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*Article*

# Intake of the Artificial Sweetener Acesulfame-Potassium Alone and in Combination with a High-Fat Diet Leads to Differential and Sex-Specific Effects on Metabolic Function in Mice

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**Abstract:** Sugar-sweetened beverages are associated with metabolic dysfunction, particularly in those with increased risk factors. Artificial sweeteners (AS) are often promoted as a healthier alternative, yet findings remain conflicting as to their effects on metabolic function. Further, there is a lack of data exploring the interaction between AS and high-fat diets (HFD). We therefore examined the effects of HFD and the AS Acesulfame-potassium (Ace-K) on glucose intolerance and adipose tissue physiology in male and female C57BL/6 mice. 40 mice were randomised to receive either a) a control diet (CDCon; standard control diet/water), b) control diet and Ace-k (CDAS; CD/7.5mM AS in drinking water), c) HFD (HFCon; HFD (45%kcal from fat)/water), or d) HF and AS (HFAS; HFD/7.5mM AS in drinking water) for 6 weeks. A HFD increased body weight in male and female mice independently of AS supplementation. AS induced sex-specific effects protecting against HFD-induced hyperglycaemia and adipocyte hypertrophy in male mice and reducing inflammatory gene expression in the adipose tissue. Conversely in females, AS induced hyperinsulinemia in HFD mice and increased expression of immune-related genes. These findings suggest that supplementation of HFD with AS exacerbates metabolic dysfunction in female mice. This work supports the importance of studying sexually dimorphic responses to an altered nutritional environment and highlights the need for further investigation into the intake of AS, particularly in those already at risk of metabolic disease such as the obese or overweight.

**Keywords:** adipose tissue; non-nutritive sweeteners; artificial sweeteners; high-fat diet; glucose intolerance

## 1. Introduction

The obesity epidemic impacts millions of individuals world-wide. Over the last two decades, the prevalence of obesity in adults (18 years and older) has increased 1.5 times globally, while the incidence of childhood obesity has more than doubled over the same period from 2000 to 2016 (from 2.9% to 6.8%) [1]. Obesity and overweight contribute to the development and progression of a number of other non-communicable diseases including type 2 diabetes mellitus (T2DM), hypertension, cardiovascular disease, non-alcoholic fatty liver disease and various cancers [2, 3]. Although genetic, physiological, social and environmental factors can influence the development of obesity, increasing sedentary lifestyles and relative ease of access to calorie dense diets, rich in saturated fat, sugar and salt, are common contributing factors in the rise of obesity in many western countries and in developing nations where western 'junk' food is becoming easily available due to nutrition transitions [4].

Sugar-sweetened beverages have long been synonymous with the 'western junk diet' and associated with increased weight gain and the development of T2DM [5]. As a result, sugar substitutes such as artificial sweeteners (AS) have rapidly increased in popularity in recent decades [6, 7]. Also known as non-nutritive sweeteners, AS are calorically light

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and promoted as the healthy alternative to their sugary counterparts. However, controversy remains as regards their impact on metabolic health with evidence to date suggesting they may even exacerbate metabolic dysfunction in the setting of obesity [8]. While some studies suggest that AS impart beneficial effects on consumers [9], others indicate increased risk of insulin resistance (IR) [10], metabolic syndrome, cardiovascular disease and, contrary to popular belief, obesity [11, 12]. Currently, >41% of adults and 25.1% of children in the United States consume AS, with consumption higher in obese individuals [7]. In the United Kingdom, 61.8% of the population report AS intake, with 67% of overweight and obese consuming AS compared to 51% of those who are of normal weight [13]. Consumption of AS occurs most commonly via diet soft drinks. However, AS are found in a range of diet or 'lite' products including in chewing gum, vitamin supplements, and toothpaste.

Acesulfame-K (Ace-K) is one of the most popular AS, with consumption frequently through diet carbonated drinks [14]. Previous work by our group has shown that a maternal intake of Ace-K impairs glucose tolerance in pregnant mice [15]. In non-pregnant mice, Ace-K has been shown to increase weight gain in male but not female mice. Further, energy metabolising pathways in the female mice were downregulated, while in males these were activated [11]. Treatment of mouse and human precursor cells by Ace-K has been shown to induce adipogenesis, indicating that these compounds influence adipocyte differentiation [16]. In obese human subjects, consumption of diet drinks containing Ace-K leads to a dysregulation in inflammatory pathways within the adipose tissue [17].

While AS are advertised as a healthier option, they in fact may pose a substantial risk to those already at risk of overweight / obesity and related metabolic disorders, with higher all-cause mortality and deaths from circulatory diseases reported in individuals consuming two or more glasses of AS soft drinks per day [18]. Given the difficulties around delineating confounding factors in human studies, animal models are critical in understanding the potential interactions involved. Further, while some studies have investigated the direct impact of AS supplementation, there are few that have examined the effects of AS and a high-fat diet (HFD) in combination. The aim of this study was therefore to determine the influence of AS consumption on metabolic health, alone and in combination with a HFD.

## 2. Materials and Methods

### *2.1 Animal procedures*

All animal procedures were approved (Code number: 001846; approved 26/04/17) by the Animal Ethics Committee at the University of Auckland in accordance with the New Zealand Animal Welfare Act, 1999. Male and female C57BL/6 mice were sourced from and housed within the Vernon Jansen Unit at the University of Auckland under standard conditions (22˚C, 12:12 light dark cycle, 40-45% humidity, and wood-shavings as bedding).

Mice were randomly assigned to one of four diets (n=5 per sex per group) as follows: a) Standard control diet (CDCon; 10% kcal from fat (D12450H, Research Diets NJ, USA) and water), b) High fat Control (HFCon; 45% kcal from fat (D12451, Research Diets NJ, USA) and water), c) CD with addition of artificial sweetener (CDAS; standard diet and 7.5 mM Ace-K in drinking water), or d) HF with addition of AS (HFAS; HF diet and 7.5 mM Ace-K in drinking water). All diets were fed ad-libitum and compositional profiles are detailed in Table 1.

Females and males were housed in same-sex cages within their assigned dietary group. Diets were maintained from 6 weeks of age to 12 weeks of age. Body weight and food and liquid intakes were measured weekly. An oral glucose tolerance test (OGTT) was undertaken at week 11, with cull at week 12 as detailed below.

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**Table 1: Composition of CD and HF diets.**

| | CD (% kcal) | HF (% kcal) |
|---|---|---|
| Protein | 20 | 20 |
| Carbohydrate | 70 | 35 |
| Fat | 10 | 45 |
| Lard | 4.44 | 39.44 |
| Soybean oil | 5.56 | 5.56 |
| Total (% kcal) | 100 | 100 |
| Energy density (kcal/gram) | 3.8 | 4.7 |

*2.2 OGTT procedure*

At 15 weeks of age, mice were fasted for 6 h from 8am. They were then weighed, followed by the snipping of the tip of the tail (<1mm). The second drop of blood was read using a glucometer (Accu-Chek Performa, Roche Diabetes Care). Mice received 2g/kg of D-glucose via oral gavage. Blood glucose concentrations were measured from the tail tip at 0, 15, 30, 60, 90, and 120 minutes. Tail blood was also collected at 0, 15, and 60 minutes into EDTA microvettes (CB300, Sarstedt). Following centrifugation for 10 mins / 2500g / 4˚C, plasma was stored at -20˚C for insulin analysis. Fasting glucose and insulin were used to calculate HOMA-IR, a validated surrogate measure of insulin sensitivity in rodents [19].

*2.3 Tissue collection*

Mice were fasted for 6 h. Tail blood and plasma samples were taken as detailed above. Mice were then culled by cervical dislocation. Mice were weighed and gonadal adipose tissue was dissected, weighed, snap-frozen, and then stored at -80˚C.

*2.4 Histological analysis*

Male and female gonadal adipose tissue was fixed in 10% neutral-buffered formalin and was paraffin embedded and sectioned (10μm) using a Leica R 2135 rotary microtome (Leica Instruments). The slides underwent haematoxylin and eosin staining, before sections were mounted with DPX mountant. A light microscope and NIS Elements-D software (Nikon 800) were used to visualise and image capture slides. Four representative images were taken of each section and analysed in a blinded manner using ImageJ software (NIH) to determine the mean adipocyte size and the size distribution.

*2.5 Plasma analysis*

Mouse-specific insulin, leptin, and testosterone ELISA kits (Ultra-Sensitive Mouse Insulin ELISA Kit (Cat. # 90080), Mouse Leptin ELISA kit (Cat. # 90030), and Mouse Testosterone ELISA Kit (Cat. # 80552), Crystal Chem Inc., IL, USA) were used according to the manufacturer's instructions.

