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Adipose tissue homeostasis orchestrates the oxidative, energetic, metabolic and endocrine disruption induced by binge drinking in adolescent rats

Inés Romero-Herrera[1], Fátima Nogales[1], María del Carmen Gallego-López[1], Javier Díaz-Castro[2,3], Jorge Moreno-Fernandez[2,3], Julio José Ochoa[2,3], Olimpia Carreras[1] and Mª Luisa Ojeda[1]

[1]*Department of Physiology, Faculty of Pharmacy, Seville University, Seville, Spain*
[2]*Institute of Nutrition and Food Technology 'José Mataix Verdú', University of Granada, Granada, Spain*
[3]*Department of Physiology, University of Granada, Granada, Spain*

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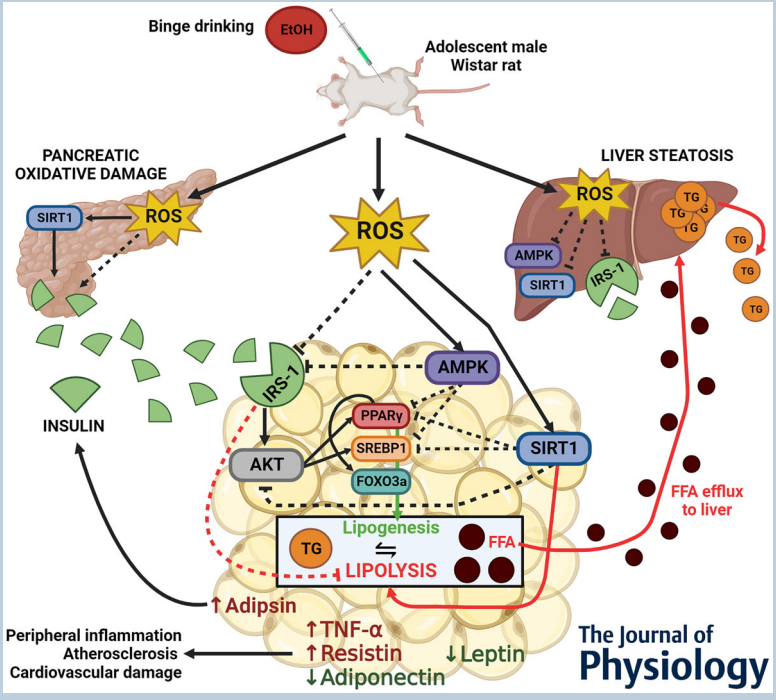

Abstract Binge drinking (BD) is the most common alcohol consumption model for adolescents, and has recently been related to the generation of high oxidation and insulin resistance (IR). White adipose tissue (WAT) is a target organ for insulin action that regulates whole-body metabolism by secreting adipokines. The present study aimed to analyse the oxidative, inflammatory, energetic and endocrine profile in the WAT of BD-exposed adolescent rats, to obtain an integrative view of insulin secretion and WAT in IR progression. Two groups of male adolescent rats were used: control ($n = 8$) and BD ($n = 8$). An intermittent I.P. BD model (20% v/v) was used during 3 consecutive weeks. BD exposure led to a pancreatic oxidative imbalance, which was joint to high insulin secretion by augmenting deacetylase sirtuin-1 (SIRT-1) pancreatic expression and serum adipsin levels. However, BD rats had hyperglycaemia and high homeostasis model assessment of insulin resistance value (HOMA-IR). BD exposure in WAT increased lipid oxidation, as well as decreased insulin receptor

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substrate 1 (IRS-1) and AKT expression, sterol regulatory element-binding protein 1 (SREBP1), forkhead box O3A (FOXO3a) and peroxisome proliferator-activated receptor γ (PPARγ), and adipocyte size. BD also affected the expression of proteins related to energy balance, such as SIRT-1 and AMP activated protein kinase (AMPK), affecting the adipokine secretion profile (increasing resistin/adiponectin ratio). BD altered the entire serum lipid profile, increasing the concentration of free fatty acids. In conclusion, BD led to an oxidative imbalance and IR process in WAT, which modified the energy balance in this tissue, decreasing the WAT lipogenic/lipolytic ratio, affecting adipokine secretion and the systemic lipid profile, and contributing to the progression of IR. Therefore, WAT is key in the generation of metabolic and endocrine disruption after BD exposure during adolescence in rats.

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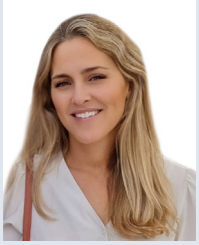
Corresponding author F. Nogales: Department of Physiology, Faculty of Pharmacy, Seville University, C/ Professor García González, no. 2, 41012. Sevilla, Spain. Email: fnogales@us.es

Abstract figure legend Binge drinking (BD) effects on the liver, pancreas and white adipose tissue (WAT) homeostasis in adolescent rats. BD-induced oxidative stress disrupts the balance in liver, pancreas and WAT of adolescent rats. In the liver, reactive oxygen species (ROS) reduce deacetylase sirtuin-1 (SIRT-1), AMP activated protein kinase (AMPK) and insulin receptor substrate 1 (IRS-1) expression, leading to hepatic insulin resistance (IR). In the pancreas, ROS stimulate SIRT-1, which promotes insulin secretion together with high serum adipsin levels. The present hyperinsulinaemia indicates a role for IR in WAT homeostasis. In WAT, ROS inhibit IRS-1, preventing its lipolysis inhibition, leading to a decreased AKT expression and subsequently reducing sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor γ (PPARγ) and forkhead box O3A (FOXO3a) expression. However, alcohol stimulates SIRT-1 and AMPK, inhibiting lipogenesis and promoting lipolysis, resulting in increased free fatty acids (FFA), a situation consistent with a hepatic accumulation of triglycerides (TG) and steatosis. BD-induced inflammation, with increased pro-inflammatory tumour necrosis factor-α (TNF-α) and resistin, and decreased anti-inflammatory adiponectin, may contribute to atherosclerosis and cardiovascular damage. Serum leptin levels were decreased. Image created with BioRender.com.

Key points

- Adolescent rat binge drinking (BD) exposure leads to hepatic and systemic oxidative stress (OS) via reactive oxygen species generation, causing hepatic insulin resistance (IR) and altered energy metabolism.
- In the present study, BD exposure in adolescent rats induces OS in the pancreas, with increased insulin secretion despite hyperglycaemia, indicating a role for IR in white adipose tissue (WAT) homeostasis.
- In WAT, BD produces IR and an oxidative and energetic imbalance, triggering an intense lipolysis where the serum lipid profile is altered and free fatty acids are increased, consistent with liver lipid accumulation and steatosis.
- BD exposure heightens inflammation in WAT, elevating pro-inflammatory and reducing anti-inflammatory adipokines, favouring cardiovascular damage.
- This research provides a comprehensive view of how adolescent BD in rats impacts liver, WAT and pancreas homeostasis, posing a risk for future cardiometabolic complications in adulthood.

Inés Romero-Herrera is a pharmacist (University-of-Seville, 2018) who completed her Master's in Clinical Research and Pharmaceutical Medicine (European University of Madrid, 2019). She worked at Janssen (Johnson & Johnson) in the Medical Affairs department (Madrid, 2019–2020). She currently is a PhD researcher and teacher in Physiology at the University of Seville (2020–2024), focused on binge drinking and adolescence, investigating oxidative stress, metabolic balance and applying a selenium antioxidant treatment in rat skeletal muscle. She recently researched at the Catholic University of Leuven (Belgium), studying aged-skeletal muscle and adiponectin. Her postdoctoral plans include exploring white and brown adipose tissue, using selenium as an antioxidant after binge drinking exposure in adolescent rats.



Introduction

Binge drinking (BD) is defined by the National Institute on Alcohol Abuse and Alcoholism (2020) as an acute ethanol (EtOH) consumption model, which brings blood alcohol concentration (BAC) to 0.08% or higher within 2 h. Nowadays, BD is a serious health issue among young people (Ojeda et al., 2022; Pompili & Laghi, 2019). This consumption is deeply related to oxidative damage as a result of the high BAC reached because, in tissues, EtOH saturates the activity of its main metaboliser enzyme, alcohol dehydrogenase (ADH), and drastically increases the activity of cytochrome P450 2E1 (CYP2E1) (Teschke, 2018). CYP2E1 induction not only transforms EtOH into the toxic acetaldehyde, but also directly generates a high amount of reactive oxidative species (ROS) (Cederbaum et al., 2009). Therefore, during chronic alcohol consumption, ADH is more competent, avoiding the extra ROS generated by CYP2E1.

