

as on liver health. Life-long exposure to a low-fat control diet (C), MF or a continuous CR diet (30% calorie reduction) were included as normal, unhealthy and healthy aging controls, respectively.

2 Materials and Methods

2.1 Ethics statement

The institutional and national guidelines for the care and use of animals were followed and the Local Committee for Care and Use of Laboratory Animals at Wageningen University approved the experiment (code number: drs-2010151b).

2.2 Animals and diets

Male C57BL/6J mice (age: 7 weeks) were purchased from Janvier (Cedex, France) and were housed in pairs of two in the light and temperature (20^{o}C)-controlled animal facility of Wageningen University (12-hour light/dark cycle, light on at 04.00). The mice received standard AIN-93G (Research Diet Services, Wijk bij Duurstede, The Netherlands) for 2 weeks upon arrival.
At the start of the diet intervention the mice were 9 weeks old, housed individually and randomly distributed into four intervention groups: 1) Control diet (C) receiving AIN-93W diet *ad libitum* (n=89); 2) Moderate-Fat diet (MF) receiving AIN-93W-MF *ad libitum* (n=127); 3) Intermittent diet (INT) receiving alternating one week AIN-93W-MF *ad libitum* followed by one week 60E% of the C diet based on the mean energy intake of the mice on the AIN-93W diet (n=155) and 4) Calorie Restricted diet (CR) receiving AIN-93W-CR in portions containing 70E% of the mean energy intake of the group of the control mice were provided each day at 15.30 (n=117). At the age of 12M we created group 5) by transferring a subset of 32 randomly selected mice from the MF-intervention group to the INT diet which they received for the second part of their life (for study design see **Figure 1A**). With the INT dietary intervention we intended to induce the maximal fluctuation in weight gain and weight loss that is allowed according to the regulations of our Local ethical committee for care and use of laboratory animals (that is 20% of the total body weight). AIN-93W is a variant of the AIN-93M (maintenance of adult mice) diet, which slightly differs on the fat source. The 10E% fat content in AIN-93M solely comes from soybean oil, while the fat source of AIN-93W is a mix of 6E% soybean oil and 4E% palm oil, in order to balance saturated and unsaturated fat composition. The AIN-93-W-MF contained 25E% fat, also from soybean oil and palm oil. AIN-93W-CR contained increased concentrations of vitamins and minerals in order to feed these mice the same concentrations of micronutrients as the mice receiving AIN-93W diet to avoid malnutrition. The complete composition of the applied diets is listed in **Supplementary Table 1S** (Research Diet Services, Wijk bij Duurstede, The Netherlands). All mice were provided with *ad libitum* access to water.
Body weight of (1) all mice was recorded every two weeks and (2) of a representative subgroup of ~24 mice of each intervention group in the in between weeks (to represent a weekly body weight development). Food intake of 20 mice of each intervention group was measured every two months, comprising one week measurement for the C, CR and MF-fed mice and two weeks measurement for the INT-fed mice. Portion sizes of the mice on the CR and INT were adjusted at the beginning of the study and at the age of 6, 12 and 18 months based on food intake of C mice.

At the age of 6, 12 and 24 months, 12-16 mice of each intervention group were sacrificed between 14.00-17.00 on 5 consecutive days (the remaining mice stayed in the experiment and were sacrificed at older ages). All mice were included in the survival analysis and censored when they were culled at the different ages. INT mice were sacrificed in their *ad libitum* MF feeding week. Similar to what we performed in the previous study [30], prior to sacrifice each mouse was first fasted for 4 hours after which they received an intragastric gavage of either solvent (0.5% carboxymethyl cellulose) or Wy-14,643 dispersed in solvent (160 mg Wy-14,643/kg body weight), then fasted again for another 6 hours. Only mock-treated animals were included in the molecular analysis, since the Wy-14,643 treatment have an immediate effect on the gene expression levels [38]. The purpose of this treatment is to examine PPARα adaptive capacity analysis, which has been covered in a separate publication [39]. After sedation with a mixture of isoflurane (1.5%) in nitrous oxide (70%) and oxygen (30%), blood samples were collected by cardiac puncture, then followed by neck dislocation. Weight of the various organs was measured and subsequently organs/tissues were snap-frozen and stored at -80^{o}C until further molecular/biochemical analysis. For histological analysis, organs/tissues were fixed in 4% paraformaldehyde.

2.3 Daily activity measurement

At 23 months of age, 19-24 mice of each intervention group were housed in new cages to monitor physical activity continuously during 3 days as previously described [40, 41]. Activity sensors (dual technology detector DUO 240, Visonic; adapted by R. Visser, NIN, Amsterdam, The Netherlands) were mounted above the cages and data were analysed with MED-PC® IV software for data collection (MED associates, St Albans, VT, USA). Activity was expressed in counts per 30 min and calculated for each mouse separately.

2.4 DEXA scan body composition analysis

Body composition was measured by Dual Energy X-ray Absorptiometry (DEXA) scan, using a PIXImus imager (GE Lunar, Madison, WI, USA) of 24 mice of each dietary intervention group. The scans produced data concerning lean mass, fat mass and bone mineral density. During the measurements the animals were under general anaesthesia (isoflurane/N_2O/O_2).

2.5 Oral glucose tolerance test

The mice sacrificed at the age of 24 months were all subjected to an oral glucose tolerance test (OGTT) two weeks prior to sacrifice. In the OGTT, the mice were fasted for 6 hours, then received 1.5 mg glucose per gram body weight via an oral gavage. Subsequently, blood glucose was measured 15, 30, 45, 60, 90 and 150 minutes following the glucose load using Accu-Check blood glucose meters (Roche Diagnostics, Almere, The Netherlands).

2.6 RNA isolation

Total RNA was isolated from the liver of each individual mouse using TRIzol reagent (Invitrogen Breda, The Netherlands) according to the manufacturer's instructions. The RNA was treated with DNAse and purified on columns using the RNAeasy microkit (Qiagen, Venlo, the Netherlands). RNA concentration was measured on a NanoDrop ND-1000 UV–vis spectrophotometer (Isogen, Maarssen, The Netherlands) and RNA integrity was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amsterdam, The Netherlands) with 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable only if samples showed intact bands of 18S and 28S

ribosomal RNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RNA integrity number (RIN) above 8.0.

2.7 Microarray hybridization and analysis

100 ng of purified RNA from the liver of the individual mice was used for the preparation of labelled cDNA, applying the Ambion Whole Transcript (WT) Expression kit (Life Technologies, Carlsbad, USA) in combination with the Affymetrix GeneChip WT Terminal Labelling kit (Affymetrix, Santa Clara, USA). All samples were hybridized at one time point to Affymetrix GeneChip Mouse Gene 1.1 ST arrays according to standard Affymetrix protocols. Microarray analysis was performed in MADMAX, a pipeline for statistical analysis of microarray data [42]. Arrays were normalized using the Robust Multiarray Average [43, 44]. Probe sets were defined according to Dai *et al.*[45]. In this method probes are assigned to unique gene identifiers, in this case Entrez IDs. The probes on the Gene 1.1 ST arrays represent 21,225 Entrez IDs. Array data will be submitted to the Gene Expression Omnibus after acceptance of the manuscript.

