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Dietary fat drives whole-body insulin resistance and promotes intestinal inflammation independent of body weight gain

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ABSTRACT

Background. The obesogenic potential of high-fat diets (HFD) in rodents is attenuated when the protein:carbohydrate ratio is increased. However, it is not known if intake of an HFD irrespective of the protein:carbohydrate ratio and in the absence of weight gain, affects glucose homeostasis and the gut microbiota.

Methods. We fed C57BL6/J mice 3 different HFDs with decreasing protein:carbohydrate ratios for 8 weeks and compared the results to a LFD reference group. We analyzed the gut microbiota composition by 16S rDNA amplicon sequencing and the intestinal gene expression by real-time PCR. Whole body glucose homeostasis was evaluated by insulin and glucose tolerance tests as well as by a hyperinsulinemic euglycemic clamp experiment.

Results. Compared with LFD-fed reference mice, HFD-fed mice, irrespective of protein:carbohydrate ratio, exhibited impaired glucose tolerance, whereas no differences were observed during insulin tolerance tests. The hyperinsulinemic euglycemic clamp revealed tissue-specific effects on glucose homeostasis in all HFD-fed groups. HFD-fed mice exhibited decreased insulin-stimulated glucose uptake in white but not in brown adipose tissue, and sustained endogenous glucose production under insulin-stimulated conditions. We observed no impairment of insulin-stimulated glucose uptake in skeletal muscles of different fiber type composition. HFD-feeding altered the gut microbiota composition

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High fat diet-induced obesity is frequently used to study obesity and related metabolic disorders in rodents. Since such a model cannot be used to distinguish whether the observed metabolic dysfunctions result from the obese state or from the high fat feeding, the health consequences of a high fat intake, in the absence of weight gain, remain largely unknown. Using rodent models to elucidate the influence of dietary fat, while eliminating weight gain as confounder, is a challenging approach as high fat diet (HFD)-feeding tends to induce obesity to various degree depending on the protein:carbohydrate ratio [1]. Nevertheless, glucose intolerance induced by conventional HFD-feeding has been shown to precede weight gain in both humans [2] and mice [3], suggesting that a HFD per se affects glucose homeostasis. In support, we have previously demonstrated how exchanging sucrose with casein in an isoenergetic HFD protects against weight gain, but not glucose intolerance [1,4]. The mechanism behind the observed decrease in glucose tolerance remains elusive. Reduced adipose plasticity affecting both local and global insulin signaling [5], and further aggravated by accompanying adipocyte inflammation, may be involved [6]. Yet, whether adipocyte inflammation is a consequence [7] or a promoter [8,9] of insulin resistance remains to be established. Apart from changes in adiposity and adipocyte function, HFDs promote intestinal alterations such as increased intestinal permeability [10] and an elevated inflammatory milieu [11]. Both factors are believed to contribute to the progression of insulin resistance [12]. Interestingly, gut anti-inflammatory agents have been shown to protect against HFD-induced glucose intolerance and insulin resistance despite significant weight gain [13], indicating intestinal inflammation as key in the development of insulin resistance.

We hypothesized that dietary fat, independent of weight gain, would change the intestinal microenvironment translating to impaired metabolic homeostasis. To disentangle the influence of HFD-feeding on intestinal inflammation and whole-body glucose disposal, we fed mice 3 experimental isoenergetic HFDs with fixed fat content and a stepwise increase in the protein:carbohydrate ratio and compared the results to a low fat diet (LFD) reference group. We took advantage of an in-house observation where mice appeared less prone to diet-induced obesity (DIO) in 1 out of our 5 animal facilities, which reflects a recent report deciphering how mouse origin and housing conditions influence weight development [14]. Using the hyperinsulinemic euglycemic clamp technique, this experimental setting enabled acquisition of information on the extent to which macronutrient composition affects whole-body metabolism without possible confounding effects of weight gain and adipose tissue inflammation.
and a glucose tolerance test (GTT), respectively. All mice were MR-scanned prior to the ITT using EchoMRI 4in1 (USA). After 7 weeks, a subset of mice in each group underwent a surgical procedure (see Section 2.4. for further details). The remaining mice were used as donor mice to ensure adequate red blood cells (RBCs) during the clamp procedure.

