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AUDREI PAVANELLO

TRANSMISSÃO PARENTAL DA OBESIDADE: O EFEITO DA MICROBIOTA FECAL

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2018

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

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PARANÁ

ATA DA BANCA EXAMINADORA DA DEFESA DE TESE DE DOUTORADO DO PÓS-GRADUANDO AUDREI PAVANELLO. Aos dezenove dias do mês de janeiro de dois mil e dezoito, sexta-feira, realizou-se no Bloco G56, Sala 201, no campus universitário, a sessão pública da defesa de tese intitulada: **"Transmissão parental da obesidade, o envolvimento da microbiota"**, apresentado pelo pós-graduando **Audrei Pavanello**, Bacharel em Ciências Biológicas pela Universidade Estadual do Oeste do Paraná, que concluiu os créditos exigidos para obtenção do grau de "Doutor em Ciências Biológicas". Os trabalhos foram instalados às 15:00 h, pelo *Prof. Dr. Paulo Cezar de Freitas Mathias*, Presidente da Banca Examinadora, constituída pelos seguintes professores: Dra. Patrícia Cristina Lisbôa da Silva, Dra. Sandra Lucinei Balbo, Dr. Marcos Ricardo da Silva Rodrigues, Dr. Giovani Marino Favero como membros. A Banca Examinadora, tendo se decidido a aceitar a tese, passou à arguição pública do candidato. Encerrados os trabalhos de arguição às 18:00 h. horas, os examinadores deram parecer final, considerando a tese APROVADO. Proclamado o resultado pelo Presidente da Banca Examinadora, foram encerrados os trabalhos e lavrada a presente ata que vai assinada pelos membros da Banca Examinadora. Maringá, aos dezenove dias do mês de janeiro dois mil e dezoito.

Prof. Dr. Paulo Cezar de Freitas Mathias

Profa. Dra. Patrícia Cristina Lisbôa da Silva

Proaf. Dra. Sandra Lucinei Balbo

Prof. Dr. Marcos Ricardo da Silva Rodrigues

Prof. Dr. Giovani Marino Favero

BIOGRAFIA

Audrei Pavanello nasceu em Francisco Beltrão/PR em 08/02/1992. Possui graduação em Ciências Biológicas Bacharelado pela Universidade Estadual do Oeste do Paraná (UNIOESTE) (2012). Realizou seu mestrado na Universidade Estadual de Maringá (UEM), onde obteu o título de mestre em Ciências Biológicas no Programa de Pós-Graduação em Ciencias Biológicas- Área de Concentração:Biologia Celular e Molecular (PBC) em 2015. Atualmente é doutorando do PBC na UEM. Tem experiência na área de Biologia Celular e Fisiologia, atuando principalmente nos seguintes temas: obesidade, secreção de insulina e microbiota intestinal.

AGRADECIMENTOS

Gostaria de agradecer aos meus pais, Creuseli Lopes Pavanello e Estevo Pavanello, pelo apoio durante todos esses anos, pela dedicação e pelo cuidado e por tudo que eles fizeram por mim.

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A todas os colaboradores com quem eu trabalhei no Laboratório de Biologia Celular e Secreção, sem os quais também esse trabalho não seria possível, tanto na teoria como na prática.

As agências de fomento, a CAPES pela concessão da bolsa de estudos e ao CNPQ pelos editais para fomento da pesquisa.

APRESENTAÇÃO

Esta tese é composta de dois artigos científicos. O primeiro manuscrito, "**Parental obesity programs pancreatic islet disfunction in obese female rat offspring"** demonstra o efeito da obesidade parental em programar disfunção metabólica em ratas fêmeas obesas. Será submetido a revista "Journal of Cellular Physiology", com fator de impacto 4.08 e qualis A2 no comitê das Ciências Biológicas 1.

O segundo artigo, "**Fecal microbiota transplantation during lactation promotes protection of pancreatic islet function in obese female rats**" descreve o efeito protetor do transplante de microbiota fecal durante o início da vida contra a disfunção pancreática em ratas obesas. Será submetido a revista "Journal Endocrinology", com fator de impacto 4.7 e qualis A2 no comitê das Ciências Biológicas 1.