2.8 Bioinformatic analysis

Of the 21,225 defined genes covered by the MA, only genes with an intensity value of ≥20 on at least 5 arrays, represented by at least 7 probes per gene on the array and an interquartile range (IQR) ≥0.1 were selected for further analysis and not annotated were removed. The top-1000 most variable genes were used for Principle Component Analysis (PCA) using MultiExperimentViewer version 4.8.1 [46, 47]. Signal log2 ratios, which represent fold changes (FC), and related significances of change were calculated from the mean signal intensities and differences between diet groups was analyzed using intensity based-moderated t-statistics (IBMT) implementing empirical Bayes correction [48]. Resulting log2 ratios and p-values were applied for further descriptive bioinformatic analysis of the data. Ingenuity Pathway Analysis (IPA, Ingenuity® Systems, www.ingenuity.com) was used to explore the canonical pathways affected by the 148 reversibly and 1510 consistently changed genes. Comparison of the expression patterns of the 148 irreversible changed genes in the MF, INT and MF/INT diet switch groups was carried out by generating a heat map using MultiExperimentViewer, version 4.8.1 [46, 47].

2.9 Histopathology

Formalin-fixed and paraffin-embedded cross-sections (5µm) of the liver lobe was stained with haematoxylin and eosin. Samples were scored blindly by a board-certified pathologist using an adapted grading method for human NASH [49] in 6-8 mice of each intervention group. Briefly, a haematoxylin and eosin stained liver cross-section per mouse was examined and the level of steatosis was determined relative to the total liver area analysed (expressed as a percentage). Hepatic inflammation was assessed by counting the number of inflammatory foci at a 100× magnification (view size 3.1 mm^2), in five non-overlapping fields.

2.10 Hepatic triglyceride and 4-hydroxyproline measurement

Liver triglycerides were determined in 5% liver homogenates prepared in buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), using the triglyceride Liquicolor Monoreagent (Instruchemie, Delfzijl, The Netherlands). 4-hydroxyproline content was determined spectrophotometrically in liver hydrolysates as previously described in Hillebrandt et al. [50]. For both assays 16 mice of each intervention group were included in the analysis.

2.11 Plasma measurements

Plasma insulin, IL-6 and leptin levels were measured using a Mouse Adipokine (MADKMAG-71K) kit, according to the manufacturer's instructions in 16 mice of each intervention group. Plasma concentration of ALT was measured with commercially available kits from Instruchemie (Delfzijl, the Netherlands) in all sacrificed mice and measured in 16 mice of each intervention group. Plasma triglyceride and free fatty acid were measured using Liquicolor (Instruchemie, Wiesbaden, Germany) and NEFA-C kit (Wako, Neuss, Germany), respectively in 6-8 mice of each intervention group. Both assays were performed according to manufacturer's instructions.

2.12 cDNA synthesis and real-time quantitative PCR

The microarray data was validated by real-time quantitative PCR (qPCR). For each individual sample, single-stranded complementary DNA was synthesized from 1 µg of total RNA using the First Strand cDNA Synthesis kit (Thermo Scientific, Landsmeer, The Netherlands), following the supplier's protocol. Q-PCR was performed using SensiMix SYBR No-ROX kit (Bioline, Alphen aan de Rijn, The Netherlands) and a CFX384 thermal cycler (Bio-Rad, Veenendaal, The Netherlands). The following thermal cycling conditions were used: 2 min at 94°C, followed by 40 cycles of 94°C for 15 s and 60°C for 45 s. PCR reactions to validate the MA results of a panel of gens were performed in duplicate and all samples were normalized to 36B4 expression. Primer sequences were retrieved from the online PrimerBank database [51].

2.13 Statistical analysis

Except for the gene expression, data were analysed with GraphPad Prism 5.04 applying 1-way ANOVA followed by a Tukey post-test analysis. Statistical significance for the survival of groups was established by the log-rank analysis of Kaplan-Meier plots.

3 Results

3.1 Intermittent calorie restriction protected against the detrimental effects of a moderate-fat diet

The first part of this study explored the long-term effects of weekly intermittent CR (for study design see **Figure 1A**). Body weight was recorded weekly during the study. The obtained results showed, as expected, that the MF-exposed mice gained the highest body weight while the lowest body weight was observed for the CR-fed animals (**Figure 1B**). Body weight of the INT-fed mice displayed a constantly fluctuating pattern, dependent on the diet the mice received in the preceding week. DEXA scan analysis showed that, in line with the body weight, the percentages of fat (f) and lean (l) body mass of the INT-exposed mice (l: 68.3%, f: 31.7%) were in between those of the CR- (l: 78.9%, f: 20.1%) and C-fed mice (l: 64.4%, f: 35.6%) (**Figure 1C**). Food intake recordings presented in **Figure 1D** show that, during the entire study, of all intervention groups the INT-exposed mice have the highest energy intake during the *ad libitum* feeding week and the lowest during their CR-restricted week. Combining the intake values of the CR and *ad libitum* MF weeks at 24 month of age revealed that the mean energy intake of the INT-fed mice was slightly lower than the amount of calories consumed by C-fed mice and substantially higher than that consumed by the CR-exposed mice (**Figure 1E**).

Furthermore, it is important to note that this figure also reveals that, although the INT-exposed mice consume less kCal/week than the control mice, their fat intake is higher. Consequently, INT-exposed mice were challenged by fluctuating amount of calories, but also by a difference in macronutrient composition of the consumed diet. We have recently shown that 12-month-old mice exposed to the INT diet demonstrate hyperphagia during the first few days of the *ad libitum* feeding week [30]. This increased eating pattern at the first days after the diet switch still occurred at old age (**supplemental Figure 1A**).

Daily activity recorded at the age of 23 months revealed that the INT-exposed animals were significantly more active than the C- and MF-exposed mice and similarly active to the life-long CR-exposed animals (**Figure 1F**). Although this increased activity pattern in the INT-exposed mice was found in both the CR and the *ad libitum* MF feeding week, the timing of the activity differed (**supplemental Figure 1B**). During the MF-week, the burst of activity was observed when the light was switched off. During the CR-week the activity increased when the food was provided, 30 minutes before the light was switched off similar to the pattern found for the life-long CR exposed mice. Mortality rates of the 4 intervention groups, presented in **Figure 1G**, show the highest survival in CR-fed animals. The survival rate of the CR intervention group was significantly higher than that of the C-fed ($p=0.001$) and MF-fed ($p<0.001$) mice. The survival rate of the INT-exposed mice was lower than that of the CR-fed mice, but still significantly enhanced ($p=0.04$) compared with the MF-exposed mice. Pathological analysis of the mice that died or were euthanized during the study revealed that most of them suffered from multiple pathologies. Various tumours, particularly in the liver and lungs, ulcerative dermatitis and eye abnormalities were often detected (**supplemental Table 2S**). No clearly distinct pathological features were detectable between the intervention groups.

An oral glucose tolerance test (OGTT), carried out 2 weeks prior to sacrifice, revealed that glucose clearance in the INT-fed mice was significantly improved compared to the MF-exposed mice and almost similar to the CR-fed mice (**Figure 1H**). In addition, fasting insulin levels in the INT-exposed mice showed a tendency to decrease compared to the MF-exposed mice, although this effect was not significant (**Figure 1I**). Similarly, the homeostatic model assessment of insulin resistance (HOMA-IR) result revealed that the mean HOMA-IR value in the INT intervention group was slightly lower than that of MF group (**Figure 1J**) but significantly higher compared to the CR-exposed mice. A significant decrease in plasma leptin levels in the INT versus the MF-exposed animals was found but no significant changes between the 4 intervention groups was found for plasma IL-6, free fatty acid (FFA) and triglyceride (TG) levels (**supplemental Figure 1C**). Weight of the epididymal white adipose tissue (eWAT) in the INT-exposed mice was found to be significantly decreased compared to the MF-exposed mice and significantly increased compared with the CR intervention group (**Figure 1K**). A similar effect was found for kidney weight but not for heart, lung, spleen and pancreas weight (**supplemental Figure 1D**).

