Increased levels of inflammatory plasma markers and obesity risk in a mouse model of Down syndrome

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Abstract

Down syndrome (DS) is caused by the trisomy of human chromosome 21 and is the most common genetic cause of intellectual disability. In addition to the intellectual deficiencies and physical anomalies, DS individuals present a higher prevalence of obesity and subsequent metabolic disorders than healthy adults. There is increasing evidence from both clinical and experimental studies indicating the association of visceral obesity with a pro-inflammatory status and recent studies have reported that obese people with DS suffer from low-grade systemic inflammation. However, the link between adiposity and inflammation has not been explored in DS. Here we used Ts65Dn mice, a validated DS mouse model, for the study of obesity-related inflammatory markers. Ts65Dn mice presented increased energy intake, and a positive energy balance leading to increased adiposity (fat mass per body weight), but did not show overweight, which only was apparent upon high fat diet induced obesity. Trisomic mice also had fasting hyperglycemia and hypoinsulinemia, and normal incretin levels. Those trisomy-associated changes were accompanied by reduced ghrelin plasma levels and slightly but not significantly increased leptin levels. Upon a glucose load, Ts65Dn mice showed normal increase of incretins accompanied by over-responses of leptin and resistin, while maintaining the hyperglycemic and hypoinsulinemic phenotype. These changes in the adipoinsular axis were accompanied by increased plasma levels of inflammatory biomarkers previously correlated with obesity galectin-3 and HSP72, and reduced IL-6. Taken together, these results suggest that increased adiposity, and pro-inflammatory adipokines leading to low-grade inflammation are important players in the propensity to obesity in DS. We conclude that DS would be a case of impaired metabolic-inflammatory axis.

Graphical Abstract

Increased adiposity leading to increased levels of inflammatory plasma markers are putative contributor factors of obesity in Down syndrome.

Keywords

Down syndrome, inflammation, obesity, leptin, ghrelin, resistin, interleukin-6, galectin-3, HSP72

Abbreviations

DS: Down syndrome, Gal-3: galectin-3, GIP: glucose dependent insulinotropic polypeptide; GLP-1: glucagon-like peptide-1(7-36) amide, HF: high fat, HSP72: heat shock protein HSP (72), IL-6: interleukin-6, ROS: reactive oxygen species, PAI-1: Plasminogen activator inhibitor 1.

Introduction

Overweight and obesity are acquiring epidemic proportions worldwide, causing alarming increases of various associated health problems. Individuals with Down syndrome (DS) are a particularly vulnerable population, since they present significantly higher prevalence of obesity than the general population. DS is caused by trisomy of chromosome 21 and is associated to multiple endocrine and nervous system alterations [1], that may contribute to obesity driving metabolic complications, insufficient exercising and poor eating habits [2] [3] [4]. DS is associated with high body adiposity [5] [6] suggesting that a propensity for fat accumulation could be a major contributor of DS obesity.

In the general population, high adiposity is associated with inflammation and oxidative stress markers [7] [8]. Indeed, visceral fat accumulation contributes to pro-oxidant and pro-inflammatory states, as well as to alterations in glucose and lipid metabolism [8].

DS individuals show increased susceptibility to autoimmune disorders, hypothyroidism, celiac disease and diabetes mellitus compared to non-DS individuals [9], all conditions having in common inefficient immune responses or abnormally increased inflammation [10]. However, the potential link between adiposity and inflammation has not been explored. Here we used the Ts65Dn

mice, a validated DS mouse model, for the study of the effect of trisomy 21 on adiposity, energy balance and inflammatory status in non-obesogenic (standard rodent chow) and obesogenic conditions (high fat chow).

Methods

Animals and housing

4-6 moths-old male Ts(1716)65Dn (Ts65Dn) and wild-type (WT) mice were used in this study. Mice were obtained through crossings of a B6EiC3Sn a/A-Ts (1716)65Dn (Ts65Dn) female to B6C3F1/J males purchased from The Jackson Laboratory (Bar Harbor, ME). Genotyping was performed by amplifying genomic DNA obtained from the mice tail as previously described [11]. It has been shown that there is a sex-dimorphism in the effect of high fat diet metabolic effects in rats and that it might be influenced by gonadal cycles [12]. Here we used males for simplifying the interpretation of the results. However, the gender dimension of the genotypes studied should be addressed in future studies. The experiments were performed in the Animal Facilities in the Barcelona Biomedical Research Park (PRBB, Barcelona, Spain, EU) and the Functional & Physiological Exploration Platform (FPE) of the Functional & Adaptive Biology (BFA) Unit of the University Paris Diderot-Paris 7, CNRS UMR 8251, in controlled conditions of temperature (22 ºC ± 1^{o}C) and humidity at 55 ± 10% on a 12 hours light/dark cycle. Food and water were available *ad libitum* except during food deprived experimental sessions. All experimental procedures were approved by the local ethical committees [Comité Ético de Experimentación Animal del PRBB (CEEA-PRBB); procedure number MDS-12-1464P3 and Animal Care Committee of the Paris Diderot University (CEEA40)].

Diets

Depending of the experiment, WT/Ts65Dn mice had *ad libitum* access to water and standard chow (Experiment 1: WT, n = 26; Ts65Dn, n = 20) or to high fat diet for 8

weeks (High Fat/DIO, Test Diet, USA; Experiment 2: WT, n = 24; Ts65Dn, n = 21). Supplementary Table 1 shows the composition of the different experimental diets.

Experiment 1: Ts65Dn phenotypes under standard chow

WT and Ts65Dn mice were given standard chow (SC; SDS, UK) for the assessment of the genotype-dependent differences in body weight, body composition, metabolic phenotyping and plasma biomarkers in non-obesogenic conditions.

Body weight and body composition analysis

Non-invasive analysis of body composition was estimated by nuclear magnetic resonance (NMR) technology using a scanner EchoMRI 900 (Echo Medical Systems, USA), that creates contrast between soft tissues taking advantage of the differences in relaxation times of the hydrogen spins and/or hydrogen density. We obtained separate measures for lean and fat mass in grams (WT, n = 10; Ts65Dn, n = 8). Fat is defined as the mass of all the fat molecules in the body expressed as equivalent weight of canola oil. Lean is muscle mass equivalent to all the body parts containing water, excluding fat, bone minerals, and other substances or tissues that do not contribute to the NMR signal, such as hair.