### 2.3. Insulin/Glucose Tolerance Tests and HOMA-IR

Prior to the ITT and the GTT mice were fasted for 2 h and 5 h, respectively, in clean cages only containing a transparent shelter, bedding and water. Insulin was diluted in succinylated gelatin (Gelofusine® B. Braun Melsungen AG, Germany). Insulin (0.75 U insulin/kg lean mass) and glucose (2 g glucose/kg lean mass) were injected intraperitoneally. Blood glucose was measured in tail vein blood before and 15, 30, 45, 60, 90, and 120 min after the glucose bolus or the insulin bolus, using Contour Next Test Strips (Bayer Contour, USA). HOMA-IR was measured 6 weeks after diet initiation in 5 h fasted mice and calculated as: (fasting plasma glucose (mmol/L) × fasting serum insulin (mU/L))/22.5.

### 2.4. Hyperinsulinemic Euglycemic Clamp Experiments

The hyperinsulinemic euglycemic clamp was performed in conscious, unrestrained mice. For the surgical catheterization of the jugular vein and the carotid artery, the mice were anesthetized with isoflurane (2.5% for induction, 1.5% for maintenance). Carprofen (10 mg/kg; Rimadyl, Pfizer, USA) was injected and blood samples were collected at variable infusion rates of human insulin (4 mU kg⁻¹ min⁻¹) followed by a constant 0.04 μCi/h infusion of 2-[1-14C]-deoxy-D-glucose (Perkin Elmer, USA) was injected and blood samples were collected at t = 122, 125, 135, 145, and 155 min, respectively. These samples were processed for determination of 2-[1-14C]-deoxy-D-glucose specific activity.

### 2.5. Clamp Sample Processing and Calculations

Plasma samples were deproteinized with Ba(OH)₂ and ZnSO₄ and aliquots of each supernatant were transferred to 2 scintillation vials. To determine plasma [3-3H]glucose, one of the aliquots was dried and resuspended in MilliQ water to remove 3H₂O and the other was counted directly by liquid scintillation counting (Hidex 300 SL). Supernatants for 2-[1-14C]-deoxy-D-glucose determinations were counted directly. Total plasma glucose concentration was determined by adding a reaction mix (200 mmol/L Tris–HCl, 500 mmol/L MgCl₂, 5.2 mmol/L ATP, 2.8 mmol/L NADP, and 148 μg of a hexokinase and GPDH mixture (Roche, Germany) pH 7.4) to each sample. Parameters related to glucose turnover rates (R₂, R₁, endoR₂, glycogenesis) were calculated as previously described [15]. Tissues were homogenized in ice-cold lysis buffer (pH 7.4, 10% glycerol, 1% IGEPAL, 50 mmol/L Hepes, 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 1 mmol/L sodium-pyrophosphate, 5 mmol/L nicotinamide, 4 μmol/L Thiamet G and protease inhibitors (SigmaFast, Sigma-Aldrich, USA)). Aliquots of each crude homogenate were transferred to two 2 ml tubes and deproteinized with perchloric acid or Ba(OH)₂ + ZnSO₄. Supernatants were transferred to scintillation vials and counted to determine 2-[1-14C]-deoxy-D-glucose content. Tissue-specific glucose uptake rates were calculated as described previously [16].

### 2.6. Western Blot Analyses

Approximately 10 mg liver tissue and 25 mg adipose tissue were homogenized (PRECELLYS® 24, USA) in ice-cold lysis buffer (pH 7.4, 10% glycerol, 1% IGEPAL, 50 mmol/L Hepes, 150 mmol/L NaCl, 10 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L EGTA, 20 mmol/L sodium pyrophosphate, 2 mmol/L sodium orthovanadate, 1 mmol/L sodium-pyrophosphate, 5 mmol/L nicotinamide, 4 μmol/L Thiamet G and protease inhibitors (SigmaFast, Sigma-Aldrich, USA)). Homogenates were incubated end-over-end for 45 min (4 °C), followed by centrifugation at 16,000 g for 10 min (4 °C). Supernatants were stored in aliquots at −80 °C until further analysis. Protein concentration was measured by BCA (#23,223 and #23,224, Thermo Scientific, USA) according to the manufacturer's instructions. Western blot analyses were performed as previously described [17] using the following antibodies: anti-ACCoA Ser79 phosphorylation (#07-303, Upstate Biotechnology, USA), anti-akt T Thr308 phosphorylation (#9275, Cell Signaling Technologies, USA), anti-akt T Thr473 phosphorylation (#9271, Cell Signaling Technologies, USA), anti-AKT Thr1719 phosphorylation (#9275, Cell Signaling Technologies, USA), anti-AKT Thr1708 phosphorylation (#9271, Cell Signaling Technologies, USA), anti-2 (3063, Cell Signaling Technologies, USA).