BD exposure during adolescence has its own problems. It is solidly associated with neurotoxic effects (Hermens & Lagopoulos, 2018), with major causes of mortality in this age range (Molina & Nelson, 2018) and a higher propensity to later adult EtOH consumption problems (Spear, 2000, 2018). Moreover, there is a new trend that defends the fact that teenager BD consumption is more hazardous than was expected (Hagström & Andreasson, 2019). Adolescence is a complex period, which involves many different hormonal pathways that orchestrate physical and biological changes, encompassing increased growth and metabolic rate, alterations in fat and muscle, and breast and genital development (Khan, 2019; Vijayakumar et al., 2018). In this context, BD consumption during adolescence has recently been associated with hepatic damage (Taylor & Miloh, 2019), increased heart rate (Ojeda et al., 2021; Ramírez-Piña et al., 2021), reduced microvasculature function (Bian et al., 2018), kidney ionic imbalance (Sobrino et al., 2019) and even insulin disturbances (Steiner & Lang, 2017), which are situations that predispose adolescents to future adult cardiometabolic problems (Ojeda et al., 2022). Furthermore, the adolescent heart is substantially more sensitive to these effects than the adult one (Ai et al., 2020).

Although the clinical effects of chronic EtOH consumption are well described, with the liver being the first organ affected by its consumption, the effects of alcohol on other organs, such as the pancreas and adipose tissue (AT), are also known to contribute to the development of liver injury, especially by altering insulin sensitivity (Gopal et al., 2021). Indeed, it is becoming clear that AT is an important site of EtOH action, which affects AT–liver axis function by influencing AT lipolysis and hepatic steatosis (Wei et al., 2013; Zhong et al., 2012). However, little is known about the effects of intermittent BD exposure during adolescence in this axis and the response of AT to insulin. In this context, our research group has described that adolescent rats exposed to intermittent BD exposure exhibit lower AMP activated protein kinase (AMPK) and NAD^+-dependent deacetylase sirtuin-1 (SIRT-1) hepatic expression (Nogales et al., 2021). Both proteins are sensors of the cellular energy status. When activated, they lead to catabolic processes to supply energy (Cantó & Auwerx, 2009). When down-regulated, they are implicated in the development of steatosis by increasing lipogenesis and avoiding lipolysis (Jiang et al., 2015). Moreover, liver SIRT-1 and AMPK depletion, via different mechanisms, inhibits some insulin signalling steps, contributing to a process of insulin resistance (IR) in BD exposed rats (Nogales et al., 2021).

Adipose tissue, together with liver and skeletal muscle, is a target organ for insulin action. It is classified into brown adipose tissue (BAT) and white adipose tissue (WAT), each with different morphological and functional profiles. WAT has energy storage as its principal function, whereas BAT is in charge of the thermogenesis process. Visceral-WAT metabolic dysregulation is related to glucose intolerance, hepatic steatosis and the IR process (Cox et al., 2021; Findeisen et al., 2011). WAT has recently been considered as a key regulator of whole-body metabolic control with actions that extend far beyond those of just an inert energy storage organ (Luo & Liu, 2016). This general effect is led by adipokines, comprising endocrine molecules secreted by adipocytes to maintain WAT homeostasis (Fantuzzi, 2005). In adult animals exposed to alcohol consumption, it has been strongly confirmed that CYP2E1 activity is increased in AT, increasing oxidative stress (OS) and inflammation, and producing changes in lipid storage and adipokine secretion (Chen et al., 2009; Gopal et al., 2020, 2021; Kang et al., 2007; Sebastian et al., 2011; Tang et al., 2012; Yoshinari et al., 2004). Therefore, after BD exposure, this increase should be even greater. In studies with adults, after heavy alcohol consumption, EtOH impairs the storage function of AT by accelerating lipolysis and reducing adipogenesis, partly by a decrease in fatty acid uptake, leading to a reduction in AT mass and to fatty acid efflux towards the liver, thus contributing to steatosis (Gopal et al., 2021; Souza-Smith et al., 2017; Steiner & Lang, 2017). Furthermore, EtOH alters the secretion of adipokines associated with inflammation, causing severe metabolic complications (Marra & Bertolani, 2009; Ouchi et al., 2011).

Not only BD consumption, but also the IR process has a higher prevalence in adolescents (Higgins & Adeli, 2017), which increases the risk of cardiometabolic disease over time (Reaven, 2012). However, until now, no data are available in adolescents related to pancreatic endocrine function and AT homeostasis after BD exposure, despite the fact that this tissue is a target of insulin

activity and has secretory attributes to counteract changes in whole-body metabolism. These alterations could be especially dangerous in a period of intense growth and during metabolic changes such as the adolescence. The present study aimed to analyse, in adolescent rats exposed to BD, oxidative balance in the pancreas and its relationship with the endocrine pancreas function. Moreover, oxidative, inflammatory, energetic and endocrine changes in WAT are also analysed, aiming to obtain an integrative view of pancreas and WAT cross-talk with respect to the IR process.

Methods

Ethical approval

Animal care procedures and experimental protocols were reviewed and approved by the Ethics Committee of the University of Seville (CEEA-US2019-4) and the Andalusian Regional Government (05-04-2019-065). All experiments carried out in the present study were conducted in accordance with the guidelines of the European Union Council (Directive 2010/63/UE) and the Spanish Royal Decree (BOE 34/11 370, 2013) and adhered to the principles and regulations described by Grundy (2015). They were meticulously planned and executed with the goal of minimising unnecessary pain and suffering, adhering to the principles of replacement, reduction and refinement wherever possible to decrease both the total number of animals and their use.

Animals

Sixteen adolescent male Wistar rats (Centre of Production and Animal Experimentation, Vice-rector's Office for Scientific Research, University of Seville) were used in the experiments. The rats were received at 21 days of age and housed in groups of two rats per cage with enrichment of the environment for 1 week to acclimate them to housing and handling conditions. The experimental protocol was conducted over a 3 week period, beginning when the rats reached postnatal day 28 and ending at 47 days of age. This period corresponds to the adolescence in Wistar rats (Ojeda et al., 2022). The animals were kept under a 12:12 h light/dark photocycle (lights on 09.00 h) at 22–23°C.

At postnatal day 28, rats were randomly assigned to two groups ($n = 8$ per group) according to their treatments: a control group (C): rats received a control diet and drinking water *ad libitum* and, on the corresponding days, a physiological saline solution (PSS) I.P.; and an intermittent BD EtOH group (BD): rats received a control diet and drinking water *ad libitum* and, on the corresponding days, an EtOH solution 20% (v/v) in saline solution (3 g kg^{-1} day^{-1}) I.P.

The standard pellet diet (LASQCdiet, Rod14-R; LASvendi, Märkische, Germany) was available *ad libitum* in the two experimental groups. Animal care procedures and experimental protocols were conducted in accordance with EU regulations (Council Directive 86/609/EEC, 24 November 1986) and were approved by the Ethics Committee of the University of Seville (CEEA-US2019-4).

Nutritional control

Body weight and the amount of food consumed by rats were monitored daily until the end of the experimental period. The amount of food ingested every day was calculated by measuring this parameter every morning and the next day; the difference between them was the amount consumed. All measurements were taken at 09.00 h to avoid changes as a result of circadian rhythms.

EtOH treatment

The intermittent BD EtOH administration protocol consists of an I.P. injection of EtOH (20 % v/v) in PSS (3 g kg^{-1} day^{-1}) at 19.00 h, when the dark cycle began, for 3 consecutive days each week for 3 weeks. No I.P. injections were given during the remaining 4 days of each week. This BD model in adolescent rats has previously been used by this research group, registering a blood alcohol concentration of almost 125.0 mg dL^{-1} 1 h after the last injection (Nogales et al., 2014). The control group received an I.P. injection of an equal volume of PSS at the same time as the injections for the alcohol BD-exposed group.