*2.6 Gene expression analysis*

Adipose tissue RNA was extracted using RNeasy Mini Kits (Cat. No 74104, Qiagen, Hilden, Germany) and a TissueLyser (Qiagen, Hilden, Germany) as per the manufacturers' instructions. RNA concentrations were assessed with a NanoDrop spectrophotometer (NanoPhotometer N60, Implen). cDNA was generated using a High Capacity cDNA

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Reverse Transcription Kit (Life Technologies Ltd, Applied Biosystems) as per the manufacturer's instructions. cDNA was mixed with Taqman Fast Advanced Master (Applied Biosystems) and pipetted into pre-designed RT² Profiler PCR Array microplates (SAbiosciences). PCR was performed using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems). Genes were normalised to the geomean of Actb, B2M, Ldha, Rplp1 and Hprt1 expression. The comparative CT method (2-ΔΔCT) was utilised to analyse the results [20].

*2.7 Statistical Analysis*

Statistical analysis was performed using GraphPad Prism and IBM SPSS Statistics Data Editor version 27. Normality was assessed using the Shapiro-Wilks test. Any data that was not normally distributed was transformed as appropriate. Data from female and males were analysed separately. Repeated measures ANOVA was performed for the OGTT data. All other data were analysed using two-way factorial ANOVA with diet (CD vs HF) and AS intake (AS vs water) as factors. Holm-Sidak post-hoc tests were performed as indicated for comparison testing between groups. Significance between groups was given at $P<0.05$. All data are presented as means ± SEM unless stated otherwise.

## 3. Results

*3.1 Body weight and caloric intake*

As expected, female and male mice fed the HFD gained more weight and were heavier at cull than their CD counterparts (Tables 2 and 3 respectively). However, there was no significant difference in body weight between AS and water groups. Absolute daily food intake was lower in both HFD groups although as expected kcal intake was significantly higher compared to CD mice. HFD increased the size of gonadal adipose tissue in a sex-specific manner. In females HFCon gonadal adipose tissue weight was significantly increased compared to CDAS (Table 2), while in male HFAS gonadal tissue weight was significantly increased compared to both CD groups (Table 3).

Table 2: Weight, cumulative weight gain, food, and energy intake, gonadal fat mass, and glucose in female mice.

| | **CDCon** | **CDAS** | **HFCon** | **HFAS** | **AS** | **HFD** | **Interaction** |
|---|---|---|---|---|---|---|---|
| Weight (g) | 21.1±0.15 | 20.78±0.37 | 22.30±0.42 | 22.72±0.63$^{*+}$ | NS | *P*=0.002 | NS |
| Cumulative weight gain (g) | 2.48±0.39 | 2.50±0.69 | 4.22±0.46 | 3.62±0.26 | NS | *P*=0.008 | NS |
| Food intake (g/d) | 2.69±0.036 | 2.98±0.099 | 2.40±0.12$^{+}$ | 2.29±.087$^{*+}$ | NS | *P*=0.00005 | *P*=0.045 |
| kcal intake (kcal/d) | 8.35±0.11 | 9.22±0.69 | 11.27±1.24$^{*+}$ | 10.75±0.9$^{*+}$ | NS | *P*=0.00003 | NS |
| Gonadal fat mass (% BW) | 1.49±0.17 | 1.17±0.13 | 1.72±0.59$^{+}$ | 1.68±0.15 | NS | *P*=0.06 | NS |

Data presented as mean ± SEM, where *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS; n=5 mice/group. NS = not significant.

Table 3: Weight, cumulative weight gain, food, and energy intake, gonadal fat mass, and glucose in male mice.

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| | CDCon | CDAS | HFCon | HFAS | AS | HFD | Interaction |
|---|---|---|---|---|---|---|---|
| Weight (g) | 27.28±0.63 | 27.26±0.84 | 29.05±0.30 | 31.44±2.02 | NS | NS | NS |
| Cumulative weight gain (g) | 6.08±0.28 | 5.44±0.52 | 7.60±0.54 | 8.14±1.18$^{+}$ | NS | *P*=0.006 | NS |
| Food intake (g/d) | 3.27±0.11 | 3.31±0.08 | 2.68±0.11 | 2.64±0.12 | NS | NS | NS |
| kcal intake (kcal/d) | 10.15±0.34 | 10.28±0.24 | 12.61±0.52$^{*+}$ | 12.40±0.59$^{*+}$ | NS | *P*=0.00004 | NS |
| Gonadal fat mass (% BW) | 1.59±0.20 | 1.65±0.26 | 2.81±0.55 | 4.10±0.73$^{*+}$ | NS | *P*=0.0003 | NS |

Data presented as mean ± SEM, where *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS; n=5 mice/group. NS = not significant.

### *3.2. Plasma analysis*

HFD increased fasting blood glucose at cull in both females and males (Tables 4 and 5 respectively), while AS reduced fasting plasma glucose in females but not males. Plasma insulin concentrations at cull displayed an interaction effect in male mice, with CDAS increased compared to CDCon, while HFCon was significantly increased compared to HFAS. In female mice HFD significantly increased plasma insulin concentrations, with HFAS trending higher in comparison to both CD groups (Table 4).

HFD increased plasma leptin concentrations in both male and female mice compared to CD, with no significant impact of AS (Table 4 and 5). Female HFCon plasma leptin concentrations were significantly increased compared to CDCon (Table 4). HFD increased HOMA-IR in female mice, but not male mice. Female HFAS was significantly increased compared to CDAS (Table 4). Male HFCon was significanly increased compared to both CDCon and CDAS (Table 5). HFD and AS resulted in increased plasma testosterone concentrations in male mice as compared to CD. In females, HFD increased plasma testosterone concentrations compared to CD, with no influence from AS. HFCon and HFAS were significantly increased compared to CDCon and CDAS (Tables 4 and 5).

Table 4: Plasma glucose, insulin, leptin and testosterone concentrations in female mice.

| | CDCon | CDAS | HFCon | HFAS | AS | HFD | Interaction |
|---|---|---|---|---|---|---|---|
| Glucose (mmol/L) | 9.10±0.61 | 8.22±0.40 | 11.64±0.48$^{+}$ | 9.22±0.81 | *P*=0.014 | *P*=0.009 | NS |
| Insulin (ng/ml) | 0.16±0.02 | 0.16±0.03 | 0.22±0.029 | 0.32±0.06 | NS | *P*=0.013 | NS |
| Leptin (ng/ml) | 0.98±0.16 | 1.42±0.55 | 4.37±1.18* | 3.42±0.68 | NS | *P*=0.002 | NS |
| HOMA-IR | 0.68±0.12 | 0.48±0.14 | 1.17±0.12 | 1.37±0.29$^{+}$ | NS | *P*=0.002 | NS |
| Testosterone (ng/ml) | 0.037±0.014 | 0.005±0.002 | 0.091±0.017$^{*+}$ | 0.091±0.003$^{*+}$ | NS | *P*=0.0004 | NS |

Data presented as mean ± SEM, where *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS, ^p<0.05 w.r.t HFCon; n=5 mice/group. NS = not significant.

Table 5: Plasma glucose, insulin, leptin and testosterone concentrations in male mice.

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| | CDCon | CDAS | HFCon | HFAS | AS | HFD | Interaction |
|---|---|---|---|---|---|---|---|
| Glucose (mmol/L) | 9.84±0.98 | 8.78±0.70 | 12.03±0.39$^{+}$ | 13.2±0.44$^{*+}$ | NS | *P*=0.0003 | NS |
| Insulin (ng/ml) | 0.14±0.013 | 0.15±0.011 | 0.18±0.012 | 0.13±0.005$^{\wedge}$ | NS | NS | *P*=0.009 |
| Leptin (ng/ml) | 1.48±0.35 | 1.53±0.43 | 7.46±2.43 | 3.76±2.18 | NS | *P*=0.044 | NS |
| HOMA-IR | 0.61±0.08 | 0.59±0.034 | 0.78±0.20$^{*+}$ | 0.77±0.05 | NS | NS | NS |
| Testosterone (ng/ml) | 0.34±0.06 | 0.40±0.06 | 3.64±0.97$^{*+\wedge}$ | 0.42±0.04 | *P*=0.006 | *P*=0.005 | *P*=0.005 |

Data presented as mean ± SEM, where *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS, ^p<0.05 w.r.t HFCon; n=5 mice/group. NS = not significant.

*3.3 Oral glucose tolerance test*

AS reduced blood glucose in the male but not female mice. In females, HFAS glucose concentrations were increased compared to CDAS at 15 minutes post glucose bolus in the OGTT (Figure 1A). HFD impaired glucose tolerance in both male and female mice, as seen in the area under the curve (AUC) (Figures 1B and 1F respectively). In male mice, HFCon and HFAS significantly increased glucose concentrations following OGTT glucose bolus at time 0, 30, 80 and 120 minutes compared to CDCon, and at time 15, 30, 60 and 90 minutes compared to CDAS. Further, HFCon increased glucose concentrations at 120 minutes compared to CDAS, and HFAS was increased at 0 minutes compared to CDAS (Figure 1E).

In female mice AS increased plasma insulin concentrations across the time challenge, with HFAS significantly increased at 15min and 60min in comparison to all other groups, and CDAS increased compared to CDCon and HFCon at 15min (Figure 1C). Conversely, there was no effect of diet on plasma insulin concentrations in response to the glucose bolus in male mice (Figure 1G). In AUC from the plasma insulin, AS exposure increased insulin in female mice, while female HFAS had greater concentrations compared to CDCon and HFCon (Figure 1D). Male mice showed no significant difference between the groups in insulin AUC (Figure 1H).