Metabolic phenotyping

Metabolic phenotyping was performed using calorimetric cages (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). Mice were individually housed in the calorimetric cage in controlled laboratory conditions with the temperature maintained at 22 ºC ± 1^{o}C on a 12 hours light/dark cycle and acclimated to the chambers for 48 h before experimental measurements. Spontaneous feeding and locomotor activity (beam breaks/hour) were measured using automated feeding and activity recording in a Labmaster apparatus (TSE Systems). Whole energy expenditure was calculated according to the Weir equation respiratory gas exchange measurements [13]. Sensors were previously calibrated with a mixture of known concentrations of O_2 and CO_2 (Air Liquide, Paris, France).

Energy intake (Kcal lean body mass/h), locomotor activity (infrared breaks/h), respiratory exchange rate (respiratory quotient (RQ) = VCO_2/VO_2) and energy expenditure (Kcal/h) were recorded during 72 h. Representative 24 h values were calculated as the mean of the three consecutive experimental days. Raw values of VO_2 consumed, VCO_2 production (expressed in ml/h), and energy expenditure (Kcal/h), were collected every 15 min. Subsequently, each value was expressed by whole lean tissue mass obtained from the EchoMRI analysis. No practical methods of estimation of the resting metabolism are available presently [14]. However, an estimation of resting metabolism (Kcal/h) can be made on the following basis: data of energy expenditure were considered as the best estimation of resting metabolism if spontaneous activity was less than 5% of the highest daily value during the 30 minutes previous to the measurement, and if animals ate less than 0.1 g of food within the last hour.

Bio-Plex Pro Mouse Diabetes Assay

Glucose tolerance test was performed in WT (n = 11) and Ts65Dn (n = 7) mice on a standard chow diet. Briefly mice were food deprived for 5 hours prior to an oral administration of 2 g/Kg of 30% glucose (Lavoisier, France). Blood was collected from the tail vein before (T_0) and 30 minutes after (T_{30}) glucose administration, to evaluate the response to glucose load of incretin activity and related metabolic parameters after food deprivation and after the glucose load. We measured the levels of basal (T_0) glucose stimulated (T_{30}) insulin, anorexigenic and orexigenic signals (leptin and ghrelin), and incretins (glucose dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1(7-36) amide (GLP-1) and glucagon). Plasminogen activator inhibitor-1 (PAI-1) and resistin were also measured since they are implicated in the release of fatty acids, are associated with insulin resistance as well as with pro-inflammatory cytokines, and are increased in obese insulin resistant individuals. Determinations were carried out using suspension bead array immunoassay kits following manufacturer's specifications (Bio-Plex Pro Mouse Diabetes Assay 8-plex, Biorad, France). Upon blood centrifugation, plasma was stored in presence of Linagliptin, a DPP-4 inhibitor (Selleckchem, USA) and

protease inhibitor (Roche, France) to inhibit the degradation of incretins. The samples were measured using a Luminex 200 apparatus (Luminex, Austin, TX). All assays were performed in duplicate.

Inflammatory markers

Galectin-3, HSP72 and interleukin-6 were measured from blood in mice feed with standard chow (WT, n = 6; Ts65Dn, n = 5). Blood was obtained by retro-orbital sinus sampling with heparinized capillaries into the tubes containing a 1/10 volume of 3.8% sodium citrate. Plasma was isolated by centrifugation (2500 x g for 15 min at 4°C). Plasma galectin-3 (Abcam ELISA kit, France), HSP72 (Enzo Life sciences ELISA kit, Germany) and IL-6 (Enzo Life sciences ELISA kit, Germany) were assessed using sandwich ELISA. After removal of unbound conjugates, bound enzyme activity was assessed by use of a chromogenic substrate for measurement at 450 nm by a microplate reader (Flex Station 3, Molecular Devices, Ltd., UK).

Hepatic toxicity assays

Alanine aminotransferase (ALT) and Triacylglycerol (TG) were measured from blood in mice feed with standard chow (WT, n = 6; Ts65Dn, n = 5). Blood was obtained by retro-orbital sinus and plasma was isolated by centrifugation (2500 x g for 15 min at 4°C). ALT activity was assayed using the Alanine Aminotransferase Activity Assay Kit (Sigma-Aldrich, France) determined by a coupled enzyme assay, which results in a fluorometric (λex = 535/λem = 587 nm) product, proportional to the pyruvate generated. One unit of ALT is defined as the amount of enzyme that generates 1.0 µmole of pyruvate per minute at 37 °C. TG levels were measured according to the manufacturer's instructions (Sigma-Aldrich, France). Briefly, the assay involves enzymatic hydrolysis by lipase of the triglycerides to glycerol and free fatty acids. All assays were performed in duplicate.

A pyruvate tolerance test was performed by injecting sodium pyruvate (Sigma, 1 g/kg, i.p.) after a 6 h of fasting. Blood samples were collected from the tail tip [15] and glucose concentrations (Diagnostic Glucometer, Lifescan Laboratories, France) were measured at 5, 10, 15, 30, 60, 90 and 120 min after the injection.

The pyruvate tolerance test was performed in SC conditions and after 8-weeks of HF exposure (WT = 10; TS = 7).

Experiment 2: high fat diet induced obesity

Some parameters were also studied as described above upon *ad libitum* access to a high fat diet (High Fat/DIO, Test Diet, USA) for 8 weeks. These experiments were performed in a separate group mice: WT, n = 24; Ts65Dn, n = 21. Specifically we analysed changes in body weight and body composition (WT, n = 6; Ts65Dn, n = 6), plasma levels of Galectin-3, interleukin-6, and HSP72, as inflammatory biomarkers previously correlated with obesity and hepatic function markers such as ALT and triacylglycerol (WT, n = 8; Ts65Dn, n = 4), to discard diet-induced liver damage.

Statistical analyses

Sample size was estimated based on our previous experience with similar experiments. Differences between genotypes and diet groups were determined using 2-tailed unpaired Student's t test. Repeated measures ANOVA was used for the analysis of the differences in locomotor activity and pyruvate tolerance test. Two-way ANOVA with Bonferroni post hoc test to correct for multiple comparisons was used to test diet and genotype interaction. Levels of statistical significance were set at $P<0.05$. The statistical analyses were performed using the by using Statistical Package for Social Science software (SPSS version 17.0, IBM Corporation, USA).

Results

1. Increased adiposity and positive energy balance in Ts65Dn mice

In Experiment 1, we observed that 5 months-old Ts65Dn mice fed with standard chow showed similar body weight than WT littermates (Figure 1 A), but Ts65Dn mice presented a significant reduction of body length ($t(1, 16) = 4.23$, $P< 0.01$, Figure 1B).

