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CAMILA BENAN ZARA

INFLUÊNCIA DA BREVE EXPOSIÇÃO A METFORMINA NA LACTAÇÃO SOBRE AS DISFUNÇÕES METABÓLICAS EM RATOS ADULTOS INDUZIDA POR DIETA RICA EM GORDURA

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (área de concentração - Biologia Celular e Molecular), da Universidade Estadual de Maringá para a obtenção do grau de Mestre em Ciências Biológicas.

Orientador: Prof. Dr. Paulo Cézar de Freitas Mathias
Coorientador: Prof. Dr. Douglas Lopes Almeida

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BIOGRAFIA

Camila Benan Zara nasceu em Maringá/PR, no dia 21/09/1996. Possui graduação em Ciências Biológicas pela Universidade Estadual de Maringá (2017) e em Nutrição pelo Centro Universitário Ingá (2020). Atualmente é mestranda do Programa de Pós-Graduação em Ciências Biológicas, área de concentração em Biologia Celular e Molecular na Universidade Estadual de Maringá, desenvolvendo seu trabalho no Laboratório de Biologia Celular e da Secreção. Atua principalmente nos seguintes temas: Obesidade, Síndrome Metabólica e Programação Metabólica.

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APRESENTAÇÃO

Em consonância com a resolução nº 056/2019 do Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, essa dissertação é composta por um resumo geral e um artigo científico, sendo este redigido de acordo com as normas da revista The Journal of Nutritional Biochemistry, com atual fator de impacto de 6,11 (Qualis CB1-A1), intitulado “Impact of a short lactational metformin exposure in metabolic dysfunction of adult diet-induced obesity rats.”. O trabalho demonstra o impacto do tratamento com metformina durante a lactação em animais que são induzidos a obesidade por uma dieta rica em gordura na vida adulta.

RESUMO GERAL

INTRODUÇÃO: A obesidade é um problema crucial de saúde, podendo desencadear diversas alterações metabólicas, como intolerância a glicose, adiposidade visceral, disfunção endotelial, dislipidemia, hipertensão, doenças cardiovasculares e certos tipos de câncer. Fatores genéticos e ambientais estão entre as causas da obesidade. Insultos estressores no início da vida parecem estar envolvidos com a predisposição a desordens metabólicas. Este vínculo entre as influências ambientais e nutricionais em períodos críticos do desenvolvimento e seus efeitos de longo prazo são abordados pelo conceito DOHaD, campo que estuda como os estresses ambientais em período de desenvolvimento podem operar para causar mudanças que protegem ou aumentam o risco de desenvolver doenças em um longo período de tempo. Desta forma, a lactação é considerada um período crítico, visto que estresses temporários podem produzir efeitos a longo prazo. A metformina é um fármaco amplamente utilizado para o tratamento do diabetes tipo 2 e vem sendo recomendado para o tratamento de diversas outras doenças, tais como: síndrome metabólica, diabetes gestacional, síndrome do ovário policístico, tratamento do câncer e prevenção do diabetes.

OBJETIVO: Avaliar se o tratamento com metformina durante os primeiros doze dias de vida é capaz de atenuar ou retardar o desenvolvimento da obesidade em ratos machos alimentados com dieta rica em gordura durante a vida adulta.

MÉTODOS: Ratas prenhes foram colocadas em caixas individuais e, ao nascimento, as ninhadas foram padronizadas para nove filhotes por mãe e foram divididas em dois grupos experimentais: Os filhotes do grupo Salina receberam injeções intraperitoneais do 1º até o 12º dia de lactação de solução salina, enquanto os filhotes do grupo metformina receberam, pelo mesmo período, injeções intraperitoneais de metformina (100 mg/kg/dia). Aos 21 dias os animais foram desmamados e mantidos com dieta comercial (Nuvital®; Curitiba/PR, Brazil) até os 72 dias de vida. Nesta idade, os animais foram rearranjados em quatro grupos experimentais: os grupos NFD-SAL e NFD-MET receberam dieta controle (normolipídica – 4.5% de gordura; Nuvital®; Curitiba/PR, Brazil), enquanto os grupos HFD-SAL e HFD-MET receberam dieta hiperlipídica (35% de gordura) até os 102 dias de idade. Aos 102 dias de idade os animais foram submetidos ao teste de tolerância à insulina intraperitoneal. No dia seguinte, os animais foram submetidos a um procedimento cirúrgico para implantação de uma cânula na veia jugular

direita para a realização do teste de tolerância à glicose intravenoso. Após os testes, os animais foram anestesiados e eutanasiados para coleta de tecidos para dosagens e análises posteriores. O teor de gordura hepática foi mensurado pelo método de Folch. Os dados estão expressos como média ± erro padrão da média, a análise estatística foi feita através do software GraphPad Prism, versão 7.01, utilizando de test T de Student ou ANOVA de duas vias seguido pelo teste de Tukey, um valor de $p<0.05$ foi considerado estatisticamente significativo.

RESULTADOS E DISCUSSÃO: No presente estudo o tratamento com metformina nos 12 primeiros dias de vida não promoveu alterações biométricas na prole até o desmame. A dieta rica em gordura na vida adulta levou aos desfechos esperados, como aumento no ganho de peso corporal e no acúmulo de gordura, intolerância à glicose, resistência à insulina, dislipidemia e aumento da gordura hepática. O tratamento com a metformina na lactação, não foi efetivo em reduzir o peso corporal, os estoques de gordura e o perfil lipídico, diferentemente de alguns estudos relatados na literatura. Contudo é necessário enfatizar que diferentemente de outros trabalhos, nosso estudo não administrou a metformina concomitantemente ao insulto obesogênico. Os dados demonstram que o tratamento no início da vida com metformina foi capaz de promover melhoras na intolerância à glicose, o que está de acordo com diversos trabalhos presentes na literatura. Também demonstramos que o tratamento lactacional com a metformina, embora não tenha reduzido os efeitos do consumo de dieta hiperlipídica por animais adultos sobre a quantidade dos triglicerídeos hepáticos e da enzima ALT, foi eficaz em atenuar o aumento na quantidade de gordura hepática de animais alimentados com HFD na vida adulta.

CONCLUSÃO: Os efeitos da exposição à metformina são mais exacerbados quando a sua administração é utilizada concomitantemente com um insulto obesogênico em comparação com a administração da droga anteriormente. Este estudo destaca a urgência de pesquisas clínicas investigando os efeitos potenciais da exposição precoce à metformina como uso profilático para o tratamento de obesidade e distúrbios metabólicos.

PALAVRAS-CHAVE: Lactação; Metformina; Obesidade; Dieta rica em gordura; DOHaD.

