SCARLETT RODRIGUES RAPOSO

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TRATAMENTO DE METFORMINA A CURTO PRAZO NA VIDA ADULTA NÃO ATENUA A OBESIDADE INDUZIDA PELA SUPERNUTRIÇÃO NEONATAL EM RATOS

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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

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SCARLETT RODRIGUES RAPOSO

BREVE TRATAMENTO COM METFORMINA EM RATOS ADULTOS NÃO ATENUA A OBESIDADE PROGRAMADA NA VIDA NEONATAL

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.

Aprovado em: 25/08/2022

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BIOGRAFIA

Scarlett Rodrigues Raposo nasceu São Paulo-SP em 17/05/1995. Possui graduação em Ciências Biológicas (Licenciatura e Bacharel) pelo Centro Universitário de Maringá – Unicesumar (2018) e Especialização em Análises Clínicas e Toxicológicas também pelo Unicesumar (2021). Atualmente é estudante de Mestrado pelo Programa de Pós-Graduação em Ciências Biológicas, área de concentração em Biologia Celular e Molecular da Universidade Estadual de Maringá – UEM. Possui como escopo de estudos a Biologia Celular aplicada aos seguintes temas: Síndrome metabólica,Diabetes tipo 2; Obesidade e Programação Metabólica.

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APRESENTAÇÃO

Esta dissertação possui um Artigo científico experimental que aborda os efeitos do tratamento com Metformina em ratos Wistar adultos, programados para a disfunção metabólica pelo modelo de redução de ninhada. O artigo foi redigido de acordo com as normas da revista *Journal of Developmental Origins of Health and Disease*, CB1 – Qualis B3, para a qual o mesmo será submetido.

Scarlett Rodrigues Raposo[1]; Lucas Paulo Jacinto Saavedra[1]; Camila Benan Zara[1]; Mariane Carneiro Mendonça[1]; Maria Natália Chimirri Peres[1]; Willian do Nascimento de Souza Rodrigues[1]; Leticia Ferreira Barbosa[1]; Gabriel Kian Guimarães Lopes[1]; Silvano Piovan[1], Anna Carolina Huppes de Souza[1], Ana Claudia Zara Couto[1], Filipe Lima dos Santos[1], Luana Marcelly Martins[1], Maiara Vanusa Guedes Ribeiro [1], Anna Rebeka Oliveira Ferreira[1], Paulo Cezar de Freitas Mathias[1]. *Breve tratamento com metformina em ratos adultos não atenua a obesidade programada na vida neonatal.*

RESUMO GERAL

INTRODUÇÃO – Segundo a Organização Mundial da Saúde, a obesidade é considerada um dos maiores riscos à saúde pública, estando associada à disfunção cardiovascular, obesidade visceral, intolerância à glicose, resistência à insulina e dislipidemia. Tem sido demonstrado que a sub ou supernutrição, durante os períodos críticos do desenvolvimento, como pré-concepção, gravidez, lactação e adolescência, levam ao desenvolvimento de obesidade e disfunção cardiometabólica mais tarde na vida, este conceito é conhecido como DOHaD (*Developmental Origins of Health and Disease*). A redução de ninhada (SL) em roedores tem sido usada como modelo animal para mimetizar os efeitos de longo prazo da superalimentação infantil. Esses animais desenvolvem uma série de alterações metabólicas durante a vida adulta, incluindo obesidade, dislipidemia, resistência à insulina e intolerância à glicose, condições comumente encontradas em indivíduos diabéticos tipo 2. O fármaco mais prescrito para o tratamento do diabetes tipo 2 é a Metformina (Met), mas além de seus efeitos benéficos no controle glicêmico, como melhora da intolerância à glicose e melhora da resistência à insulina, estudos têm demonstrado outros efeitos favoráveis como a melhora do controle do peso corporal, diminuição da adiposidade visceral e hiperfagia. Pensando nos diversos efeitos benéficos que a Metformina tem mostrado, surge nossa hipótese de que um tratamento de curto prazo com Metformina na vida adulta não atenua a obesidade induzida pela supernutrição neonatal em ratos.

OBJETIVOS – Avaliar os efeitos de tratamento curto de Met na vida adulta, sobretudo em relação ao seu peso corporal, ingestão alimentar, perfil lipídico e glicêmico além de parâmetros bioquímicos, em ratos queforam programados para o desenvolvimento de disfunção metabólica em um modelo de redução de ninhada.

MÉTODOS – Ratos Wistar adultos fêmeas (N=48) e machos (N=24) foram adquiridos do Biotério Central da Universidade Estadual de Maringá (UEM), e colocados no Biotério Setorial do Laboratório de Biologia Celular da Secreção. Os animais foram mantidos em ambiente climatizado com temperatura de 22±2ºC, ciclo de fotoperíodo de 12 horas (7:00-19:00 horas, período de luz), com livre acesso a água e ração (Nuvital®, Curitiba, Brasil). Após uma semana de aclimatação (adaptação), os animais foram colocados em

caixas para cruzamento, na proporção de duas fêmeas para cada macho. Uma vez prenhas, as fêmeas permaneceram em caixas individuais com livre acesso a ração comercial e água até o nascimento da prole, que foi considerado dia 0. No dia 1, todas as ninhadas foram padronizadas para 9 filhotes por lactante e no 3º dia após o nascimento, as ninhadas foram ajustadas para 3 filhotes porlactante no grupo ninhada reduzida (*small littter*, SL), e os animais da ninhada normal (*normal litter*, NL), permaneceram com o mesmo número de filhotes. A redução da ninhada é necessária para o modelo de indução de disfunção metabólica induzidas pela superalimentação neonatal que altera a oferta de leite aos filhotes durante a lactação, devido a essa menor competição alimentar, os animais acabam se alimentando mais levando a um rápido ganho de peso e distúrbios metabólicos na vida adulta. Após o desmame, que ocorre aos 21 dias de vida, os animais foram desmamados, tiveram livre acesso à água e ração comercial. Foram utilizados apenas ratos machos para este estudo, dado o dimorfismo sexual entre machos e fêmeas, bem como as flutuações hormonais que as fêmeas sofrem durante o ciclo estral, o que altera seu metabolismo e comportamento, apesar das evidências que demonstram que existem diferenças biológicas fundamentais importantes entre os sexos, e a falha em elucidar essas diferenças. Aos 70 diasde idade, os animais foram separados em 2 novos grupos: Salina (SAL) e Metformina (MET). Durante 12 dias, os animais NL e SL foram tratados diariamente com soro fisiológico (NaCl 0,9%), dando origem aos grupos NL-SAL e SL-SAL, ou foram tratados com Met 100mg/kg, dando origem aos grupos NL-MET e SL-MET. Após o término do tratamento (82 dias) os animais foram mantidos até os 142 dias onde foram eutanasiados e analisados peso corporal e ingestão alimentar, estoques de gordura, peso do fígado e homeostase glicêmica.