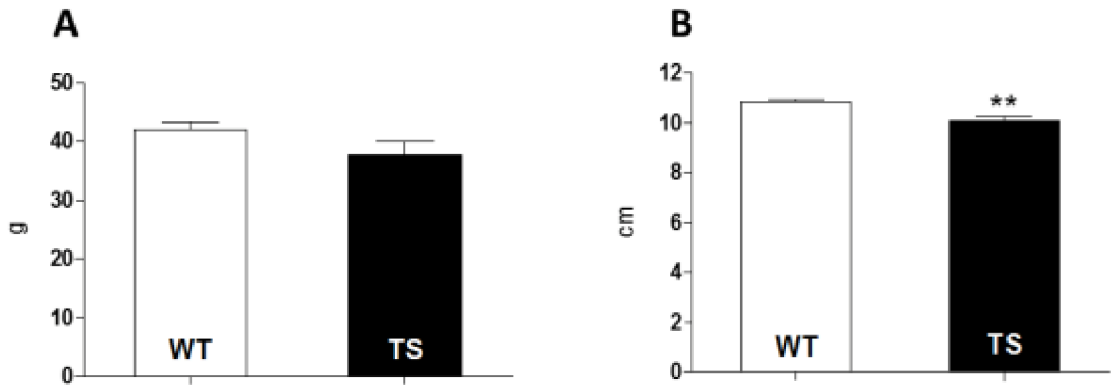

Figure 1. **Somatometric characteristics of wild type (WT) and Ts65Dn (TS) mice fed with standard chow (SC). (A)** Body weight (grams) and (**B)** body length (cm) in WT mice (n=10) and TS mice (n=8) fed with standard chow (SC). Student's t test for independent samples ** P<0.01.

Using Nuclear Magnetic Resonance (NMR) we detected significantly reduced lean mass (t (1, 16) = 2.92; P = 0.01; Figure 2A) and higher fat mass per body weight in Ts65Dn as compared to WT (t (1, 16) = -2.96, P<0.01; Figure 2B), replicating human studies that report individuals with DS having lower height and higher adiposity than typical development peers [6] [16].

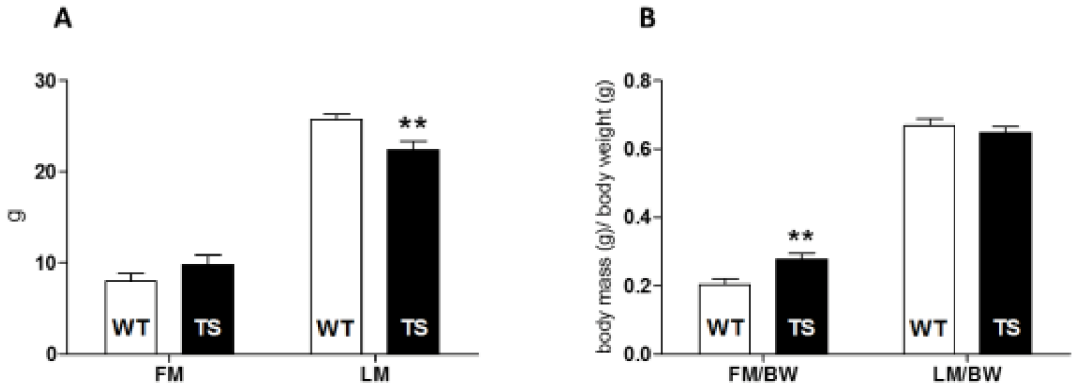

Figure 2. NMR analysis of body mass composition in wild-type (WT) and Ts65Dn (TS) mice. Bar blots depict **(A)** lean mass (LM) and fat mass (FM) represented in grams, and **(B)** LB and FB corrected for body weight (BW) in WT (n = 10) and TS (n = 8) mice. Data expressed as mean ± SEM. Student's t test for independent samples ** P<0.01.

Regarding energy intake, Ts65Dn mice showed significantly higher mean daily energy intake compared to WT (t (1, 9) = -3.23; P<0.01) due to significantly

increased energy intake during the active (dark) (t (1, 9) = -3.58; P<0.01; Figure 3A). Locomotor activity was similar in both genotypes during the resting phase, but we detected a slight hyperactivity in Ts65Dn mice during specific periods of the dark phase that did not reach statistical significance (Figure 3B ANOVA (dark phase) F (1,8) = 3.017; P>0.05). Energy expenditure (EE) was significantly higher in Ts65Dn mice both in the light and the dark phases of the circadian cycle compared to WT (t (1,9) = -3,40; P<0.01 (light) and t (1,9) = -3.52; P<0.01 (dark); Figure 3C). This increased EE in Ts65Dn mice might be related to a non-significant higher total oxygen consumption and carbon dioxide production (ml/Kg LM/h) compared to WT mice (TS [4567,49 ± 383,44] and WT [4039,91± 84,24]; (VO_2) t (1,8) = -1.35; P<0.05; TS [3993,52± 352,13] and WT [3541,52 ± 59,56]; (VCO_2), t (1,8) = -1.27; P<0.05), but the respiratory quotient (RQ = VCO_2/VO_2) was similar between both genotypes (Figure 3D). Estimated resting metabolism (ERM) of Ts65Dn mice was significantly higher compared to WT mice, (t (1, 9) = -3.47; P<0.05; Figure 3E), [17]. Even so, Ts65Dn mice still showed a significantly more positive energy balance during the dark phase than WT mice (t (1, 9) = -2.43; P<0.05, Figure 3F).