### 2.7. Isolation of Intestinal Epithelial Cells (IECs)

The small intestine was sectioned in 8 equal segments. The 5th and the 8th segments, starting from duodenum, were
collected as representative of the jejunum and the ileum [18] for isolation of the intestinal epithelial cells. These segments were flushed with cold PBS buffer, cut open in the length and fractioned in 0.2–0.5 cm pieces. Samples were placed on ice and separately incubated in 5 ml of buffer (Matrisperse cell recovery solution, Corning, USA) for 40 min allowing epithelium detachment [19,20]. Efficient recovery of crypt and villi and viability of the epithelial cells were controlled on a microscope following a trypan blue (Sigma-Aldrich, USA) staining. Cells were centrifuged at 10,000 g for 2 min and snap frozen in liquid nitrogen. Total RNA was extracted with TRIzol reagent (Invitrogen, USA), and RNA quality was assessed on a Bioanalyzer (Agilent, USA) by RIN value (285/18S ratio). Samples with a RIN value below 7 were excluded.

### 2.8. Quantitative PCR

Total RNA of adipose tissues was extracted by TRIreagent® (Sigma-Aldrich, USA) according to manufacturer’s protocol using PRECELLYS® 24 for homogenization. One microgram of RNA was transcribed into cDNA by reverse transcriptase (Invitrogen, USA). Quantitative PCR analyses were performed using the SYBR Green qPCR Master mix (Thermo Scientific, USA) and the Stratagene Mx3000P qPCR System. Primer sequences are summarized in Supplementary Table S1.

### 2.9. 16S rRNA Amplicon Sequencing and Bioinformatics

Bacterial DNA from cecal and colon samples was extracted using a NucleoSpin soil kit (Macherey-Nagel, Germany) according to manufacturer’s instructions. DNA yield and integrity were assessed using a Nanodrop (Thermo Scientific, USA) and agarose gel electrophoresis, respectively. PCR-based library formation targeting the 16S rRNA gene’s variable region 4 (V4) was performed as previously described [21]. The PCR products were purified using Agencourt AMPure XP (Beckman Coulter, USA) beads and pooled in equal concentrations. Sequencing was performed using an Illumina MiSeq with V2 PE500 cartridge (500 cycles). The generated data was analyzed using QIIME [22] v1.9.1 with default settings. UCHIME [23] was used for chimera checking and UCLUST [24] for de novo OTU-picking based on 97% sequence similarity. Bacterial taxonomy was assigned using the RDP-classifier [25] and Greengenes [26] database v13.8. Subsequent analysis was performed in R v3.2.3 using the Phyloseq [27], metagenomeSeq [28] and Vegan [29]. Low-abundance operational taxonomic units (OTUs) were filtered from the data by removal of OTUs present in fewer than 3 of the samples or with a relative abundance across all samples of less than 0.005%. The data used for the analysis contained on average 18.494 ± 3.422 (SD) sequences per sample after filtering. Analysis of bacterial differential abundance was performed on data normalized with metagenomeSeq using cumulative-sum scaling. In addition, the statistical analyses using metagenomeSeq were performed on data filtered based on effective sample sizes, where taxa were not included if they had fewer than X effective number of positive samples, where X is the median of estimated effective samples per feature.

### 2.10. Histology Examination and Adipose Tissue Histology

Liver and adipose tissues were fixed in 4% phosphate buffered paraformaldehyde, dehydrated, and paraffin embedded. Five micrometer sections from 6 to 8 mice of epididymal white adipose tissue (eWAT) and inguinal white adipose tissue (iWAT) were stained with hematoxylin and eosin (H&E). One representative micrograph of each group is shown, and 2 sections of adipose tissue depots from each mouse were measured by drawing and used for quantification of mean cell diameter using Image J open source software.