Audrei Pavanello[1], Isabela Peixoto Martins[1], Laize Peron Tófolo[1], Carina Previate[1], Ana Maria Praxedes[1], Camila Cristina Ianoni Matiusso[1], Paulo Henrique Olivieri da Silva[1], Kelly Valério Prates[1,] Vander da Silva Alves[1], Douglas Lopes de Almeida[1], Ananda Malta[1] Paulo Cezar de Freitas Mathias[1]; **Parental obesity programs pancreatic islet disfunction in obese female rat offspring**

Audrei Pavanello[1], Isabela Peixoto Martins[1], Laize Peron Tófolo[1], Carina Previate[1], Camila Cristina Ianoni Matiusso[1], Ana Maria Praxedes[1], Paulo Henrique Olivieri da Silva[1], Kelly Valério Prates[1], Ananda Malta[1], Vander da Silva Alves[1], Douglas Lopes de Almeida[1], Ananda Malta[1], Paulo Cezar de Freitas Mathias[1]; **Fecal microbiota transplantation during lactation promotes protection of pancreatic islet function in obese female rats**

RESUMO GERAL

INTRODUÇÃO

Manuscrito 1: A obesidade é uma doença multifatorial, o estado nutricional e o sedentarismo são implicados como as maiores causas do aumento da mesma. Um novo conceito ligando o início da vida a doenças metabólicas na vida se mostrou como uma nova via de explicar a epidemia da obesidade. Seguindo esse conceito, intitulado "Developmental Origins of Health and Disease" (DOHaD), insultos durante o início da vida podem levar a doenças metabólicas na vida adulta. A gestação e a lactação são janelas onde esses insultos podem programar o metabolismo e a saúde metabólica de mulheres é especialmente importante durante essa fase. A obesidade materna durante essas fases pode programar o fenótipo da prole para o desenvolvimento da obesidade durante a vida adulta.

Manuscrito 2: A microbiota intestinal corresponde a um conjunto de células que vive de forma simbiótica com o hospedeiro. Há pouco tempo foi descoberto que a microbiota apresenta efeitos no metabolismo do hospedeiro, influenciando a deposição de gordura e também a secreção de hormônios entéricos. Insultos no início da vida podem programar o metabolismo, mas a contribuição da microbiota para isso ainda não é clara.

OBJETIVOS

Manuscrito 1: Investigar o efeito da programação metabólica causada por pais obesos no fenótipo da vida adulta em ratas Wistar obesas.

Manuscrito 2: Investigar o efeito do transplante de microbiota fecal durante o início da vida em ratas Wistar fêmeas.

MÉTODOS

Manuscrito 1: Para induzir obesidade, ratos Wistar foram cruzados e a prole teve seu

tamanho de ninhada ajustado para 9 filhotes por mãe (NL) e 3 filhotes por mãe (SL). Esses filhotes foram cruzados com 90 dias de vida e a sua prole teve novamente o tamanho de ninhada ajustado, somente a prole fêmea foi usada, criando quatro grupos experimentais: Pais ninhada normal com prole ninhada normal (NLNL); Pais ninhada normal com prole redução de ninhada (NLSL); Pais ninhada reduzida com prole ninhada normal (SLNL); Prole ninhada reduzida com prole ninhada reduzida (SLSL).

Manuscrito 2: Machos e fêmeas de ninhada normal foram cruzados com 90 dias de vida, ninhada normal (NL) com ninhada normal e ninhada reduzida com ninhada reduzida (SL). No 3° dia após o nascimento, a prole teve seu tamanho de ninhada ajustado para 9 filhotes (NL) ou 3 filhotes (SL), somente fêmeas foram utilizadas no experimento. Do 10° ao 25° dia de vida a prole recebeu gavagem de uma solução contendo as fezes diluídas da mãe oposta ou solução salina na mesma concentração. Quatro grupos experimentais foram criados de acordo com a condição de obesidade no início da vida e o transplante de microbiota fecal: Prole de ninhada normal que recebeu solução salina (NLS); prole de ninhada normal que recebeu microbiota fecal diluída (NLM); prole de ninhada reduzida que recebeu salina (SLS); prole de ninhada reduzida que recebeu solução de microbiota fecal diluída (SLM).