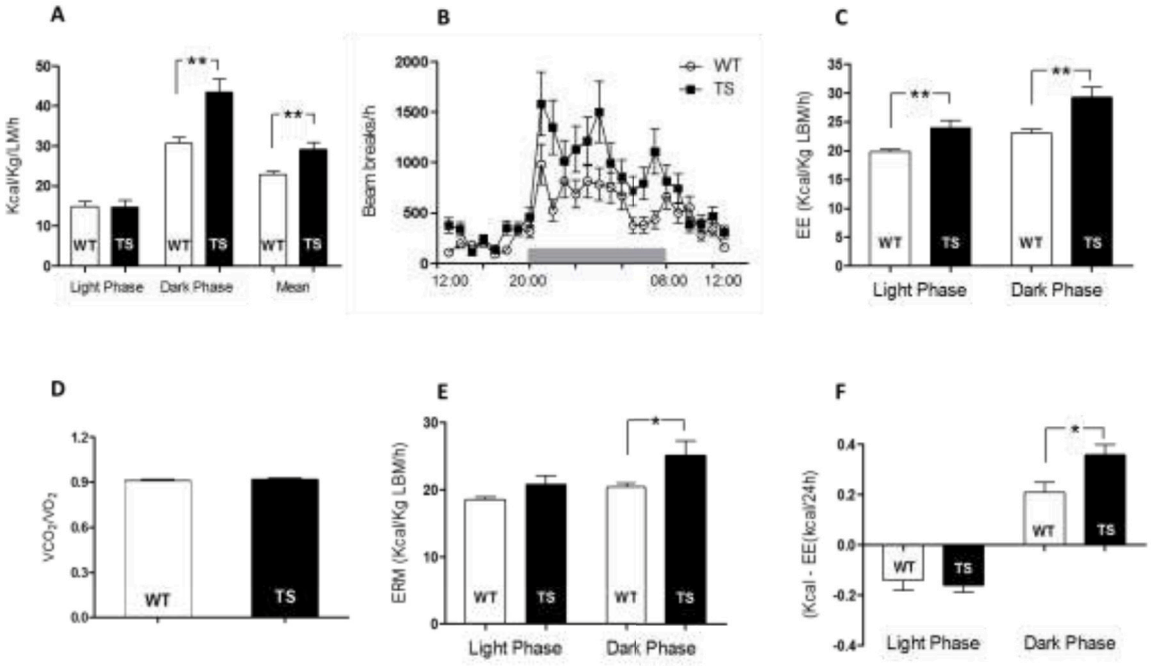

Figure 3. Indirect calorimetry in wild-type (WT) and Ts65Dn (TS) mice fed with standard chow
Bar blots depict **(A)** energy intake (kcal/ LM/h) during the inactive (light phase), active (dark phase) period of the 12 hours light/dark cycle and the daily mean intake. **(B)** Locomotor activity (beam breaks/h) during 24 h. **(C)** Energy expenditure**,** EE (Kcal/Kg LM/h). **(D)** Respiratory exchange ratio (VCO_2/CO_2). **(E)** Estimated resting metabolism, ERM (Kcal/Kg LM/h). **(F)** Energy balance (energy intake – energy expenditure). WT (n = 5) and TS (n = 6) data are expressed as mean ± SEM. Student's t test for independent samples * P<0.05; ** P<0.01. LM: lean mass; EE: energy expenditure; ERM: estimated resting metabolism**.**

2. Altered levels of metabolic and inflammatory biomarkers in Ts65Dn mice

To study the effect of the trisomy on energy balance, inflammation and oxidative stress, we obtained the basal (t_0, 5-hour after starvation) and postprandial (t_{30}, 30 minutes after an oral glucose load) glycemic phenotype and metabolic profile of selected markers in Ts65Dn and WT (Table 1).

In basal conditions (t_0), Ts65Dn mice showed higher plasma glucose compared to WT mice (t (1, 17) = -2.59; P<0.05) along with significantly lower insulin levels (t (1, 17) = 2.40; P<0.05) suggesting an insulin-resistant phenotype in trisomic mice or a deficient insulin secretion. We also analysed ghrelin and leptin, which are key appetitive and satiety signals. The plasma levels of ghrelin were significantly lower in Ts65Dn than in WT mice (t (1, 16) = 3.17; P<0.01) while leptin levels were similar (Table 1) suggesting that the increased energy intake in trisomic mice could be related to an altered balance of anorexigenic and orexigenic signals. After glucose load (t_{30}), the levels of glucose increased similarly in both genotypes (Figure 4), and thus remained significantly higher (t (1, 17) = -2.26, P<0.05) in Ts65Dn compared to WT mice (Table 1). The levels of insulin also increased similarly in both genotypes (Figure 4). Both genotypes increased plasma leptin levels that were higher in Ts65Dn (t(1,17) = -2.40; P <0.05) though the absolute levels were not significantly different from those of WT mice (t (1, 17) = -2.09; P = 0.052; Table 1).

Incretins are implicated in glucose homeostasis since they stimulate insulin secretion. We did not observe genotype-related differences in the levels of glucose dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1(7-36) amide

(GLP-1) or glucagon at t_0 or t_{30} nor in their percentage of change upon glucose load (Table 1, Figure 4). Plasminogen activator inhibitor-1 (PAI-1) and resistin were also measured since they are implicated in the flow of fatty acids from plasma and are associated with insulin resistance as well as with pro-inflammatory cytokines [18] [19]. Ts65Dn and WT mice showed similar levels of PAI-1 and resistin at t_0 and t_{30}. However, Ts65Dn mice showed a significantly higher increase of resistin upon glucose load ($t(1,17) = -2.26$; $P < 0.05$) that have been previously associated with impaired insulin-stimulated glucose intake in in vitro adipocytes studies [20].

Table 1. Plasma levels of obesity biomarkers markers in wild type (WT) and Ts65Dn (TS) mice in basal conditions (t_0) and 30 minutes after a glucose load (t_{30}). Data are expressed as mean ± SEM. WT (n = 11) and TS (n = 7). WT vs. TS; Student's t test for independent samples.

		Glucose (mg/dl)	Insulin (ng/ml)	Ghrelin (pg/ml)	Leptin (pg/ml)	Glucagon (pg/ml)	GIP (pg/ml)	GLP-1 (pg/ml)	PAI-1 (pg/ml)	Resistin (pg/ml)
T_0	WT	195.5 ± 5.86	3658.11 ± 122.07	5595.74 ± 360.27	7193.91 ± 541.18	387.07 ± 15.62	518.16 ± 12.48	55.43 ± 2.28	2876.12 ± 154.90	50159.32 ± 1696.01
	TS	251.33 ± 4.55	2528.17 ± 135.78	1540.14 ±2.41.39	10173.25 ± 848.14	386 ± 19.51	584.42 ± 30.78	54.53 ± 2	2605.35 ± 221.52	48765.63 ± 1810.21
		P< 0.05	P< 0.05	P< 0.01						
T_{30}	WT	340 ± 13.72	6332.15 ± 200.37	6382.22 ± 476.85	12763.55 ± 1004.05	696.22 ± 37.63	575.37 ± 11.42	121.15 ± 7.41	5155 ± 254.42	79604.79 ± 2515.79
	TS	470.67 ± 22.03	4709.02 ± 193.55	3421.21 ± 327.81	20783.37 ± 1174.96	511.03 ± 22.28	673.40 ± 31.77	135.86 ± 6.26	4416.92 ± 635.62	97005.72 ± 3480.49
		P< 0.05								