Samples and anthropometric measurements

At the end of the experimental period, the rats were fasted for 12 h using individual metabolic cages and, afterwards, the adolescent rats were anaesthetised with an I.P. injection of 28% w/v urethane (0.5 mL/100 g of body weight). Immediately, the cranium-caudal length was measured using a metric calliper, and body mass index was calculated using the corresponding formula: body weight (g)/length^2 (cm^2). Blood was obtained by heart puncture and collected in tubes. The serum was prepared using low-speed centrifugation for 15 min at 1300 ***g***. The abdomen was opened by a midline incision in order to obtain organ samples. Pancreas and retroperitoneal WAT from both kidneys were removed, weighed, frozen in liquid nitrogen and stored at −80°C prior to biochemical determinations. The somatic index of WAT and pancreas was calculated by dividing the organ weight by the body weight.

Biochemical measurements in serum

In serum, insulin and glucose levels as well as the lipid profile [triglycerides (TG), cholesterol and high-density lipoprotein (HDL)] were measured with an automated analyser (Technicon RA-1000; Bayer Diagnostics, Leverkusen, Germany). Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) serum values were estimated as: VLDL = TG/5 and LDL = cholesterol – HDL – VLDL. The HDL/LDL ratio was calculated from these data. Fasting glucose and insulin serum concentrations were used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR), according to the formula: (fasting glucose concentration × fasting insulin serum concentration)/2430. The homeostatic model for the assessment of β-cell function was calculated using the formula: (fasting insulin serum concentration × 360)/(fasting glucose concentration – 63). Free fatty acids (FFA) were also measured, using an enzyme-linked immunosorbent assay (ELISA) technique [Rat Free Fatty Acid ELISA Kit; MyBioSource, San Diego, CA, USA].

Antioxidant enzyme activity and oxidative stress markers in the pancreas and WAT

To measure the activity of the antioxidant enzyme glutathione peroxidase (GPx), as well as oxidative stress markers, pancreas and WAT samples of the adolescent rats were homogenised (100 ***g*** for 1 min, 1:4 w/v) using a Potter homogeniser (#245432; Pobel, Madrid, Spain) in a sucrose buffer (15 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA and 1 mM dithiothreitol) in an ice bath. The homogenates were centrifuged at 900 ***g*** for 10 min at 4°C. Then, the resulting supernatant was employed for the biochemical assay. GPx activity (mU mg^{-1}) was determined in homogenates tissues according to the technique described by Lawrence & Burk (1976), in which GPx catalyses the oxidation of glutathione by hydrogen peroxide, and the absorbance decrease as a result of the oxidation of NADPH is measured at 340 nm for 3 min. Activity of the antioxidant enzyme superoxide dismutase (SOD) was determined using the method of Fridovich (1985). It was measured by the inhibition of the superoxide anion reduction of cytochrome *c*, produced by the xanthine/xanthine oxidase system. One unit of SOD activity was defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50% under the present measurement conditions. As for antioxidant enzyme catalase (CAT) activity, this was determined using the methods of Beers & Sizer (1952) by measuring the decrease in the H_2O_2 concentration at 240 nm. The activity values of SOD and CAT were expressed as units per milligram of protein (U mg^{-1}) (Beers & Sizer, 1952). The oxidative stress status in WAT was evaluated by the lipid oxidation levels. Lipid peroxidation was determined by the colorimetric method described by Draper & Hadley (1990), where malondialdehyde (MDA) (mol mg^{-1} protein), the end-product of the oxidative degradation of lipids, reacts with thiobarbituric acid and the final product is quantified at 535 nm.

Immunoblotting assays

Tissues samples were homogenised (1:10 w/v) in 50 mM phosphate buffer [K_2HPO_4 50 mM, KH_2PO_4 50 mM, EDTA 0.01 mM, protease inhibitor 1:10 (Complete Protease Inhibitor Cocktail Tablets; Roche, Madrid, Spain)] using a Potter homogeniser (245432; Pobel). Then, the homogenates were centrifuged at 500 *g* at 4°C for 10 min, and the final supernatant was aliquoted and frozen at −80°C until analysis.

The expression of SIRT-1 in the pancreas and WAT, as well as the expression of insulin receptor substrate 1 (IRS-1), AKT, sterol regulatory element-binding protein 1 (SREBP1), peroxisome proliferator-activated receptor γ (PPARγ), forkhead box O3A (FOXO3a), total AMP-activated protein kinase (AMPKt), phosphorylated AMP-activated protein kinase (pAMPK) and β-actin (as load control) in WAT homogenates, was determined by the protein immunodetection technique or western blotting. The protein content of the samples was analysed by the method of Lowry et al. (1951) and the samples for western blotting contained 100 or 25 μg of protein to pancreas or WAT homogenates, respectively. Proteins were separated on a polyacrylamide gel (9%) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) using a blot system (Transblot; BioRad). Non-specific membrane sites were blocked for 1 h with a blocking buffer, TBS-M:TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and milk powder 5% (Bio-Rad). Then, they were probed overnight at 4°C with the corresponding specific primary antibodies diluted in TBS-M. The antibodies (all of them from Santa Cruz Biotechnology, Santa Cruz, CA, USA) except pAMPK and β-actin, were SIRT-1 mouse monoclonal antibody IgG (dilution 1:500 in blocking buffer; catalogue number sc-74 465); IRS-1 mouse monoclonal IgG (dilution 1:500 in blocking buffer; catalogue number sc-8038); AKT (B-1) mouse monoclonal IgG (dilution 1:750 in blocking buffer; catalogue number sc-5298); SREBP1 (A-4) mouse monoclonal IgG (dilution 1:1000 in blocking buffer; catalogue number sc-365 513); PPARγ (E-8) mouse monoclonal IgG (dilution 1:750 in blocking buffer; catalogue number sc-7273); FOXO3a mouse monoclonal IgG (dilution 1:500 in blocking buffer; catalogue number sc-48 348); tAMPK alpha 1/2 antibody (D-6) (dilution 1:4000 in blocking buffer; catalogue number sc-74 461);

pAMPK rabbit monoclonal IgG (dilution 1:4000 in blocking buffer; catalogue number #2535; Cell Signaling Technology, Beverly, MA, USA); and mouse monoclonal anti β-actin IgG (dilution 1:10 000 in blocking buffer; catalogue number A2228; Sigma-Aldrich, Madrid, España). The next day, the probed membranes were washed 5 times during 4 min each wash with TBS-T [TBS with 0.1% (v/v) Tween 20] and incubated with the corresponding secondary antibody diluted in TBS-M: goat Anti-Rabbit IgG (H + L) Horseradish Peroxidase Conjugate (170-6515; Bio-Rad) for tAMPK and pAMPK (dilution 1:10 000), as well as goat Anti-Mouse IgG (H + L)-HRP Conjugate, (170-6516; Bio-Rad) for SIRT-1 (dilution 1:1500), IRS-1 (dilution 1:1500), AKT (dilution 1:2000), SREBP1 (dilution 1:2500), PPARγ (dilution 1:2000), FOXO3a (dilution 1:1500) and β-actin (dilution 1:8000). Subsequently, the membranes were washed five times over 4 min for each wash with TBS-T, then incubated for 1 min with the commercial developer solution Luminol ECL reagent (GE Healthcare, Chalfont St Giles, UK; Lumigen Inc., Southfield, MI, USA) and finally analysed with the Amersham Imager 600 (GE Healthcare). The quantification of the blots was performed by densitometry with ImageJ (NIH, Bethesda, MD, USA). The results were expressed as percent arbitrary relative units, referring to values in control animals, which were defined as 100%.

Adipocyte size

To measure adipocyte size, a scanning electron microscope operating under an ultra-high vacuum (Phenom Pro Desktop SEM; Thermo Fisher Scientific, Waltham, MA, USA) was used. With this microscope, information about the surface topography and composition of WAT can be recorded in 3D. The size of 100 adipocytes from heterogeneous areas was measured in each of the groups.

Adipokines

Serum adipokines such as adiponectin, resistin, adipsin and tumour necrosis factor-α (TNF-α) were measured using the MILLIPLEX® MAP Rat Adipokine Panel (Millipore Corp., St Charles, MO USA), based on immunoassays on the surface of fluorescent-coated beads (microspheres) in accordance with the manufacturer's instructions (50 events per bead, 50 μL sample, gate settings of 8000–15,000, time out of 60 s, melatonin bead set of 34). The plate was read on a LABScan 100 analyser (Luminex Corp., Austin, TX, USA) with xPONENT software for data acquisition. The average values for each set of duplicate samples or standards were within 15% of the mean. Adipokines concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay. The leptin adipokine was determined by an ELISA, using Rat Leptin ELISA Kit (MyBioSource, San Diego, CA, USA).