GENERAL ABSTRACT

INTRODUCTION: Obesity is a crucial health problem and can trigger several metabolic changes, such as glucose intolerance and dyslipidemia, associated to hypertension, type 2 diabetes and cardiovascular disease. Genetic and environmental factors are among the causes of obesity. Early life stresses seem to be involved with the predisposition to metabolic disorders. This link between environmental influences in critical periods of development and their long-term effects is addressed by the DOHaD concept, a field that studies how early life stresses can operate to cause changes that may protect or increase the risk of developing later diseases. Thus, lactation is a critical period of development, and a temporary stress in this developmental window can produce long-term health effects. Metformin is a drug widely used for the treatment of type 2 diabetes and has been recommended for the treatment of several other diseases, such as: metabolic syndrome, gestational diabetes, polycystic ovary syndrome, cancer treatment and diabetes prevention.

AIM: Evaluate if the treatment with metformin during the first twelve days of life is able to attenuate or hold back the development of obesity in male rats feeding with a high fat diet during the adult life.

METHODS: Pregnant rats were placed in individual cages and, at birth, the litters were standardized to nine pups per dam and divided into two experimental groups: the pups in the saline group received intraperitoneal injections of saline solution from the 1st to the 12th day of lactation, and the pups in the metformin group received, trough the same period, intraperitoneal injections of metformin (100 mg/kg). kg/day). At 21 days-old, the animals were weaned and maintained on a commercial diet (Nuvital®; Curitiba/PR, Brazil) until 72 days-old, when the animals were rearranged into four experimental groups: The NFD-SAL and NFD-MET groups that received a control diet (normolipidic - 4.5% fat; Nuvital®; Curitiba/PR, Brazil) while the HFD-SAL and HFD-MET groups received a high-fat diet (35% fat) until 102 days-old. At 102 days-old, the animals were submitted to an intraperitoneal insulin tolerance test. On the next day, the animals underwent a surgical procedure to implant a cannula in the right jugular vein to perform the intravenous glucose tolerance test. Following the glucose homeostasis tests, the animals were anesthetized and euthanized for tissue collection for later measurements. The liver was used to measure the hepatic fat content by the Folch method. Data were

analyzed using GraphPad Prism, version 7.01 software and are expressed as mean ± standard error of mean. Student's T test or two-way ANOVA followed by Tukey's test, was used, $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION: The treatment with metformin in the first 12 days of life didn't promote biometrical changes during lactation. The HFD in adulthood led to the expected outcomes, such as increased body weight gain and fat accumulation, glucose intolerance, dyslipidemia, and hepatic steatosis. Although the metformin early treatment was not effective in reducing body weight, fat accumulation or change the lipid profile in HFD animals, it was able to promote improvements in glucose tolerance and attenuate the hepatic fat accumulation in the HFD-MET group. However, the last was not accompanied by changes in hepatic triglycerides or the ALT enzyme activity. It is important to emphasize that, unlike other studies with metformin and obesogenic stresses, our study did not administer metformin concomitantly with the diet-induction of obesity.

CONCLUSION: The effects of exposure to metformin are more exacerbated when diet is used concomitantly with an obesogenic insult compared to administration of the drug previously, this study highlights the urge for clinical researches investigating the potential effects of early exposure to metformin as prophylactic use for the treatment of obesity and metabolic disorders.

KEYWORDS: Lactation; Metformin; Obesity; High fat diet; DOHaD.

SUMÁRIO

Impact of a short lactational metformin exposure in metabolic dysfunction of adult diet-induced obesity rats.

Keywords: short metformin treatment, lactation, high fat diet, obesity, metabolic programming.

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Abstract

High caloric and high fat diet (HFD) consumption is associated to obesity. Early life is shown to be a window of plasticity that may predispose to health or to metabolic disorders. Metformin is used for the treatment of type 2 diabetes and recommended for the treatment of several other diseases. We evaluated if the treatment with metformin during the first twelve days of life is able to attenuate the development of obesity in male rats feeding with a HFD during the adult life. Two experimental groups were performed: saline (SAL) and metformin (MET), which received from the 1st to the 12th day an intraperitoneal injection of saline and metformin at a concentration of 100mg/kg, respectively. At 72 days, four groups were defined: metformin (NFD-MET) and saline (NFD-SAL), fed a normolipidic diet (4.5% fat) and saline (HFD-SAL) and metformin (HFD-MET), fed with a HFD (35% fat). Biometric and metabolic patterns of the animals were determined. The HFD in adulthood led to increase in body weight gain and fat accumulation, glucose intolerance, insulin resistance, dyslipidemia, and liver fat accumulation. Treatment with metformin during lactation wasn't effective in reducing mostly of those deleterious effects, but caused a modest improvement glucose intolerance and decreased liver fat. The beneficial effects of metformin observed when used concomitantly to the obesogenic stress may not be as effective when administered before. This study highlights the urge for clinical researches investigating the potential effects of early exposure to metformin as prophylactic use for the treatment of metabolic disorders.

Key words: Obesity; Lactation; Metformin; High fat diet; Metabolic disorders.

1. Introduction

Obesity is a critical Public Health issue of our time. Almost 2 billion of adults in the world are overweight, and approximately 650 million are obese [1-2]. Obese individuals are more susceptible to the development of metabolic syndrome [3], which consists of a range of metabolic alterations, including at least 3 of the following: glucose intolerance, visceral adiposity, endothelial dysfunction, dyslipidemia, and hypertension. All of these metabolism conditions increase the risk of developing cardiovascular diseases, type II diabetes, and some types of cancer [3-4]. Genetic, metabolic and environmental factors, such as overfeeding and sedentary lifestyle, can influence the obesity onset [5-6]. Recent epidemiological and experimental studies indicate that environment stressors, especially those that occur in early postnatal life, play a significant role in the obesity pandemic [7].

This relation between early life stress and increased predisposition to metabolism disorders is approached by the concept of the Developmental Origins of Health and Disease (DOHaD), which refers to a mechanism of plasticity that plays a role in humans and animals. DOHaD "critical" periods are windows during which specific environmental influences and nutritional perturbations may operate to cause changes that can protect or increase the risk of developing diseases in a long time. The most vulnerable periods and critical times for intervention are of growth and development, from preconception to adolescence [8]. The lactation is a window in which temporary insults could yield long-term effects, as there is the development of tissues and organs, and the maturation of cellular functions [9-10]. Previously, a study of our group reported that metformin exposure during lactation in early overfed animals improved glucose tolerance and insulin sensitivity in adult life, decreasing body weight and food intake, with improved function of the endocrine pancreas [11].

The metformin is a derivate from guanidine, an active compost of *Galega officinalis*. This substance is well known as an antihyperglycemic drug and it is the first choice for the treatment of type 2 diabetes mellitus (T2D) [12-13]. Additionally, as metformin has been shown to reduce inflammation and oxidative stress in a systemic level [14], it has been indicated for the treatment and prevention of other diseases as metabolic syndrome [15], gestational diabetes [16], polycystic ovary syndrome [17], cancer treatment [18], diabetes prevention [19] and Alzheimer's diseases[20]. Although the underlying mechanism of

metformin action has not yet been fully elucidated, the effect is to decrease hepatic gluconeogenesis and glycogenolysis, and the stimulation of glycogenesis.