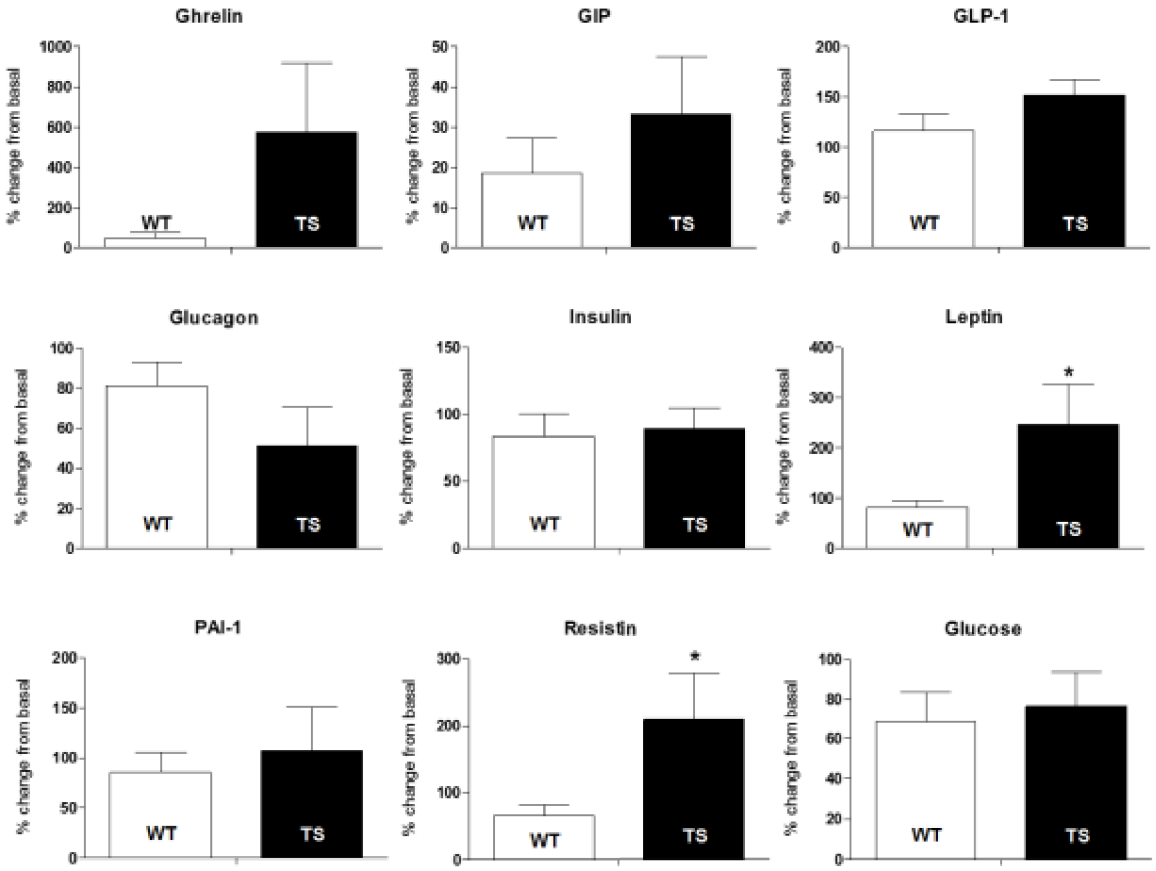

Figure 4. Percentage of change of obesity biomarkers in plasma levels in wild type (WT) and Ts65Dn (TS) mice from basal (t_0) levels to 30 minutes after a glucose load (t_{30}). Data are expressed as mean ± SEM. WT (n = 11) and TS (n = 7). WT vs. TS; Student's t test for independent samples * P< 0.05.

3. Body weight increase, body composition and indirect calorimetry upon high fat diet access

In Experiment 2, we studied the effect of 8-weeks of exposure to high fat (HF) diet in obesity development and inflammatory status. We first observed that both genotypes gained body weight and increased fat mass as shown by NMR, being significantly higher in Ts65Dn mice (body weight gain: t (1, 10) = -3.32; P<0.01, Figure 5A; fat mass: (t (1, 10) = -3.40; P< 0.01; Figure 5B).

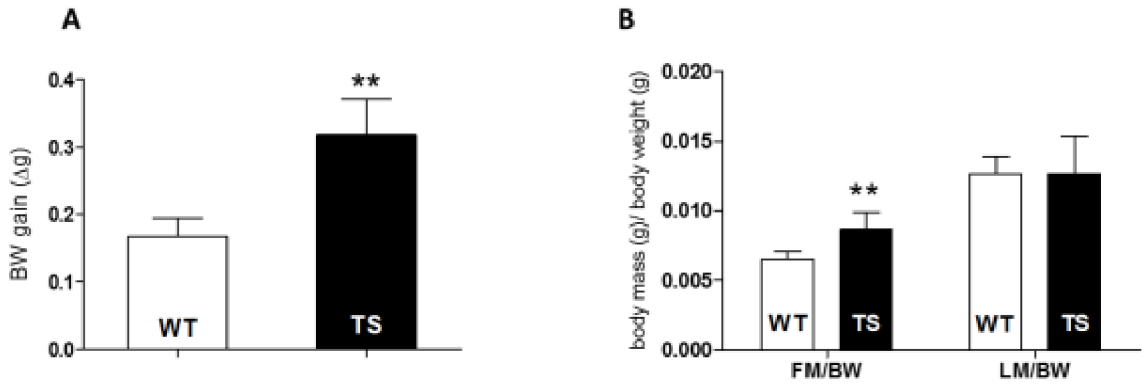

Figure 5. Body weight gain and body composition in wild type (WT) and Ts65Dn (TS) mice fed with high fat diet. (A) Body weight gain upon 8 weeks of HF (expressed as the difference from baseline body weight in grams). **(B)** Lean mass (LM) / body weight (g) and fat mass (FM) /body weight (g) in the 8th week. WT (n = 6) and TS (n = 6). Data expressed as mean ± SEM. Student's t test for independent samples ** P<0.01.