### 2.11. Histopathological and Immunohistochemical Analysis of Mouse Liver Tissue

Preparation of liver sections and histopathological techniques were performed according to standard protocols. Two independent observers performed all histological assessments in a blinded fashion. We used the established NAFLD activity score (NAS) for evaluation of H&E stained random liver sections as described elsewhere [30]. Briefly, a score of 0 to 2 excludes non-alcoholic steatohepatitis (NASH), a score of 3–4 defines “borderline NASH”, whereas a score of 5 and higher assures the full picture of NASH.

### 2.12. Lipid Profile and Thin-Layer Chromatography

Diacylglycerol (DAG) and triacylglycerol (TAG) were measured on 5 mg liver and ceramide content was measured on 20 mg liver by thin-layer chromatography (TLC). Lipids were extracted in chloroform–methanol (2:1) [31] and dissolved in chloroform as previously described [32]. DAG, TAG, and ceramides were separated on silica-gel coated plates using two different separate mobile phases consisting of chloroform–methanol–acetic acid–water (50:50:5:5) followed by petroleum ether–diethyl ether–acetic acid (120:25:1.5) for DAG and TAG and chloroform–methanol–acetic acid (98:2:0.5) for ceramides. Butylated hydroxytoluene (50 mg/L) was added to both of the mobile phases. The lipids were developed by a 10% copper sulfate pentahydrate and 8% phosphoric acid solution at 120 °C for 15 min for DAG and TAG and at 140 °C for 10 min for ceramides. The silica coated plates were visualized on a Typhoon FLA 7000 IP fluorescent scanner and analyzed according to weight using ImageQuant TL (GE Healthcare Life sciences, United Kingdom).

### 2.13. Immuno Assays

Plasma insulin, adiponectin, and tumor necrosis factor alpha (TNF-α) were analyzed on the MSD platform (Mesoscale, USA), while serum amyloid A (SAA) was analyzed by standard ELISA (Abcam, USA) according to the manufacturers’ protocol.

### 2.14. Statistical Analyses

Data are presented as mean ± SEM. All time-dependent analyses were evaluated by two-way repeated measures (RM) ANOVA, Dunnett post hoc test. Time-independent
analyses were evaluated by one-way ANOVA, Dunnett post hoc test. Bacterial composition was examined using the metagenomeSeq package and adonis test in R and correlation analyses were examined by Spearman rank correlation test, adjusted for multiple comparisons by Benjamin and Hochberg false discovery rate (FDR) test. qPCR data on IECs were log (Ln) transformed prior statistical analyses due to a non-Gaussian distribution. Statistical significance was set at $p < 0.05$ and the different levels of significance were set at $^* = p < 0.05$, $^{**} = p < 0.01$, $^{***} = p < 0.001$.

3. Results

3.1. High-Fat Diets Induce Glucose Intolerance Independent of Protein and Sucrose Content

We used 3 isoenergetic HFDs designated HFHP, HFIP and HFNP with matched fat content (45% energy from fat) and a stepwise increased protein:carbohydrate ratio, and compared the results to a LFD reference group. To determine the influence of dietary