RESULTADOS E DISCUSSÃO – Os animais oriundos de ninhadas reduzidas apresentaram diferença significativa, mostrando que a metformina não atenuou o modelo SL. O SL-SAL aumentou o ganho depeso corporal quando comparado ao NL-SAL (AUC, $p<0,001$) e não foi observada diferença entre SL-SAL e SL-MET, mostrando que não houve atenuação do tratamento com Met neste parâmetro. Ao analisar o consumo alimentar,os animais SL-SAL e SL-MET tiveram um aumento significativo no consumo quando comparados aos grupos NL-SAL e NL-MET ($p<0,001$). O grupo SL-SAL apresentou gordura retroperitoneal

significativamente maior do que o grupo NL-SAL, o mesmo foi observado ao compara SL-MET com NL-MET ($p<0,001$), a gordura mesentérica no SL-SAL foi significativamente maior para o grupo NL -SAL, o mesmo foi observado ao compara SL-MET com NL-MET ($p<0,001$). O peso do fígado no grupo SL-SAL é significativamente maior do que o grupo NL-SAL, onde o mesmo foi observado ao compara SL-MET com NL-MET ($p<0,001$). A análise sobre as concentraçõesde glicose durante o teste de tolerância a glicose, os animais SL tiveram concentrações de glicose significativamente mais altas durante o teste quando comparados aos animais NL, onde o mesmo foi observado ao compara SL-MET com NL-MET (AUC, $p<0,001$). Durante o teste de tolerância insulina, o grupo SL comparado ao grupo NL são resistentes à insulina ($p<0,001$). Animais SL apresentam mais triglicerídeos e colesterol total (ANOVA, $p<0,0001$) do que os animais do grupo NL, enquanto que os animais NL têm mais colesterol HDL que os animais SL ($p<0,0001$).

CONCLUSÕES – Em conclusão, o tratamento de curta duração durante a vida adulta com Met não atenuou a disfunção metabólica induzida pela superalimentação neonatal em ratos.

GENERAL ABSTRACT

INTRODUCTION – According to the World Health Organization, obesity is considered one of the greatest risks to public health, being associated with cardiovascular dysfunction, visceral obesity, glucose intolerance, insulin resistance and dyslipidemia. It has been shown that under or overnutrition during critical periods of development such as preconception, pregnancy, lactation and adolescence lead to the development of obesity and cardiometabolic dysfunction later in life, this concept is known as DOHaD (Developmental Origins of Health and Disease). Litter reduction (SL) in rodents has been used as an animal model to mimic the long-term effects of infant overfeeding. These animals develop a series of metabolic changes during adulthood, including obesity, dyslipidemia, insulin resistance and glucose intolerance, conditions commonly found in type 2 diabetic individuals. The most prescribed drug for the treatment of type 2 diabetes is Metformin (Met), but in addition to its beneficial effects on glycemic control, such as improving glucose intolerance and improving insulin resistance, studies have shown other favorable effects such as improved body weight control, decreased visceral adiposity and hyperphagia. Considering the many beneficial effects that Metformin has shown, our hypothesis arises that a short-term treatment with Metformin in adult life does not attenuate obesity induced by neonatal overnutrition in rats.

AIMS – To evaluate the effects of short-term Met treatment in adulthood, particularly in relation to body weight, food intake, lipid and glycemic profile and biochemical parameters, in rats that were programmed to develop metabolic dysfunction in a litter reduction model.

METHODS – Adult female (N=48) and male (N=24) Wistar rats were acquired from the Central Animal Facility of the Universidade Estadual de Maringá (UEM), and placed in the Sectorial Animal Facility of the Laboratory of Cell Biology of Secretion. The animals were kept in an acclimatized environment with a temperature of 22±2ºC, 12-hour photoperiod cycle (7:00-19:00 hours, light period), with free access to water and feed (Nuvital®, Curitiba, Brazil) . After a week of acclimatization (adaptation), the animals were placed in boxes for mating, in the proportion of two females for each male. Once pregnant, the females remained in individual boxes with free access to commercial food and water until the birth of the offspring, which was considered day 0. On day 1, all

litters were standardized to 9 pups per lactant and on the 3rd day after birth. At birth, litters were adjusted to 3 pups per lactant in the reduced litter group (small littter, SL), and animals from the normal litter (normal litter, NL) remained with the same number of pups. Litter reduction is necessary for the model of induction of metabolic dysfunction induced by neonatal overfeeding that alters the milk supply to pups during lactation, due to this lower food competition, the animals end up feeding more leading to rapid weight gain. and metabolic disorders in adulthood. After weaning, which occurs at 21 days of age, the animals were weaned and had free access to water and commercial feed. Only male rats were used for this study, given the sexual dimorphism between males and females, as well as the hormonal fluctuations that females undergo during the estrous cycle, which alters their metabolism and behavior, despite evidence demonstrating that there are fundamental biological differences. between the sexes, and the failure to elucidate these differences. At 70 days of age, the animals were separated into 2 new groups: Saline (SAL) and Metformin (MET). For 12 days, the NL and SL animals were treated daily with saline (0.9% NaCl), giving rise to the NL-SAL and SL- SAL groups, or were treated with Met 100mg/kg, giving rise to the NL-SAL groups. MET and SL-MET. After the end of the treatment (82 days) the animals were kept until 142 days where they were euthanized and analyzed body weight and food intake, fat stores,liver weight and glycemic homeostasis.

RESULTS AND DISCUSSION – Animals from reduced litters showed a significant difference, showing that metformin did not attenuate the SL model. SL-SAL increased body weight gain when compared to NL-SAL (AUC, $p<0.001$) and no difference was observed between SL-SAL and SL-MET, showing that there was no attenuation of Met treatment in this parameter. When analyzing food consumption, the SL-SAL and SL-MET animals had a significant increase in consumption when compared to the NL-SAL and NL-MET groups ($p<0.001$). The SL-SAL group presented significantly higher retroperitoneal fat than the NL-SAL group, the same was observed when comparing SL-MET with NL-MET ($p<0.001$), the mesenteric fat in the SL-SAL was significantly higher for the SL-SAL group. NL-SAL, the same was observed when comparing SL-MET with NL-MET ($p<0.001$). Liver weight in the SL-SAL group is significantly higher than the NL-SAL group, where the same was observed when comparing SL-MET with NL-MET ($p<0.001$). The analysis of glucose concentrations during the glucose tolerance test, the SL animals had significantly higher glucose concentrations during the test when compared to the NL animals, where the same was observed when comparing

SL-MET with NL-MET (AUC, $p<0.001$). During the insulin tolerance test, the SL group compared to the NL group are insulin resistant ($p<0.001$). SL animals have more triglycerides and total cholesterol (ANOVA, $p<0.0001$) than animals in the NL group, while NL animals have more HDL cholesterol than SL animals ($p<0.0001$).