RESULTADOS E DISCUSSÃO

Manuscrito 1: Superalimentação no início da vida causou aumento do peso corporal do 21° ao 90° dia de vida. Obesidade parental causou aumento na deposição de gordura em SLNL e SLSL. Também causou diminuição nos níveis de colesterol total e HDL-C em SLNL e SLSL, levando a um aumento do risco de aterosclerose medido pelo índice casteli I e II. SLSL teve hiperinsulinemia de jejum comparado com todos os grupos experimentais e resistência à insulina. NLSL e SLSL tiveram diminuída secreção de insulina em 5.6,8.3 e 16 mmol/L de glicose. NLSL teve aumento da resposta colinérgica a acetilcolina e responda diminuída ao antagonista do receptor M3 4-DAMP. Todos os grupos tiveram resposta adrenérgica diminuída a adrenalina e a um antagonista do receptor α2 comparado a NLNL.

Manuscrito 2: O transplante de microbiota fecal levou a uma diminuição do ganho de peso durante a vida e aumento dos depósitos de gordura no grupo SLM. Esses animais tiveram aumento do HDL-C e níveis totais de colesterol, refletindo num risco menor de aterosclerose estimado pelo índice castelli I e II. Animais NLM mostram o efeito oposto. Obesidade no início da vida causou intolerância a glicose nos grupos SLS e SLM, mas o transplante de microbiota fecal protegeu contra a resistência a insulina em animais SLM. Todos os grupos tiveram aumento da resposta secretória em 5.6 e 8.3 mmol/L de glicose comparados com NLS, o transplante de microbiota fecal diminuiu esse valor em 16.7 mmol/L de glicose nos grupos NLM e SLM. O transplante de microbiota fecal levou a uma diminuída resposta insulinotrópica colinérgica. Animais NLM mostraram aumento da resposta insulinoestática adrenérgica, animais SLM mostraram uma resposta contrária no mesmo parâmetro.

CONCLUSÕES

Manuscrito 1: Obesidade Parental programou disfunção metabólica em ratas Wistar, com menor resposta colinérgica e adrenérgica.

Manuscrito 2: Transplante de microbiota fecal durante o inicio da vida protegeu contra disfunção das ilhotas pancreáticas causadas por obesidade em ratas Wistar.

GENERAL ABSTRACT

INTRODUCTION

Manuscript 1: A new prospect to study obesity is that early life insults program metabolic diseases later in life. Obesity is transmitted to the following generation, and females were especially important in this transmission, whereas the offspring can be programmed to obesity because of the parental metabolic health.

Manuscript 2: Intestinal microbiota is involved in many physiological process. Recently, it has been implied that the microbiota is involved in obesity onset. The first contact happens during early life but the effects of microbiota in metabolic programming at adulthood are still not understood.

AIMS

Manuscript 1: The aim of this work was to evaluate the effects of parental obesity metabolic programming in the metabolic health of female lean and obese rats.

Manuscript 2: The aim of this work was to evaluate the transplantation of fecal microbiota during lactation to female offspring rats from lean and obese mothers. Obese parents were induced by litter reduction during the lactational period.

METHODS

Manuscript 1: To induce obesity, wistar rats were mated and the offspring had their litter size adjusted to 9 pups per dam (NL) and 3 pups per dam (SL). Those were mated at 90th days old and the offspring had their litter size adjusted, only female offspring were used, creating four experimental groups: Normal Litter parents with normal litter offspring (NLNL); Normal Litter parents with Small Litter offspring (NLSL); Small Litter parents with Normal Litter offspring (SLNL) and Small Litter Parents with Small Litter offspring (SLSL). Early overfeeding caused increased body weight at 21th and 90th days old.