Ts65Dn mice fed with HF showed higher energy intake than WT mice both during the light and dark periods, being significant the mean daily energy intake (t (1,10) = -4.01; P<0.01; Figure 6A). While the ambulatory activity was similar between genotypes (Figure 6B), with only a slight hyperactivity in specific time-points, again trisomic mice showed increased energy expenditure in both the light and dark phases (t (1,10) = -3.07; P<0.05 (light) and t (1,10) = -2.97; P< 0.05 (dark); Figure 6C). It might be related to the significant higher oxygen consumption and carbon dioxide production in Ts65Dn relative to WT mice (TS vs. WT (VO_2) t (1,8) = -3.7; P<0.01; TS vs. WT (VCO_2), t (1,8) = -3.81; P<0.01) although the metabolic exchange (VCO_2/VO_2) was similar between genotypes (Figure 6D). Again, Ts65Dn mice showed a significant increased estimated resting metabolism (t (1,10) = -3.57; P< 0.01 (light) and t (1,10) = -2.50; P<0.05 (dark; Figure 6D) but both genotypes had negative energy balance during light phase and positive during the dark period (Figure 6C).

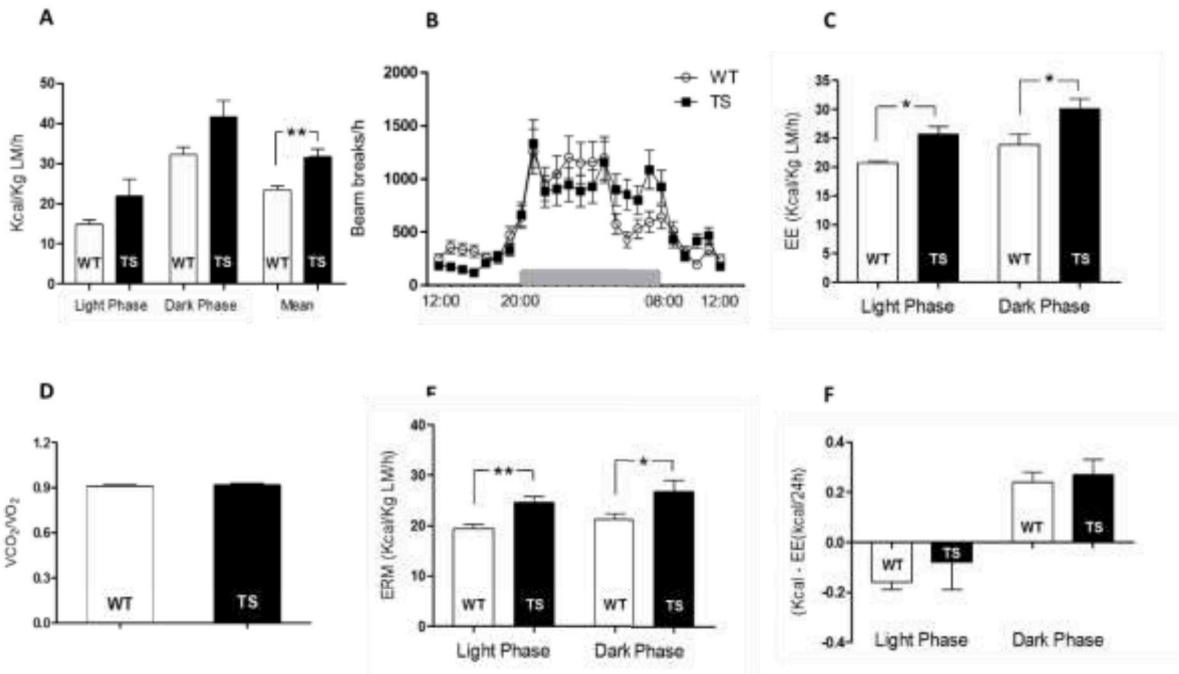

Figure 6. Indirect calorimetry in wild-type (WT) and Ts65Dn (TS) mice fed with high fat diet (HF). Bar blots depicted **(A)** intake (Kcal/ Kg LM/h) during the inactive (light phase) and active (dark phase) period of the 12hours light/dark cycle and the daily mean intake. **(B)** Daily locomotor activity (beam breaks/h). **(C)** Energy expenditure**,** EE (Kcal/Kg LM /h). **(D)** Respiratory exchange ratio (VCO_2/CO_2). **(E)** Estimated resting metabolism, ERM (Kcal/Kg LM /h). **(F)** Energy balance (food intake – energy expenditure). WT (n = 5) and TS (n = 6) data are expressed as mean ± SEM. Student's t test for independent samples ** P <0.01. LM: lean mass; EE: energy expenditure**;** ERM: estimated resting metabolism.

4. Ts65Dn show higher plasma levels of galectin-3 and HSP72 and diet-associated changes in interleukin-6

Chronic low-grade inflammation is observed in subjects with obesity and represents a mechanistic link between obesity, insulin resistance and type 2 diabetes mellitus [21]. Hence, plasma levels of molecules such as galectin-3, HSP72, and interleukin-6 (IL-6) have been shown to positively correlate with human obesity [22].

Independently of the diet (Experiment 1 and 2), Ts65Dn showed higher levels of galectin-3 and HSP72 compared to WT (genotype effect for galectin 3, ANOVA, F

(1, 23) = 23.31, P<0.001; Figure 7A; and for HSP72, ANOVA, F (1, 23) = 7.2, P<0.05; Figure 7B). As previously shown in DS patients [23] plasma levels of IL-6 were lower in Ts65Dn compared to WT mice (Figure 7C) but increased significantly upon HF diet in both genotypes (diet effect, ANOVA F (1, 23) = 18.25, P<0.001).

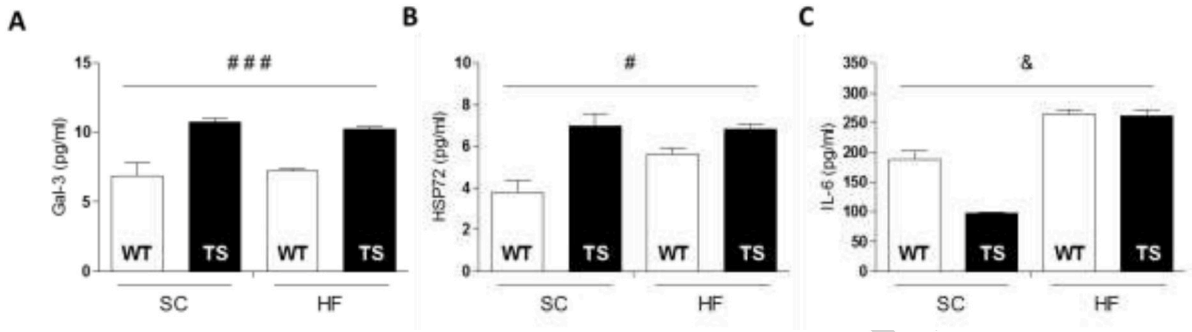

Figure 7. Levels of circulating markers of inflammation in wild type (WT) and Ts65Dn (TS) mice in non-obesogenic (standard chow, SC) and obesogenic (high fat diet, HF) conditions. **(A)** galectin-3 (Gal-3), **(B)** Heat shock protein HSP72 (HSP72) and **(C)** interleukin-6 (IL-6). D-E) SC: standard chow; HF: high fat diet. Data are expressed as mean ± SEM. WT (SC) = 6, WT (HF) = 8; TS (SC) = 5, TS (HF) = 4. Two-way ANOVA. # P< 0.05; ## < 0.01; ### P< 0.001 genotype effect; & P< 0.05 diet effect.