CONCLUSIONS – In conclusion, short-term treatment during adulthood with Met did not attenuate the metabolic dysfunction induced by neonatal overfeeding in rats.

Title: Short treatment with Metformin in adult rat does not inhibit metabolic dysfunction programed by early overnutrition.

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Abstract

Interventions occurring during critical periods of development can program the individual for health or disease, this is known as the DOHaD (Developmental Origins of Health and Disease) concept. Studies have shown its beneficial effect such as improved control of body weight, decreased visceral adiposity and hyperphagia. Considering the improvements in metabolic parameters of treatment with Metformin (MET) and the necessity to clarify whether this effect is only observed if the exposure occurs during the critical periods, we have hypothesized that a brief treatment with MET during adulthood would not be able to attenuate obesity and metabolic dysfunction induced by early overnutrition. On day 1, all litters are standardized to 9 pups per dam and on day 3 after birth, litters were adjusted to 3 pups per dam in the small litter (SL) group and the NL group remained with 9 animals. At 70 days-old, the animals were separated into 2 new groups: Saline (SAL) and Metformin (MET). For 12 days, NL and SL animals were treated daily with saline, giving rise to NL-SAL and SL-SAL groups, or were treated with Met 100mg/kg/day (NL-MET and SL-MET). Analyzes of body weight gain during the lactation period show a significant increase when comparing SL animals with NL ($p<0.0001$); body weight remained significantly heavier in SL animals compared to NL until 142 days-old $p<0.0001$); with no significant difference was observed between when comparing NL-SAL vs NL-MET, and SL- SAL vs SL-MET. When analyzing the insulin and glucose tolerance test the results show that SL groups compared to NL are glucose intolerant and insulin resistant ($p<0.0001$). When analyzing the treatment has not effect, confirming our hypothesis that the brief treatment during adulthood with Metformin did not attenuate the metabolic dysfunction induced by neonatal overfeeding.

Keywords: Small Litter, adulthood, obesity, metformin, rats.

Introduction

According to the World Health Organization, obesity is considered one of the greatest public health risks, being associated with cardiovascular dysfunction, glucose intolerance, insulin resistance and dyslipidemia. About 59% of adults are overweight or obese and approximately 1.2 million of people die every year because of this disease [1, 2].

The cause of obesity is multifactorial and several epidemiological and experimental data have been pointing to the role of early life events in the development later disease. It has been shown that under or overnutrition, during the critical periods of development, such as pre-conception, pregnancy, lactation and adolescence, lead to the development of obesity and cardiometabolic dysfunction later in life, this concept is known as DOHaD (Developmental Origins of Health and Disease), the small litter (SL) in rodents have been used as an animal model to mimic long-term programming effects of overnutrition during lactation. Reduction in litter size is a relatively on invasive experimental manipulation that results in increased consumption due to increased maternal milk availability, these animals develop a range of metabolic alterations during adulthood, including visceral obesity, dyslipidemia, insulin resistance and glucose intolerance, conditions commonly found in type 2 diabetic individuals [3-6].

Currently, the most prescribed drug for the treatment of type 2 diabetes is Metformin (Met), but in addition to its beneficial effects on glycemic control, such as improvement in glucose tolerance and insulin sensitivity resistance, studies have shown other favorable effects such as improved control of body weight, decreased visceral adiposity and hyperphagia [7-19].

Moreover, our group reported that a short treatment with Met in SL rats during lactation was able to attenuate the development of obesity and metabolic dysfunction induced by early overfeeding [4]. Although many experimental studies explore the long-term effects of a brief exposure to a stressor during a critical period of development, there is scarce evidence that exposure to the same stressor or treatment during adulthood would have any effects on the metabolism after a therapeutic recovery. In order to affirm the observed phenotype, it is imperative the proof of the determine if far from a critical phase of development, namely adulthood, a similar phenotype would be observed after a period of recuperation, using the Met. Considering previous work from our laboratory that observed improvements in metabolic parameters from early treatment with Met and the necessity to clarify whether this effect is only observed if the exposure occurs during the critical period of lactation, we have hypothesized that a brief treatment with Met during adulthood would not be able to attenuate obesity and metabolic dysfunction induced by early overnutrition.

Methods

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 3220080620).

Experimental design and treatment

Adult female (N=48) and male (N=24) Wistar rats were obtained from the Central Animal Facility of the State University of Maringá (UEM), and placed in the Sectorial Animal Facility of the Laboratory of Cell Biology of Secretion. The animals were kept in polypropylene boxes measuring 15 cm; 30cm; 45 cm, for height, width and length respectively. All boxes were lined internally with wood shavings, being changed three times a week for clean boxes and bedding. The animals were kept in an acclimatized environment with a temperature of 22±2ºC, photoperiod cycle of 12 hours (7:00-19:00 hours, light period), with free access to water and food (Nuvital®, Curitiba, Brazil). After a week of acclimatization (adaptation), the animals were placed in boxes for crossing, in the proportion of two females for each male. Once pregnant, the females remained in individual boxes with free access to commercial food (Nuvilab® Curitiba, Brazil) and water until the offspring were born, which was considered day 0.

On day 1, all litters are standardized to 9 pups per dam and on day 3 after birth, litters were adjusted to 9 pups per dam in the Normal Litter (NL) group and 3 pups per dam in the Small Litter (SL) group, this induction of metabolic by de small litter model dysfunction increases milk supply to pups during lactation [20-22]. After weaning, which occurs at 21 days-old, the animals had free access to water and chow, only males rats were used in this study.

At 70 days-old, the animals were separated into 2 new groups: Saline (Sal) and Met. During 12 days, NL and SL animals were treated daily with saline (NaCl 0.9%), thus giving rise to the NL-Sal and SL-Sal groups, or Met 100mg/kg [4] giving rise to the NL-MET and SL-MET groups. Thus, the treatment ended at 82 days of life. At 142 days after carrying out the experimental procedures,

the animals were euthanized with thiopental i.p. at a lethal dose (150mg/kg), associated with lidocaine (10mg/mL). The figure 1 shows the experimental design.