Fig. 1 – High-fat diets induce glucose intolerance independently of background diet and obesity. 8-week-old male mice were fed either a high-fat-high-protein (HFHP), a high-fat-intermediate-protein (HFIP) or a high-fat-normal-protein (HFNP) diet for 8 weeks. A) Body weight development. B) Lean body mass 5 weeks post diet initiation. C) Fat mass 5 weeks post diet initiation. D) and E) feed consumption. F) HOMA-IR 6 weeks post diet initiation. G) Insulin tolerance test 5 weeks post diet initiation. H) Glucose tolerance test 6 weeks post diet initiation. I) Plasma insulin change from prior to 5 min after a glucose bolus in 5-h fasted mice. Data are presented as mean ± SEM. n = 6 mice per group. A–E, G–H: One representative out of 3 independent experiments. F and I: One representative out of 2 independent experiments. 1- and 2-way ANOVA where appropriate, Dunnett post hoc test. In H: # indicates difference between HFHP and LFD; $^\dagger$ indicates difference between HFIP and LFD, * indicates difference between HFNP and LFD.
fat, carbohydrate, and protein, we used an animal facility where mice empirically are known to be less prone to DM. Accordingly, body weight and fat composition were similar between groups (Fig. 1A–C). LFD-fed mice had a higher feed intake (grams of feed consumed) than HFD-fed mice. This was most likely a consequence of a less energy dense diet (Table 1), as the calorie intake was similar between the groups (Fig. 1D and E, respectively). These findings were replicated in 3 independent experiments (n = 6). In this experimental setting, we were able to obtain information on the extent to which macronutrient composition affected whole-body metabolism without confounding effects of weight gain. Compared to LFD reference mice, 5 weeks of HFD feeding did not affect the blood glucose response to a single insulin bolus regardless of the protein:carbohydrate ratio (Fig. 1G). However, all HFD-fed groups had reduced glucose tolerance by 6 weeks of HFD feeding (Fig. 1H), suggesting that HFDs impaired glucose homeostasis independent of weight gain and protein:carbohydrate ratio. The observed discrepancy between glucose intolerance (Fig. 1H) and systemic insulin sensitivity (Fig. 1F–G) could indicate impaired insulin secretion capacity. However, glucose-stimulated insulin secretion measured 5 min post glucose bolus was indistinguishable between groups (Fig. 1I).

3.2. Intestinal Alterations in the Microbiome Potentially Translate to Increased Gluconeogenesis in the Small Intestine of HFD-Fed Mice

The difference between glucose tolerance and insulin sensitivity prompted us to investigate whether alterations in the gut might be involved. A possible scenario would involve diet-dependent alterations in the composition of the gut microbiota, which subsequently could affect intestinal gene expression disturbing metabolic homeostasis. Consequently, we analyzed the intestinal microbiota composition based on 16S rDNA amplicon sequencing. We observed a pronounced separation between the colonic and cecal microbiota in all HFD-fed mice and the LFD-fed reference mice (Figs. 2A–C and S1A–C). Among the HFD-fed mice only the HFHP and HFIP groups separated significantly (adonis unweighted unifrac p = 0.023). A higher relative abundance of a single family, namely S24-7 from the Bacteroidetes phylum was the main difference comparing the HFHP with the HFIP group (Figs. 2D and S1D). Despite the observed changes in the S24-7 abundance, our data strongly suggest that in these settings, dietary fat is a stronger driver of alterations in the composition of the gut microbiota than the amount and/or ratio between dietary carbohydrate and protein.

Intestinal epithelial cells (IECs) are ‘first line responders’ to dietary constituents and key in intestinal gluconeogenesis [33]. Intestinal gluconeogenesis accounts for up to 20% of host glucose production [34] and can therefore significantly affect host glucose homeostasis [35,36]. Moreover, IECs have immune modulatory potential [37] releasing pro-inflammatory cytokines to the portal circulation, thereby potentially aggravating hepatic insulin resistance. We therefore analyzed gene expression in ileal and jejunal IECs. Compared to the LFD-fed reference mice, we observed significant induction of expression of Nos2, Tnfa, and Il12a in the jejunum of HFHP fed mice, whereas mice fed the HFNP diet exhibited induction of Nos2 and Il1b. In contrast, expression of Il10 and Il13 tended to decrease in mice fed either of the HFDs. In the ileum, no significant changes in expression of these cytokines were observed (Fig. 2E–F). Interestingly, in all HFD-fed mice, irrespective of the ratio between carbohydrate and protein, we observed a striking induction of Pck1 and G6pc both in the jejunum and the ileum (Fig. 2H), suggesting that increased intestinal gluconeogenesis may contribute to the observed impaired glucose tolerance.

3.3. Protein:Carbohydrate Ratio Modulates Liver Histology

To further investigate possible mechanisms contributing to the observed impaired glucose tolerance, we investigated whether cytokines released from the intestine had affected liver metabolism, including accumulation of lipids. None of the HFD-fed mice exhibited ectopic lipid accumulation as determined by analysis of hepatic levels of TAG, DAG, and ceramides (Fig. 3A). Furthermore, serum amyloid A1, an acute phase protein with high dynamic range, primarily reflecting hepatic secretion, and a potential biomarker of insulin resistance [38], did not differ between groups (Fig. S2a). However, immunohistochemical analyses revealed a borderline increase in the NAFLD activity score in mice fed the HFNP diet (Fig. 3B–C). This finding suggested that the protein:carbohydrate ratio in connection with HF-feeding may affect hepatic metabolism in a manner not detectable by biochemical analysis of lipid accumulation, and that the HFDs with high and intermediate protein:carbohydrate ratio counteracted this increase in the NAFLD activity score.