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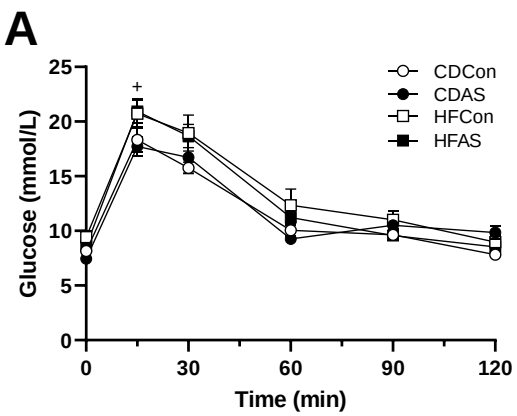
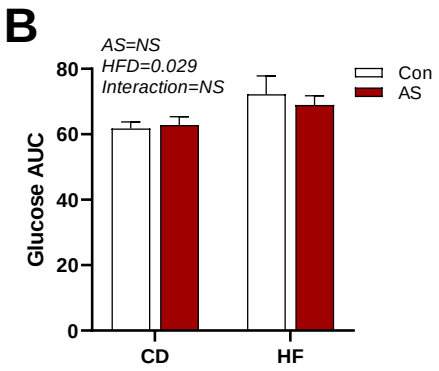
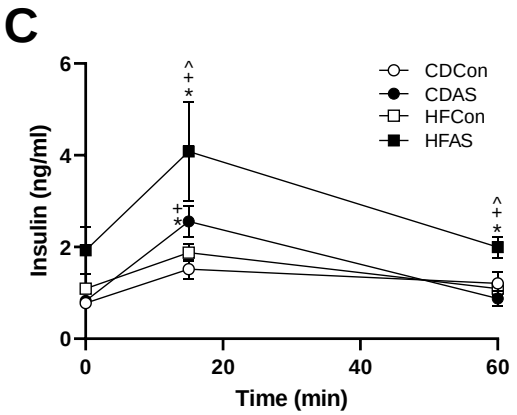
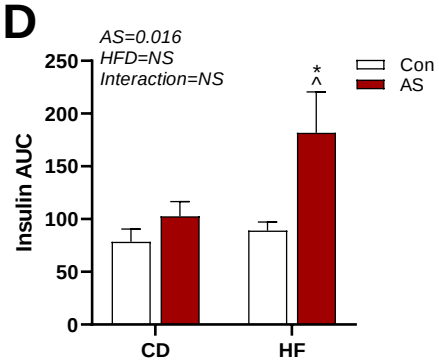
# Females

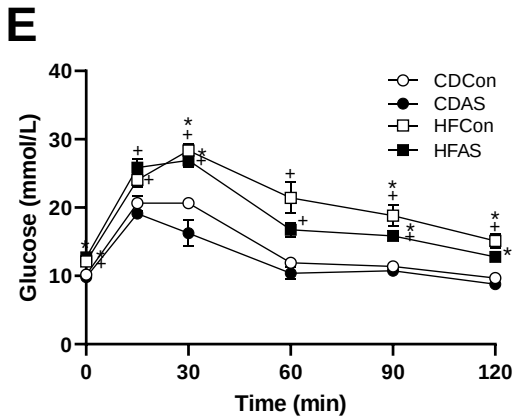


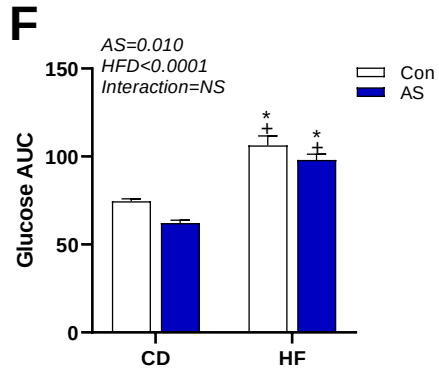



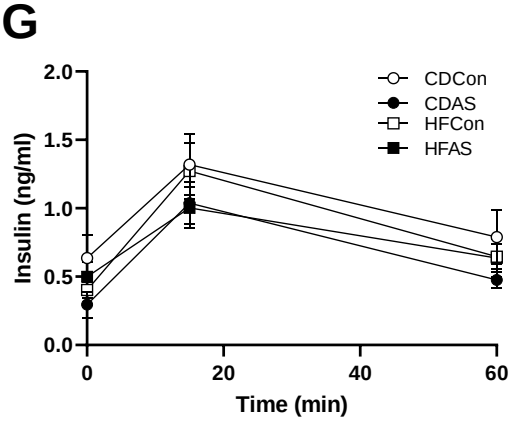




# Males



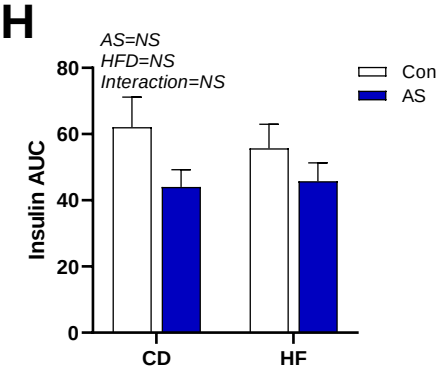



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**Figure 1.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on glucose homeostasis at 11 weeks of age in female and male C57BL/6 mice. (A) OGTT (2g/kg) in female mice, (B) Area under the OGTT curves in female mice, (C) Plasma insulin secretion curve at 0, 15, and 60min post OGTT in female mice, (D) Area under the curve in insulin in female mice, (E) OGTT (2g/kg) in male mice, (F) Area under the OGTT curves in male mice, (G) Plasma insulin secretion curve at 0, 15, and 60min post OGTT in male mice, (H) Area under the curve in insulin in male mice. Data were analysed using 2-way ANOVA. Data are expressed as mean ± SEM. *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS, ^p<0.05 w.r.t HFCon; n=5/group.

### *3.4. Adipocyte hypertrophy*

In female mice, average adipocyte size was significantly increased in both HF groups compared to CDCon (Figures 2A and C). This is in agreement with analysis looking at the distribution of adipocytes by size where adipocyte size was skewed toward those larger than 4-5000µm in the HF groups while adipocytes in the CD groups were skewed towards those <4-5000µm (Figure 2B).

In males, average adipocyte size was increased as a result of HFD, with HFCon significantly increased compared to CDCon (Figures 2D and 2F). The percentage of adipocytes in male mice >10000µm was significantly increased in the CDAS and both HF groups as compared to CDCon (Figure 2E). There was a significant interation between AS and HFD for both males and females with AS increasing adipocyte size in CD animals, the reverse being true with the HFD (Figures 2C and F).