Biometrical parameters

Pups body weight, abdominal diameter, skull diameter and nasoanal length were measured at 7 - 14 - 21 days of age. After weaning, body weight and food intake were determined weekly until 142 days-old. At 142 days of life, laparotomy was performed to collect the liver, pancreas, perigonadal fat, mesenteric fat and retroperitoneal fat (all collected tissues were weighed). Plasma was collected after 12 hours of fasting before the glucose tolerance test via the intravenous route, the used to store the blood at the time of collection were heparinized to prevent blood clotting, blood samples from were centrifuge (10,000 rpm for 5 min) for plasma collected, then frozen at -20°C and collected tissues were frozen at -80°C.

Intravenous glucose tolerance test (ivGTT)

The animals (N=54) were anesthetized with a mixture of Ketamine (75 mg/Kg of body weight) / Xylazine (15 mg/Kg of body weight). Then they underwent surgery for the implantation of a cannula of silicone in the right jugular vein, through an incision in the anterior cervical region, tissue dissection took place until the vein was visualized. Then, a silicone cannula was inserted into the vein and fixed to the pectoralis major muscle through a simple suture. The cannula was filled with a 10% heparin solution (Liquemine®) diluted in saline (0.9% NaCl) to prevent the ingress of blood and the consequent formation of clots in its inside. After surgery the animals received a subcutaneous injection of analgesic (Acetylsalicylic Acid, Aspirin®, 20mg/kg of body mass, 2 times/day); a dose soon after surgery and another 8 hours later, they were accommodated in the sectorial vivarium, remaining in individual boxes for24 h after performing the procedure.

After surgery, with overnight fasting for 12 h, the animals were submitted to the removal of a blood sample (400µL), directly from the jugular vein through the cannula to obtain fasting blood

glucose. During the test, a glucose solution was administered at a dose of 1g/kg of animal weight. After the administration of the glucose solution, blood samples (400 µL) were taken at 5, 15, 30 and 45 minutes. The corresponding volume was replaced with 0.9% saline solution. Blood samples were collected and transferred to in an ice bath. Subsequently, the samples were centrifuged at 4,500 rpm for 5 minutes, and the plasma was used to determine the blood glucose. After undergoing the test, all animals were euthanized by an anesthetic overdose of thiopental (150mg/kg), associated with lidocaine (10mg/mL).

Insulin tolerance test (ITT) 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 were measured using a glucometer (FreeStyle OptimumH®, Abbott Laboratories), before the injection of insulin (1 g/kg bw) (0 min) and 5, 15, 30, 45, and 60 min afterwards. Subsequently, the rate of blood glucose decay constant (kITT) was calculated.

Biochemical analyzes

Basal plasma values of glucose, total cholesterol, HDL-cholesterol and triglycerides were quantified from plasma samples obtained from whole blood by ivGTT (time 0). Values were quantified by spectrophotometry in a microplate reader (Kasuaki), with using commercial kits (Gold Analise®, Belo Horizonte/MG, Brazil).

Statistical analyses

Using GraphPad Prism Software version 9.00 for Windows (GraphPad Software Inc.,La Jolla California USA, www.graphpad.com), the results were presented as mean ± standard error of the mean (SEM). The data were analyzed through Student's t-test, two-way ANOVA and Tukey post-test. Values of $p<0.05$ were considered as the significance level.

Results

Biometric parameters during lactation small litter model in 21 days

Figure 1A show the effect of small litter model on body weight (BW) gain in 21 days. The analysis of the AUC, reveal that small litter model presented a significant increase in BW gain (SL 66.23 ± 1.72 vs NL 44.27 ± 1.41, $p<0.0001$).

Figure 1B, 1C and 1D show nasoanal length, abdominal diameter and skull diameter, respectively, SL group shows higher nasoanal length (SL, 115.1 ± 1.22 vs 109.9 ± 0.55, $p<0.05$), abdominal diameter (SL, 33.40 ± 1.06 vs NL, 27.47 ± 1.03, $p<0.05$) and in skull diameter (SL, 19.44 ± 0.32 vs NL, 17.43 ± 0.40, $p<0.05$).

Food intake and body weight gain

Figure 2 shows the evolution and the AUC BW evolution of all groups. There was a significant effect of litter ($p<0.001$), and treatment ($p<0.05$) in BW gain, but no interaction was observed. When comparing NL-SAL vs SL-SAL and SL-MET, there was a significant difference, showing that metformin did not able to prevent BW gain ins SL rats ($p<0.001$). SL-SAL increased body weight gain when compared to NL-SAL (42366.1 ± 558.28 vs 34870.76 ± 473.24, $p<0.001$) and no difference was observed between SL-SAL and SL-MET, showing no attenuation of treatment with Met in this parameter. Also, no difference was observed between NL-SAL vs NL-MET.

Figure 3 shows the AUC food intake evolution of all groups. In the AUC, it was observed a significant increase of food intake in SL-SAL group when compared with NL-SAL group (3354.6 ± 70.21 vs 2966.2 ± 102.9, $p<0.001$). In NL-SAL vs SL-SAL and SL-MET, there was a significant difference, showing that metformin did not attenuate the SL model ($p<0.001$).

Adiposity and liver mass

Figure 4A, 4B and 4C, shows the effects of Met treatment on mass of the, perigonadal, retroperitoneal and mesenteric tissues, respectively. SL-SAL group had significantly higher retroperitoneal fat than NL-SAL group (SL-SAL, 9.49 ± 0.51 vs NL-SAL, 6.29 ± 0.34, $p<0.001$) and mesenteric fat weight in the SL-SAL group was significantly higher than NL-SAL group (SL-SAL, 5.54 ± 0.40 vs NL-SAL, 3.73 ± 0.33 g, $p<0.001$), there were no differences in fat pad stores among Met-offspring with saline groups. Liver weight in SL-SAL group is significantly higher than NL-SAL

group (SL-SAL, 16.99 ± 0.43 vs NL-SAL, 14.28 ± 0.26, $p<0.001$), in SL-SAL group the perigonadal

fat is significantly higher than NL-SAL group (SL-SAL, 8.11 ± 0.36 vs NL-SAL, 5.18 ± 0.23, p<0.001). Despite there being an effect of treatment (p<0.05), this effect is not observed when comparing treated animals with their respective controls.