Statistical analysis

The results are expressed as the mean ± SD with symbols representing individual datapoints. The data were analysed using Prism, version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA). Student's unpaired *t* test was used to analyse the difference between C and BD groups. $P < 0.05$ was considered statistically significant. The Shapiro–Wilk test was used to validate the assumption of normality.

Results

Intermittent BD exposure during adolescence leads to a lower increase in body weight ($P = 0.0327$), which is not related to food intake (Table 1). Relative retroperitoneal-WAT weight is drastically decreased ($P < 0.001$), without affecting its protein content. However, pancreas somatic index is increased after BD exposure ($P = 0.0469$) and protein content is markedly increased ($P < 0.001$). During adolescence, BD exposure also alters the whole serum lipid profile (Table 2). This consumption increases TG ($P = 0.0061$), total cholesterol ($P = 0.016$), and HDL ($P = 0.0042$) and VLDL ($P = 0.0040$) serum levels, and also decreases LDL values ($P = 0.0398$). Therefore, the ratio HDL/LDL is increased ($P = 0.0011$). Furthermore, FFA in serum are also greatly increased in the BD group compared to control rats ($P < 0.001$).

Fig. 1 shows that acute EtOH administration during adolescence affects the antioxidant balance in the pancreas. It increases SOD ($P = 0.0133$) and decreases CAT ($P = 0.0334$) and GPx ($P = 0.0158$) activities, contributing to an unproper profile of the SOD/CAT + GPx ratio ($P = 0.0011$), and leading to lipid oxidation as shown by the analysis of MDA levels ($P = 0.0023$).

BD exposure during adolescence increases insulin ($P = 0.0021$) and glucose serum levels ($P = 0.0126$), contributing to significantly increasing the HOMA-IR value ($P < 0.001$). BD also increases adipsin serum levels ($P < 0.001$) and SIRT-1 pancreatic expression ($P < 0.001$) (Fig. 2).

During adolescence, BD exposure alters the antioxidant balance in WAT (Fig. 3). It increases SOD ($P = 0.0022$), CAT ($P = 0.0235$) and GPx ($P < 0.001$) activities. However, the ratio SOD/CAT + GPx is increased ($P < 0.001$), indicating that ROS generation is not efficiently coped. Consequently, MDA levels are markedly increased ($P < 0.001$). Moreover, Fig. 4 represents

Table 1. Morphology parameters in adolescent rats

	C (n = 8)	BD (n = 8)	P value
Initial body weight (g)	60.4 ± 2.7	63.2 ± 4.9	0.2020 (ns)
Increased body weight (g day^{-1})	5.9 ± 0.3	5.3 ± 0.5*	0.0327
Food intake (g day^{-1})	15.2 ± 1.1	14.6 ± 0.3	0.1677 (ns)
BMI (g cm^{-2})	0.517 ± 0.002	0.501 ± 0.001	0.1321 (ns)
WATSI [g wet tissue g^{-1} body weight (%)]	0.73 ± 0.07	0.45 ± 0.11***	<0.001
Proteins in WAT (mg g^{-1} wet tissue)	5.6 ± 0.7	6.1 ± 0.5	0.1600 (ns)
PSI (g wet tissue g^{-1} body weight (%))	0.72 ± 0.07	0.85 ± 0.17*	0.0469
Proteins in the pancreas (mg g^{-1} wet tissue)	159 ± 42	284 ± 74***	<0.001

BMI, body mass index; WATSI, white adipose tissue somatic index; PSI, pancreas somatic index. The results are expressed as the mean ± SD and analysed by Student's t test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a P value: C *vs.* BD: *$P < 0.05$, ***$P < 0.001$. ns, non-significant difference.

Table 2. Serum lipid profile in adolescent rats

	C (n = 8)	BD (n = 8)	P value
TG (mg dL^{-1})	73.8 ± 3.4	87.9 ± 11.8**	0.0061
Cholesterol (mg dL^{-1})	70.1 ± 5.9	79.8 ± 8.2*	0.0160
HDL (mg dL^{-1})	34.7 ± 7.9	46.9 ± 6.2**	0.0042
VLDL (mg dL^{-1})	14.9 ± 0.8	17.5 ± 1.7**	0.0040
LDL (mg dL^{-1})	20.1 ± 4.5	15.7 ± 3.1*	0.0398
HDL/LDL (ratio)	1.7 ± 0.3	2.9 ± 0.8**	0.0011
FFA (μmol L^{-1})	70.1 ± 0.3	90.3 ± 0.6***	<0.001

TG: triglycerides; HDL: high-density lipoprotein; VLDL: very low-density lipoprotein; LDL: low-density lipoprotein; FFA: free fatty acids. The results are expressed as the mean ± SD and analysed by Student's t test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a P value: C *vs.* BD: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

different proteins for which the activity can modulate the adipogenesis/lipolysis ratio in WAT. Intermittent BD decreases IRS-1 ($P = 0.0042$), AKT ($P = 0.0028$), SREBP1 ($P = 0.0070$), PPARγ ($P = 0.0017$) and FOXO3a ($P = 0.0013$) expression in WAT during adolescence.

Fig. 5 shows the energy balance in WAT. pAMPK ($P = 0.0174$) and SIRT-1 ($P < 0.001$) are both increased, resulting in an augmented pAMPK/AMPKt ratio ($P = 0.0068$). On the other hand, BD exposure during adolescence affects adipokine secretion, which leads to an increase in resistin ($P < 0.001$) and TNF-α ($P < 0.001$), as well as a decrease in adiponectin ($P = 0.015$) and leptin ($P = 0.0444$) (Fig. 6).

Finally, Fig. 7 shows that, during adolescence, intermittent BD exposure alters WAT morphology. It leads to significantly smaller adipocytes (58.1 ± 20.9 *vs.* 84.1 ± 25.0, in BD *vs.* control groups), with a lower amount of fat depots and a lower ratio adipocyte/collagen fibres. An immune response is observed because immune cells are disposed into a crown-like structure surrounding adipocytes.

Discussion

BD-exposure during adolescence decreases fat depots in WAT, affecting the serum lipid profile, which contributes to liver steatosis induction

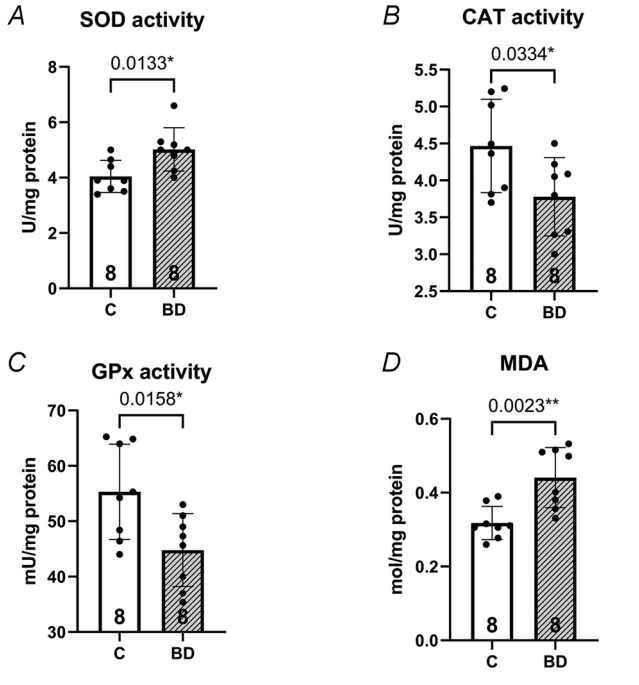
Intermittent BD exposure during adolescence by an I.P. route does not affect solid intake; however, it leads to a lower increase in body weight, indicating that body catabolic routes could be established. In this context, WAT mass has decreased in depth. This implies that an imbalance among lipolysis/lipogenesis towards catabolism is appearing. Indeed, after BD exposure, serum FFA levels are increased to TG serum levels, possibly because, as in chronic EtOH exposure during adulthood (Wei et al., 2013), the liver is processing too many FFA, accumulating TG in the liver, and mobilising them into the bloodstream in form of VLDL. Moreover, HDL is upregulated. This lipoprotein, commonly known as 'good cholesterol', carries cholesterol to the liver. Nonetheless, in this case, it is contributing to fat storage in the liver, which, together with FFA mobilisation, could promote ectopic fat deposition and steatosis. This clearly contributes to the severity of alcoholic liver disease (ALD). This increase in HDL has recently been recognised as an important step in the ALD evolution in adults exposed to chronic EtOH (Furtado et al., 2022; Wilkens et al., 2023).