Considering the experimental evidence that metformin when administered during lactation can have long term influence in the metabolism of early obese animals, the present study aimed to evaluate whether the treatment with metformin during the first twelve days of life is able to attenuate or hold back the development of obesity and associated metabolic disorders in male rats feeding with a HFD during the adult life.

2. Materials and Methods

2.1 Ethical approval

All experimental procedures were performed in accordance with the standards of the Brazilian National Council for Control of Animal Experimentation (CONCEA) and were approved by the Ethic Commission in The Use of Animals (CEUA) from State University of Maringá, Brazil (Protocol Number 4021080620).

2.2 Animals and experimental design

Male and female Wistar rats (80 and 70 days-old, respectively) were obtained from the Central Animal House of the State University of Maringá and housed in the Sectorial Animal House of the Laboratory of Cell Biology of Secretion, kept under controlled conditions of temperature (22±2ºC) and photoperiod (7:00 a.m. to 7:00 p.m., light cycle). The animals had free access to water and commercial food (Nuvilab® CR-1, Curitiba, Paraná, Brazil). After five days of adaptation, animals were mated in the ratio of 3 females to each male. When pregnancy was detected, females were housed individually until delivery, when the litters were standardized to nine pups *per* dam (5-6 males and 3-4 females) and divided into two groups: saline (SAL- 12 litters) and metformin (MET-11 litters). All offspring received intraperitoneal injections from the 1st until the 12th day of lactation of saline or Metformin diluted in saline solution at 100 mg/kg/day [11-21]. Animals were weaned at 21 days-old, only male pups were used and housed in four *per* cage. Both groups received water and commercial diet (4.5% fat; Nuvilab® CR-1, Curitiba, Paraná, Brazil) until 72 days-old. From 72 days-old to 102 days-old, offspring's subsets of the saline and metformin groups were fed with the commercial normal-fat diet (NFD-Sal: 24 rats from 6 litters; NFD-Met: 20 rats from 5 litters) or a high-fat diet (35%

fat) (HFD-Sal: 24 rats from 6 litters; HFD-Met: 24 rats from 6 litters). The HFD was manufactured in our laboratory, using lard as a lipid source that were reported previously [22-23].

2.3 Biometric markers

Body weight (bw) of the pups was measured ever day until 21 days-old, also, the abdominal diameter and nasoanal length were measured on 7, 14 and 21 days-old using a digital pachymeter.

After weaning bw and food intake were determined every week until 102 days-old (from 20-24 rats/5-6 litters of group). Spillage not accounted, the food intake was calculated as the difference between the total food provided ($D_{initial}$) and the amount of food remaining (D_{final}), divided by the number of days and the number of animals in the cage: [food intake (in grams) = ($D_{initial} - D_{final}$)/7/4] [24]. Considering that the energetic values of the diets are different, food intake was presented in calories (Kcal/g bw). At 104 days-old, all rats were anaesthetized with thiopental associated to Lidocaine (150 mg/kg bw) intraperitoneally (10 mg/kg bw) and laparotomized to remove their retroperitoneal, periepididymal and mesenteric fat pad stores, pancreas and liver. The weight of fat pads and liver were expressed in relation to the bw of each animal (g/100g bw).

2.4 Intraperitoneal insulin tolerance test (ipITT) and glucose decay constant (kITT)

After a six-hour fast, all groups (20-24 rats/5-6 litters *per* group) received an intraperitoneal injection of insulin (1U/kg bw). Blood samples were collected through a small cut at the tip of the tail and blood glucose was measured using a glucometer (FreeStyle OptimumH®, Abbott Laboratories), before (0 min) the injection of insulin (1 g/kg bw) and 5, 15, 30, 45, and 60 min afterwards. Subsequently, the rate of the blood glucose decay constant (kITT) was calculated [25].

2.5 Intravenous glucose tolerance test (ivGTT)

At 103 days-old, the animals (20-24 rats/5-6 litters of group) were weighed and anesthetized using a ketamine and xylazine (75 and 15 mg/kg of body weight intramuscularly) solution, prior to the surgical implantation of a silicone cannula into the right external jugular vein, attached to the dorsal region of the neck. The animals were given a day for recovery from surgery, as previously described [26]. After a 12-hour fast, blood samples were removed before the injection of glucose (2 g/kg bw) (0 min) and 5,

15, 30 and 45 min afterwards. The samples were centrifuged and the plasma collected. Blood glucose concentration was determined using the glucose oxidase method with a commercial kit (Gold Analisa®, Belo Horizonte, Brazil)

2.6 Biochemical analyzes

After a 12-hour fast, the animals were anesthetized using a ketamine and xylazine (75 and 15 mg/kg of body weight intraperitoneally) solution for the collection of blood samples for evaluation of plasma glucose values. After centrifuging the blood taken from the animals, the plasma was stored in a -20°C freezer. Glucose was measured using the glucose oxidase method by Elisa, using a commercial kit (Gold Analisa®; Belo Horizonte, Brazil).

Plasma was also used to measure plasma levels of triglycerides, total cholesterol and HDL cholesterol using a commercial kit (Gold Analisa®; Belo Horizonte, Brazil). LDL and VLDL cholesterol values were calculated using the Friedewald calculation: LDL (mg/dL) = Total cholesterol – (HDL + VLDL) and VLDL (mg/dL) = Triglycerides/ 5.

The liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the TGO and TGP kits, respectively, using commercial kits (Gold Analisa®; Belo Horizonte, Brazil), both readings were carried out on spectrophotometry equipment (Semi-automatic biochemical analyzer, BIO200FL, Bio Plus®, 151 São Paulo, Brazil).

2.7 Liver fat extraction

The liver total fat was extracted by using the Folch method [27]. Samples of ~500mg of the liver was homogenized with 10 ml of a solution composed by chloroform: metanol (2:1) to extract the fat from liver samples. After the extraction, the solution was filtered and mixed with 0.9% saline. The sample was left to rest for 1 hour and then centrifuged at 1.000 rpm for 05 minutes to facilitate the separation of the upper or aqueous phase, dissolved in the methanol, and the lower or chloroform phase, which contained the fat extracted from the liver. Most of the aqueous phase was then removed and the chloroform phase adjusted to a final volume of 1.5 ml. This phase was transferred into a tube that had been previously weighed and the solution was evaporated to dryness using a forced air-drying oven. Finally, the tube was weighed again and the amount of fat calculated. The liver total cholesterol and triglycerides were measured using a commercial kit after

dissolving in isopropanol the previous extract of liver fat (GoldAnalisa®; Belo Horizonte, Brazil).