Glycemic homeostasis

The AUC of glucose concentrations during ivGTT shows that SL animals had significantly higher glucose concentrations during the test when compared with NL animals (SL-SAL, 7584,5 ± 484,7, SL-MET, 7117.583 ± 461.272; NL-SAL, 4148,4 ± 194,8, NL-MET, 4719.091 ± 268.024, p<0.001, Fig.5). In the kITT test the results show that SL group as compared to NL group insulin resistant (SL-SAL, 0,814 ± 0,06, SL-MET, 0.682 ± 0.065; NL-SAL, 1,337 ±0,11, NL-MET, 1.172 ± 0,104 p<0.001, Fig. 6). These analyses show that there was no treatment effect.

Lipid profile

Figure 7A shows that SL-SAL had a significantly higher triglyceride in NL-SAL group (SL-SAL, 143.39 ± 7.52 vs NL-SAL, 75.65 ± 4.79, p<0.001) and in NL-SAL vs SL-MET, had significant difference, that show the metformin not attenuated the SL model (p<0.001). Figure 7B shows that SL-SAL group had significantly less HDL cholesterol than NL-SAL group (SL-SAL, 17.47 ± 1.27 vs NL-SAL, 34.56 ± 1.53, p<0.001) and in NL-SAL vs SL-MET, had significant difference, that show the metformin not attenuated the SL model (p<0.001). Figure 7C shows that the SL-SAL group had a significantly higher total cholesterol than NL-SAL group (SL-SAL, 88.43 ± 5.12 vs NL-SAL, 70.42 ± 2.40, p<0.001).

Discussion

In the present study, we demonstrated for the first time that short-term treatment with Met in adulthood was not able to attenuate obesity and metabolic dysfunction induced by early overnutrition. As expected, the SL rats showed by increases in body weight associated with the increases of food intake, fat mass and liver weight, glucose intolerance, insulin resistance and dyslipidemia.

Due to great plasticity found in the lactation period, a stress in this stage can program the Individual for both health and disease. This can be demonstrated in a neonatal overfeeding model, where the animals are undergoing a process of development of tissues and organs, and the maturation, proliferation and differentiation of cellular functions [23]. Furthermore, the great

availability of milk of milk in this period, can lead the animal to overweight [4,5,24-31].

Previous works from our laboratory showed that a short treatment with Met during lactation was able to attenuate the effects generated by SL in adult animals, reducing body weight and improving glycemic parameters similar data it was observed with brief treatment with scopolamine butyl bromide during lactation which reduces adipose tissue in adult rats [4,32].

These findings suggest that lactation is a critical and sensitive plastic period to environmental disturbances, and interventions that take place during this period also aim to prevent the development of metabolic disorders. These stresses modify brain functions, synaptic plasticity and cognitive functions, which may impair the development of hypothalamic neural systems that are involved in the regulation of energy balance. Therefore, during this period, the organism may be affected, taking all these changes for the rest of life. Differently during adult life the organs are fully mature and little or none plasticity is observed [33,34].

Most of the studies that use Met, they treat for long time animals and analyze just after ending the treatment to check the effects of the drug soon after its use [35,36]. However, regarding metabolic programming using DOHaD concept it is necessary to wait a period of time to perform the analyzes in order to identify whether the effect was long term programmed [37,38].

Malta *et al.* 2016 showed that a low protein diet given during adulthood fails to programming Rats to lean phenotype, again suggesting that adulthood is not considered a programming window [39].

We admit that we did not analyze the animals after the short treatment in order to confirm That the beneficial effects of Met we lost after the recovery period, but studies in the Literature showed that the evaluation soon after treatment with Met, shows beneficial effects in decreasing the adipocyte diameter, overall adiposity, body weight and food consumption in addition to beneficial effects on cardiovascular disease risk profile [40,41].

In conclusion, considering the lack of studies testing the adulthood as a programming window, this work points out that short exposure to Met does not mitigate the effects of neonatal overfeeding in rats.

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Conflicts of Interest

The authors declare no conflict of interest

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (CONCEA), and has been approved by the institutional committee (CEUA - 3220080620).

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Figure

Figure 1 shows the entire experimental protocol.

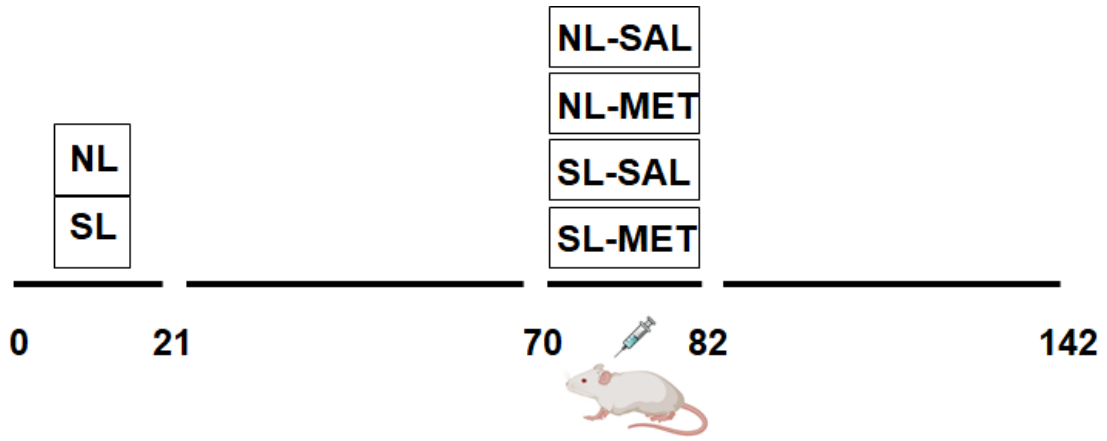

Figure 1 - Experimental design – NL means normal litter and SL means small litter, SAL is saline and MET is Metformin.

Day 21 refers to weaning day, 70-82 day refers to treatment period and 142 day is the end of the protocol.