Taken together, intermittent CR significantly improved overall health compared with the MF-fed mice, indicating a protection against the detrimental effects of a MF diet. However, the beneficial effects were not as pronounced as induced by continuous CR exposure.

3.2 Life-long weekly intermittent calorie restriction reduced most but not all detrimental effects caused by a MF diet in the liver

To evaluate the effects of life-long INT feeding on the liver, a panel of different markers were examined in 24-month-old mice. Liver weight in the INT-fed mice was significantly lower compared

with the MF-exposed mice and did not differ significantly from the CR-fed mice (**Figure 2A**). However, when normalized to the body weight, the relative liver weight of INT diet group was significantly lower than that of the CR diet (**Figure 2B**). A significant decrease in the intrahepatic triglyceride (IHTG) levels was found in the INT-fed mice compared with the MF-exposed mice, but the levels were significantly higher compared with the CR-exposed mice (**Figure 2C**). The correlation between IHTG and plasma insulin levels was assessed, and revealed that high IHTG levels did not necessarily correlate with high plasma insulin levels (**Figure 2D**). Histological analysis of the livers of the mice from the different intervention groups confirmed the results of the IHTG measurements (**Figure 2E** and **supplemental Figure 2**). Moreover, intermittent CR caused significant improvement of plasma alanine aminotransferase (ALT) levels, a well-established marker of liver injury/damage compared to the mice exposed to the MF diet (**Figure 2F**). In addition, histological scoring of inflammatory aggregates (**Figure 2G**) and quantification of liver fibrosis by measuring 4-hydroxyproline levels (**Figure 2H**) showed significant improvement of liver health of the INT-fed mice compared to the MF-exposed mice. These last three markers represent the more advanced stages of NAFLD and revealed no significant differences between the INT- and CR-exposed mice.

In summary, despite the fact that INT-fed animals had been exposed to the MF diet for half of their life, apart from displaying slight accumulation of liver triglycerides, they performed equally well as CR-exposed mice for all other NAFLD markers tested.

3.3 Gene expression profiles of 24-month-old INT-fed mice were more similar to CR than to MF-exposed animals

Next, we studied the differences in hepatic gene expression patterns of the INT-exposed mice in comparison to the CR and MF intervention groups. For this purpose, microarray (MA) analysis was applied on RNA isolated of the livers from the mice. As shown in **Figure 3A**, the number of significantly ($p<0.01$) differentially expressed genes between INT- and MF-exposed animals increased with age and the highest number of differentially expressed genes was found at the oldest time point. In contrast, the difference between INT- and CR-exposed mice was most abundant in 6-month-old mice and decreased during aging. At the age of 24 months, in total 2815 genes displayed differential expression between INT- and MF-exposed animals while 569 genes were found to be differential expressed between INT- and CR-fed mice. Principal Component Analysis (PCA) of the top-1000 most variable genes in the 24-month-old animals, showed higher similarity between the INT and the CR-fed animals than between the INT- and MF-exposed mice (**Figure 3B**). Ingenuity pathway analysis (IPA) revealed that the differentially expressed genes in the two comparisons are related to different canonical pathways. The results presented in **Figure 3C** show that that hepatic fibrosis/ hepatic stellate cell activation and other immune pathway-related genes dominate the difference between INT- and MF-exposed animals. This result confirms the biochemical data presented in **Figure 2**. RXR activation was identified as the major difference between INT- and CR-fed mice by IPA. Furthermore, the IPA results indicated IPA upstream regulator analysis suggested that the factors driving the differential gene expression between the INT and MF diet were TGFB1 and a number other cytokines including TNF, IL4 and IFNγ. On the contrary, differential gene expression between INT and CR- exposed mice was due to NFE2L2 (or NRF2) activity well-known for its antioxidant response [52], XBP1, acknowledged to mediate ER stress/apoptosis [53, 54], and the well-established liver the liver transcription factor HNF4A [55]. Remarkably, our data do not point towards the involvement of one or more of the four well established pathways involved in mediating the CR effect including 1) the insulin like growth factor

(IGF-1)/insulin signalling pathway, 2) the sirtuin pathway, 3) the adenosine monophosphate (AMP) activated protein kinase (AMPK) pathway and 4) the target of rapamycin (TOR) pathway [6, 56], as upstream regulators. The expression profiles of the strongest differentially expressed genes in INT-exposed mice are depicted in **Figure 3D-G** and listed in **supplemental Table 3S**. *Cyp2b9*, *Igkv4-57-1* and *Acot3* are the strongest up-regulated genes when compared to the CR-fed mice (**Figure 3D**). *Moxd1*, *Cyp4a12b* and *Gldn* exhibit the strongest down-regulation in the same comparison (**Figure 3E**). The results presented in **Figure 3D+E** reveal that in INT-fed mice the genes that are differentially expressed compared to the CR mice are highly similar to the expression patterns in the MF-exposed mice. The genes showing the strongest increased expression in INT-fed compared to MF-exposed mice are *Hsd3b5*, *Serpina4-ps1* and *Slco1a1* (**Figure 3F**) while *Cyp2b13*, *Cidea* and *Pls1* exhibit the strongest decrease (**Figure 3G**). The genes presented in **Figure 3F+G** show, apart from differential expression compared to the MF-exposed mice, highly similar expression compared to CR-exposed mice.

In conclusion, these results indicate that weekly intermittent CR alters gene expression profiles induced by the MF diet at old age and are, significant but to a lesser extent, distinct from the continuously CR-exposed mice.

3.4 Phenotypic plasticity observed in the MF/INT diet switch group

In the final part of our analysis we explored the plasticity of the long-term effects induced by the MF diet. For this purpose, a subset of 12-month-old mice was transferred from the MF to the INT diet (MF/INT diet switch group). The mice of the diet switch group received the INT diet till sacrifice at the age of 24 months. As shown in **Figure 4A**, the results of the OGTT analysis revealed that glucose metabolism of the mice in the diet switch group was similar to that of the life-long INT-exposed mice. Furthermore, survival of the diet switch group markedly increased compared to the life-long MF-exposed animals, although this effect did not reach the level of significance (**Figure 4B**). Compared with the life-long MF-exposed mice body weight decreased significantly in the diet switch mice (**Figure 4C**). A reduction in eWAT (**Figure 4D**) and liver (**Figure 4E**) weight was observed but both adaptations were not significant. Analysis of a panel of liver health markers (**Figure 4F**) revealed no change in IHTG levels after the diet switch and a marked but not significant decrease in plasma ALT, lymphocyte infiltration and 4-hydroxyproline levels in the MF/INT diet switch mice compared to the life-long MF-exposed animals.