BD leads to oxidative stress in the pancreas of adolescent rats, affecting insulin secretion

The hormone insulin, secreted by the pancreas, plays a pivotal role in maintaining WAT mass homeostasis

by inducing adipogenesis/lipogenesis and avoiding lipolysis. The most critical physiological functions of insulin action in WAT, setting aside the glucose uptake stimulation, is the suppression of lipolysis by decreasing the hormone-sensitive lipase, which is responsible for catalysing the hydrolysis of stored TG towards FFA release (Petersen & Shulman, 2018). In this context, intermittent BD exposure in rats during adolescence clearly affects the pancreas exocrine/endocrine physiology because it increases pancreatic mass, protein concentration and lipid peroxidation, and also affects insulin secretion.

Clinically, it is well accepted in adults that EtOH consumption is often accompanied by the onset of pancreatitis (Apte et al., 1998) as a result of the toxic



E

	CONTROL (n=8)	BINGE DRINKING (n=8)	p value
RATIO SOD/(CAT+GPx)	0.845 ± 0.098	1.165 ± 0.198**	0.0011

Figure 1. Antioxidant activity of endogenous enzymes and lipidic oxidation in the pancreas of adolescent rats after binge drinking exposure
A, superoxide dismutase enzyme (SOD) activity. *B*, catalase enzyme (CAT) activity. *C*, glutathione peroxidase enzyme (GPx) activity. *D*, malondialdehyde levels (MDA). *E*, ratio of SOD/(CAT+GPx). The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs.* BD: $^{*}P < 0.05$, $^{**}P < 0.01$.

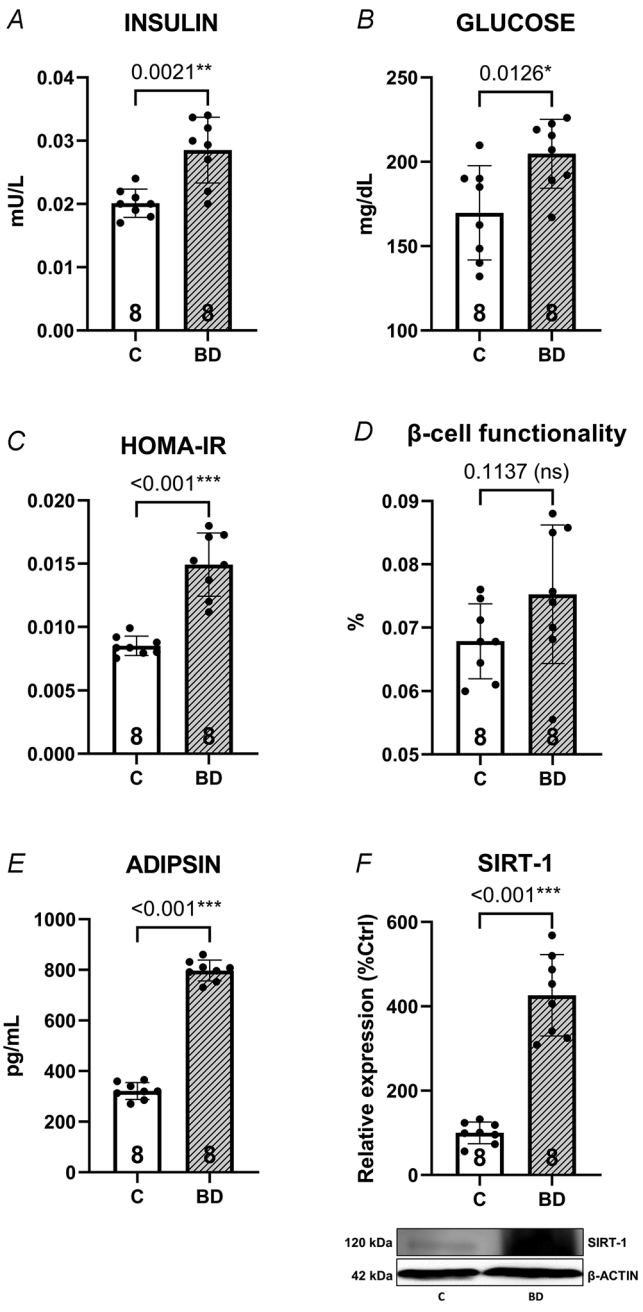
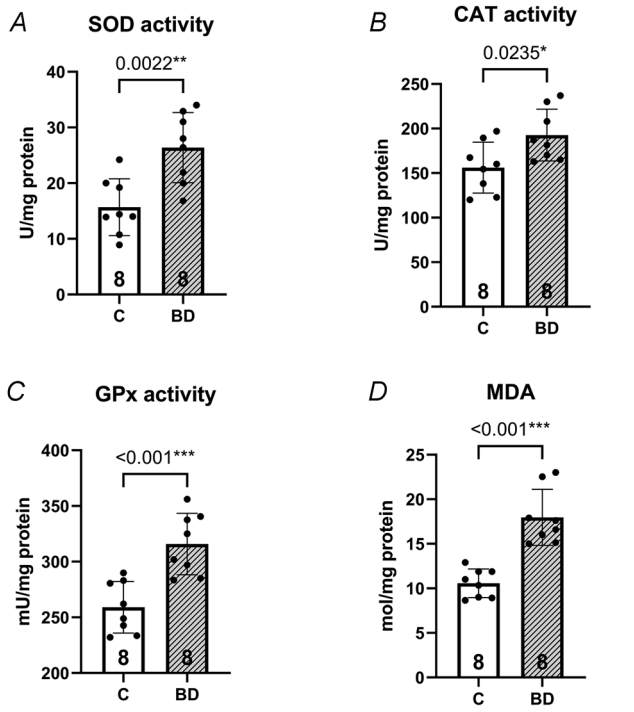


Figure 2. Pancreas-related parameters in adolescent rats after binge drinking exposure
Insulin (*A*) and glucose (*B*) serum levels; homeostasis assessment of insulin resistance index (HOMA-IR) (*C*). *β*-cell functionality (pancreas functionality parameter) (*D*); adipsin serum levels (*E*); and NAD+-dependent sirtuin deacetylase 1 (SIRT-1) expression in the pancreas (*F*) are shown. The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs.* BD: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, non-significant difference.

effects that EtOH produces on acinar cells. Acinar cells metabolise EtOH such as hepatocytes; therefore, after an acute exposure such as BD, CYP2E1 activation leads to ROS generation, OS (Wilson & Apte, 2003), necroinflammation and fibrosis in the exocrine pancreas, together with a premature activation of digestive enzymes (Vonlaufen et al., 2007). Repeated episodes of tissue inflammation and death lead to periductular obstructive scarring, increased digestive enzyme concentration and an increase in the release of litostatin and glycoprotein 2 by acinar cells, which contribute to the characteristic protein



E

	CONTROL (n=8)	BINGE DRINKING (n=8)	p value
RATIO SOD/(CAT+GPx)	9.362 ± 0.424	13.683 ± 1.697***	<0.001

Figure 3. Antioxidant activity of endogenous enzymes and lipidic oxidation in WAT of adolescent rats after binge drinking exposure
A, superoxide dismutase enzyme (SOD) activity. *B*, catalase enzyme (CAT) activity. *C*, glutathione peroxidase enzyme (GPx) activity. *D*, malondialdehyde levels (MDA). *E*, ratio of SOD/(CAT+GPx). The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs*. BD: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