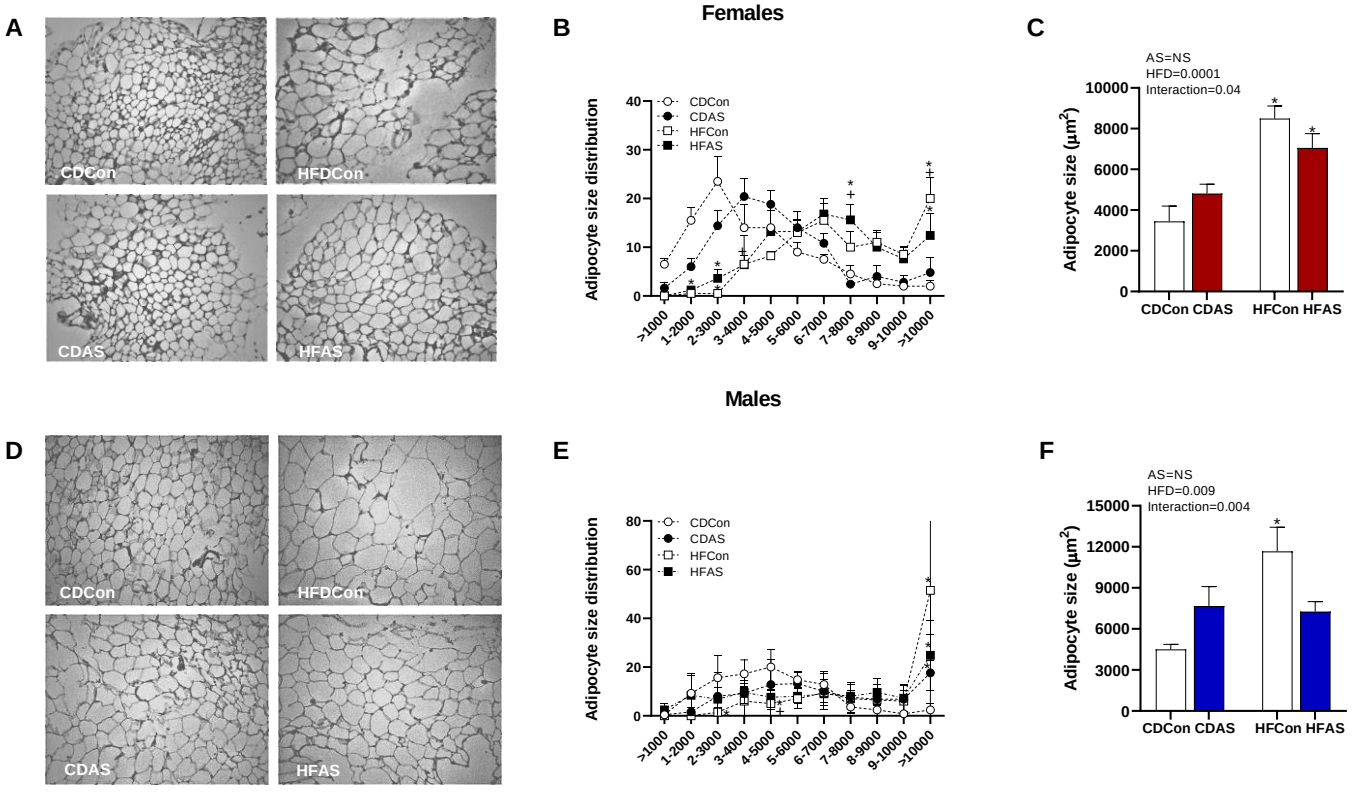


**Figure 2.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on adipocyte size in female and male C57BL/6 mice. Representative gonadal adipose tissue sections

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by haematoxylin and eosin (scale bars=200μm) in female (A) and male (D) mice. (B) Adipocyte size distribution in female mice. (C) Average adipocyte size in female mice. (E) Adipocyte size distribution in male mice. (F) Average adipocyte size in male mice. Data were analysed using 2-way ANOVA and repeated measures ANOVA as required. Data are expressed as mean ± SEM. *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS; n=5/group.

### *3.5. Adipogenic gene expression*

In female mice, AS increased expression of insulin receptor substrate 1 (*Irs1*) (Figure 3A). Phosphodiesterase 3B (*Pde3b*) was increased with AS exposure, with CDAS significantly increased compared to CDCon (Figure 3C). Peroxisome proliferator activated receptor gamma (*Pparγ*) expressed interactive effects, where CDCon trended higher than CDAS, while HFAS was increased compared to HFCon (Figure 3E). Pparg coactivator 1 alpha (*Ppargc1*) was increased overall in response to AS, particularly in the CDAS group compared to CDCon (Figure 3G).

In male mice, HFD and AS both increased adiponectin (*Adipoq*) expression in male mice, with HFAS significantly increased compared to CDCon (Figure 3B). Adiponectin receptor 1 (*Adipor1*) was influenced by AS and had interactive effects, where HFCon was increased compared to CDCon and CDAS groups, and HFAS was reduced significantly compared to HFCon (Figure 3D). HFD exposure decreased *Pparg* exposure, with an interactive effect also noted where CDAS was significantly increased versus CDCon, and HFAS reduced compared to HFCon and significantly reduced versus CDAS (Figure 3E). There was a reduced expression of insulin-like growth factor 1 (*Igf1)* as a result of HFD exposure with expression in both HFD groups significantly reduced compared to CDAS, and HFAS reduced compared to CDCon as well (Figure 3F). *Ppargc1* was reduced as a result of HFD and AS, with HFAS significantly reduced compared to both CD groups (Figure 3G). AS increased lipase E, hormone sensitive type (*Lipe*) expression in male mice, with CDAS trending higher than CDCon (Figure 3H). There was no significant difference between groups with *Irs1* and *Pde3b* in male mice, and no difference between female groups with *Igf1*, *Adipoq*, *Adipor1*, and *Lipe*.

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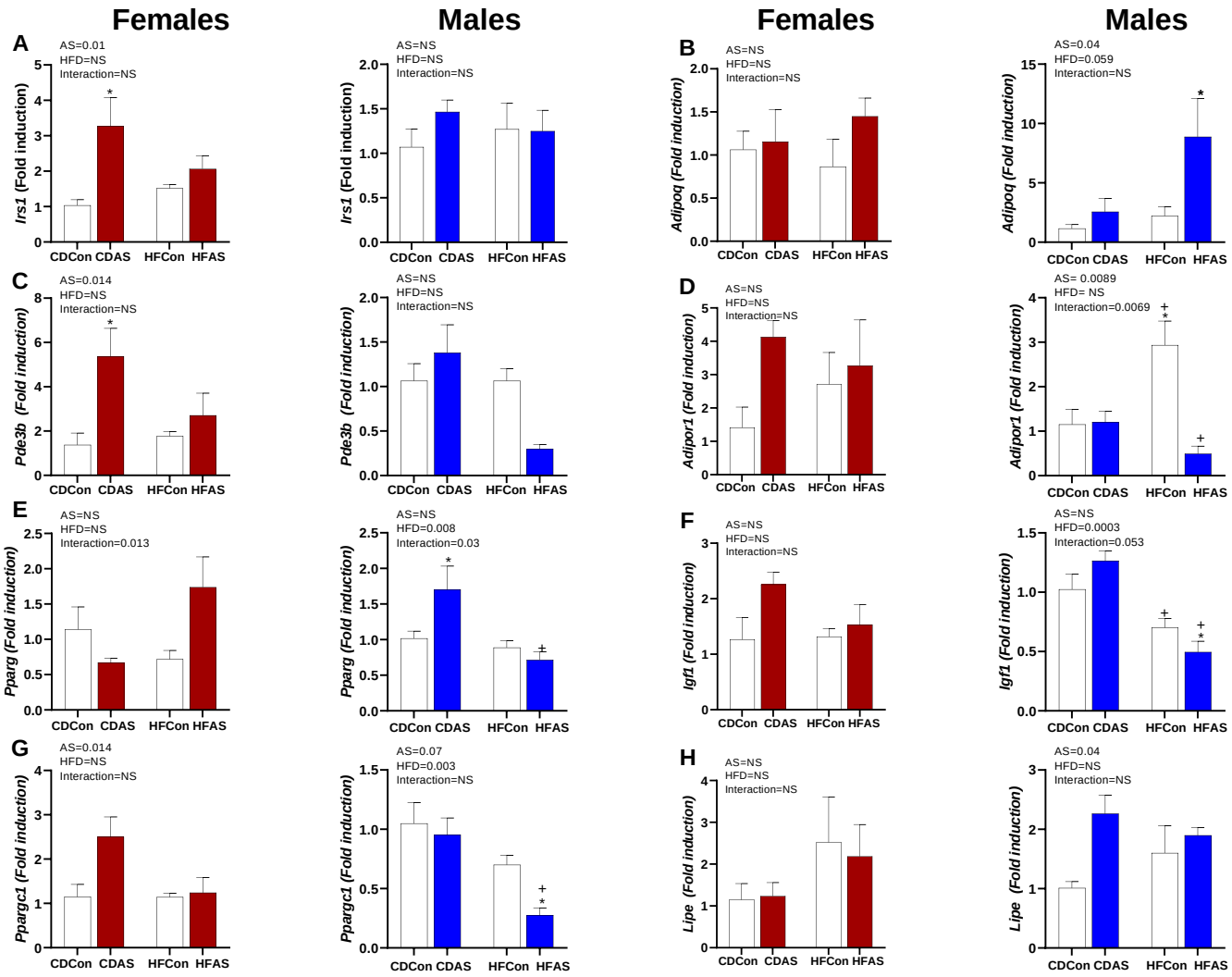


**Figure 3.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on adipose tissue adipogenic gene expression in female and male C57BL/6 mice. (A) *Irs1*, (B) *Adipoq*, (C) *Pde3b*, (D) *Adipor1*, (E) *Pparg*, (F) *Igf1*, (G) *Ppargc1*, (H) *Lipe*. Data were analysed using 2-way ANOVA. Data are expressed as mean ± SEM.*p<0.05 w.r.t CDCon. +p<0.05 w.r.t CDAS; n=5/group.

### *3.6 Glucose metabolism gene expression*

AS increased AKT Serine Kinase 3 (*Akt3)* expression in female mice (Figure 4A). Glycogen synthase 1 (*Gys1*) was reduced by HFD, while AS increased expression, with CDAS increased compared to all other groups (Figure 4C). Solute carrier family 27 member 1 (*Slc27a1*) displayed increased expression as a result of AS, as did solute carrier family 2 member 4 (*Slc2a4*) (Figures 4B and 4D).

Conversely to what was seen in the female mice, male mice displayed a reduction in expression of *Gys1* as a result of HFD and AS exposure, with expression in all groups reduced compared to CDCon (Figure 4A). HFD increased *Slc27a1* expression, with HFCon significantly increased compared to CDCon (Figure 4B). HFD exposure reduced Hexokinase 2 (*Hk2*) expression in male mice, with a significant interaction shown with CDAS and HFAS increased and reduced respectively compared to CDCon and HFCon (Figure 4E). There was no significant difference between male groups with *Akt3* and *Slc2a4*, and no difference between groups with *Hk2* in female mice.