3.5 Molecular adaptations and irreversible changes in the liver of the MF/INT diet switch group

As shown in **Figure 3A**, microarray analysis revealed that, at the age of 24 months, 2815 genes displayed significant differentially expression between INT- and MF-exposed mice. We compared the expression levels of these 2815 genes between the diet switch group with either the life-long MF- or INT-exposed animals, respectively (see **Figure 5A**). Expression levels of 1510 genes were found to be similar in the MF/INT and the life-long INT-exposed animals and distinct from the MF-exposed animals (MF/INT vs INT $p>0.01$; MF/INT vs MF $p<0.01$). This result indicated that expression levels of these 1510 genes adapted to the INT diet that the mice had received during the last 12 months of their life. Expression levels of a second subset of 1157 genes did not differ significantly from either the MF or from the INT-fed animals (MF/INT vs INT $p>0.01$; MF/INT vs MF $p>0.01$). Interestingly, expression of a relative small subset of 148 genes differed significantly between the MF/INT diet switch and the INT-exposed mice, but not from the MF-exposed mice (MF/INT vs INT $p<0.01$; MF/INT vs MF $p>0.01$). This result implies that the differential expression induced by the MF diet of these

148 genes did not adjust to the INT diet during the last 12 months of life, thus indicating an irreversibility of the MF-induced effects. The heatmap of the hierarchical clustering of this selection of 148 genes presented in **Figure 5B** shows clustering of the INT-exposed mice and a distinct expression profile compared to mice of the MF and MF/INT intervention groups. IPA applied to compare the canonical pathways of the 148 irreversible (**Supplemental table 4S**) and the 1510 reversible genes (**Supplemental table 5S**), revealed that these subsets of genes represented distinct canonical pathways. The 148 consistently altered genes were found to affect RXR-mediated processes and xenobiotic metabolism signalling (**Figure 5C**). In contrast, the 1510 adaptive genes were found to be involved in a variety of immune response and inflammation-related pathways and in hepatic fibrosis/stellate cell activation (**Figure 5D**). IPA was applied to obtain insight into the mechanisms regulating the expression of the 148 consistently altered genes and found that PXR was the strongest regulator of this selection of genes (**Table 1**).

In **Figure 5E** expression profiles of *Hsd3b5*, *Cd36* and *Pparγ* are shown, representing 3 examples of genes of the subset of 148 consistently altered genes (qPCR validation and additional examples are presented in **supplemental Figure 3A** and **B**). Interestingly, for most of the genes displaying consistent changes induced by the MF diet, significant differential expression between the INT- and MF-exposed mice started at a younger age (6 or 12 months), e.g. *Cd36, Pparγ* and *Cidea*. **Figure 5E** presents the expression levels of *Cyp2u1*, *Lpl* and *Clec10a*, showing that the mean expression levels in the MF/INT-exposed animals differs strongly from the life-long MF-exposed mice. For these genes of which the expression levels adapted to the INT diet, no marked changes were found between young MF- and INT-exposed animals (qPCR validation and additional examples are presented in **supplemental Figure 3C** and **D**). By analysing the age-related effect of the 148 consistently altered genes in more detail we found that 43% of these genes showed a MF-induced change in gene expression at young age (6 or 12 months) while this was found for only 9% of the 1510 adaptive genes.

In conclusion, differential expression regulation of irreversible altered genes might have an onset earlier in life than the genes of which expression levels adapt to the INT diet.

4 Discussion

In this study we explored whether life-long intermittent CR could prevent against the adverse health effects induced by a MF diet in aging mice. During the last decade the health-promoting and life-extending effects of a wide variety of variants of a standard CR diet have been examined in both humans and models species [29, 57-62]. The major differences between the dietary variants of intermittent calorie restriction are 1) the extent of calorie reduction varying from complete fasting to a mild decrease in calorie intake and 2) the time window when the fasting/CR is applied in combination with the period in between the fasting/CR cycles. We define our INT dietary regimen as periods of one week of CR and refeeding, in order to allow the body to adapt to the two dietary

conditions. Our results obtained from the food intake measurements at middle [30] and old age (**supplemental Fig 1C**) revealed that the mice displayed hyperphagia during the *ad libitum* feeding but returned voluntarily to normal rations at the end of the week, indicating that this adaptive response was achieved. To attain the strongest adaptation in weight loss that did not exceed the guidelines of the "Committee for Care and Use of Laboratory Animals" (less than 20% loss of body weight/week) we have included a 40E% calorie reduction. During the experiment the INT-fed mice weekly lose or gain ~15% of their body weight. This fluctuating pattern is maintained from 9 weeks till 24 months of age, which is much longer than the time interval applied in most other studies examining intermittent fasting/CR [9, 17, 30, 63, 64] and allows us to explore the effects induced at both young and old age. To check whether this dietary variant could prevent against the adverse health effects caused by an obesogenic diet during the *ad libitum* feeding week we included a MF instead of a control diet. The results we present show that, although the mean energy intake of the INT-exposed over a two-week time interval mice was only marginally (7%) reduced compared to the MF-exposed mice, the 24-month-old mice display 1) a significant decrease in body weight, 2) a better fat/lean body mass ratio, 3) an improved glucose metabolism and 4) an increased survival. However, it should be noted that, apart from glucose metabolism, continuously CR-exposed mice performed better on all features compared with the INT intervention group. This result indicates the INT diet applied in this study is insufficient to reach the same health and life-span promoting effects as achieved by the CR diet, but largely restores the serious health effects induced by the MF diet. This is in agreement with previous findings, in which the application of intermittent CR provides protection against prostate cancer, mammary tumorigenesis and pancreatic cancer [65-68].

It is important to note that with the relatively extreme variant of this INT diet we exposed the mice to multiple challenges. In line with previous publications [26-29, 69] it can be hypothesized that the health- and life-span extending effects are not necessarily derived from the energy reduction only, but that intermittent exposure to challenges might have an additional health-promoting effect. Apart from exposure to alternating energy availability in this study the gastrointestinal tract as well as metabolic organs like the liver, were challenged to handle variations in carbohydrate and fat content. Another challenge the INT mice were exposed to was the food exposure time. During the CR week one portion of food was offered 30 minutes prior to the initiation of the dark-phase and the mice consumed the whole portion (almost all) at once. In contrast, during the MF-week the mice had continuous access to food. Furthermore, it is important to take into consideration that the daily activity of the INT-exposed mice appeared to differ significantly from the MF-exposed mice. We have previously reported an increase in daily activity in life-long CR-exposed mice in this cohort [41] in line with what has been reported earlier [6]. Since exercise is an important factor regulating health, this feature might very likely contribute to the health improving effects induced by the INT and CR diet. Intriguingly, the increase in daily activity in the INT-fed compared to the MF-exposed mice was not only observed in the restricted feeding week, due to foraging activity when hungry, but the same enhanced level of activity was also measured during the *ad libitum* feeding week. This result suggests that the increased activity can be seen as a habit more than a response to the lack of availability of food. These data also indicate that performing physical activity measurements in animal studies exploring the effects of dietary interventions is of utmost important to determine whether altered daily activity might play a (causal) role in diet-induced health- and lifespan promoting effects.