5. Ts65Dn mice do not present compromised liver function

Insulin resistance is usually associated to intra- or extra-hepatic injury that can be determined by measuring the circulating levels of alanine amino transferase (ALT) activity [24] and triglycerides levels in plasma [25]. Several human reports have shown an altered lipid profile associated to trisomy 21 [26]. Therefore, we here checked the ALT and triacylglycerol (TG) in standard chow and high fat diet conditions to determine the possible implication of hepatic dysfunction in the insulin-resistance and increased pro-inflammatory profile detected in Ts65Dn mice (Experiment 1 and 2). In non-obesogenic conditions, ALT levels were similar in WT and Ts65Dn mice and upon HF access, mice of both genotypes showed higher ALT circulating levels (diet effect, ANOVA F(1,1)) 5.45, P<0.05; Figure 8A). The opposite was detected for triacylglycerol plasma levels with HF mice showing lower

circulating TG in both genotypes compared to SC fed mice, without differences between genotypes (diet effect, ANOVA F(1,1)) 13.34, P<0.01;Figure 8B). Finally, we performed a pyruvate tolerance test to address the possible effect of the trisomy on hepatic glucose homeostasis. It has been shown that the development of insulin resistance in the liver is accompanied by a progressive deterioration of the control of hepatic glucose production leading to excessive hepatic glucose output through increased glycogenolysis and gluconeogenesis [27]. In non-obesogenic conditions, the injection of pyruvate led to a similar rise of plasma glucose levels in both WT and Ts65Dn. Upon HF diet, both genotypes displayed a greater increase in plasma glucose levels upon pyruvate which indicates increased gluconeogenesis (diet effect, ANOVA F(1,32) = 6.48; P<0.05; Figure 8C).

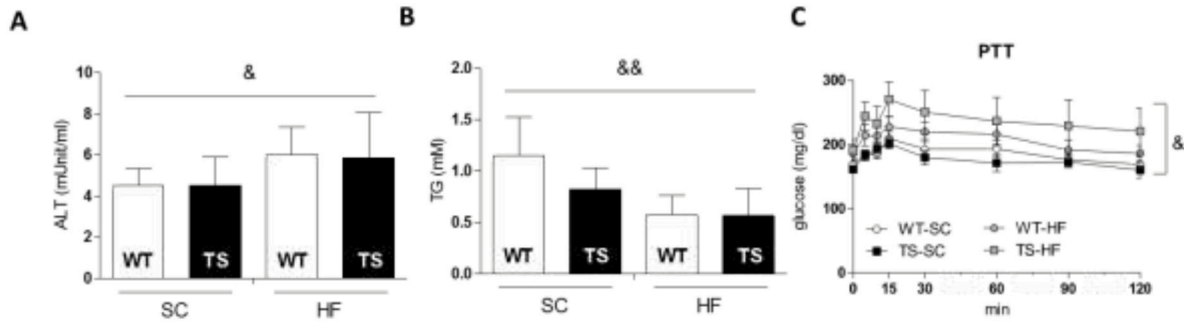

Figure 8. Hepatic glucose homeostasis though plasma biomarkers of liver injury and in vivo glycogenesis determination in wild type (WT) and Ts65Dn (TS) mice in non-obesogenic (standard chow, SC) and obesogenic (high fat diet, HF) conditions. A-B) Hepatic markers: **(A)** alanine aminotransferase (ALT) activity and **(B)** triacylglycerols **(TG). (C)** Pyruvate tolerance test (PTT). SC: standard chow; HF: high fat diet. Data are expressed as mean ± SEM. For metabolic parameters: WT (SC) = 6, WT (HF) = 8; TS (SC) = 5, TS (HF) = 4. For PTT: WT (SC) = 10, WT (HF) = 10; TS (SC) = 7, TS (HF) = 7. A,B) Two-way ANOVA; & P< 0.05; && P< 0.01 diet effect. C) ANOVA Repeated measures.

Discussion

Obesity in Down syndrome (DS) has been mainly attributed to insufficient exercising and reduced energy metabolism or to poor eating habits [28] [29]. Here

we show that visceral fat accumulation contributes to pro-inflammatory states, as well as to alterations in glucose metabolism in Ts65Dn mice that show increased plasma levels of inflammatory biomarkers previously correlated with obesity such as galectin-3 and HSP72.

In the general population, obesity and propensity to weight gain have been associated with low resting metabolism and reduced energy expenditure [30]. In our experiments, Ts65Dn mice showed higher energy intake but also energy expenditure (EE), and higher estimated resting metabolism (ERM) compared to WT. Even so, trisomic mice present a marked positive energy balance. The contribution of energy metabolism and energy expenditure in DS obesity is controversial. Some reports have shown that resting metabolism is reduced in DS [31] [32] [33], possibly due to defects in thyroid gland function, which is prevalent in this population [34]. Instead, in our experiments, Ts65Dn mice presented an increased resting metabolism, which may contribute to their hyperphagic phenotype, which could lead to the significantly increased fat mass we observed in trisomic mice. Also, we replicated previous observations in Ts65Dn mice that showed hyperglycemia with concomitantly reduced insulin plasma levels in fasting and glucose loaded conditions. Approximately 60–70% of insulin secretion is dependent on the release of the incretin GLP1 and GIP [35] but in our experiments, we did not detect trisomy-dependent changes in fasting or glucose-stimulated incretin plasma levels. However, other factors could be contributing to this hypoinsulinemic phenotype. Perturbed glucose homeostasis has been previously described in Ts65Dn that was ascribed to functional changes in pancreatic β-cells possibly dependent of RCAN1 triplication [36], but recent studies also point to the adipose tissue, that we found increased in Ts65Dn, as a highly active endocrine organ secreting a range of hormones such as resistin and leptin. Resistin is an adipocyte-specific hormone that has been related to impaired glucose tolerance, and reduced insulin plasma levels [19]. Studies in adipocytes have shown that resistin antiserum enhances insulin-stimulated glucose uptake and blunts insulin action [20]. Conversely, exogenous administration of resistin impairs glucose tolerance upon a glucose load in mice [36]. We found significantly higher increases

in resistin levels upon glucose load in Ts65Dn compared WT mice. This resistin over-response could contribute to hypoinsulinemic phenotype of trisomic mice through insulin-secretion independent mechanisms. We only found one report in human showing that the levels of resistin in amniotic fluid of DS pregnancies are lower compared to non-affected pregnancies [37].