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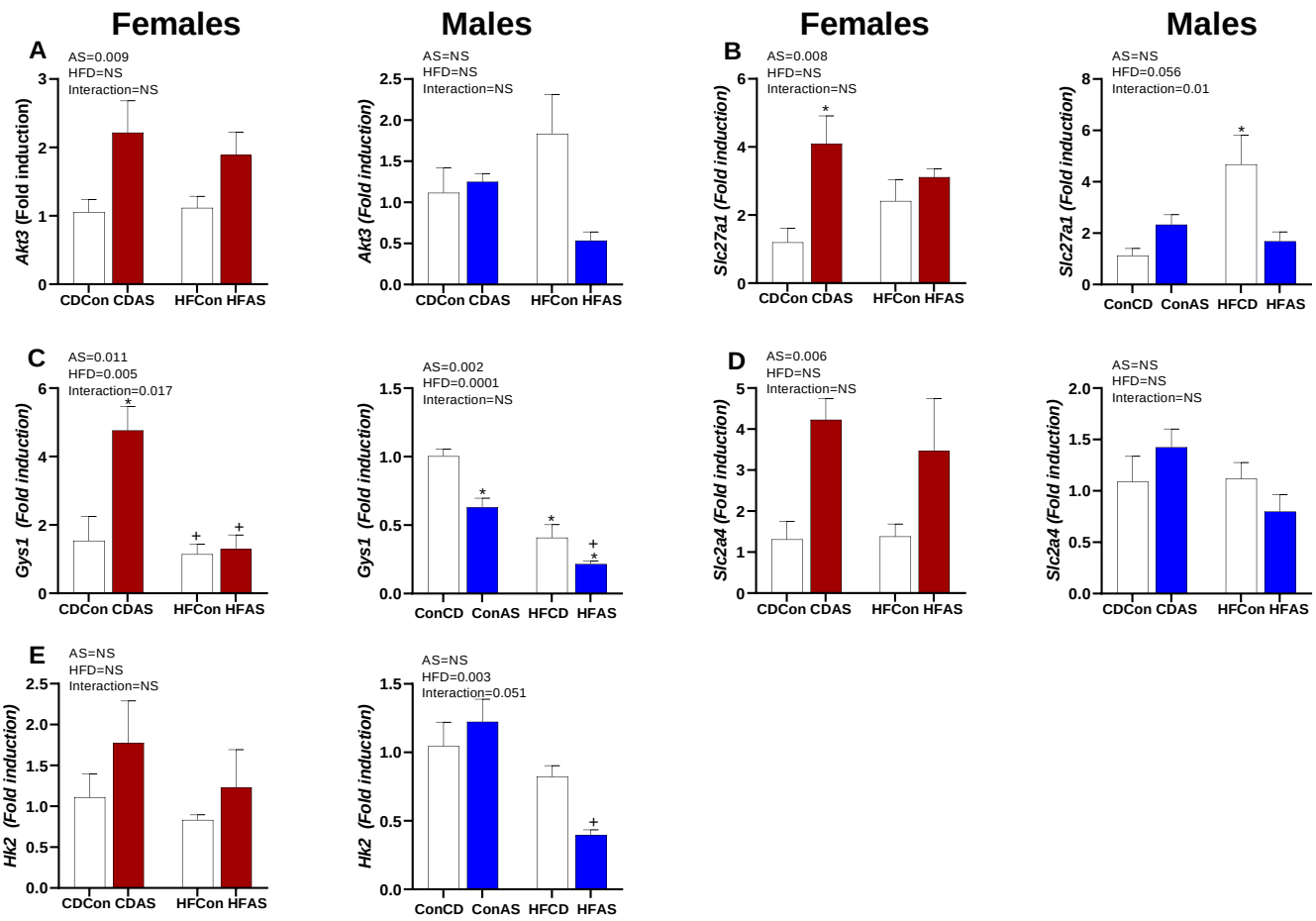


**Figure 4.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on adipose tissue glucose metabolism gene expression in female and male C57BL/6 mice. (A) *Akt3*, (B) *Slc27a1*, (C) *Gys1*, (D) *Slc2a4*, (E) *Hk2*. Data were analysed using 2-way ANOVA. Data are expressed as mean ± SEM. *p<0.05 w.r.t CDCon, +p<0.05 w.r.t CDAS; n=5/group.

*3.7 Lipid and fatty acid gene expression*

AS increased expression of acyl-CoA synthetase long chain family member 1 (*Acsl1*) in female mice (Figure 5A). Meanwhile, HFD heightened expression of arachidonate 5-lipoxygenase (*Alox5*) and fatty acid binding protein 4 (*Fabp4*) in female mice (Figure 5C and D). Lipoprotein lipase (*Lpl*) was increased by AS (Figure 5F). As expected, leptin receptor (*Lepr*) was increased in reaction to HFD, however HFAS was reduced compared to HFCon, while CDAS was increased compared to CDCon in an interactive effect (Figure 5G).

In male mice, HFD reduced expression of acyl-CoA synthetase long chain family member 4 (*Acsl4*) with HFAS significantly reduced compared to both CD groups (Figure 5B). *Fabp4* mirrored effects in the female mice with HFD increasing expression in the males (Figure 5D). While fatty acid synthase (*Fasn*) saw no difference in female mice, there was a reduction in expression as a result of HFD, with HFAS significantly reduced compared to both CDCon and CDAS (Figure 5E). *Lpl* was increased in response to both AS and HFD

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exposure, with HFCon significantly increased compared to CDCon and HFAS increased versus CDAS (Figure 5F). As with the female mice, *Lepr* was increased by HFD, and HFAS was significantly increased compared to CDAS (Figure 5G). There was no significant difference between groups for *Alox5* in male mice, and no difference in female mice for *Fasn.*

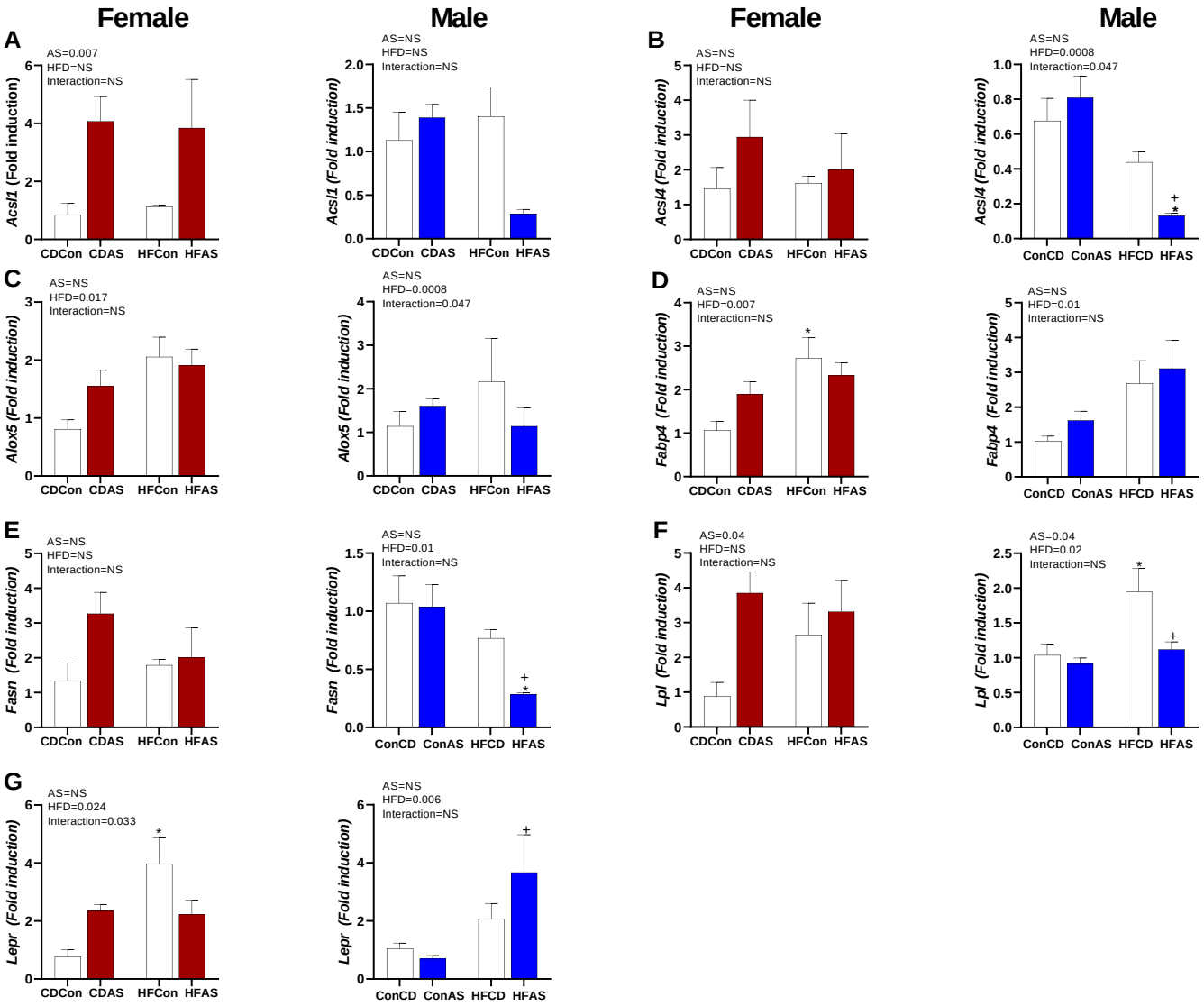


**Figure 5.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on adipose tissue lipid metabolism gene expression in male and female C57BL/6 mice. (A) *Acsl1*, (B) *Acsl4*, (C) *Alox5*, (D) *Fabp4*, (E) *Fasn*, (F) *Lpl*, *(G) Lepr.* Data were analysed using 2-way ANOVA. Data are expressed as mean ± SEM. *p<0.05 w.r.t CDCon. +p<0.05 w.r.t CDAS; n=5/group.