The effects of the INT diet were analysed in more detail in the liver, a central organ in the regulation of metabolic health. NAFLD frequently occurs in obese individuals and is recognized as the

hepatic manifestation of metabolic syndrome [70]. In the 24-month-old MF-exposed mice, increased IHTG, greater liver inflammation, enhanced liver 4-hydroxyproline levels and elevated plasma ALT levels indicate the presence of advanced stages of NAFLD. Life-long exposure to the CR diet fully protected against NAFLD development. INT-fed mice display significant decreases in markers representing the advanced stages of NAFLD (ALT, liver inflammation and liver fibrosis) compared with the MF-exposed mice. INT-exposed mice, however, display mild steatosis with IHTG levels significantly lower than the MF-exposed mice. This result differs from what we previously have observed in 12-month-old mice where the INT diet improved liver health even beyond the effect achieved by the CR diet [30]. **Figure 6** presents an overview of physiological markers measured in the INT-exposed animals during aging. In this figure the degree of similarity of the INT-exposed animals at the age of 12 and 24 month compared to the continuous CR and MF diets is depicted for all markers apart from survival for which only 24 months value is depicted (since almost all mice were alive at 12 months of age). At both 12 and 24 months of age, the body weights of INT-exposed animals were between those of CR- and MF-fed animals wile food intake levels increased. With aging, plasma insulin and IL-6 levels of animals in the INT diet group became more comparable with the profiles of the CR group while relative leptin levels did not alter. Furthermore, unlike in the continuous MF-exposed animals, NAFLD in the INT diet group did not progress to severe pathology, as indicated by the liver 4-hydroxyproline content. A small increase in relative plasma ALT levels was observed. In contrast, IHTG levels substantially elevated during aging, reaching a level similar to that of the MF group at the age of 24 months. The results we present here indicate that, in the long run, an INT diet protects the liver for the advanced stages of NAFLD but does not fully prevent MF-induced lipid accumulation at older age. This suggests that the metabolic capacity required for the modulation of liver fat content by catabolism and redistribution at old age might be impaired. This result is in line with previous studies that have indicated that aging is a risk factor for fat accumulation in the liver [71, 72].

Gene expression profiles generated from the liver of the 24-month-old INT-exposed mice revealed that gene expression in these animals appeared to be more similar to the CR than to the MF-exposed mice, underscoring the beneficial effects of the INT diet on metabolic health. This size of effect is remarkably large, considering that previous findings have shown that the effect of CR diet on the liver transcriptomic profile is rapidly blunted following an ad libitum re-feeding [73, 74]. This suggests that the effects of long-term repeated exposure to CR might accumulate and eventually result in a profile more similar to CR diet, instead of the MF diet. Functional analysis of the differentially expressed genes further confirmed that intermittent CR affects pathways involved in liver fibrosis and cirrhosis and the advanced stages of NAFLD. Taken together, strong improvement in total body and liver health was caused by regular, short-term exposure to a CR diet. The INT diet almost completely counteracts the adverse health effects of the MF diet, which these mice consumed for half of their life. However, despite of the additional challenges these mice have been exposed to in addition to the reduction in energy intake, the effects did not reach the standards achieved by a life-long CR diet.

CR interventions are often applied to achieve weight loss in overweight and obese subjects [75-77]. In the second part of our study we explored into what extent, the molecular effects induced by a MF diet in the liver during the first 12 months of life, can be reversed by exposure to the INT diet during the second 12 months of life. Glucose tolerance is markedly increased in the diet switch group which is in agreement with a recent study where obese mice were exposed to an high-fat alternating-day fasting regimen that showed improved glucose tolerance [78].

Microarray analysis exhibited significant differential expression of 2815 genes between 24-month-old MF and INT intervention groups. Analysis of the gene expression levels in the MF/INT diet switch group revealed that the majority of these 2815 differentially expressed genes partially (1157 genes) or fully (1510 genes) adopted to the INT expression profile. IPA analysis showed that the subset of 1510 reversible genes represent predominantly inflammation-related pathways. Similarly, a decrease in plasma ALT levels and liver lymphocyte aggregates and fibrosis was observed in the diet switch mice, although these effects were not significant. Expression levels of a relative small subset of genes (148), however, appeared consistently changed by exposure to the MF diet during the first 12 months of life. IPA showed that this subset of 148 genes encompasses genes involving lipid and xenobiotic metabolic processes. The observation that PXR (or NR1I2*)* was the strongest predicted upstream regulator, suggests a connection between these two functions. PXR is a ligand-activated nuclear receptor that, upon activation, forms a heterodimer with RXR. This complex is not only activated by exogenous toxins but has also been shown to responds to endobiotics like bile acids and steroid hormones [79, 80]. Previous studies have shown that PXR activation in mice induces fatty acids uptake via up-regulation of *Cd36*, which is one of the 148 consistently regulated genes. Additionally, other genes involved in lipid metabolism including *Pparγ, Cyp7b1, Cidea* and *Cidec* are consistently up-regulated in the MF/INT diet switch mice. This persistently regulated lipid metabolism and/or storage genes might "set" the homeostasis to be either inefficient in transporting and oxidizing fatty acids or susceptible to fat accumulation. With respect to the PXR target genes involved in xenobiotic metabolism, it should be noted that a large number of genes are consistently upregulated by the MF diet including *Gstm1, Gstm5, Fmo1, Fmo2, Fmo3, Abcc3, Cyp3a5*, etc., indicating strongly enhanced xenobiotic and/or endobiotic metabolism in both the MF-exposed and the diet switch mice. Another example of a gene persistently altered by the MF diet that might contribute to the development of liver dysfunction is Ubiquitin-conjugating enzyme 2c (*Ube2c*). *Ube2c* has been identified as a hepatocellular carcinoma gene [81, 82] and we observed elevated expression of *Ube2c* already at the age of 6 months in the MF-exposed mice (**supplemental Figure 3B**) but the INT diet appears to reverse this effect at young age. Importantly, overexpression of *Ube2c* have been shown to induce loss of genomic stability, since the cells neglect the mitotic spindle checkpoint signals [83]. Therefore, the persistent effect of the MF diet on the expression of this gene might have serious consequences on liver health and might enhance the risk on hepatocellular carcinoma development. Additional research is required to validate this observation. Taken together these results suggest that, after a strong weight loss, robust reductions in the advanced stages of NAFLD can be achieved but that hepatic steatosis might not be fully reversible at old age.

In conclusion, our data indicate that intermittent CR offers significant health improving effects and largely counteracts the adverse effects of a MF diet on the liver, but does not reach the health- and life span improving effects of a CR diet. Although the number of consistent molecular changes induced in the liver by a MF diet is small, they might have potentially important adverse effects on health.

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Author contribution

Conceived and designed the experiments: FR, MM and WTS. Performed the experiments: FR, CL, MVB, MvD, ALM and WTS. Analyzed the data: WTS, FR, MVB, MvD, ALM, KvN. Assessed quality control of microarrays: MVB. Wrote the paper: FR and WTS. Provided valuable feedback on manuscript: KvN, MVB, MvD, ALM, MM. All authors read and approved the final manuscript.

Conflict of interest statement

Miriam van Dijk and Klaske van Norren are affiliated with Nutricia Research. Aswin L Menke is affiliated with Triskelion. The other authors declare that they have no competing interests.

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Figure legends

Figure 1: Intermittent calorie restriction (INT) strongly increases life- and health-span. A) Study design scheme. **B)** Weekly body weight measurements show a fluctuating body weight of the INT-exposed mice dependent on the diet the mice received in the preceding week and a mean body weight in between that of the mice fed a control (C) - or calorie restricted (CR) diet. Body weight development results up till 12 months of age have been published before [30]. **C)** INT-exposed mice display an increase in lean body mass but not to the same extent as found in the CR-fed animals. **D)** During the whole study the highest food intake was found for the *ad libitum* fed mice from the INT group while the during the CR week this intervention group had the lowest food intake. **E)** Mean energy intake of the INT-fed mice is slightly lower compared to the Moderate-fat (MF)-exposed

animals and significantly higher compared to the CR-fed mice. **F)** Mean daily activity levels in the INT-fed mice were highly similar to the CR-exposed animals and differ significantly from the MF-exposed mice. **G)** Weekly intermittent CR causes a significant increase in survival compared to the MF-exposed mice but not to the same extent as found for the CR-fed animals. **H)** An oral glucose tolerance test (OGTT) showed that glucose clearance in the INT-exposed mice is similar to the CR-fed mice. **I)** Fasting insulin levels and HOMA-IR index of the INT-fed mice were in between the levels found in the MF- and CR-exposed mice. **J)** eWAT weight of the INT-exposed animals was significantly lower compared to the MF-fed mice but significantly higher than found for the CR-exposed mice. Results are means ± SEM, *p < 0.01, **p < 0.001, ***p < 0.0001.