3.4. Tissue-Specific Alterations in Insulin-Stimulated Glucose Uptake in Response to HFD Feeding

To further examine the metabolic alterations associated with the HFD-feeding, we determined whole-body insulin sensitivity and tissue-specific insulin-stimulated glucose uptake by performing a hyperinsulinemic euglycemic clamp experiment in cohorts of mice challenged by the 4 different diets (Fig. 4A). In parallel to the decreased glucose tolerance observed during GTT, the glucose infusion rate under insulin-stimulated conditions (Fig. 4B) was decreased in all HFD-fed mice compared to LFD-fed reference mice. This observation was marginally different under steady state conditions (Fig. 4C), where only HFHP and HFIP reached the level of statistical significance. For glucose disappearance rate (Fig. 4D), only the HFIP group was significantly different from the LFD reference mice, albeit all HFD-fed mice tended towards decreased glucose disappearance. All HFD-fed groups appeared to have sustained EGP under clamped condition (Fig. 4E), indicating decreased insulin-mediated suppression of gluconeogenesis. The apparently sustained EGP was, however, not significantly different from the LFD reference group, presumably due to insufficient power (n = 4–7 mice per group). Still, sustained EGP was unlikely to be the only mechanism behind the observed glucose intolerance. Accordingly, we examined insulin-stimulated glucose disposal in peripheral tissues. Irrespective of the diet composition, clamped mice had comparable insulin-stimulated glucose uptake in soleus, extensor digitorum longus (EDL), and tibialis anterior (TA) muscles (Fig. 4F). However, we observed a significant
decrease in insulin-stimulated glucose uptake in iWAT of all HFD-fed mice compared to LFD reference mice, and a tendency to decreased glucose uptake in eWAT, but no intergroup variation in brown adipose tissue of clamped mice (Fig. 4G).

3.5. Insulin Signaling is Impaired in iWAT of HFD Fed Mice

The above results prompted us to investigate adipose tissue morphology and function. All HFD-fed groups displayed enlarged adipocytes in both eWAT and iWAT (Fig. 5A–B) despite a lean phenotype and uniform fat mass between groups. Serum fatty acids and TAG concentrations were not altered between groups; neither under basal nor clamped conditions (Fig. S2D–E). In keeping with the marked decrease in glucose uptake particularly in iWAT, we focused our subsequent analyses on this depot. AKT phosphorylation at Thr308 and Ser473 under insulin-stimulated conditions did not differ between groups when the statistical analyses were run on data from each phosphorylation site separately (Fig. 5C). However, performing a two-way ANOVA on the combined data from both phosphorylation sites revealed a significant main effect of "diet" ($p = 0.028$), and subsequent posthoc tests using the Dunnett’s method with the LFD group as control group showed a significant difference between LFD and the HFNP groups ($p = 0.021$), and borderline significant differences between LFD and HFHP ($p = 0.055$) and LFD and HFIP ($p = 0.068$). Thus, effects of insulin on AKT signaling are generally reduced in all HFD-fed groups.