2.8 Statistical analysis

The results are presented as mean ± S.E.M. Statistical analysis were performed using Student's t-test or two-way ANOVA (analysis of variance - for the effects of metformin treatment, high-fat diet or the interaction (I) of metformin treatment and HFD followed by Tukey's test. Data was analyzed for normality using the D'Agostino & Pearson's test. A p value < 0.05 was considered statistically significant. Data analyses were performed using GraphPad Prism v7.00 for Windows (GraphPad Software Inc., San Diego, CA - USA).

3. Results

3.1 Body weight gain, caloric intake and fat pad store measurements

As shown in Table 1, the metformin treatment during lactation not affect the offspring bw at 21 days-old. When analyzing the effect of metformin treatment on abdominal diameter and nasoanal length, no statistical changes were observed at 7, 14 and 21 days-old when compared the group SAL and MET. Table 2 shows the results of bw, the HFD increases the bw of both, HFD-SAL ($p<0.0001$) and HFD-MET ($p<0.0001$), when compared to the NFD-SAL and NFD-MET groups, respectively, the metformin treatment did not affect bw, this is evidenced by the post-test result. As indicated by the AUC, for the caloric intake, the diet factor was able to significantly increased the offspring's caloric consumption and the treatment factor with metformin didn't interfere in this parameter, evidenced by pos-test result, showed when compared HFD-SAL ($p< 0.0001$) to NFD-SAL and HFD-MET ($p<0.0001$) to NFD-MET (Figure 2B).

As shown in Table 2, adipose tissue mass was equal between the NFD-SAL and NFD-MET groups. As expected, HFD-SAL animals had larger stocks of fat than NFD-SAL as demonstrated by the increases in periepididymal ($p<0.0001$), retroperitoneal ($p<0.0001$) and mesenteric fat pads ($p<0.0001$). The HFD-MET group either showed an increase in body fat composition when compared with NFD-MET, as demonstrated by the increases in periepididymal ($p<0.0001$), retroperitoneal ($p<0.0001$) and mesenteric fat pads ($p<0.0001$). These results show main that diet factor had a statistical significance. Except

in the periepididymal fat, the treatment factor didn't interfere in this parameter. As presented in the Table 2, only the diet factor was able to increases the brown adipose tissue, evidenced by pos-test result, showed when compared HFD-SAL to NFD-SAL($p<0.0001$) and HFD-MET to NFD-MET($p<0.0001$).

3.2 Intravenous glucose tolerance test, Intraperitoneal insulin tolerance test and Glucose decay constant

Both factors didn't affect the fasting glycemia on 102 days-old, either HFD or NFD (Figure 3A). During the ivGTT, as demonstrated by the AUC, the glycemia of HFD-SAL was increased when compared to the NFD-SAL ($p<0.0001$). The group HFD-MET demonstrated increases in glycaemia when compared with the NFD-MET ($p<0.002$), but when we compared the HFD-SAL and HFD-MET, we observed a reduction in glycemia over the test period, this is evidenced by the interaction between the diet factor and treatment factor (Figure 2A). Analyzing the ipITT curve through kITT index, the HFD in adulthood increases the insulin resistance of the HFD-SAL ($p<0.044$) and HFD-MET ($p<0.04$) groups when compared to the NFD-SAL and NFD-MET rats, respectively, the metformin treatment did not effective to change this parameter. However, it is not significant between individual groups using the posttest (Figure 2B).

3.3 Biochemical parameters

As show in the Figure 3, only the diet factor caused an interference in the parameters on free fat in the blood. Both groups fed with a HFD in adulthood had an increased in total cholesterol at 102 days-old, when compared HFD-SAL ($p<0.004$) with NFD-SAL and HFD-MET ($p<0.005$) with NFD-MET. However, only the animals of HFD-SAL group showed a significant increase ($p<0.014$) in plasma triglycerides compared to the NFD-SAL. There was no statistical significance in the treatment factor, as shown by the comparison between the control NFD-SAL and NFD-MET groups.

Regarding the lipid fractions, only the diet factor caused an interference in those parameters. The group HFD-SAL had an increase in the LDL cholesterol ($p<0.04$) and VLDL cholesterol ($p<0.023$) compared with NFD-SAL. But there wasn't significantly difference in the HDL cholesterol. However, the group HFD-MET had an increase in the LDL cholesterol of ($p< 0.0086$) and a decrease in the HDL cholesterol ($p<0.04$), without a significantly difference in the VLDL cholesterol when compared NFD-MET. No

significant changes we found in the comparison between NFD-SAL and NFD-MET groups, this is evidence in the pos-test (Figure 4).

3.4 Liver fat content and profile

As presented in the Figure 4A, the diet factor was able to increase the liver fat in animals fed with a HFD in adulthood and the treatment factor with metformin was statistically significant to reverse this alteration, evidenced by pos-test result, showed when compared HFD-SAL to NFD-SAL($p<0.0066$) and HFD-MET to NFD-MET($p<0.046$). Both groups fed with a HFD showed that this factor leads to an increase of the hepatic triglycerides content when compared HFD-SAL ($p<0.0001$) with NFD-SAL and HFD-MET ($p<0.014$) with NFD-MET (Figure 4B), without an alteration in the hepatic cholesterol content (Figure 4C). Regarding the liver enzymes AST and ALT, only the ALT activity was increased in relation to the diet factor, when compared HFD-SAL ($p<0.007$) with NFD-SAL and HFD-MET ($p<0.002$) with NFD-MET (Figure 4E). However, no significant difference between NFD-SAL and NFD-MET was observed in post-test.

4. Discussion

Studying the effects of neonatal short metformin exposure in obese rats induced by a HFD in adulthood, we showed that early exposure to metformin most didn't change the deleterious effects caused by the HFD, such as increase in body weight gain associated with the increase of periepididymal, retroperitoneal and mesenteric fat pad accumulation, insulin resistance and the elevation of triglycerides, cholesterol total, liver triglycerides and ALT. However, metformin attenuated the effects of the HFD on glucose intolerance and the accumulation of fat in the liver.

The effect of lactational exposure to metformin on pups' body weight is still controversial. Similar to our results, it has been reported that the treatment with metformin during lactation did not reduce the litters body weight, even when the metformin is used in higher concentrations (200mg/kg/day) than in the present study [28]. In addition, it has been demonstrated that the maternal and lactational treatment with metformin also did not interfere on body weight gain of the pups [29]. On the other hand, it has been described that offspring of dams exposed to metformin presented overall lower body weight and were shorter than control offspring starting at 7 days-old [9]. According to Novi *et al.* [29] these differences can be related to the experimental models evaluated.

It has been observed in other studies that metformin treatment decreases body weight, fat pad stores and food intake [11-30-31] in short and long term, but those studies evaluated the effects of metformin in people or animals that had metabolic disorders or that received the drug concomitantly with an obesogenic stress. In the present study we demonstrated that the early-life short treatment with metformin is not able to alters the caloric intake, body weight and adipose tissue mass when the animals received the obesogenic diet later in adulthood. Similarly, Salomaki *et al.* described that the mice offspring of dam's treated with metformin (300 mg/kg/day) that were fed with HFD in adult life didn't presented decreases of body weight, food intake, retroperitoneal and periepididimal fat pad stores [32].