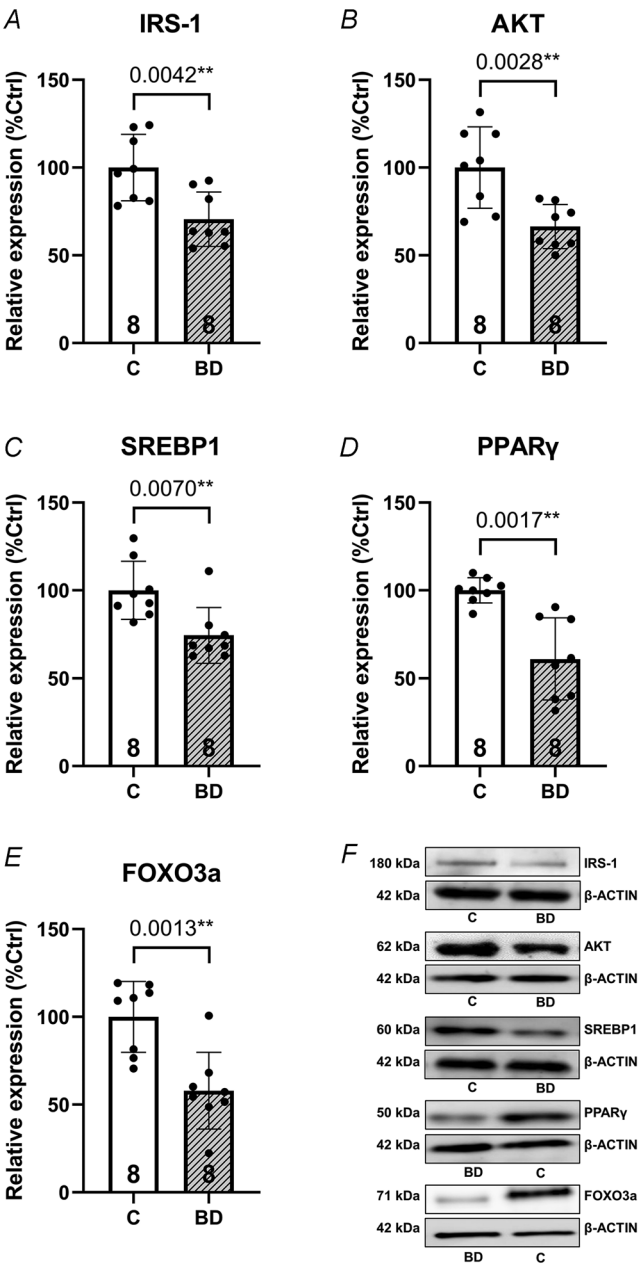


Figure 4. Protein expressions of adipogenesis/lipolysis ratio modulators in WAT of adolescent rats after binge drinking exposure
A, insulin receptor substrate 1 (IRS-1). *B*, AKT. *C*, sterol regulatory element-binding protein 1 (SREBP1). *D*, peroxisome proliferator-activated receptor gamma (PPARγ). *E*, forkhead box O3A (FOXO3a). *F*, western blot images with β-actin as load control. The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs*. BD: $^{**}P < 0.01$.

plug formation, aggravating the situation (Vonlaufen et al., 2007). In this context, the higher protein content and the pancreatic lipid oxidation found in BD adolescent rats is a worrying finding because it is indicative of pancreatic damage progression.

Pancreatic exocrine damage and severity correlate with the severity of concomitant pancreatic endocrine insufficiency (Boreham & Ammori, 2003). It is important to remember that only in pancreatic islets was the gene expression of antioxidant enzymes such as SOD, GPx and CAT substantially lower than that in other tissues (Lenzen et al., 1996), providing an explanation for the extraordinary sensitivity of pancreatic β-cells towards OS. OS has been shown to play a central role in promoting β-cell dysfunction (Eguchi et al., 2021). Therefore, in the present study, BD exposure during adolescence also affects the endocrine pancreas function, but not exactly as expected. Despite the lipid peroxidation generated, BD leads to a significant release of insulin into the blood, without decreasing the functionality of β-cells. However, hyperglycaemia appears, indicating that insulin is not functioning properly, probably because resistance to its action is stablished, rather than being a problem related to its secretion. The high HOMA-IR value found reinforces this theory. A study has confirmed that BD alters insulin production, causing IR, inhibiting glucose transport and decreasing exposure to it, making an increase in insulin secretion necessary (Shelmet et al., 1988). Recently, in male adolescent rats exposed to the same experimental intermittent BD protocol as that used in the present study, a downregulation of IRS-1 in the liver and skeletal muscle was described (Nogales et al., 2021; Romero-Herrera et al., 2023). Therefore, during adolescence in rats, insulin does not actively reach these tissues and, consequently, its physiological action decreases.

To understand the possible mechanism by which BD exposure, despite the pancreatic-oxidative damage generated, could be increasing β-cell function, pancreatic

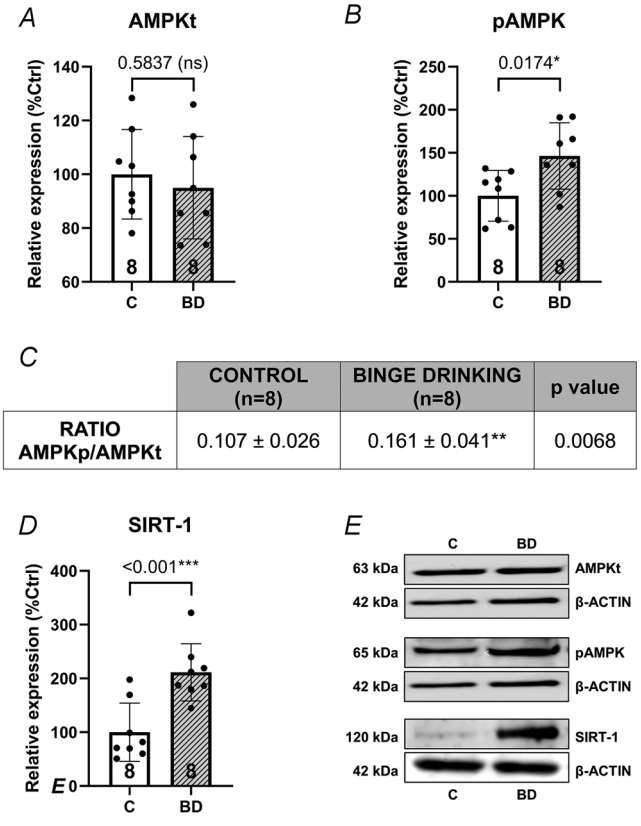


Figure 5. Energy balance in WAT of adolescent rats after binge drinking exposure
A, total AMP-activated protein kinase (AMPKt) expression. *B*, phosphorylated AMP-activated protein kinase (pAMPK) expression. *C*, ratio pAMPK/AMPKt. *D*, NAD+-dependent sirtuin deacetylase 1 (SIRT-1) expression. *E*, western blot images with β-actin as load control. The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs*. BD: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, non-significant difference.

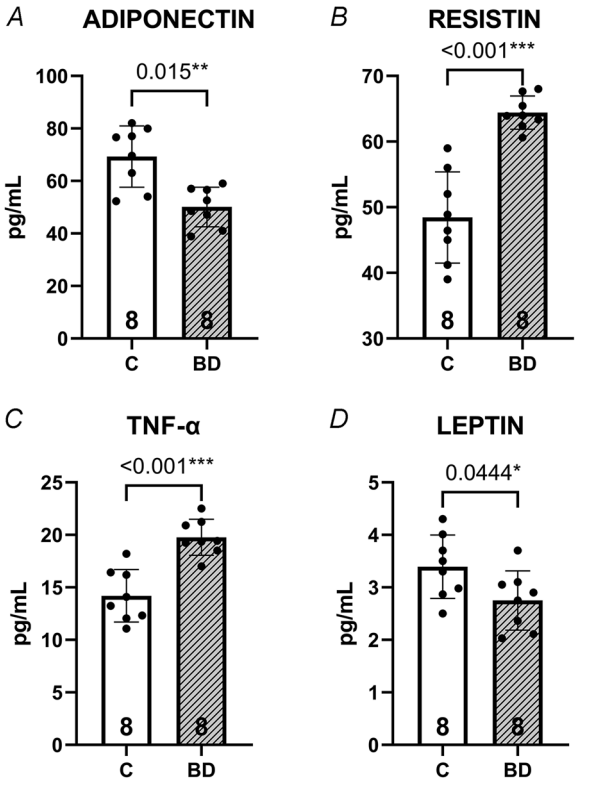


Figure 6. Adipokines related to inflammation serum concentrations
A, adiponectin. *B*, resistin. *C*, tumour necrosis factor alpha (TNF-α). *D*, leptin. The results are expressed as the mean ± SD and analysed by Student's *t* test. The number of animals in each group is eight. Groups: C, control group; BD, binge drinking group. A statistical difference between groups is expressed as a *P* value: C *vs*. BD: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

SIRT-1 expression was measured. SIRT-1 is a protein involved not only in energy homeostasis, but also in the prevention of inflammatory and oxidative processes. Indeed, it has been described as an essential protein in the formation and maintenance of pancreatic β-cells (Singh & Ubaid, 2020; Wang et al., 2013). SIRT-1 increases the cell mass of the endocrine pancreas and participates in insulin secretion by regulating uncoupling protein 2 (Bordone et al., 2006; Prud'Homme et al., 2014). In BD adolescent rats, pancreatic SIRT-1 expression is upregulated, coinciding with a higher pancreas mass and insulin secretion. Therefore, this SIRT-1 could also be preventing the effects of OS on β-cells and maintaining their insulin secretion function. In this context, more studies in isolated β-cells should be conducted.