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Figures

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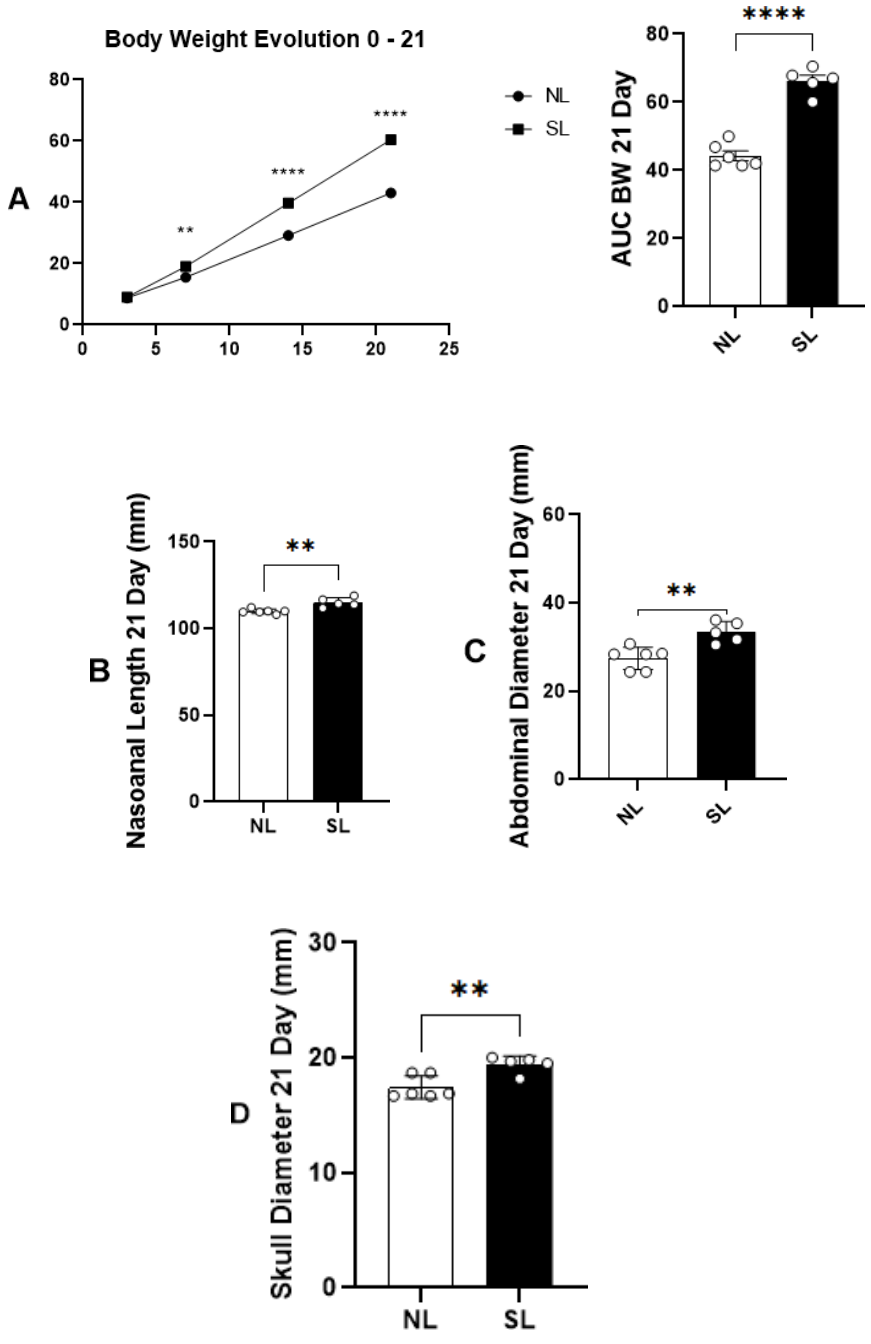

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Fig. 1 – Biometric parameters during lactation in 21 days. Bodyweight evolution and area under the curve of BW evolution (A), PN 21; nasoanal length (B), abdominal diameter (C), skull diameter (D), (N= NL - 6; SL - 5 litters). Data are presented as mean ± SEM. To compare the

experimental groups were used Student's t test, where ** $p<0.05$ and **** $p<0.0001$.

Fig. 2

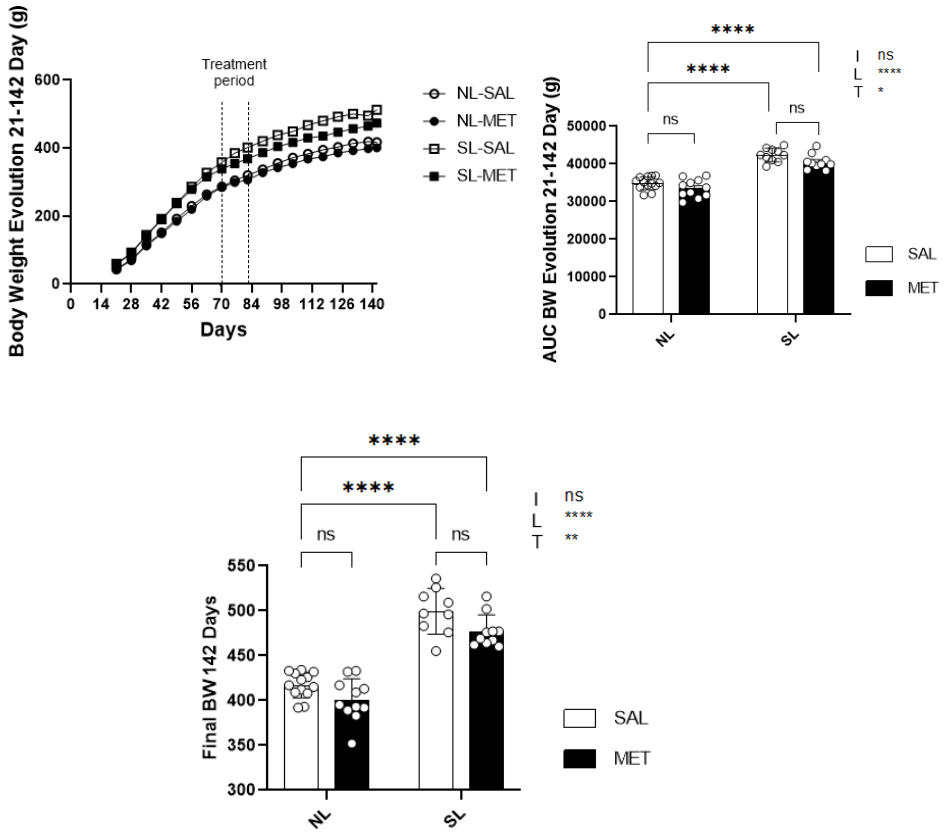

Fig. 2 – Body weight gain. Body weight evolution and area under the curve (AUC) of BW evolution PN 21 – 142. (N= NL-SAL=13; NL-MET=11; SL-SAL=10; NL-MET=10 animals). Data are presented as mean ± SEM. To compare the experimental groups was used two-way ANOVA test, where **** *p<0.0001*.