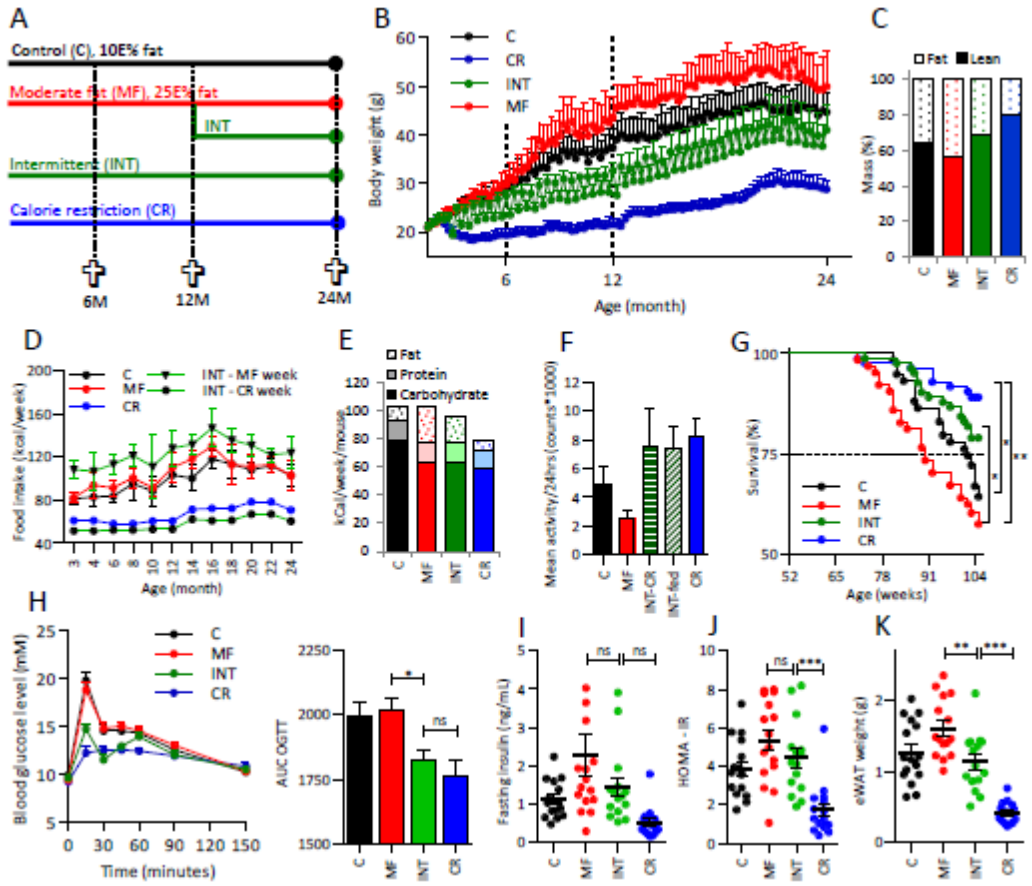


Figure 2: Intermittent CR counteracts the adverse health effects of the MF diet on the liver. The adverse effects caused by a MF diet on **A)** liver weight, **B)** relative liver weight, **C)** intrahepatic triglyceride (IHTG) levels, and **D)** Correlation between IHTG and plasma insulin. **E)** 4 representative examples of haematoxylin and eosin stained sections of the different intervention groups are presented. Markers of the severe stages of NAFLD including **F)** ALT, **G)** lymphocyte infiltration and **H)** 4-hydroxyproline levels were significantly counteracted by intermittent CR in the INT-exposed animals. Results are means ± SEM, *p < 0.01, **p < 0.001, ***p < 0.0001.

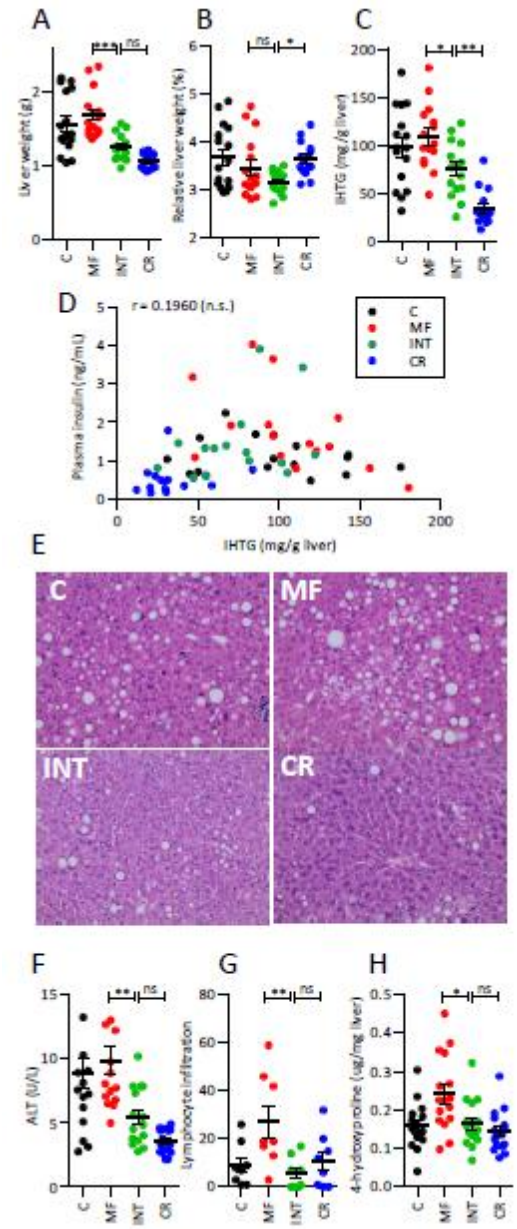
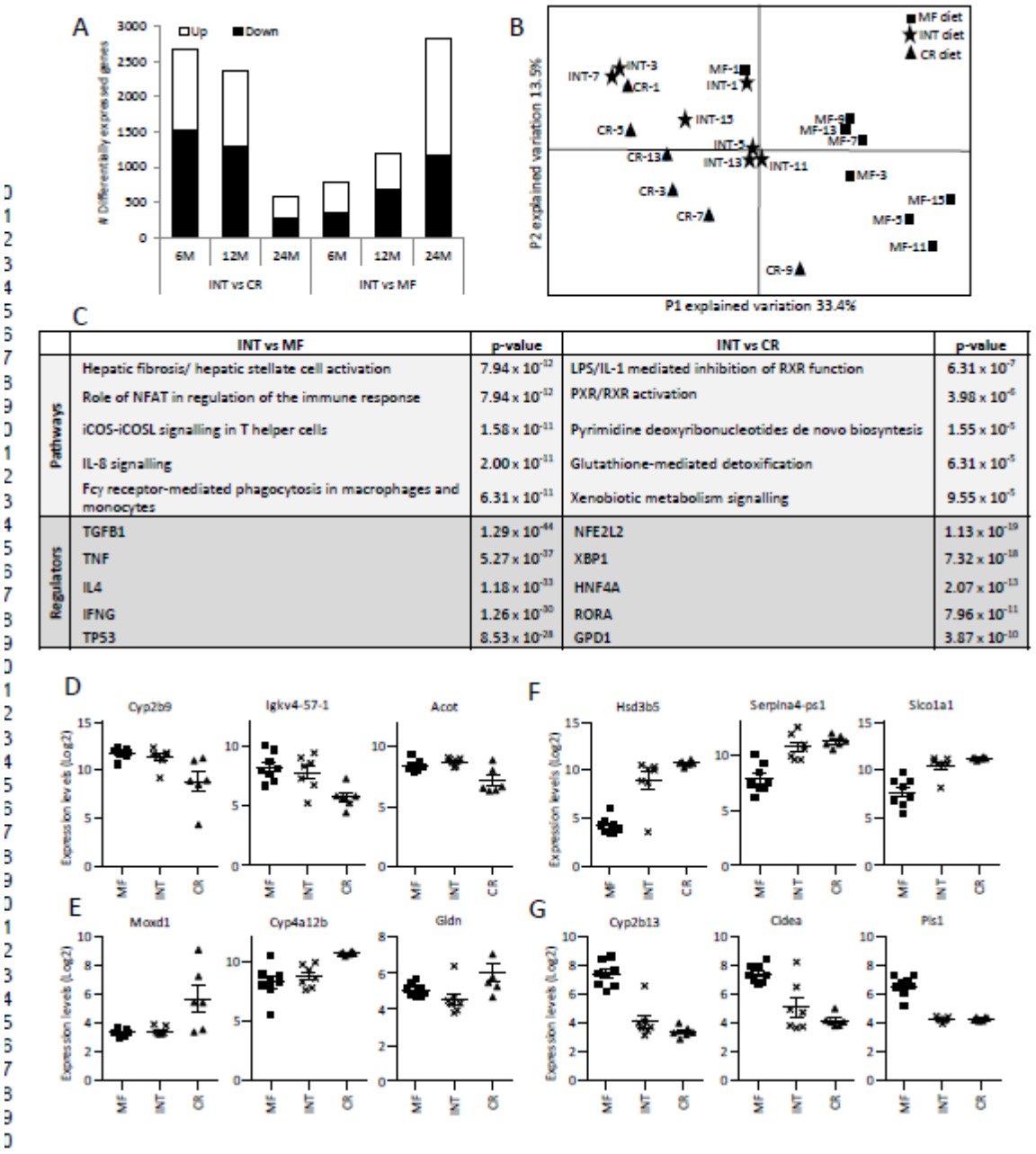