Second, serum adipsin secretion could also be contributing to improve β-cell function and insulin secretion. Adipsin is an adipokine secreted by WAT and it is involved in its homeostasis; however, it also controls the generation of the C3a component of the complement and participates in the insulin secretion of the pancreatic β-cells (Wang et al., 2018). Adipsin is not a simple insulin secretagogue, but rather prevents dedifferentiation and death of pancreatic islet cells by inhibiting dual-specificity phosphatase 26, enabling the improvement of blood glucose and protecting β-cells (Gómez-Banoy & Lo, 2019). BD exposure to adolescent rats leads to a significant increase in adipsin secretion, consistent with the high insulin values found.

The oxidative stress in WAT of adolescent rats exposed to BD has lipolytic repercussions

Despite the hyperinsulinaemia found after BD exposure, it appears that insulin is not acting properly in WAT, which is in a catabolic state. These results point to a WAT dysfunction in the insulin signalling process. It is important to note that a correct ROS balance is necessary for this signalling process because moderate ROS levels are necessary secondary messengers in this pathway. However, excessive ROS production can impair insulin signalling by directly modifying key signalling proteins,

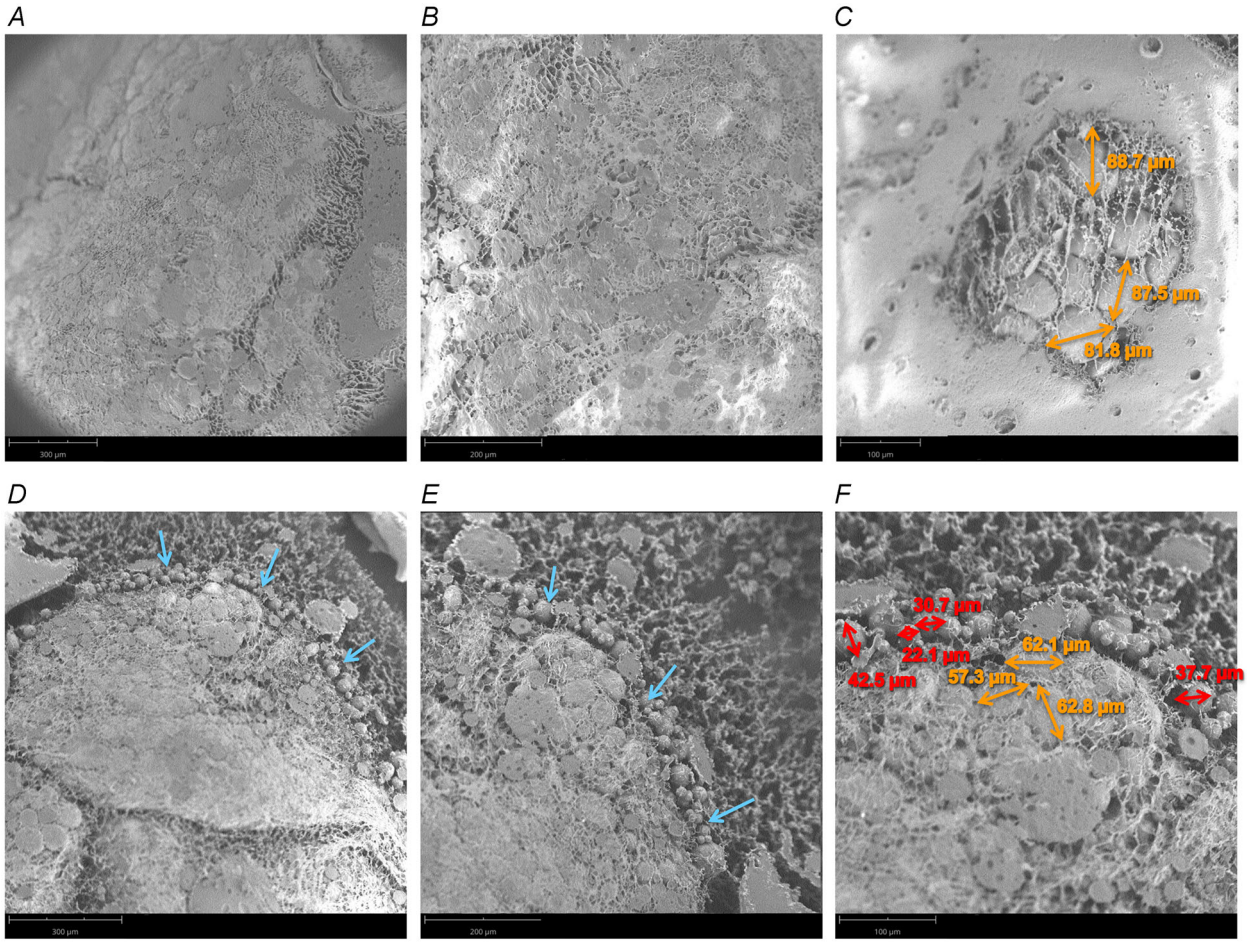


Figure 7. Scanning electron microscopy images of WAT
A–C, control group. *D–F*, binge drinking group.

such as IRS-1 and AKT, by oxidising critical cysteine residues, contributing to the WAT-IR process (Rains & Jain, 2011).

In the present study, impaired lipid homeostasis in adolescent adipocytes after BD exposure is accompanied by an increase in lipid peroxidation and a disturbed endogenous antioxidant enzyme profile. BD increases CAT and GPx activities; however, they cannot adequately counteract the effects of the increased SOD activity, as indicated by the SOD/CAT + GPx ratio, suggesting the establishment of OS. Consistently, IRS-1 and AKT levels are decreased, compromising the insulin signalling process in WAT. AKT activation not only promotes glucose uptake, but also TG storage (lipogenesis) and adipogenesis by increasing SREBP1 and PPARγ (Guru et al., 2021; Jia et al., 2016). SREBP1 stimulates the expression of enzymes involved in FFA and TG synthesis, such as fatty acid synthase and acetyl-CoA carboxylase, which leads to lipogenesis (Shimano & Sato, 2017). PPARγ plays a central role in adipocyte differentiation and increases the expression of genes involved in lipid storage, being a master regulator of adipogenesis, as well as a potent modulator of whole-body lipid metabolism and insulin sensitivity (Ahmadian et al., 2013). Recently, FOXO3a, a member of the forkhead box O transcription factor family, has been described as a protein having a direct relationship with the activity of PPARγ (Luo et al., 2008) and with the modulation of inflammation in adipocytes through autophagy in WAT (Zhang et al., 2021). Adolescent rats exposed to BD present low IRS-1, AKT, SREBP1, PPARγ and FOXO3a expression in WAT, as well as high FFA serum levels, indicating that the lipogenesis/lipolysis balance in WAT is clearly shifted towards lipolysis. Indeed, it has been reported that a high amount of EtOH consumption during adolescence decreases adipogenesis, but mainly increases lipolysis (Gopal et al., 2021; Steiner & Lang, 2017). Thus, as in chronic EtOH exposure, the disruption in the insulin-dependent signalling in adipocytes is probably one of the main pathways through which EtOH promotes WAT lipolysis (Kang & Nagy, 2006).