*3.8 Immune-related gene expression*

Females

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Chemokine receptor 6 (*Ccr6*) expression was increased in response to HFD in female mice. *Cd3e* displayed an interactive effect where AS increased expression in CD female mice, but was decreased between the HFD and CD mice, while chemokine receptor 3 (*Cxcr3)* expression was increased in response to AS in female mice (Figure 6A).

Adhesian G protein-coupled receptor E1 (*Emr1)* was increased by HFD with HFAS significanly increased compared to CDCon in female mice. Interferon gamme (*Ifng*) experienced increased expression due to AS, and an interactive effect due to CDAS which was significantly increased compared to all others for *Ifng*. HFD and AS increased interleukin-1 receptor 1 (*Il-1r1*) expression in female mice. Further, *Il-1r1* HFAS was significanly increased compared to CDCon. Mechanistic Target Of Rapamycin Kinase (*mTor*) illustrated increased expression followed HFD exposure, with an interactive effect where HFCon was increased compared to CDCon. HFD reduced and AS increased NLR family pyrin domain containing 3 (*Nlrp3*) expression in female mice. *Nlrp3* CDAS was increased compared to all other groups in female mice. RELA Proto-Oncogene, NF-KB Subunit (*Rela*) displayed interactive effects in female mice, where CDAS was increased compared to CDCon, while HFCon was increased compared to HFAS. Serpin Family E Member 1 (*Serpine1*) was similarly increased by HFD and AS exposure, with CDAS significantly increased compared to all other groups (Figure 6B). There was no difference between groups in the female mice for *Cxcr4, Ccl12, Chuk, Cnbp,* and *Rps6kb1 (Figure 6).*

Males

In male mice, Chemokine ligand 12 (*Ccl12*), component of inhibitor of nuclear factor kappa B kinase complex (*Chuk*), and CCHC-type zinc finger nucleic acid binding protein (*Cnbp*) expression were all increased following HFD exposure, with HFAS reduced compared to CDAS. Further, *Ccl12* and *Cnbp* experienced interactive effects, where CDAS was increased compared to CDCon, while HFAS was reduced compared to HFCon. CDAS was also increased significantly compared to CDCon for *Cnbp* in male mice. *Cxcr3* was increased by HFD exposure. A decrease in expression was found in chemokine receptor 4 (*Cxcr4*) following HFD intake, which also showed a significant interaction whereby expression was increased in CDAS versus CDCon, and reduced in HDAS compared to HFCon. Both HF groups were also increased compared to CDCon and CDAS (Figure 6C).

*Emr1* expression was increased by HFD, with an interactive effect where HFAS was reduced compared to CDAS. *Nlrp3* was increased by AS exposure, with HFCon heightened compared to CDCon, and HFAS reduced compared to CDAS (Figure 6D). As with the female mice, *Rela* experienced interactive effects, however HFD exposure also reduced *Rela* expression, while HFAS was signficiantly reduced compared to all other groups. Ribosomal protein S6 kinase B1 (*Rps6kb1*) was reduced by HFD, and influenced by AS with a signficant interaction effect, where both HFD groups were reduced compared to CDAS. *Serpine1* was reduced overall with AS exposure, due to the increased expression of HFCon. Male mice also displayed interactive effects in *Serpine1* expression, with HFCon increased versus HFAS and CDCon (Figure 6D). In the male mice, there was no difference between the groups for *Ccr6, Cd3e, Ifng, mTor,* and *IL-1r1.*

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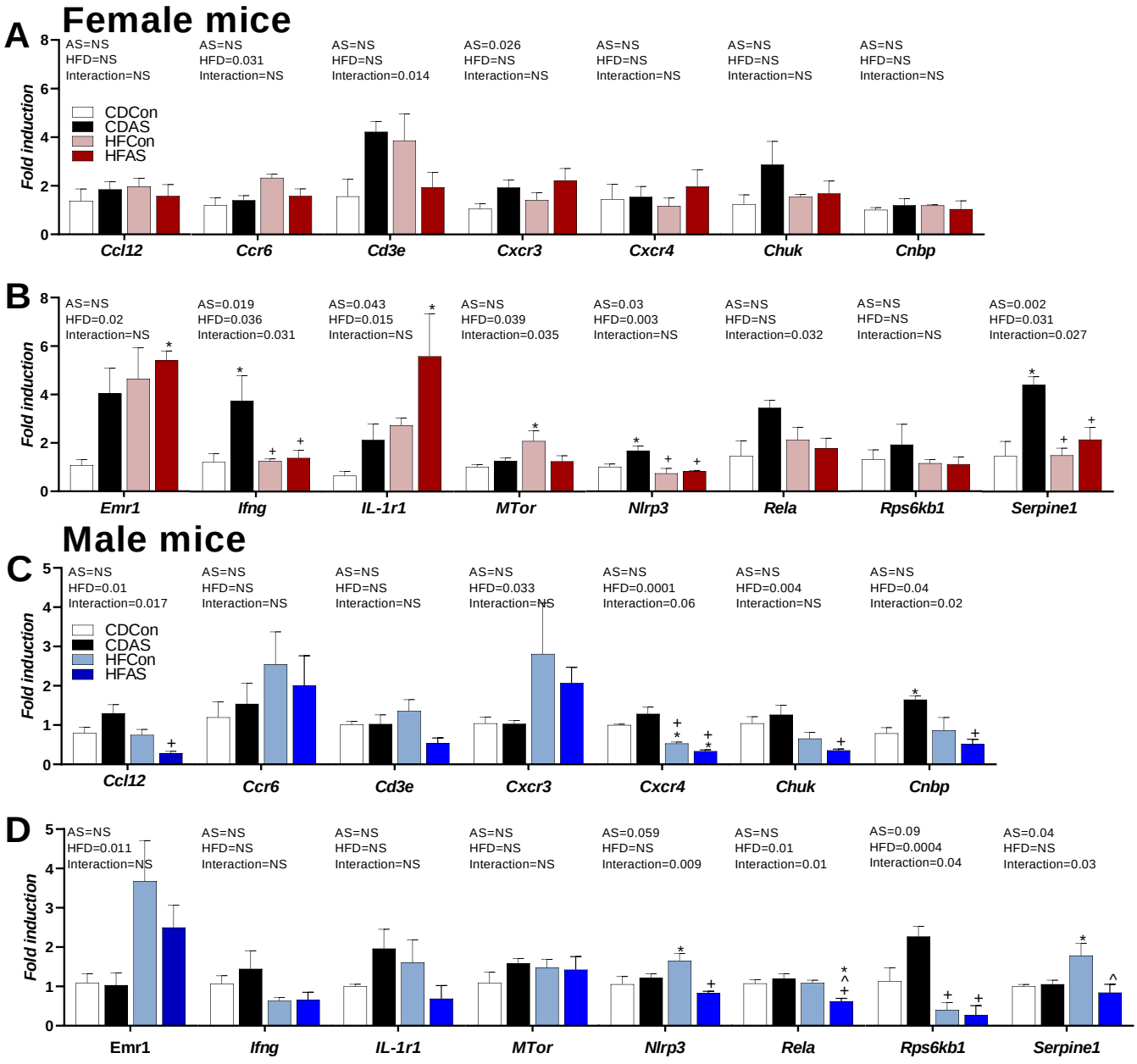


**Figure 12.** The effects of Ace-K (AS) and high-fat diet (HFD) compared to regular drinking water (Con) and standard control diet (CD) on immune-related gene expression in adipose tissue. Expression of *Ccl12, Ccr6, Cd3e, Cxcr3, Cxcr4, Chuk,* and *Cnbp* in (A) female mice and (C) male mice. Expression of *Emr1, Ignf, Il-1r1, MTor, Nlrp3, Rela, Rps6kb1, Serpine1* in (B) female mice and (D) male mice. Data were analysed using 2-way ANOVA. Data are expressed as mean ± SEM. *p<0.05 w.r.t CDCon. +p<0.05 w.r.t CDAS. ^p<0.05 w.r.t HFCon; n=5/group.