Figure 3: Gene expression measured in the liver of 24-months-old INT-exposed mice is more similar to that of CR than MF-fed animals. A) The number of significantly differentially expressed genes between the INT and MF intervention groups increase during aging while the INT versus CR comparison revealed an aging-related decrease of differentially expressed genes. **B)** The principal component analysis (PCA) plot generated from the top-1000 most variable genes showed that the expression profiles of the INT-fed animals is more similar to the CR than to the MF-exposed animals. The numbers displayed in the PCA plot represent the numbers of the individual mice of the 3 intervention groups. **C)** IPA analysis revealed distinct canonical pathways and upstream regulators for the two subsets of differentially expressed genes. MA expression profiles of the 3 genes encompassing the strongest **D)** up-regulation or **E)** down-regulation in INT compared to CR-exposed mice or **F)** up-regulation or **G)** down-regulation compared to MF-exposed mice.



	INT vs MF	p-value	INT vs CR	p-value
Pathways	Hepatic fibrosis/ hepatic stellate cell activation	7.94×10^{-12}	LPS/IL-1 mediated inhibition of RXR function	6.31×10^{-7}
	Role of NFAT in regulation of the immune response	7.94×10^{-12}	PXR/RXR activation	3.98×10^{-6}
	iCOS-iCOSL signalling in T helper cells	1.58×10^{-11}	Pyrimidine deoxyribonucleotides de novo biosyntesis	1.55×10^{-5}
	IL-8 signalling	2.00×10^{-11}	Glutathione-mediated detoxification	6.31×10^{-5}
	Fcγ receptor-mediated phagocytosis in macrophages and monocytes	6.31×10^{-11}	Xenobiotic metabolism signalling	9.55×10^{-5}
Regulators	TGFB1	1.29×10^{-44}	NFE2L2	1.13×10^{-19}
	TNF	5.27×10^{-37}	XBP1	7.32×10^{-18}
	IL4	1.18×10^{-33}	HNF4A	2.07×10^{-13}
	IFNG	1.26×10^{-30}	RORA	7.96×10^{-11}
	TP53	8.53×10^{-28}	GPD1	3.87×10^{-10}

Figure 4: Effects of exposure to a MF diet are partially reversed after transferring the mice to the INT diet for the last 12 months of their life. Strong adaptation to the INT diet in the MF/INT diet switch group was found by measuring **A)** glucose clearance **B)** survival and **C)** body weight. The decrease in **D)** eWAT weight, **E)** liver weight, **F)** IHTG levels, liver inflammation, ALT and 4-hydroxyproline levels after the diet switch were not significant. The data of 12-month-old mice are parts of our previous publication [30]. Results are means ± SEM,*$p < 0.01$, **$p < 0.001$, ***$p < 0.0001$.

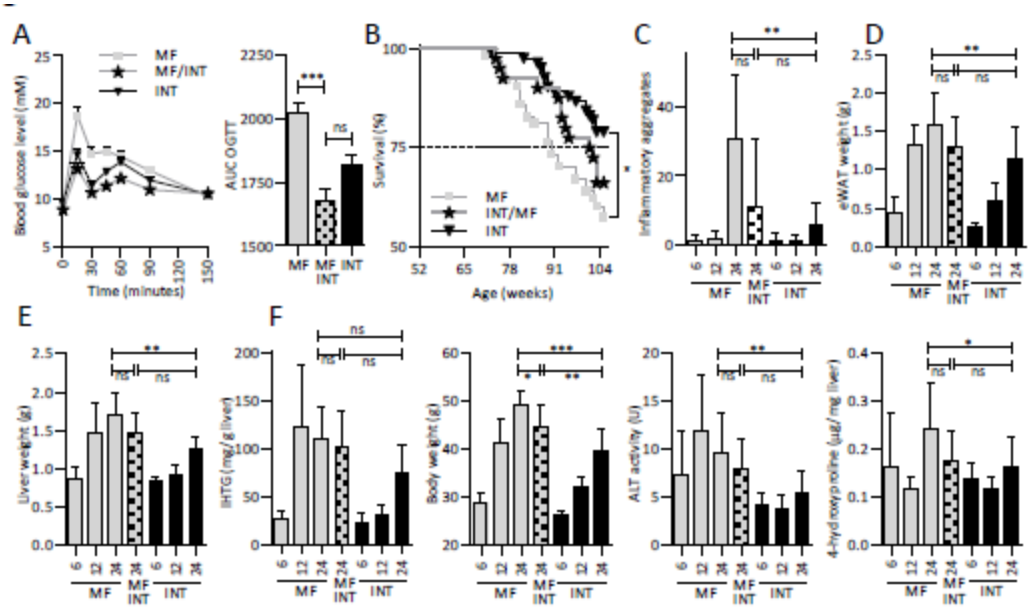


Figure 5: **A small fraction of the MF-induced genes during the first 12 months of life are consistently altered. A)** Schematic overview of the different gene groups revealing full (1510 genes) or partial (1157) adaptation to the INT diet and a subset of 148 genes of which the expression levels remained similar to the life-long exposed MF mice. **B)** A heatmap of the 148 consistently altered genes show clustering of the MF and MF/INT diet switch mice and distinct expression profiles for most of the INT-exposed 24-month-old mice. Ingenuity pathway analysis (IPA) of the **C)** 148 genes consistently changed by the MF diet and the **D)** 1510 adaptable genes revealed that they present different functional categories. **E)** Gene expression profiles obtained from microarray data of 3 examples of consistently affected genes and **F)** genes that adapt to the INT diet after the diet switch. The data of 12-month-old mice are parts of our previous publication [30]. Results are means ± SEM.