On the other hand, leptin, a key hormone secreted by adipocytes that leads to higher energy expenditure and reduces food intake [38] was increased, though not significantly, in Ts65Dn mice, whereas the level of the orexigenic hormone ghrelin was decreased compared to WT. Ts65Dn consumed more calories, suggesting that the satiating effects of leptin would be inefficient. Leptin levels correlate with adiposity in DS children and adolescents [39] [40] and in free feeding conditions, and leptin levels are higher in DS compared to unaffected siblings [41] [42] possibly due to the greater leptin production per unit of adipose tissue [41]. Two studies have reported lower leptin levels in DS adults [42] and foetus [43], but the majority of the existing reports support a leptin resistance phenotype. We only found one report of ghrelin levels in DS individuals showing no differences compared to typical developing peers [41] but in the general population obese individuals usually have lower plasma levels of ghrelin than lean individuals [44]. Even though we did not explore directly the leptin resistance in our mice, the finding of obese-like hyperleptinemia and hypoghrelinemia along with hyperphagia in Ts65Dn mice lead to speculate that diminished anorexigenic/orexigenic sensitivity might increase the risk of obesity development when exposed to an obesogenic environment. In fact, we detected a higher body weight gain and increased energy intake in Ts65Dn mice compared to WT littermates upon exposure to high fat diet. This result would be in line with previous reports showing that rats having greater absolute leptin response to intravenous glucose administration, also had increased energy intake and gained more weight upon 6 weeks of high fat diet [45].

It may be argued that leptin/insulin resistance could be driven by hepatic dysfunction. Diet-induced obese mice show elevated blood levels of alanine

aminotransferase (ALT) activity and triglycerides [46] that are indicators of liver damage. Thus, we measured ALT activity, as a marker of hepatic injury, and circulating triacylglycerols (TG) that might reflect a high fat-induced partitioning of lipids in the liver, either by reduced hepatic triglyceride production or by increased triglyceride clearance [47]. In non-obesogenic conditions, ALT activity was similar in WT and Ts65Dn mice and upon HF access, mice of both genotypes showed higher ALT activity. The opposite was detected for plasma TG in HF mice, showing lower circulating TG compared to SC fed mice, without differences between genotypes. Finally, it has been shown that the development of insulin resistance in the liver is accompanied by a progressive deterioration of the control of hepatic glucose production leading to excessive hepatic glucose output through increased glycogenolysis and gluconeogenesis [27]. We explored this phenotype by performing the pyruvate tolerance test in WT/Ts65Dn mice in both non-obesogenic and obesogenic conditions. According to our results, gluconeogenesis is neither impaired as a result of the trisomy nor trisomy leads to an increased vulnerability to hepatic injury upon diet-induced obesity.

Leptin is a pro-inflammatory adipokine, implicated in the release of inflammatory cytokines [48] while ghrelin acts as an anti-inflammatory signal [49]. We thus speculated that hyperleptinemia and hypoghrelinemia could produce increased inflammation that may contribute to obesity development. In the general population there is a strong association between inflammation and increased propensity for fat accumulation and obesity [21] [50]. Also in DS there is a link between adiposity and autoimmune diseases [9, 48]. Specifically, we found that Ts65Dn mice had higher levels of pro-inflammatory and pro-obesogenic markers such as plasma galectin-3 and HSP72 compared to WT mice both in non-obesogenic conditions and upon high fat diet. Galectin-3 is a lectin which levels are highly increased during inflammation in both humans and obese mice [51]. Moreover, its administration causes insulin resistance and glucose intolerance in mice [52]. Therefore, our result suggests that galectin-3 could be a new interesting target for DS. Also, high levels of the stress heat shock protein 70 (HSP72) in plasma has been associated with autoimmunity [53] [54] and accumulating data show that HSP72 extracellular

levels correlate with oxidative damage [55] [56]. According to our results, trisomy 21 seems the first natural example of increased plasma level of HSP72, which is in accordance with the high prevalence of autoimmune diseases in DS individuals. Finally, Interleukin-6 (IL-6) is positively correlated with fat mass and inflammation in the general population and also in DS individuals [57] [58] [59]. However, in our hands, Ts65Dn fed with SC had lower plasma IL-6 level compared to WT mice. This would parallel the depressed levels of IL-6 described before in DS children [23] [60]. In fact, it has been shown that mice lacking IL-6 expression develop systemic insulin resistance and late-onset obesity [61] [62]. Taken together, our results suggest that trisomy 21 is associated to a disrupted adipoinsular axis leading to abnormally increased levels of pro-inflammatory mediators sustaining a low-grade inflammatory state.

In conclusion, in this study we found that Ts65Dn mice have increased fat mass and energy intake and impaired glucose homeostasis. We also detected an increased glucose-stimulated response of pro-inflammatory adipokines along with significant increases of plasma galectin-3 and HSP72 that might potentially explain their increased obesity propensity. Taken together, these results suggest that increased adiposity, and pro-inflammatory adipokines leading to low-grade inflammation are important players in the propensity to obesity in DS. We conclude that DS would be a case of impaired metabolic-inflammatory axis.

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Highlights

In non-obesogenic conditions, Ts65Dn mice present increased body adiposity and hyperglycemic/hypoinsulinemic phenotypes.

Upon high fat free choice access, Ts65Dn mice gained more weight than WT mice.

Ts65Dn mice present increased glucose-stimulated responses for pro-inflammatory adipokines, resistin and leptin that might account for the observed increase of energy intake.

Independently of the diet, Ts65Dn mice show increased plasma levels of galectin-3 and HSP72, two inflammatory biomarkers previously correlated with obesity.