Manuscript 2: At 90th days old, NL and SL males and females (parents), from different litters, were mated, NL male vs NL female; SL male vs SL female. At birth, the litter was standardized in the 3rd day of life to NL or SL, only females were used. From the 10th until the 25th day of life the offspring received gavage of a solution containing the diluted feces of the opposite dam or saline solution in the same concentration. Four experimental groups were created using early life obesity condition and transplantation of fecal microbiota; normal litter offspring that received saline (NLS), normal litter offspring that received diluted fecal microbiota (NLM), small litter offspring that received saline (SLS), small litter offspring that received diluted fecal microbiota (SLM).

RESULTS AND DISCUSSION

Manuscript 1: Early overfeeding caused increased body weight at 21th and 90th days old. Parental obesity caused increased fat deposition at adulthood in SLNL and SLSL. It also caused decreased total cholesterol and HDL-C levels in SLNL and SLSL groups, leading to increased atherosclerosis risk as evaluated by castelli index I and II. SLSL had fasting hyperinsulinemia compared with all groups and insulin resistance. NLSL and SLNL had decreased insulin secretion at 5.6, 8.3 and 16.7 mmol/L of glucose. NLSL had increased cholinergic response to acetylcholine and decreased response to M3 antagonist 4-DAMP. All groups had lower adrenergic response compared with NLNL to adrenaline and an α2 antagonist.

Manuscript 2: Fecal microbiota transplantation caused decreased body weight gain during life and increased fat deposition in the SLM group. SLM animals had increased HDL-C and total cholesterol levels, reflecting in lower atherosclerosis risk assessment by the castelli index I and II. By the other hand, NLM animals showed the opposite effect. Early life obesity caused glucose intolerance in SLS and SLM groups, but fecal microbiota transplantation protected against insulin resistance in SLM animals. All groups had increased secretory response of insulin in 5.6 and 8.3 mmol/L of glucose compared with NLS, but fecal microbiota transplantation lowered this value in 16.7 mmol/L of glucose in NLM and SLM groups. Fecal microbiota transplantation lead to decreased cholinergic

insulinotropic response. NLM animals showed increased adrenergic insulinostatic response, SLM animals showed an opposite response to the same parameter.

CONCLUSIONS

Manuscript 1: Parental obesity programmed metabolic dysfunction in female wistar rats, with lower cholinergic and adrenergic pancreatic response.

Manuscript 2: Fecal microbiota transplantation caused protection against pancreatic islet dysfunction caused by obesity in early life.

TEXTOS REFERENTES AOS MANUSCRITOS

MANUSCRITO 1

Parental obesity programs pancreatic islet disfunction in obese female rat offspring

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Keywords: metabolic programming, parental obesity, insulin secretion, postnatal nutrition.

Abstract: A new prospect to study obesity is that early life insults program metabolic diseases later in life. Obesity is transmitted to the following generation, and females were especially important in this transmission, whereas the offspring can be programmed to obesity because of the parental metabolic health. The current study tested the influence of parental obesity to program female offspring to metabolic diseases in adulthood . To induce obesity, wistar rats were mated and the offspring had their litter size adjusted to 9 pups per dam (NL) and 3 pups per dam (SL). Those were mated at 90th days old and the offspring had their litter size adjusted, only female offspring were used, creating four experimental groups: Normal Litter parents with normal litter offspring (NLNL); Normal Litter parents with Small Litter offspring (NLSL); Small Litter parents with Normal Litter offspring (SLNL) and Small Litter Parents with Small Litter offspring (SLSL). Early overfeeding caused increased body weight at 21th and 90th days old. Parental obesity caused increased fat deposition at adulthood in SLNL and SLSL. It also caused decreased total cholesterol and HDL-C levels in SLNL and SLSL groups, leading to increased atherosclerosis risk as evaluated by castelli index I and II. SLSL had fasting hyperinsulinemia compared with all groups and insulin resistance. NLSL and SLNL had decreased insulin secretion at 5.6, 8.3 and 16.7 mmol/L of glucose. NLSL had increased cholinergic response to acetylcholine and decreased response to M3 antagonist 4-DAMP. All groups had lower adrenergic response compared with NLNL to adrenaline and an α2 antagonist. Parental obesity programmed metabolic dysfunction in female wistar rats, with lower cholinergic and adrenergic pancreatic response.