It is well established that HFD consumption leads to obesity associated with glucose intolerance and insulin resistance and impaired endocrine-pancreatic function in rats [33-34-35]. In our study, the animals fed with a HFD presented glucose intolerance during the ivGTT and the early treatment with metformin caused a modest improvement in glucose tolerance. Previate *et al.* and Carlson *et al*. demonstrated that the early exposure to metformin causes an improvement of the glucose intolerance [9-11]. Additionally, the treatment with metformin in adult life was able to promote improvement in glucose intolerance [36], including in HFD-induced obese rodents [37].

We demonstrated that animals fed with a HFD present insulin resistance and treatment with metformin didn't change the insulin resistance. Similarly, Novi *et al.* showed that treatment with metformin during pregnancy and lactation didn't promote changes in insulin resistance in the offspring [29]. However, it has been reported that metformin exposure during lactation in early overfed animals lead to improved insulin sensitivity in adult life [11]. Theses discordances probably due to the marked methodological differences of the studies.

It has already been reported in the literature that treatment with metformin improves lipid profiles and alleviates the severity of high-fat-induced hepatic steatosis in obese humans and mice [38]. For example, Al-Ani *et al.* demonstrated that rats receiving an obesogenic insult concomitantly with metformin treatment (200 mg/kg/day) present a decrease in total cholesterol, triglycerides, LDL cholesterol and an increase in HDL cholesterol [39]. In our study, we observed that the treatment with metformin during lactation does not affect these parameters when the rats are submitted an obesogenic insult in a later phase

of life. Similar to our results, but using the gestational treatment with metformin (300 mg/kg/day) in mice, a study showed that there is no decrease in plasmatic levels of cholesterol and triglycerides in the offspring exposed to a high-fat diet in adulthood [32].

The liver is the first local of action of metformin [40] and the concentration of metformin is higher in the portal circulation than elsewhere in the body [41]. In the current study, the early metformin treatment was able to reduce fat liver accumulation in the HFD fed animals. Metformin leads to AMPK activation in the liver that, among other effects, results in the inhibition of hepatic lipogenesis and hepatic gluconeogenesis and increased local fatty acid oxidation [42]. Although, the early metformin intervention did not promote changes in the hepatic cholesterol and triglycerides, attenuating total fat liver is a positive effect, as non-alcoholic fatty liver disease is a chronic disease marked by lipid accumulation in the liver [43], which is associated with the increase of mortality particularly due to cardiovascular disease, hepatocellular carcinoma and multisystemic complications [44].

ALT and AST are enzymes present in hepatocytes involved in hepatic metabolism and related to lipid and glucose metabolism. These enzymes are considered markers of liver damage, as elevated activity in plasmatic levels have been associated with hepatic fat infiltration and can be used to identify hepatic steatosis [45-46-47-48]. We showed that consumption of HFD lead to an increases of ALT activity in the plasma, without any alteration in the AST activity. The early treatment with metformin did not change these parameters. Tajima et al. showed that mice fed HFD had an increase in plasma ALT level and treatment with metformin (250mg/kg/day) was not able to change this parameter [49]. However, Yasmin *et al.* demonstrated that animals fed with a HFD in adult life exhibited an increase in AST and ALT and the treatment with metformin (200mg/kg/day) concurrently the diet administration attenuated the plasma activities of AST and ALT enzymes [50]. Zamani-Garmsiri *et al.* demonstrated that HFD diet significant increase the AST and ALT and the treatment with metformin (2.3g/kg diet) led to decrease in these parameters [51]. These factors indicate that metformin can be efficient if used concomitantly with the administration of the diet or evaluated soon after the treatment in these parameters. Yet, in a programming context (12 days of lactation) with evaluation of the long-term effect indicate that metformin may not be efficient.

In summary, the early short metformin administration, during the first 12 days of lactation, was able to promote a modest improvement in glucose tolerance and in the accumulation of fat liver caused by a HFD in adulthood. However, the treatment was ineffective in alter the other deleterious effects of HFD, such as increased body weight gain and fat accumulation, insulin resistance, dyslipidemia and the increase of plasmatic activity of ALT. Although, the effects of exposure to metformin are more marked when diet is used concomitantly with an obesogenic insult compared to previous administration of the drug, this study highlights the urge for clinical researches investigating the potential effects of early exposure to metformin as prophylactic use for the treatment of obesity and metabolic disorders.

Author Contributions:

C.B.Z., P.C.d.F.M., D.L.A., V.M.M. and A.M. were responsible for the conception and design of the experiments. C.B.Z., W.d.N.d.S., S.R.R., R.V., M.N.C.P., C.C.I.M., M.C., M.V.G.R., F.L.d.S., A.C.Z.C., L.F.B., A.C.H.Ds., G.K.G.L., L.P.J.S., worked in the collection, analysis and interpretation of the data. All authors were involved in drafting the article and critically revising it for intellectual content. All authors approved the final version of the manuscript submitted for publication.

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502 disease in high-fat diet-fed mice. The Journal of Nutritional Biochemistry.
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504

505 **Table 1-** Effect of the treatment of metformin in the offspring on body weight, abdominal
506 diameter and nasoanal length.

Parameters	SAL	MET	P Value
AUC of Body weight gain (0-21 days)	24.85±2.62	24.94±2.65	p<0.9178
Abdominal diameter 7 days-old	1.26±0.017	1.29±0.015	p<0.2794
Abdominal diameter 14 days-old	1.50±0.025	1.45±0.021	p<0.2109
Abdominal diameter 21 days-old	1.63±0.021	1.64±0.31	p<0.8056
Nasoanal length 7 days-old	6.41±0.049	6.46±0.041	p<0.4127
Nasoanal length 14 days-old	8.16±0.051	8.041±0.038	p<0.066
Nasoanal length 21 days-old	10.01 ± 0.102	9.798 ± 0.066	p<0.0910

507

508 Data are expressed as the mean ± SEM from at 12 litters. SAL: Offspring treat with saline;
509 MET: Offspring treat with metformin. The inset represents the area under the curve
510 (AUC) for the student's test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

511 **Table 2-** Effect of a high fat diet consumption on body weight and adipose tissue mass
512 of adult animals programmed by metformin treatment in lactation.