Fig. 3 – The dietary protein:carbohydrate ratio affects NAFLD development. Mice were fed experimental diets for 8 weeks before tissues were either fixed in paraformaldehyde and prepared for paraffin embedding, or snap frozen in liquid nitrogen. All mice were euthanized in the morning. They had free access to feed. A) Hepatic lipid accumulation. B) NAFLD activity score evaluated by inspection of images from C. C) H&E stains of liver sections, one representative out of 4–6 images per group. A–B: Data are represented as mean ± SEM. 1-way ANOVA, Tukey post hoc test. * depicts difference between the labeled group and the HFIP-fed group. n = 6–8 mice per group.
Reduced AMP-activated protein kinase (AMPK) activity in WAT has been linked to insulin resistance in humans [39], and acetyl-CoA carboxylase (ACC) phosphorylation mirrors AMPK activity in both muscles and adipose tissue [40,41]. We found significantly decreased phosphorylation of ACC at Ser79 ($p = 0.0015$ and $p = 0.0017$, respectively, Fig. 5D) in HFHP- and HFIP-fed mice, suggesting a decreased activity of AMPK in iWAT of HFHP- and HFIP-fed mice.
Adiponectin is an insulin-enhancing adipokine [42] exerting both autocrine and paracrine functions. At the autocrine level, adiponectin stimulates lipid storage and enhances both insulin-stimulated glucose uptake [43] and AMPK activation [44]. HFHP- and HFIP-fed mice had reduced levels of circulating adiponectin compared to LFD reference mice, while HFNP-fed mice only trended towards a reduction in serum adiponectin (Fig. 5E). Interestingly, the pro-inflammatory cytokine, TNF-α, has been shown to induce a lowering of adiponectin release in adipose tissue of lean, but not obese individuals [45]. As neither circulating TNF-α protein nor WAT mRNA levels of Tnfα (Fig. S2B and C-D, respectively) were different between LFD and HFD-fed groups, TNF-α may not explain the observed reduced adiponectin levels. Inspection of H&E stained tissue sections revealed no increase in macrophage infiltration and crown-like structures (CLSs) into white adipose tissue (data not shown), and we did not observe alterations in mRNA expression of macrophage chemo-attractant protein-1 (Mcp-1), or the inflammatory regulators, interleukin (Il)1β and Il6 (Fig. S2C–D). Finally, we asked if the serum protein levels of another macrophage chemo-attractant, C-X-C motif chemokine 10 (CXCL10), an anti-inflammatory molecule, IL10, as well as
resistin were altered between HFD-fed mice and LFD reference mice. Both resistin and CXCL10 have been reported to be increased in human type 2 diabetic patients [46,47], whereas serum IL10 levels appear to be decreased in type 2 diabetic patients [48]. However, none of these markers differed between the four groups of mice (Fig. S2B).

3.6 Dietary Fat is a Predominant Driver of Diet-Induced Immuno-Metabolic Alterations

Investigating the correlation between jejunal gene expression, adipocyte size, adiponectin secretion and weight change by Spearman correlation analysis indicated a correlation between intestinal inflammatory profile and expression of gluconeogenic genes (Table 2). Bridging intestinal immunity to whole-body metabolism, the inducible isoform of nitric oxide synthases, NOS2, involved in pro-inflammatory immune responses, was found to positively correlate with adipocyte size in iWAT (Table 2, \( r = 0.52, p = 0.033 \)), whereas the anti-inflammatory cytokine IL10 correlated negatively with adipocyte size in iWAT (Table 2, \( r = -0.54, p = 0.021 \)). Spearman correlation analyses including dietary constituents revealed fat as the most prominent driver, correlating positively with weight change (Table 2, \( r = 0.39, p = 0.035 \)), adipocyte size in eWAT (Table 2, \( r = 0.32, p = 0.009 \)), and also with jejunal G6pc expression (Table 2, \( r = 0.32, p = 0.008 \)), while correlating negatively with serum adiponectin (Table 2, \( r = -0.47, p = 0.033 \)). Sucrose was inversely correlated with jejunal transcripts of Il13 (Table 2, \( r = -0.51, p = 0.021 \)), and protein intake was, like fat intake, inversely correlated with serum adiponectin (Table 2, \( r = -0.54, p = 0.012 \)). Interestingly, serum adiponectin levels were not correlated with expression of markers for intestinal gluconeogenesis (Pck1: \( r = -0.27, p = 0.257; \) G6pc: \( r = -0.33, p = 0.166 \)) or adipocyte size, albeit a borderline correlation between serum adiponectin and eWAT cell size (\( p = 0.053, n = 18 \)) but not iWAT cell size (\( p = 0.204, n = 17 \)) was observed.

In contrast, adipocyte size correlated with both inflammatory and gluconeogenic jejunal gene expression (Table 2).