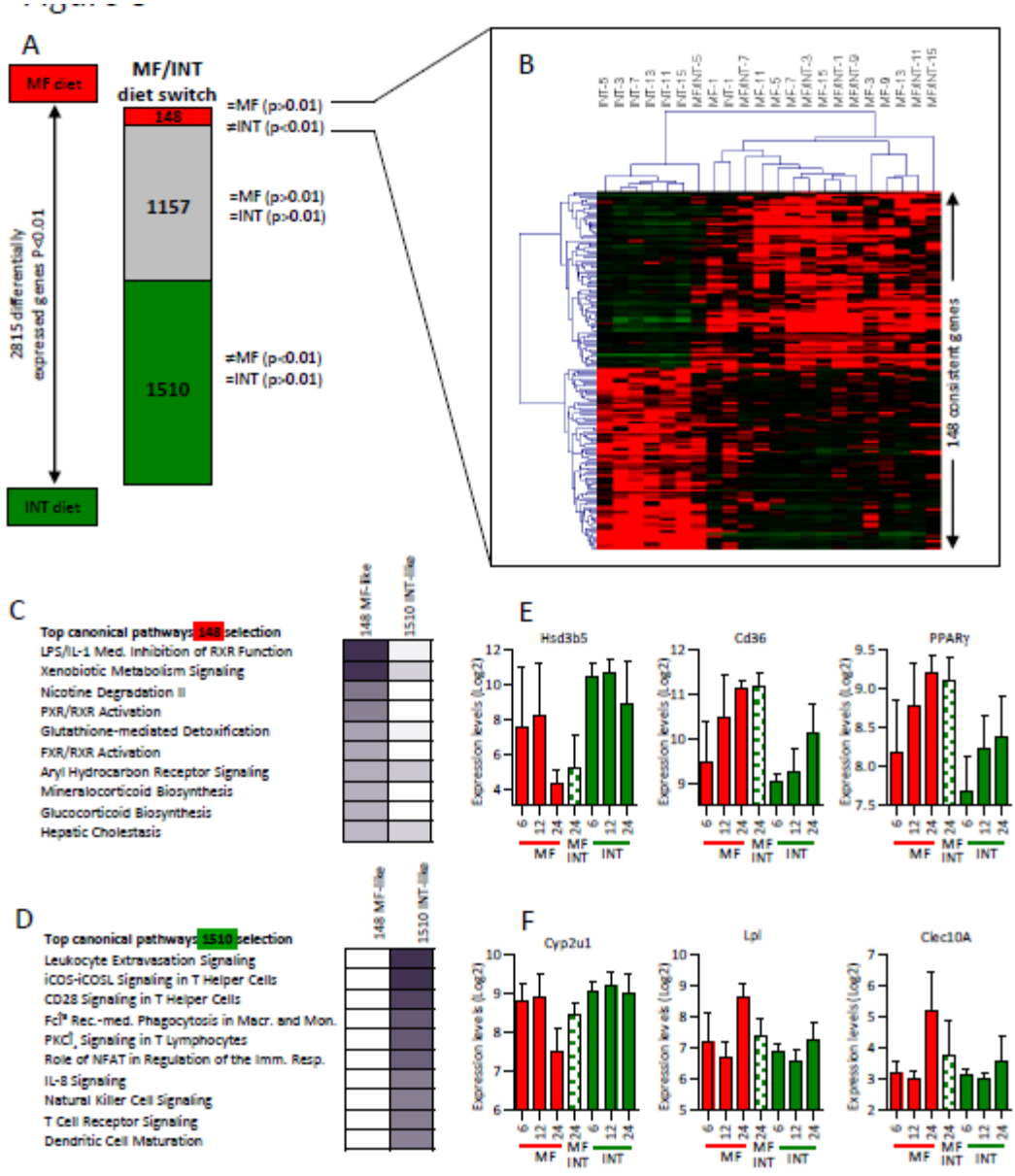
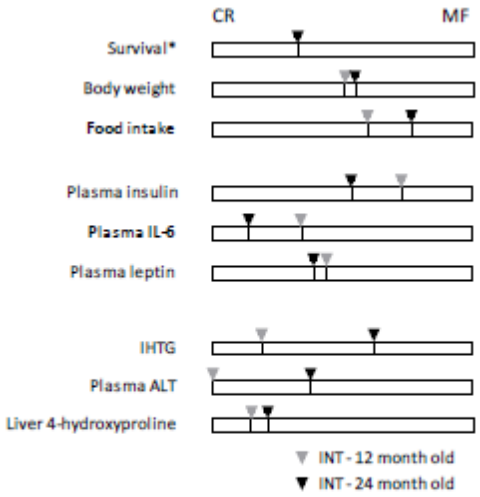
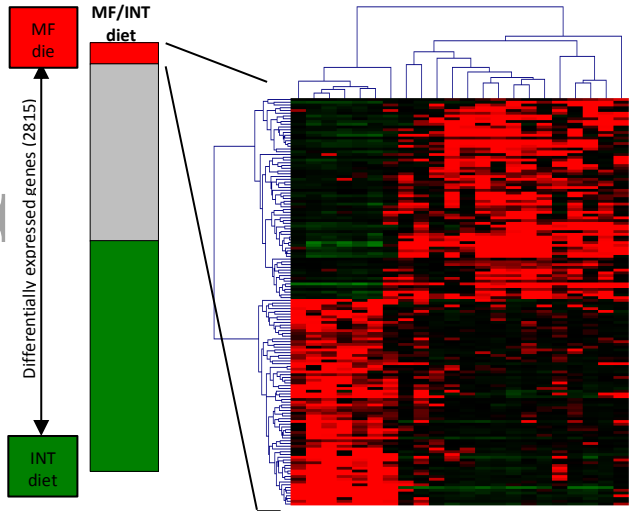


Figure 6. Feature similarity of the INT diet group at 12 and 24 months in comparison to the CR and MF groups. The bars represents the difference between the CR and MF groups, which was set as 100% for each time point. Then, the position of the INT-exposed group within the 100% scale was determined and represented by the line and pointer. The survival bar included only the 24M pointer since hardly any mice died at this age.





Graphic Abstract

Table 1: Upstream regulators of the 148 MF-consistent genes with a p-value of overlap<0.001 and an activation score>2 or repression score<-2

Upstream Regulator	Exp Fold Change	Molecule Type	Activation z-score	p-value of overlap
PXR		ligand-dependent nuclear receptor	3.20	2.28E–13
Ncoa-PXR-Rxra		complex	2.00	4.50E–06

PXR ligand-PXR-Retinoic acid-RXRα		complex	2.20	4.58E–06
MED13		transcription regulator	–2.00	8.38E–06
NFE2L2		transcription regulator	2.80	2.83E–05
PPARG	1.76	ligand-dependent nuclear receptor	2.60	4.00E–04
mir-223		microrna	2.00	9.24E–04