Parameters	NFD		HFD		Factors		
	NFD-SAL	NFD-MET	HFD-SAL	HFD-MET	D	T	I
Body weight (g) at 102 days-old	391.9±8.322^{a}	403.2±5.844^{a}	441.2±5.954^{b}	451.6±5.153^{b}	****	ns	ns
Liver weight (g/100g bw)	3.808±0.076^{a}	3.589±0.073^{a}	3.620±0.043^{a}	3.623±0.079^{a}	ns	ns	ns
Periepidydimal fat pad (g/100g bw)	1.134±0.067^{a}	1.315±0.085^{a}	1.705±0.053^{b}	1.984±0.057^{b}	****	**	ns
Retroperitoneal fat pad (g/100g bw)	1.277±0.066^{a}	1.422±0.069^{a}	2.325±0.056^{b}	2.359±0.073^{b}	****	ns	ns
Mesenteric fat pad (g/100g bw)	0.736±0.030^{a}	0.71±0.039^{a}	1.243±0.054^{b}	1.249±0.054^{b}	****	ns	ns
Brown fat pad (g/100g bw)	0.057±0.002^{a}	0.058±0.002^{a}	0.074±0.003^{b}	0.074±0.003^{b}	****	ns	ns

513

Data are expressed as the mean ± SEM of 16-24 rats from at least 5 different litters. NFD-SAL: Saline offspring fed with normal fat diet; NFD-MET: Metformin offspring fed with normal fat diet; HFD-SAL: Saline offspring fed with high-fat diet and HFD-MET: Metformin offspring fed with high-fat diet. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance. Factors: diet (D), treatment (T), interaction between drug and treatment (I). Letters (A and B) indicates the post-test results when the difference among groups were significant ($p<0.05$).

6. Figure legends

Figure 1- Body weight gain and caloric intake. Body weight gain (A) and caloric intake (B) from 21 to 102 days-old. The data are expressed as the means ± SEM and were obtained from 20-24 rats/5-6 litters of group (A and B). The inset represents the area under the curve (AUC). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance. Factors: diet (D), treatment (T), interaction between drug and treatment (I). NFD-SAL: Saline offspring fed with normal fat diet; NFD-MET: Metformin offspring fed with normal fat diet; HFD-SAL: Saline offspring fed with high-fat diet and HFD-MET: Metformin offspring fed with high-fat diet.

Figure 2– Plasma glucose during ivGTT and Kitt. ivGTT (A) and Kitt (B). The data are expressed as the means ± SEM and were obtained from 12 rats of each group (from 5/6 different litters). The inset represents the area under the curve (AUC). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance. Factors: diet (D), treatment (T) interaction between drug and treatment (I). NFD-SAL: Saline offspring fed with normal fat diet; NFD-MET: Metformin offspring fed with normal fat diet; HFD-SAL: Saline offspring fed with high-fat diet and HFD-MET: Metformin offspring fed with high-fat diet.

Figure 3- Biochemical Parameters. Fasting Glycemia (A), Triglycerides (B), Total Cholesterol (C), HDL Cholesterol (D), VLDL Cholesterol (E) and LDL Cholesterol (F). The data are expressed as the means ± SEM and were obtained from 8/12 rats of each group (from 4/5 different litters). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance. Factors: diet (D), treatment (T), interaction between drug and treatment (I). NFD-SAL: Saline offspring fed

with normal fat diet; NFD-MET: Metformin offspring fed with normal fat diet; HFD-SAL: Saline offspring fed with high-fat diet and HFD-MET: Metformin offspring fed with high-fat diet.

Figure 4- Liver fat accumulation, Activity of AST and ALT in the plasma. Liver fat (A), Liver triglycerides (B), Liver cholesterol (C), AST (D) and ALT (E). The data are expressed as the means ± SEM and were obtained from 8 rats of each group (from 4/5 different litters). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 and ns, no significant difference, based on a two-way analysis of variance. Factors: diet (D), treatment (T), interaction between drug and treatment (I). NFD-SAL: Saline offspring fed with normal fat diet; NFD-MET: Metformin offspring fed with normal fat diet; HFD-SAL: Saline offspring fed with high-fat diet and HFD-MET: Metformin offspring fed with high-fat diet.