## 4. Discussion

The association between high sugar, HFD and metabolic dysfunction is now well recognised [20] with sugar and fat-enriched diets inducing increases in weight gain, IR, and glucose, and thus heightening the risk for a range of metabolic disorders. One strategy utilized as a possible approach to combat obesity and the deleterious effects of HFDs is the substitution of sugar-sweetened beverages with AS beverages, the majority of which

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include Ace-K. However, evidence to date suggests that these non-nutritive sweeteners may not be as harmless as previously thought [18], and may themselves increase the risk for obesity and metabolic syndrome through alteration of the gut microbiome, glucose homeostasis, satiety, and increases in caloric consumption [8]. Further, there remains a lack of research on the interaction between AS and HFD in combination, and their influence on metabolic health. We investigated AS and HFD in a rodent model in female and male mice, using Ace-K, a commonly consumed AS [14], but one that remains poorly studied. We demonstrated a protective effect of AS in combination with HFD from glucose intolerance in male but not female mice. This was potentially mediated by reductions in HFD-induced inflammatory gene expression in the male adipose tissue, effects that were not seen in the females

As expected, HFD increased body weight gain in both males and females. However, AS did not significantly reduce weight gain or alter energy intake, alone or in conjunction with HFD and thus demonstrates that the results of our study are independent of body weight. These findings are consistent with a majority of current literature indicating no change in body weight following low doses of AS [21-23], and align with previous work by our group in pregnant mice [15]. Studies which contradict these findings and report weight gain following AS consumption in rodents vary in their mode of AS administration and dose used. Swithers et al., who suggested AS consumption impairs an animal's ability to predict caloric intake based on sweet taste, thereby disrupting caloric intake, fed rats AS-treated yoghurt, which potentially masked the sweetness of the AS and may have influenced gut microbiota independently of AS [24]. Our results also contrast with Bian et al., who reported an increase in body weight in male but not female mice following Ace-K administration via oral gavage, a mode of administration which can in itself induce a stressor. Further, their dose was significantly higher than that used in the present study and thus likely also reflected the differences in body weight gain observed [11].

Female HFD-induced glucose intolerance was prevented by AS intake at the time of cull, though AS did not protect against HFD-mediated increases in insulin secretion at cull. Notably, in females AS independently and in a synergistic manner with HFD increased insulin concentrations following the OGTT compared to controls. This was further confirmed via the HOMA-IR index, where female HFD increased readings and HFAS even more so compared to CDAS. Hyperinsulinemia has similarly been seen in rats fed the artificial sweetener sucralose [25], with a HFD shown to induce abnormalities associated with sucralose intake. Human studies have demonstrated that hyperinsulinemia is linked to an increased risk of IR and T2DM [26]. The negative influence of AS in combination with HFD on insulin secretion, compared to the potential protective effect from AS on the fasting glucose concentrations, may indicate a mechanism independent of glucose signalling on insulin in female mice.

While AS exacerbated hyperinsulinemia in females, a positive effect was seen in male mice where AS protected against HFD-induced glucose intolerance following OGTT and reduced HFAS insulin secretion at cull compared to HFCon. Similarly, insulin concentrations following OGTT trended lower following AS intake. This contrasts with some studies where Ace-K has been shown to increase the uptake of glucose in human and rat enterocytes via translocation of GLUT2, independently of sweet-taste receptors on the tongue [27]. However, while *in vitro* observations allow insight at the cellular level, the results may differ in an animal model where complex interactions occur between various systems that may alter glucose regulation. While current evidence suggests Ace-K is not digested as it passes through the gut, it is clear from animal models that Ace-K still imparts an influence on the gastrointestinal tract, through both its influence on glucose uptake via Glut2 [27], and an impact on the gut microbiome [11, 28]. The significant reduction in insulin secretion at cull in HFAS male mice compared to their HFD counterparts

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may suggest that, in male mice, AS supplementation confers partial protective effects in the setting of a HFD.

One possible mechanism for the observed hyperinsulinemia in females could be through testosterone. Testosterone was assessed in the current study given previous reports linking AS with alteration in key reproductive markers [29] and possible impact on metabolic function [30]. HFD elevated testosterone concentrations in female mice. Evidence in humans implicates testosterone in the induction of IR and further metabolic dysfunction [31]. Differing from its influence in women, testosterone in human males has been implicated in the regulation of insulin sensitivity, with lower concentrations of testosterone associated with diabetes and obesity [32]. A decrease in testosterone concentrations with rising obesity was not seen in HFCon male mice, only in HFAS and both CD groups, potentially contrasting with previous evidence and indicating further mechanisms influencing the metabolism of androgens.

Given the HFD-induced increase in weight gain, it was unsurprising to see an overall increase in adipocyte size following HFD in male and female mice. In females, adipocyte size distribution analysis demonstrated a shift towards larger cells in both the HFCon and HFAS groups, while in male mice HFAS trended towards reduced adipocyte size compared to HFD alone. Adipocyte hypertrophy is seen as a marker of adipose tissue dysfunction and is typically associated with macrophage recruitment, cellular stress and decreased metabolic flexibility, all factors which contribute to reduced sensitivity to insulin. Evidence points to hypertrophy, even in the absence of obesity, being a predictor of metabolic dysfunction, IR, and the development of T2DM [33, 34]. Conversely, smaller adipocytes can indicate a more metabolically healthy environment, even in an obese state. Leptin, a satiety hormone produced primarily by adipocytes, displays a strong correlation with adipocyte size [35]. It was therefore unsurprising that both male and female mice displayed increased blood leptin concentrations at cull following HFD. Further, the leptin receptor (Lepr) was upregulated by HFD in male and female adipose tissue. Both leptin and adipocyte size may be indicators of altered metabolism within the adipose tissue, with leptin implicated in increases to lipolysis and modification of insulin sensitivity [36], and adipose tissue Lepr knockdown associated with reduced weight gain and altered glucose metabolism [37].

In order to further investigate the influence of HFD and AS on metabolic health, a panel of genes associated with adipogenic pathways within the adipose tissue were investigated. There was a reduction of Ppargc1 expression from AS and HFD intake in male but not female mice, who instead saw an increase following AS intake mediated by CDAS. Ppargc1 encodes for PGC-1alpha, a co-activator of Pparγ and essential in the regulation of adipogenesis and fatty acid oxidation pathways. Reduction of PGC-1alpha within the adipose tissue has been associated with IR in humans [38] and reduced mitochondrial function, fatty acid oxidation, impaired glucose intolerance and insulin secretion in mice fed a HFD [39]. Increased levels of PGC-1alpha may protect against glucose intolerance as mice age. Pparγ, a vital component in adipose tissue insulin sensitivity, was reduced following HFD, mediated largely by the increased expression in CDAS males. In female mice, CDCon and HFAS expression were increased compared to CDAS and HFCon. Sucralose has been demonstrated to reduce Pparγ expression in male rats fed a HFD [25]. However, it should be noted that Pparγ is a transcription factor and activation is mediated via a series of post-translational modifications, with effects in relation to adipocyte hypertrophy. It is therefore possible that there is decreased activation of this pathway that is not observable via analysis of gene expression alone.

In male mice, AS alone and in combination with HFD elevated Adipoq gene expression. Adiponectin is involved in maintaining energy homeostasis and typically decreases

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with obesity. Adipose gene expression of Adipor1, a receptor for adiponectin, was reduced by AS intake, while HFD alone increased expression in male mice. Female mice saw no difference in either Adipoq or Adipor1. This contrasts a previous study in mice, where Adipor1 was shown to be inversely regulated by insulin, with obesity-induced IR leading to downregulation of the receptor [40]. Further sex-specific effects were seen, with AS consumption upregulating expression of Irs1 and Akt3 in female, but not male mice. In males, Akt3 HFAS expression trended lower compared to HFCon. Irs1 and Akt are involved in insulin-stimulated signal transduction pathways. In a mouse model of IR, Irs1 degradation was associated with impaired glucose uptake [41], while dysregulation of Akt3 has been shown to increase adipogenesis and predispose mice to obesity. Activation of Akt3 is indicated in the inhibition of lipolysis [42], a result similarly seen following stimulation of Akt by the AS saccharin [16]. Insulin itself has also been indicated in the downregulation of lipolysis [16]. These results suggest a possible protection against glucose intolerance from AS intake in females and indicates a complex mechanism through which Ace-K influences metabolic health in mice.

HFD also reduced expression of Igf1 in male mice, which in adipose tissue is associated with differentiation and metabolic regulation of adipocytes. Overall, these changes may indicate dysregulation of adipogenesis following HFD with no significant effects in response to AS supplementation in male mice. In female mice, Igf1 expression trended higher following AS intake. As adipocytes increase in size, their production of Igf1 is reduced, which is compensated by macrophage-mediated production of Igf1 [43]. Fabp4 is also expressed in both adipocytes and macrophages, and plays an important role in relation of metabolic inflammation and adipogeneic processes. Fabp4-induced lipid accumulation in macrophages induces inflammatory pathways which can shift macrophage polarisation towards the pro-inflammatory phenotype that is associated with IR. Fabp4 was upregulated following HFD intake in male and female mice, which matches with data linking increased expression with obese, diabetic individuals.