Fig. 3

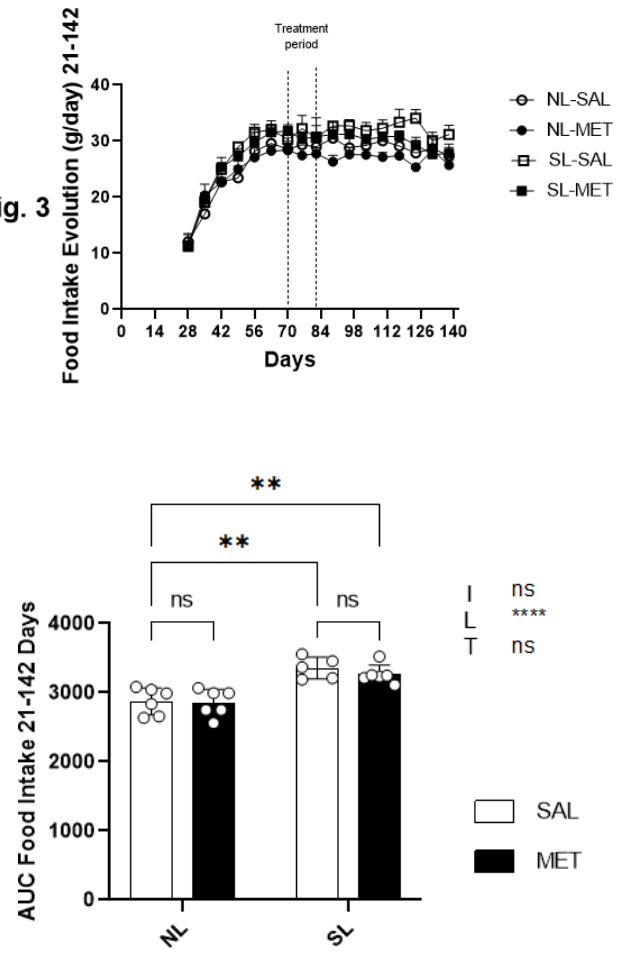

Fig. 3 – Food intake. Food intake evolution and area under the curve of food intake during 21 – 142. (NL-SAL=6; NL-MET=6; SL-SAL=5; NL-MET=6 litter). Data are presented as mean ± SEM. To compare the experimental groups was used two-way ANOVA test, where * *p<0.05*.

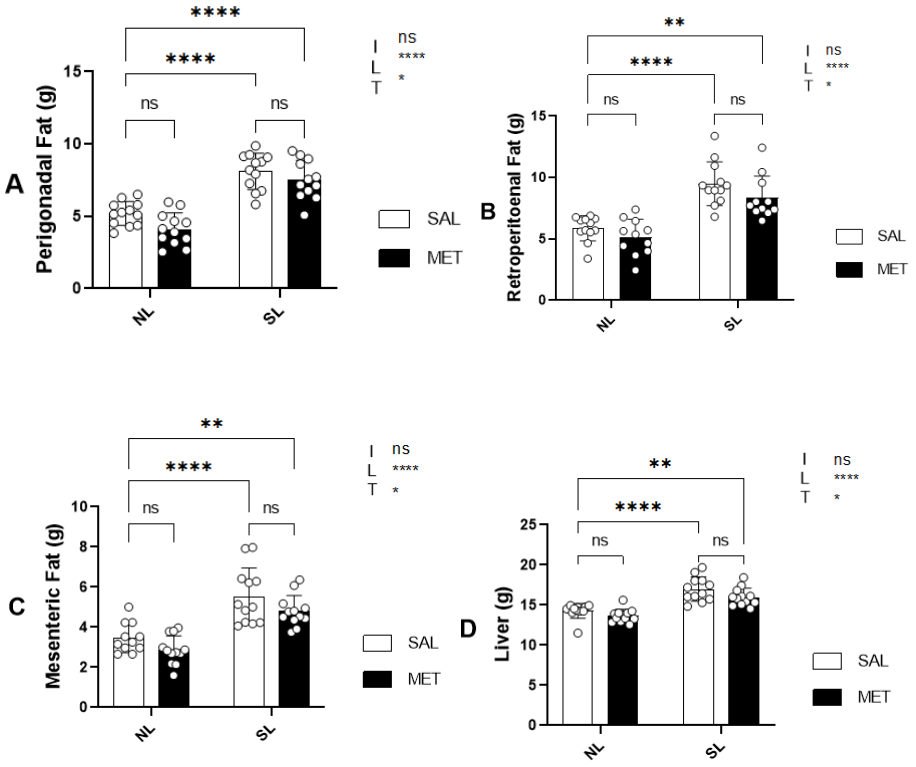

Fig. 4 – Adiposity and liver mass. Weight tissues of perigonadal fat (A), retroperitoneal fat (B) and mesenteric fat (C), liver weight (D) (N= NL-SAL=12; NL-MET=12; SL-SAL=12; NL-MET=12 animals). Data are presented as mean ± SEM. To compare the experimental groups was used two-way ANOVA test, where *** *p<0.001* and **** *p<0.0001*.