7. Figures

Figure 1

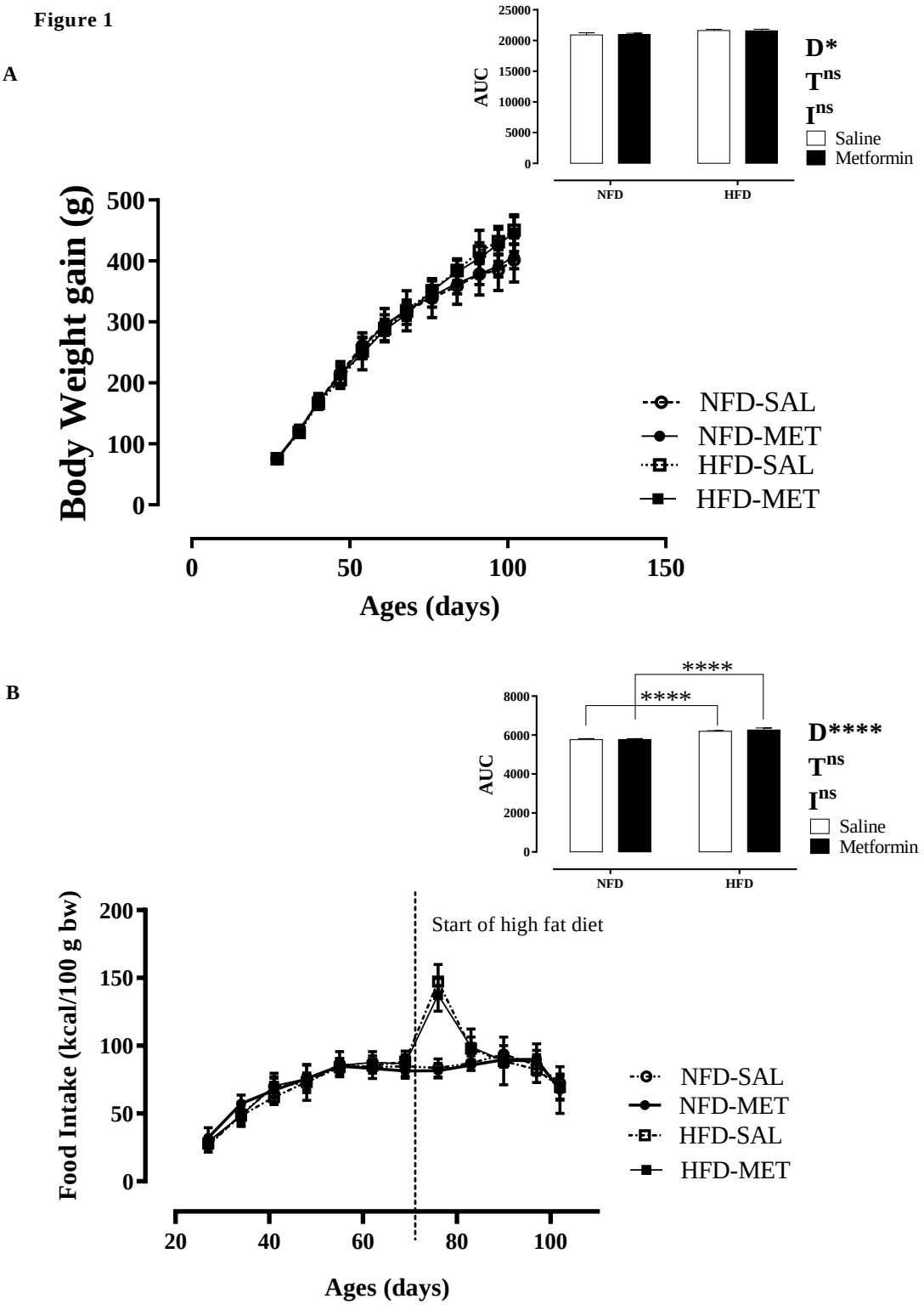
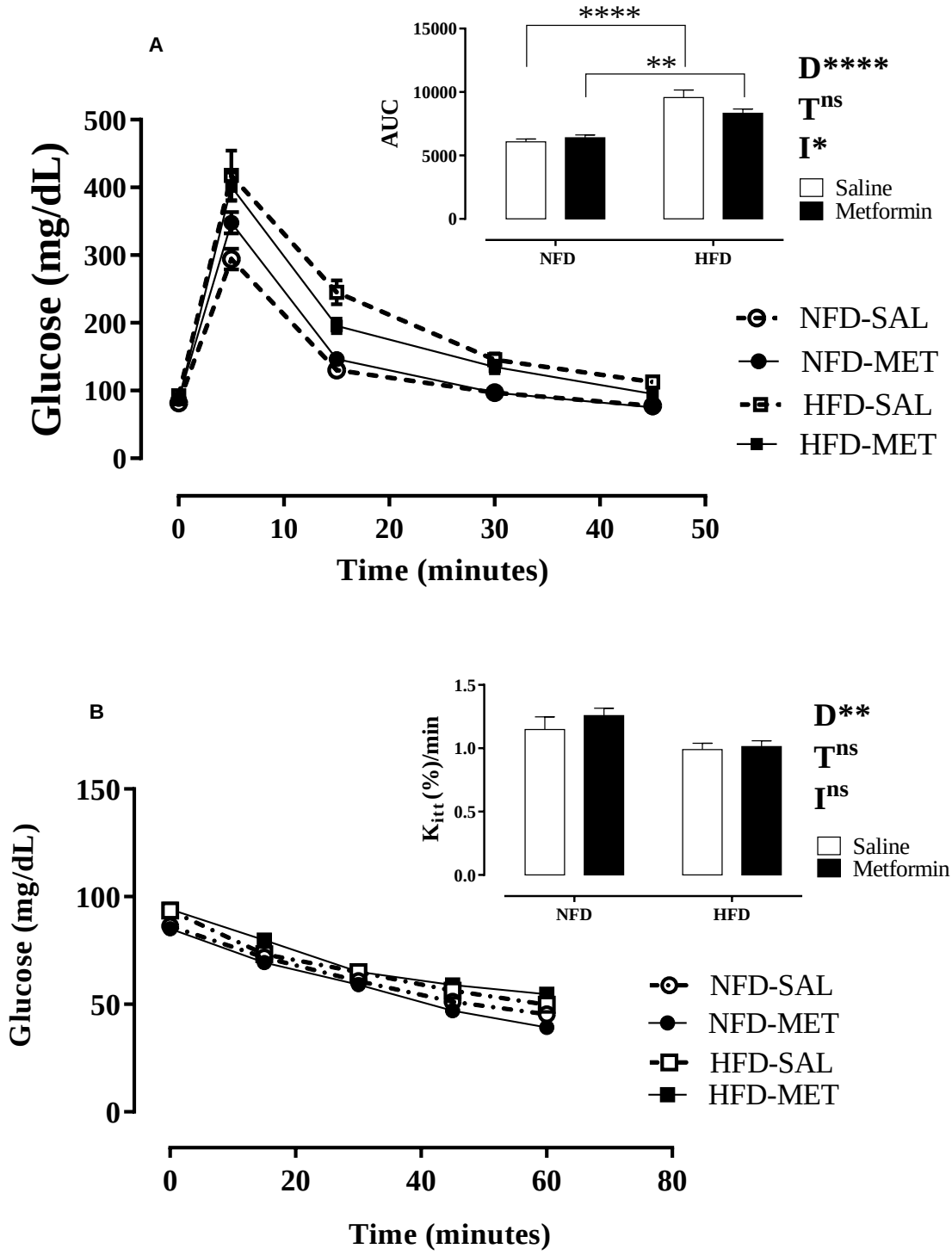


Figure 2


A
Glucose (mg/dL)
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I*
Saline
Metformin
NFD
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NFD-SAL
NFD-MET
HFD-SAL
HFD-MET
B
Glucose (mg/dL)
Time (minutes)
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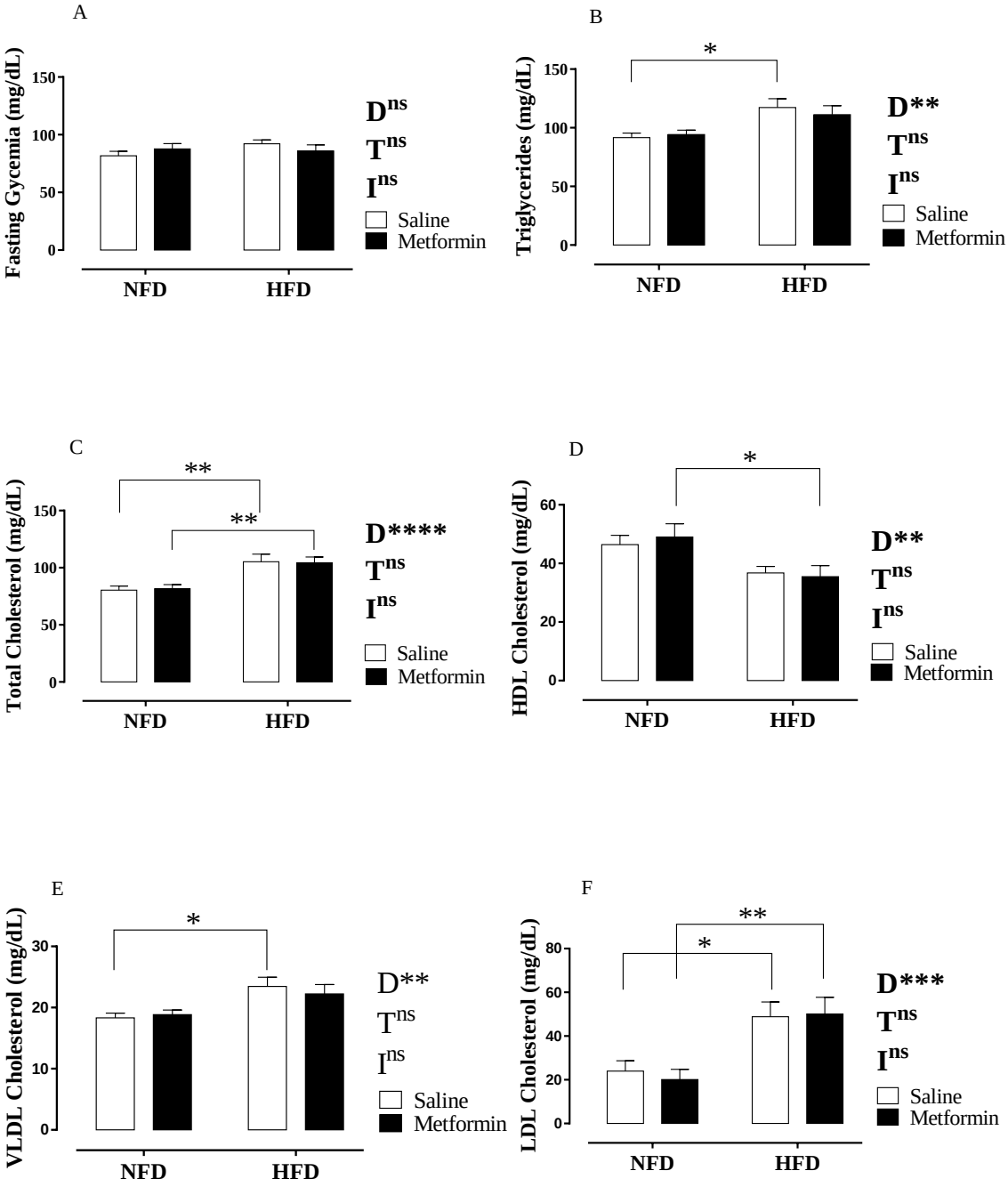
Saline
Metformin
NFD
HFD
NFD-SAL
NFD-MET
HFD-SAL
HFD-MET