In addition to changes in adipogenic pathways, obesity is associated with increased lipolysis and the distribution of excess lipids from adipose tissue to other areas of the body, which in turn may contribute to IR. Therefore, we assessed lipid metabolism gene expression in adipose tissue. AS intake in male but not female mice increased expression of Lipe, a gene responsible for the transcription of hormone sensitive lipase (HSL) and adipose lipolysis. HSL plays a prominent role in lipid metabolism and energy homeostasis, where distortion of this enzyme may be related to abnormalities in triglyceride breakdown and increased vulnerability to obesity and T2DM [44, 45]. Lpl is the rate-limiting step in the triglyceride catabolism and absorption of free fatty acids [46, 47], where overexpression in adipose tissue can lead to increased fatty acid uptake, contributing to hypertrophy and inflammation. In our female mice, Lpl was increased following AS intake, while male HFAS expression was reduced compared to HFCon. There was also a reduction in expression of Acsl4, a key regulator of lipid biosynthesis, by HFD intake in male mice, which is further reduced with the combination of AS and HFD. Again, female mice differed, with no change in Acsl4 expression. Killion et al., demonstrated that HFD-fed mice had increased expression in adipose tissue and determined that knockout Acsl4 mice were protected against HFD-induced adipose tissue inflammation and IR [48]. They utilised a 60% kcal HFD, maintained their diet across 12 weeks, and cull therefore occurred at an older age compared to the present study using a 45% kcal diet for 6 weeks. Further, the Killion study utilised a chow control diet as compared to a matched control diet as used in the present study. As such, differences between these studies are therefore likely due to differences in experimental approaches used. Further experiments in mice models have demonstrated that a Slc27a1 knock-out improves insulin sensitivity and protection from diet-induced obesity [49, 50]. In our study, male mice displayed increased expression following HFD exposure, driven by HFCon. Conversely, in female mice, Slc27a1 expression was increased following AS consumption.

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Genes associated with glucose metabolism, such as Slc2a4, help to explain some of the metabolic effects observed in our mice. Also known as Glut4, Slc2a4 is an insulin sensitive glucose transporter. Obesity is linked to decreased expression of Slc2a4, leading to hyperglycaemia, an effect reported in rats by Sanchez-Tapia et al. following supplementation of the AS sucralose [25]. In female but not male mice, Slc2a4 was upregulated by AS. Differences in ASs may explain the variation seen between findings of the present study versus that of Sanchez-Tapia et al. However, AS female mice displayed further upregulation of glucose metabolism-related genes; Gys1, mediated by an increase in CDAS, and Hk2, where expression trended higher. For both Gys1 and Hk2, HFD decreased expression in male mice, an effect exacerbated by AS in combination with HFD and, with Gys1, independently. Gys1 encodes enzymes in the glycogen synthesis pathway within adipose tissue. Knockout of Gys1 impairs the accumulation of glycogen and lipid droplet biogenesis within human and mice preadipocytes during differentiation [51], while down-regulation of the gene Hk2 by a HFD impairs fatty acid synthesis and induces hyperglycaemia [52]. Overall, in male mice these results point to dysregulation of adipocyte glycogen pathways from HFD, with AS further impairing this homeostasis. In females, AS appears to impact the processes associated with glucose transport and metabolism and may therefore confer a protective effect with respect to glucose metabolism as compared to males. However, given other physiological impairments noted in the female mice, including hyperinsulinemia, this potential effect may not be enough to protect female mice from further metabolic dysfunction.

The most pronounced sex-specific differences were those observed for immune-related genes. In humans, while obesity is commonly associated with low-grade chronic inflammation, Ace-K has been shown to dysregulate inflammatory homeostasis in adipose tissue [17], effects of which in our study were more pronounced in females as compared to males. Cxcr3, for example, contributes to T-cell recruitment into adipose tissue in obese mice [53]. HFD-induced increases in Cxcr3 expression in male mice were reduced with AS intake, however in female mice AS increased expression. Pde3b, which plays a role in the obesity-induced inflammatory response through activation of Nlrp3 and other proinflammatory genes [54], was increased following AS intake in female but not male mice, as was Nlrp3, driven largely by female CDAS. Serpine1 and Ifnγ followed this pattern, with AS intake increasing expression, largely driven by CDAS. Ifnγ is a key mediator in adipose tissue inflammation. Obese mice lacking Ifnγ display reduced inflammatory marker infiltration into adipose tissue and improved glucose intolerance [55]. Il-1r1, a primary receptor involved in inflammation, was also upregulated by AS and HFD separately and in combination in female but not male mice. Previous studies have established that a knockout of Il-1r1 protected male mice from diet-induced obesity, though these effects appeared to be reversed in older aged mice [56]. Taken together, this provides strong evidence that AS results in a dysregulation of adipose tissue immune responses in females, potentially leading to increased immune-cell recruitment and inflammation.

Further immune gene expression results illustrate a potential protective effect by AS in combination with HFD in male mice. For example, HFAS expression was reduced in comparison to CDAS in Ccl12, Cxcr4, Chuk, Cnbp, Rela, and Rps6kb1, and trended lower in genes such as Alox5 and IL-1r1 compared to HFCon, effects not seen in female mice. Obesity typically leads to increased inflammation, led in part by increased infiltration of macrophages into adipose tissue, which in turn contributes to IR [57]. Ccl12 and Cxcr4 promote recruitment of macrophages into the adipose tissue and are essential for the inducement of obesity-induced inflammation and IR [58]. Alox5 is involved in fatty acid metabolism, inflammatory pathway activation and secretion of proinflammatory adipokines such as IL-6 and TNF-$\alpha$ [59]. In male mice, AS in combination with HFD potentially limits the recruitment of immune-related cells and adipokines, despite the noted adipocyte hypertrophy. This may be one mechanism through which AS improved glucose

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and insulin sensitivity in male mice. However, male mice clearly display other impairments within the adipose tissue, suggesting that while some protection occurs, HFAS male mice may be yet to progress to a stage of inflammation which negatively influences metabolic health, particularly given alterations to genes negatively impacting glucose and lipid metabolism as previously detailed.

This study utilised a mouse model whereby AS intake was the equivalent of a human consuming one standard can of diet soda a day. In some previous models, supraphysiological concentrations of AS have been used to induce specific metabolic effects. We have shown that even at this lower concentration, AS can assert an effect on the metabolic health of both male and female mice, independently and in combination with HFD. It would be of value to further investigate the effects of other ASs, such as the newer plant-based sweeteners, and to investigate the long-term effects of AS consumption. This study did not look at the influence of AS consumption on the gut microbiome, however it could be a valuable avenue to investigate as alterations may indicate increased risk of weight gain and other metabolic derangements. AS has been shown to influence the gut microbiota, such as Ace-K's involvement in destabilizing Escherichia coli, a common habitant of the gut [28]. Previous work by Bian et al., investigating the influence of Ace-k administration on weight gain in male and female mice, showed altered expression of gut microbiota that could contribute to increased chronic inflammation [11]. Further, due to limited samples, we were unable to conduct comprehensive analysis of circulating plasma proteins which may have provide more mechanistic insight into the altered inflammatory profiles seen in our animals. Nonetheless, the use of PCR analysis to investigate the expression of inflammatory markers within the adipose tissue provided valuable insights into the inflammatory environment in these mice.

## 5. Conclusions

HFD-induced obesity is known to induce metabolic dysfunction, which can lead to IR and T2DM. Little information exists on the interaction effects between intake of AS and a HFD. We sought to investigate this by utilising a mouse model of HFD and AS supplementation in male and female mice. Our results show that AS did not prevent HFD-induced weight gain in males or females, but reduced glucose intolerance and adipocyte hypertrophy in male but not female mice. This metabolic improvement in male mice may have been mediated by reduced recruitment of key components related to HFD-induced inflammation as a result of AS supplementation. This was not seen in female mice, who experienced increased immune-related gene expression following AS intake. Despite this, male but not female mice experienced deregulation of genes associated with adipogenesis, and glycogen pathways. This further supports the importance of studying sexually dimorphic responses to an altered nutritional environment and highlights the need for further investigation into the intake of AS, particularly in those already at risk of metabolic disease such as the obese or overweight.

**Supplementary Materials:** Not applicable.

**Author Contributions:** Conceptualization, CMR; methodology, CMR, PBC and MHV; formal analysis, CMR and PBC; writing—original draft preparation, PBC; writing—review and editing, CMR, PBC and MHV; supervision, CMR and MHV; project administration, CMR and MHV; funding acquisition, CMR and MHV. All authors have read and agreed to the published version of the manuscript."

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**Institutional Review Board Statement:**

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All animal work was approved by the Animal Ethics Committee at the University of Auckland in accordance with all appropriate institutional and international guidelines and regulations of animal research.

**Informed Consent Statement:**

Not applicable.

**Data Availability Statement:**

The data detailed in this publication are available from the authors upon reasonable request.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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