Finally, the oxidative imbalance also affects energy sensors such as SIRT-1 and AMPK, which lead cells to catabolism and energy supply. In WAT, it has been confirmed that ROS can induce the activation of AMPK even without changes in the cellular ATP present (Gallego-Lopez et al., 2022; Quintero et al., 2006; Wu et al., 2012). Relative to SIRT-1, OS in WAT is also inversely related to SIRT-1 expression (Manna et al., 2017). Therefore, BD exposed rats present higher expression of AMPK and SIRT-1 in WAT, indicating that WAT is in a catabolic state.

AMPK and SIRT-1 have been deeply related to WAT homeostasis and function, by directly influencing the lipolysis and lipogenesis function, and by indirectly influencing the insulin-signalling pathways (Herzig & Shaw, 2018; Zhou et al., 2018). In this context, regulation of lipid metabolism is the first known function of AMPK (Ahmad et al., 2020), contributing to low lipid storage (Bijland et al., 2013). AMPK inhibits *de novo* synthesis of cholesterol, TG and fatty acids, and β-oxidation (Jeon, 2016; Seo et al., 2015). It also inhibits the regulation of various lipogenic enzymes by downregulating SREBP-1c and modulating PPARγ and the differentiation of white pre-adipocytes (Daval et al., 2006). Relative to insulin functioning, most of the studies demonstrate that activation of AMPK in white adipocytes inhibits insulin-stimulated glucose uptake (van Dam et al., 2015). Therefore, it mainly acts by decreasing adipogenesis. BD exposure during adolescence enhances AMPK activity, and therefore adipogenesis depletion is reinforced.

In WAT, SIRT-1 activation clearly triggers lipolysis and fat loss (Picard et al., 2004). Under SIRT-1 activation, SREBP-1c and PPARγ are repressed and FOXO1-adipose triglyceride lipase signalling is activated, which avoids adipogenesis and promotes WAT lipolysis, markedly increasing the FFA efflux to the circulation (Bai et al., 2016; Peredo-Escárcega et al., 2015; Zhou et al., 2018). SIRT-1 also decreased AKT activation and all its actions in different types of cells, leading to IR (Lee et al., 2022). BD exposure during adolescence greatly increases SIRT-1 expression and this effect of EtOH on WAT is addressed for the first time.

BD exposure during adolescence in rats leads to a dangerous profile of catabolic AMPK and SIRT-1 expression in WAT, which together with their anabolic profile found in the liver of BD adolescent rats (Nogales et al., 2021), triggers the AT–liver axis, exacerbating WAT lipolysis and increasing the amount of circulating FFA. All of this could contribute to liver steatosis accompanied by an IR process, comprising a clear risk factor for the development of future cardiovascular diseases.

BD exposure during adolescence highly increases inflammation in WAT: role of adipokines

Because WAT energy balance is disrupted towards lipolysis, and IR and lipid peroxidation appear after BD exposure during adolescence, the pro-inflammatory and anti-inflammatory adipokine profile is compromised. According to the damage generated in WAT, BD leads to a clear pro-inflammatory secretion profile of adipokines, increasing pro-inflammatory TNF-α and resistin, and decreasing the anti-inflammatory serum adiponectin values (Ajmo et al., 2008; Shen et al., 2010; Yu et al., 2010).

In general terms, these adipokines are related to the inflammatory balance in WAT, but they have additional actions. Resistin not only contributes to generate inflammation in WAT, but also to the IR

process, decreasing insulin–glucose uptake by adipocytes and promoting lipolysis, producing an inappropriate release of fatty acids into the circulation (Gopal et al., 2021; Steiner & Lang, 2017). Its increment has peripheral repercussions because it is solidly related to atherosclerosis and heart damage by promoting systemic inflammation and endothelial dysfunction (Opatrilova et al., 2018; Ouchi et al., 2011). The increase in serum TNF-α is in line with this trend because high serum TNF-α values are clearly related to peripheral and hepatic-IR generation. It impairs AT metabolism by increasing lipolysis and decreasing lipogenesis (Steiner & Lang, 2017). The lower serum values of adiponectin found in adolescent rats after intermittent BD exposure contribute to the loss of WAT mass because adiponectin increases glucose uptake and adipogenesis, and also decreases inflammation in WAT. Moreover, it specifically contributes to augment IRS-1 expression in WAT (Wang et al., 2007). Increasing evidence suggests that low adiponectin production in AT and impaired expression of hepatic adiponectin receptors are associated with the development of alcoholic liver steatosis in rodent models. This hepato-protective effect of adiponectin is attributed to different co-ordinated pathways in the liver, enhancing fat oxidation and reducing lipogenesis (Rogers et al., 2008). This adiponectin imbalance is especially dangerous because, in addition to being anti-inflammatory in WAT, it exerts several systemic beneficial effects on glucose and lipid metabolism, avoiding atherosclerotic progression (Yanai & Yoshida, 2019).

Another important adipokine, leptin, is decreased after BD exposure. The degree of synthesis and secretion of leptin is directly proportional to fat mass. Its main known function is to decrease food intake, although it also plays an important role in maintaining serum FFA homeostasis (Yadav et al., 2013). The lower leptin levels found after BD exposure in adolescent rats are directly proportional to the lower fat mass and the higher serum FFA levels found in these animals.

According to the above adipokine profile, WAT morphology is deeply modified, presenting BD exposed adolescent rats with a lower number of adipocytes that are significantly smaller, with lower fat depots, indicating that a correct adipogenic process is not established. The balance between adipogenesis/lipolysis is disrupted towards catabolism. Furthermore, a pro-inflammatory process is well established because immune cells are disposed as crown-like structures surrounding adipocytes. A similar disposition of macrophages as crown-like structures that surround necrotic adipocytes for engulfment has been described after long chronic alcohol exposure (Kang et al., 2007; Sebastian et al., 2011); however, in male mice exposed to a chronic-binge alcohol intoxication model, these effects were not observed (Fulham & Mandrekar, 2016). In the present study, the intermittent BD model used clearly expressed this inflammatory response, indicating the crucial repercussion of BD exposure on WAT homeostasis.

Conclusions

The present study reports that intermittent BD exposure during adolescence in rats is a crucial metabolic problem, where WAT plays a pivotal role. The results of this consumption are more hazardous than initially expected. The intense lipolytic action of this alcoholic intake in WAT is the result of a multifactorial process that implies an oxidative imbalance related to the instauration of an IR process and an energy imbalance in which SIRT-1 and AMPK activities are upregulated, contributing to WAT catabolism and a pro-inflammatory adipokine secretion profile. Despite oxidative damage, the pancreas, partially stimulated by the high amount of adipsin secreted by WAT, stimulates insulin secretion. However, this hormone is not acting properly because hyperglycaemia materialised, pointing to IR as a key point in WAT lipolysis. Finally, the increased amount of circulating FFA derived from WAT could alter the AT-liver cross-talk towards liver steatosis onset, as indicated by the lipoprotein profile. The results obtained in rats unequivocally demonstrate that BD consumption during adolescence, when endocrine and energy functions are highly active, is a hazardous trend to avoid because the cross-talk between the pancreas and WAT is redirected towards a disbalance in the lipid profile, an IR process and potential future cardiometabolic complications.

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Additional information

Data availability statement

Data are available as supporting information for review purposes (original western blot images). The rest of the data are all presented in this article.

Competing interests

The authors declare that they have no competing interests.

Author contributions

I.R.H., M.L.O. and O.C. were responsible for the conception and design of experiment. I.R.H., F.N., M.D.C.G.L. and J.M.F. were responsible for development of the methodology. I.R.H., O.C., J.D.C. and J.O. were responsible for the analysis and interpretation of data (e.g. statistical analysis). I.R.H., M.L.O. and F.N. were responsible for the writing the original draft. O.C., M.D.C.G.L., J.M.F. and J.O. were responsible for reviewing the manuscript. I.R.H., F.N. and M.D.C.G.L. were responsible for editing the manuscript. M.L.O. and I.R.H. were responsible for visualisation. O.C., J.D.C. and J.M.F. were responsible for the supervision. I.R.H. and F.N. were responsible for management of references. I.R.H., M.L.O. and F.N. were responsible for the design of figures. O.C. and J.D.C. were responsible for funding acquisition. All authors have read and agreed to the final version of the manuscript submitted for publication.

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Keywords

adipokine, adipose tissue, binge drinking, insulin resistance, oxidative stress, pancreas

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

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