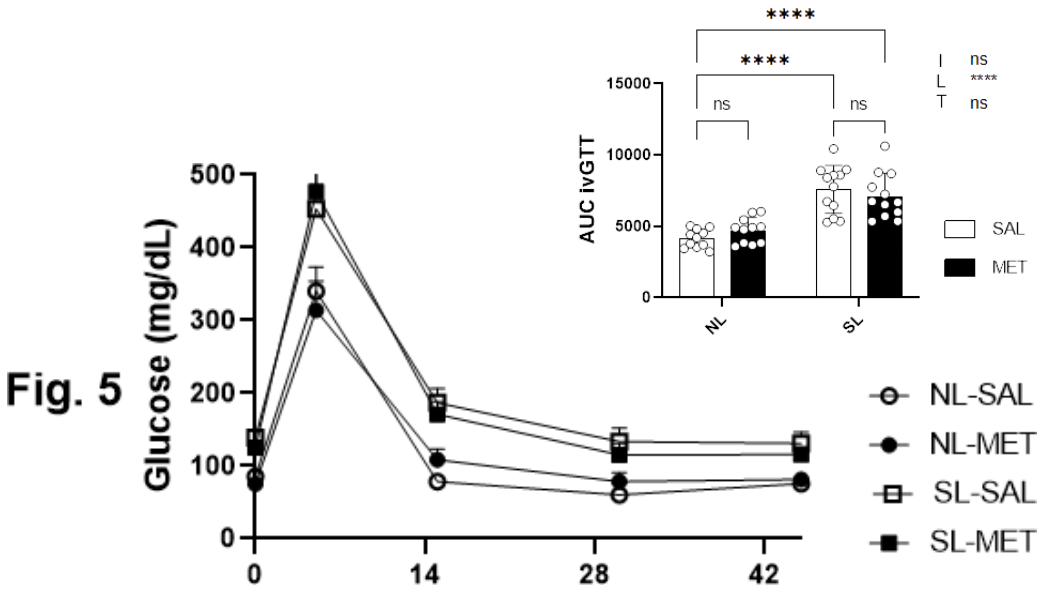

Fig. 5 – Glycemic homeostasis. The glucose concentrations during ivGTT, AUC and the fasting glucose (NL-SAL=11; NL-MET=11; SL-SAL=12; NL-MET=12 animals). Data are presented as mean ± SEM. To compare the experimental groups was used two-way ANOVA test, where *** *p<0.001* and **** *p<0.0001*.

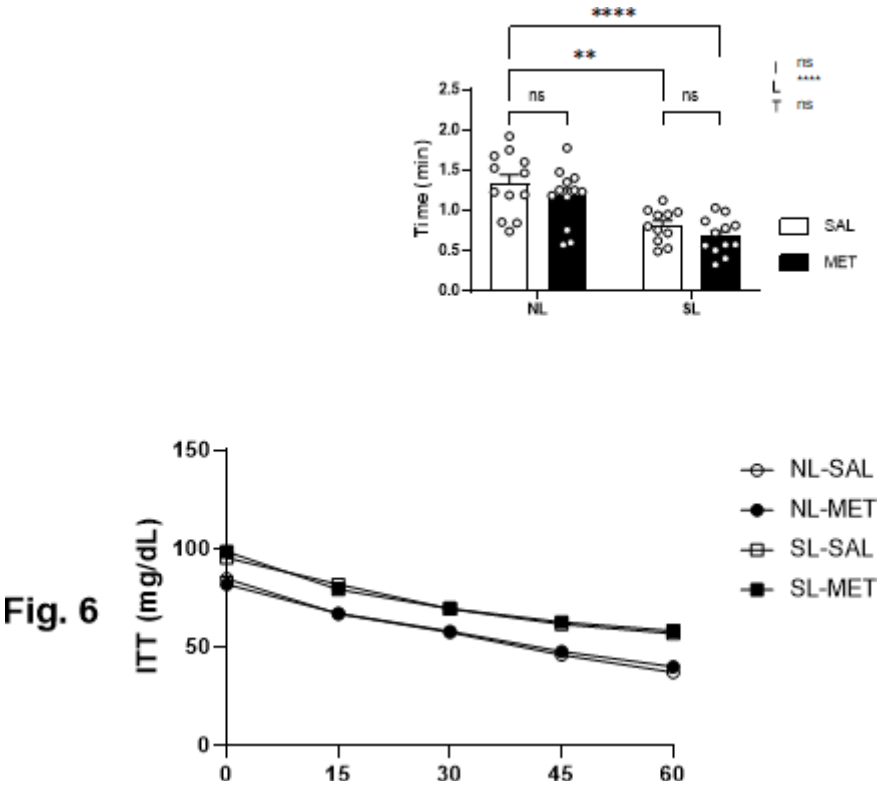

Fig. 6

Fig. 6 – The glucose concentrations during ipITT and the kITT. (NL-SAL=12; NL-MET=12; SL- SAL=12; NL-MET=12 animals). Data are presented as mean ± SEM. To compare the experimental groups was used two-way ANOVA test, where ** *p<0.01*.

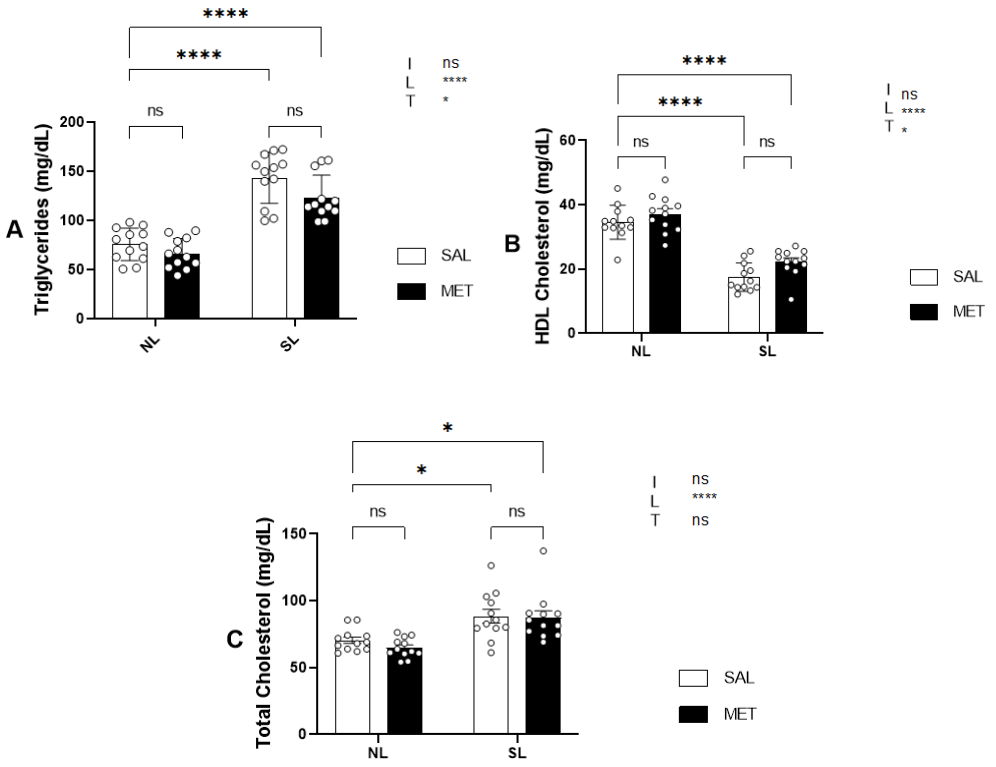

Fig. 7 – Lipid profile. Triglyceride (A), HDL cholesterol (B), total cholesterol (C). (NL-SAL=12; NL-MET=12; SL-SAL=12; NL-MET=12 animals). Data are presented as mean ± SEM. To compare the experimental groups were used two-way ANOVA test, where * *p<0.05 and* **** *p<0.0001*.