Introduction

Cardiometabolic diseases such as obesity nowadays are the principal source of mortality in the world (Global Burden of Metabolic Risk Factors for Chronic Diseases, 2014). Although, at first this epidemic was confined in developed countries, the levels of obesity have increased worldwide in a worrying trend (Ng et al., 2014). Globally its estimated that 2.1 billion people are overweight or obese (Smith and Smith, 2016). Obesity has a complex etiology and many factors are involved in it onset. Poor diets including high density energy foods such as industrialized food and behavioral factors like a sedentary lifestyle are commonly associated as the principal factors involved in the genesis of obesity and its associated comorbidities(Smith and Smith, 2016).

Those factors alone and other influences, such as genes, cannot explain alone the epidemic proportions of the increase in obesity and overweight levels. New epidemiologic and experimental data suggests that early life is strongly important in shaping metabolic health at adulthood (Szyf, 2009). The first study showing this relationship were made by professor David Barker that showed that low birth weight has an association with increased blood pressure during adulthood (Barker et al., 1990). This relationship leads to the concept of “Developmental Origins of Health and Disease” (DOHaD) (Hanson, 2015). According with this concept, early life insults during specific periods such as pregnancy, lactation and adolescence programs the metabolism to metabolic diseases in later life (Vickers, 2014).

Gestation and lactation maternal nutritional and metabolic status is a strong predictor of future health in the offspring (Poston, 2012). According with this, its observed in humans

and animal models that offspring of obese parents have an increased risk for obesity in childhood and also in adulthood (Catalano and deMouzon, 2015).

Early overfeeding induced by litter size reduction during lactation to three pups per lactating dam programs the metabolism of rats to develop overweight, hyperinsulinemia, hyperglycemia, hiperleptinemia and higher fat pad deposition. (de Almeida et al., 2013; Plagemann et al., 2009; Plagemann et al., 1992). Its a widely animal model of metabolic programming to study early life insults and its effects at adulthood.

The increased focus on studies in pregnant women, lead to many studies investigating the pathophysiological effects of gestation in obese mothers. Still, there is a gap of knowledge regarding the effects that those mothers pass to female offspring, and how the metabolism of those females develops until adulthood. The aim of this work was to evaluate the effects of parental obesity metabolic programming in the metabolic health of female lean and obese rats.

Methods

Ethical approval

All experiments were undertaken according to the norms established by the Brazilian Association for Animal Experimentation (COBEA) and were approved by the Ethics Committee in Animal Research of the State University of Maringá (protocol number 9648231014).

Animal model and experimental design

Obtainment of parental obesity

Obese parents were induced by litter reduction during the lactational period. For this, control Wistar rats were mated (three 70 days-old females for one 80 days-old male). Pregnant females were separated and placed in individual cages with free access to water and food. At birth, the litter was standardized in the 3rd day of life to 9 pups per dam (normal litter, NL) or 3 pups per dam (small litter, SL).

Offspring

At 90th days old, NL and SL males and females (parents), from different litters, were mated, NL male vs NL female; SL male vs SL female. Pregnant female were separated and placed in individual cages with free access to water and food. At birth, the litter was standardized in the 3rd day of life to NL or SL. A mix of male and female offspring was utilized. Thus, four groups were obtained according to parental conditions: Normal Litter parents with normal litter offspring (NLNL); Normal Litter parents with Small Litter offspring (NLSL); Small Litter parents with Normal Litter offspring (SLNL) and Small Litter Parents with Small Litter offspring (SLSL). After that, female offspring were weaned at 21th days of life (3 animals per cage) and were kept under controlled temperature (22±2 C°), photoperiod (12 hours dark and 12h hours of light – 07:00 a.m. – 07:00 p.m.) and had *ad libitum* access to water and the standard chow (Nuvital®, Curitiba/PR, Brazil).