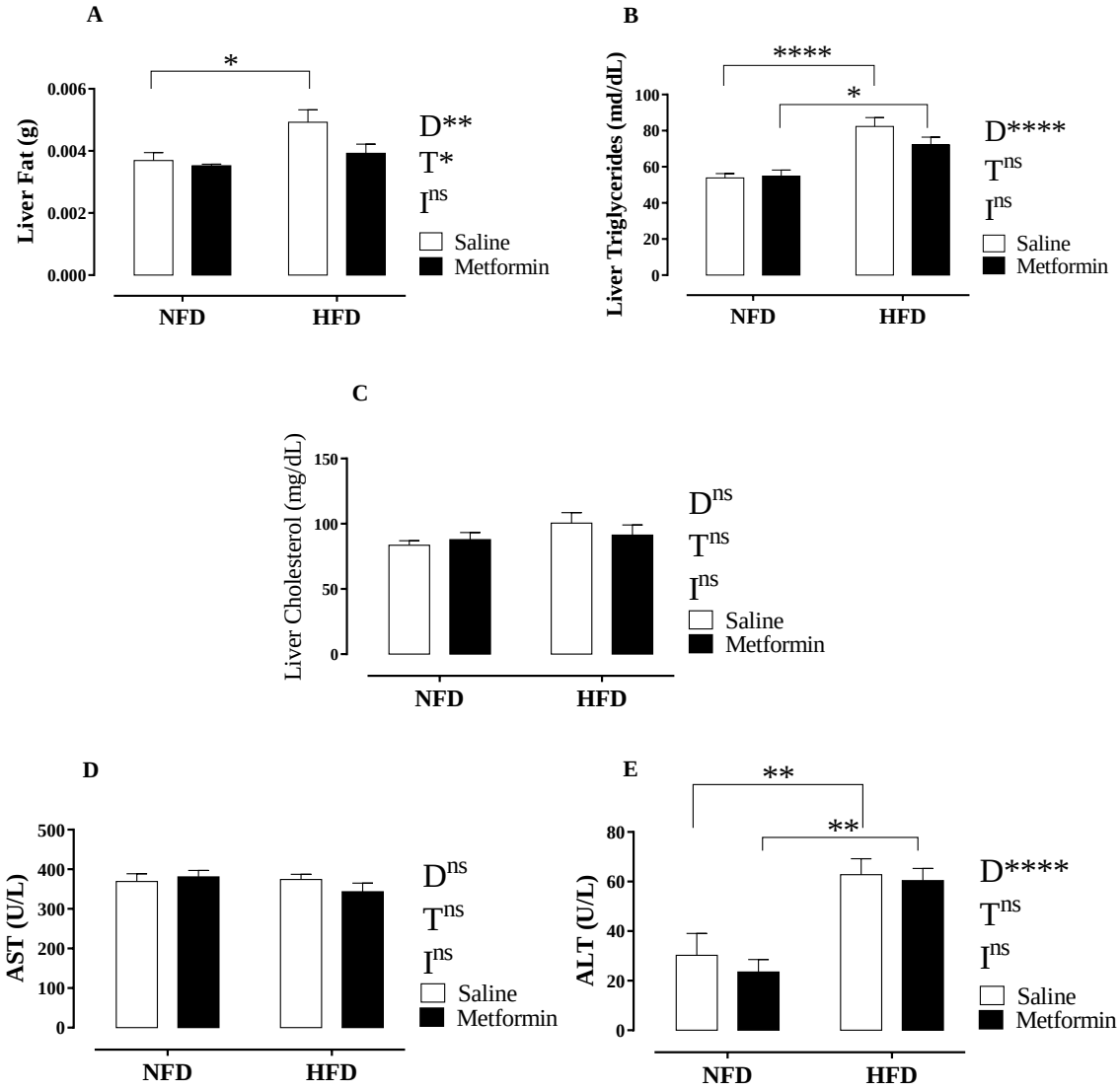
560

Figure 3


A
Fasting Gycemia (mg/dL)
150
100
50
0
NFD
HFD
D^ns
T^ns
I^ns
Saline
Metformin
B
Triglycerides (mg/dL)
*
D**
T^ns
I^ns
C
Total Cholesterol (mg/dL)
**
**
D****
T^ns
I^ns
D
HDL Cholesterol (mg/dL)
60
40
20
*
D**
T^ns
I^ns
E
VLDL Cholesterol (mg/dL)
30
20
10
*
D**
T^ns
I^ns
F
LDL Cholesterol (mg/dL)
80
60
40
20
**
*
D***
T^ns
I^ns

561

Figure 4


A
Liver Fat (g)
0.006
0.004
0.002
0.000
*
D**
T*
Ins
Saline
Metformin
NFD
HFD
B
Liver Triglycerides (md/dL)
100
80
60
40
20
0
****
*
D****
Tns
Ins
Saline
Metformin
NFD
HFD
C
Liver Cholesterol (mg/dL)
150
100
50
0
Dns
Tns
Ins
Saline
Metformin
NFD
HFD
D
AST (U/L)
500
400
300
200
100
0
Dns
Tns
Ins
Saline
Metformin
NFD
HFD
E
ALT (U/L)
80
60
40
20
0
**
**
D****
Tns
Ins
Saline
Metformin
NFD
HFD

562