4. Discussion

In the present study we demonstrate that HFD consumption impaired whole-body glucose homeostasis independent of weight gain and irrespective of the protein:carbohydrate ratio. The observed glucose intolerance of HFD-fed animals may be a consequence of elevated gluconeogenic potential in intestinal epithelial cell which is supported by our finding of increased expression of gluconeogenic genes in the epithelial cells of the jejunum and ileum, and the sustained EGP during the hyperinsulinemic euglycemic clamp experiments in these animals. We did not observe any impairment of glucose-stimulated insulin secretion as measured 5 min after administration of a glucose bolus, and inflammation markers in adipose tissues were not elevated. However, insulin-stimulated glucose uptake in iWAT was impaired and was accompanied by decreased insulin-dependent phosphorylation of AKT. Collectively, our data support that diminished insulin-dependent glucose uptake in adipose tissues and increased intestinal gluconeogenesis contribute to the observed impaired glucose tolerance.

Diets high in protein have been suggested to promote leanness through increased satiety mediated by an augmented intestinal gluconeogenesis [36]. Yet, we did not find changes in gene expression of gluconeogenic genes, or changes in feed intake as a function of dietary protein content. Rather, we observed that induction of key genes involved in intestinal gluconeogenesis correlated with high fat intake. Dietary fat intake has previously been found to be a more powerful driver of changes in the composition of the gut microbiota than the protein:carbohydrate ratio [49]. Our analyses confirm this finding and further show that fat intake correlated with more changes than protein and sucrose intake combined, pointing to fat intake independent of weight gain as a key determinant for metabolic regulation.

Because dietary long chain fatty acids, protein and simple carbohydrates are digested in the small intestine, particularly in jejunum, we hypothesized that diet-induced jejunal immune alterations might orchestrate subsequent host responses of glucoregulatory origin. In support of this hypothesis, HFD-induced intestinal Tnfa expression precedes both obesity and insulin resistance [50], while gut anti-inflammatory agents protect against insulin resistance, but not obesity [13]. Moreover, our multivariate analysis revealed that the expression of...
several jejunal inflammatory gene transcripts correlated with expression of jejunal gluconeogenic gene transcripts.

Despite a similar reduction in glucose tolerance across all three HFDs examined, our data suggest that background diet, in this case protein:carbohydrate ratio, may have affected the ‘route of action’. Both serum adiponectin and ACC phosphorylation in I.WAT were diminished in HFHP and HFIP-fed mice compared to LFD reference mice, while HFNPF-fed mice were less affected. In contrast, HFNPF-fed mice had decreased adipose insulin signaling, as determined by AKT phosphorylation, compared to the LFD reference group, and increased NAFLD activity score compared to the HFIP and HFHP fed mice. HFIP-fed mice had a reduced abundance of OTUs belonging to the bacterial family S24-7 compared to any of the tested diets. Furthermore, Spearman correlation analyses showed that both dietary protein and fat but not sucrose negatively correlated with serum adiponectin levels. This is of particular interest since low carbohydrate intake counterbalanced by a high intake of protein and fat is a popular tool in weight losing regimes.

One limitation of the current study is the relative low n size in several of the experiments. Obviously, this affected the statistical power in the subsequent analysis of the data, and thereby may have promoted type 2 errors and the risk of accepting differences as non-significant. Nonetheless, the unique experimental settings applied in this study have enabled us to decipher how diets high in fat affect whole-body glucose homeostasis in mice independent of a confounding weight gain. The detailed physiological assessment of whole-body insulin sensitivity and tissue-specific glucose uptake, as well as comprehensive biochemical and histological analyses of a wide range of tissues and cell types, have provided us with valuable data that support a role of dietary fat per se to increase the gluconeogenic potential of intestinal epithelial cells, to contribute to development of glucose intolerance, and to decrease insulin-stimulated glucose uptake in adipose tissue. To our knowledge, no human studies have been conducted in which these effects of dietary fat have been investigated in the absence of a confounding weight gain. Results from such studies would be very interesting and obviously important for proper dietary guidance of people in need of maintaining or losing weight.

Author Contributions
A. Experimental conception and design: BAHJ, JTT, KB, LM, KK.
B. Data collection, analysis and interpretation: BAHJ, TSN, AMF, JBH, EF, AKS, SR, SIP, IS, AP, MP, TM, BS, LM, KK, JTT.
C. Drafting the manuscript: BAHJ, KK, JTT.
D. Editing, revision and approval of final manuscript: All.

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Conflicts of Interest
The authors declare no conflicts of interest.

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Appendix A. Supplementary Data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2016.09.002.

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