Body weight and food intake evaluation

After weaning, offspring from all groups were weighed and food intake was determined tree times per week. Food intake was calculated for each cage as $\frac{\frac{(Chow\ placed - chow\ eaten)}{number\ of\ animals}}{number\ of\ days}$ for the absolute food intake (expressed in g) and the relative food intake was calculated by

$\left(\frac{(absolute\ food\ intake*100)}{mean\ of body\ weight}\right)$ and expressed as g/100g of bw. The total area under the curve (AUC) of food consumption throughout experimental protocol was calculated.

Obesity evaluation

At 90th days old, animals from all groups were weighted, anaesthetized by an intraperitoneal injection of pentobarbital sodium (thiopental 45 mg/kg bw) and euthanized by decapitation to had blood samples collected,centrifuged and the plasma was stored at -20 C° for biochemical analyses. Insulin concentration was determined by radioimmunoassay (RIA). The limit of detection was 0.006 ng/ml. The measurements were performed in a single assay. The retroperitoneal, perieuterin, periovarian and mesenteric fat pads were removed and weighed, and the results were expressed in relation to the bw of each animal (g/100 g bw).

Intravenous glucose tolerance test (ivGTT)

At 91thdays old, rats from all groups underwent a surgery for the silicone cannula implantation into the right jugular vein, as previously described (de Oliveira et al., 2011). At 24 hours after the surgery, and an overnight fasting, the rats received a glucose infusion (1 g/kg bw). Blood samples were collected in heparinized syringes at 0 (before glucose administration), 5, 15, 30 and 45 min after the glucose administration. Plasma samples were stored at -20°C for determination of glucose concentration. The glucose concentration was evaluated by the glucose oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The AUC of glycemia and throughout the ivGTT was calculated.

Intraperitoneal insulin tolerance test (ipITT)

The animals were fasted for 6h prior to an intraperitoneal insulin tolerance test (1 U/kg bw), as previously described (Miranda et al., 2014). The blood was collected in the tail of the animals with capillary tubes. After the test, the blood was centrifuged and plasma samples were samples were stored at -20°C for determination of glucose concentration .The absorption rate of glucose by the tissue (rate constant for the disappearance of plasma glucose, K_{itt}) was calculated as already described (Lundbaek, 1962). The plasma glucose t1/2 was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decline.

Lipid Profile

The plasma samples were used to determine the concentrations of the Total Cholesterol, HDL-Cholesterol and Triglycerides. Triglyceridemia was determined by the lipase lipoprotein – glycerol-3 phosphate oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The Total Cholesterol was determined by the cholesterol oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil) and HDL-Cholesterol was precipitated and determined by the cholesterol oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The VLDL-Cholesterol and LDL-Cholesterol was evaluated using the Friedwald equation (Fukuyama et al., 2008), whereas the VLDL concentration was $trygliceridemia/5$ and LDL was $total\ cholesterol - (HDL - VLDL)$. The Castelli Index was determined as showed by (Millan et al., 2009), with the Castelli Index I equal to $total\ cholesterol/HDL$ and Castelli Index II equal to LDL/HDL.

Pancreatic islets isolation and insulin secretion measurements

Pancreatic islets were isolated by collagenase digestion and washed with Hank's solution as previously described (Gravena et al., 2002). Groups of four isolated islets were pre-incubated for 60 min at 37 °C in Krebs solution containing 5.6 mmol/l glucose in a mixture of 95% O_2: 5% CO_2 at pH 7.4 to stabilize insulin secretion. After that, these islets were used in one of the experimental condition to assess the responsivity to glucose as well as autonomic agents. To study the glucose-insulinotropic response, a batch of islets were incubated for 60 min more under the different glucose concentrations (5.6, 8.3 and 16.7 mmol/l). To study the muscarinic acetylcholine receptor (mAChR) function, another batch of the islets were incubated with a solution containing either 8.3 mmol/l glucose or 8.3 mmol/l glucose plus, 10 µmol /l acetylcholine in the presence of 10 µmol/l neostigmine to avoid acetylcholinesterase action in the islets. To study the insulinotrpic fucntion of the mAChR subtype M3, 100 µmol/l of the selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) were used. To study adrenergic receptor function in the islets, another batch of the islets were incubated in the presence of 16.7 mmol/l of glucose plus 1 µmol/l of adrenaline. To block α2 adrenergic receptor, we used an antagonist, Yohimbine (10 µmol/l) with 16.7 mmol/l of glucose plus 1 µmol/l of adrenaline. The supernatants from the incubations were collected and stored at –20°C for further insulin measurements by RIA.

Statistical analysis

The results are expressed as the mean ± SEM. Data were submitted to Student t-test or variance analysis (two-way ANOVA) with the factors: early overfeeding (e), parental obesity (p) and early overfeeding x parental obesity (i). In the case of analyses with a significant F, the differences between the means were evaluated by Tukey's test; p<0,05) were considered statistically significant. Tests were performed using GraphPad Prism version 7 for Windows (GraphPad Software Inc., San Diego/CA, USA).

Results

Biometric Parameters

Weight gain and food intake throughout life

No group of animals showed increased body weight throughout life (**figure 1**). The same pattern was observed in the relative food intake (**figure 2**).

Body weight and fat pad deposition

At 21th days old, early overfeeding lead to increased body weight in NLSL and SLSL groups (p^e<0.0001) when compared with NLNL (p<0.001 vs NLSL, p<0.0001 vs SLSL) and SLNL (p<0.01 vs NLSL, p<0.0001 vs SLSL). At 90th days old the same pattern was observed, but with lower intensity (p^e<0.01). NLSL and SLSL had increased bodyweight compared with NLNL (p<0.05 and p<0.05, respectively) (**figure 3A and 3B**). No differences in fat pad deposition were observed in the periuterin fat pad. Regarding the periovarian fat pad, early overfeeding was the predominant factor leading to increased fat pad deposition (p^e<0.0001). All groups had higher deposition compared with SLNL (p<0.01 vs NLNL, p<0.0001 vs NLSL, p<0.01 vs SLSL). The retroperitoneal and

mesenteric fat pads had parental obesity as the predominant factor ($p^p<0.001$ and $p^p<0.0001$, respectively). leading to increased fat deposition in SLNL and SLSL groups compared to NLNL and NLSL (**figure 3C,3D,3E and 3F**).

Biochemical Parameters

Lipid Profile

No differences between the groups were observed in the trygliceridemia and VLDL-cholesterol (**figure 4A and 4E**). Parental obesity lead to decreased total cholesterol ($p^p<0.0001$) and HDL-cholesterol ($p^p<0.0001$) (**figure 4B and 4C**). By the other hand, early overfeeding lead to increased levels of LDL-cholesterol ($p^e<0.01$) (**figure 4D**).

Atherosclerosis risk indexes

Parental obesity lead to an increased castelli index I ($p^p<0.001$). The SLSL group had an increased index compared with NLNL ($p<0.01$) and NLSL ($p<0.01$). The same pattern was observed in the castelli index II, but the increased levels of the SLSL group caused an interaction of the factors ($p^i<0.05$), still, the predominant factor was parental obesity ($p^p<0.0001$) (**figure 5**).

Glycemic Profile

No differences were observed in the fasting glycemia, but the SLSL group had an increased fasting insulinemia compared with all groups ($p^i<0.05$) ($p<0.05$ vs NLNL, $p<0.01$ vs NLSL, $p<0.05$ vs SLNL) (**figure 6**). Regarding the ivGTT, parental obesity offspring had an increased glucose intolerance ($p^p<0.05$) compared with lean parents

232 animals (**figure 7**). Early overfeeding lead to decreased peripherical insulin sensitivity as
233 assessed by the ITT (p^e<0.01) (**figure 8**).
234
235 *Pancreatic isolated islets insulin secretion*
236 The secretory response of NLSL and SLNL was impaired compared with NLNL and SLSL
237 at 5.6 mmol/L of glucose (p^i<0.05) and was progressively decreased in higher glucose
238 concentrations (8.3 mmol/L p^i<0.001; 16.7 mmol/L p^i<0.001) (**figure 9A**). When the islets
239 were challenged with acetylcholine in 8.3 mmol/L of glucose, the NLSL group had a higher
240 response compared with all groups (pi<0.05; p<0.01 vs NLNL, p<0.0001 vs SLNL,
241 p<0.0001 vs SLSL). Nevertheless, when those islets were added a muscarinic M3
242 antagonist (4-DAMP), the response of the NLSL group was inverted, with all groups
243 having a higher response (p^i<0.0001; p<0.0001 vs NLNL, p<0.0001 vs SLNL, p<0.0001
244 vs SLSL) (**figure 9B and 9C**). Finally, the insulinostatic effect of adrenaline was
245 decreased in all groups compared with NLNL (p^i<0.0001; p<0.0001 vs NLSL, p<0.0001
246 vs SLNL, p<0.0001 vs SLSL) and the same pattern was observed when the islets were
247 added an α2 receptor antagonist (Yohimbine) (p^i<0.05; p<0.0001 vs NLSL, p<0.0001 vs
248 SLNL, p<0.0001 vs SLSL) (**figure 9D and 9E**).
249
250 **Discussion**
251
252 The present study demonstrate that parental obesity programming is strongly affected by
253 postnatal nutrition. Is of note that being programmed by parental obesity alone have a
254 different effect on the outcome at adulthood, with a different glycemic and pancreatic

255 profile. Early overfeeding during lactation programs by increasing insulin levels during
256 early life (Plagemann et al., 1992). The effect of insulin on the hypothalamus affects the
257 neural circuitry involving the maturation of the feeding behavior and leads to increased
258 food intake during lactation (Plagemann et al., 2009). Insulin is a growth factor and
259 increased insulin levels are related with increased body weight (Marcovecchio and
260 Chiarelli, 2013). This effect is evident at an early age in animals, with increased body
261 weight at early life, and a lower difference at adulthood. This is in agreement with the
262 literature (Argente-Arizon et al., 2016).
263 Decreased levels of HLD-C were found on parental obesity programmed rats. This
264 reflected an increased atherosclerosis risk as assessed by the castelli index. This index
265 is a very useful marker for estimating the risk of cardiac diseases (Millan et al., 2009).
266 HDL-C levels are lower in obese individuals and are related with increased cardiovascular
267 risk (Barter, 2011). The relationship between how HDL-C levels decrease in an obesity
268 state are complex. Obesity has a strong inflammatory component, this is especially
269 important for the adipose tissue, where inflammation lead to the release of
270 proinflammatory cytokines that leads to low grade systemic inflammation in the body
271 (Wensveen et al., 2015). HDL-C has antiinflammatory and antioxidant effect on the body
272 (Kontush and Chapman, 2006). Some evidence suggests that the increased reactive
273 oxygen species on obese individuals associated with increased inflammation leads to
274 lower levels of HDL-C (Kontush and Chapman, 2006).
275 Inflammation has an effect on pancreatic function, leading to B-cell apoptosis and
276 impaired insulin secretion (Donath et al., 2013). This could be the case for SLNL animals,
277 that displays lower levels of glucose induced insulin secretion (GSIS). The autonomic

nervous system modulates insulin secretion via parasympathetic and sympathetic activity on the pancreas (Kiba, 2004). Acetylcholine has an insulinotropic effect on GSIS via the M3 muscarinic receptor (M3achR) (Ruiz de Azua et al., 2012) and adrenaline has an insulinostatic effect via the α2 adrenergic receptor (Liggett, 2009). The modulation of those branches dictates how much insulin will be secreted during GSIS. Parental obesity programmed a lower response on M3 receptor on pancreatic islets associated with decreased adrenergic inhibitory response. This could explain the higher levels of insulin observed and the increased fat deposition on those animals and suggests that parental obesity programming has an effect on the autonomic nervous system as a whole.

Parental obesity programmed metabolic dysfunction in female wistar rats, associated with lower cholinergic and adrenergic pancreatic response and those pathways can be a target to look for possible approaches in treating obesity.

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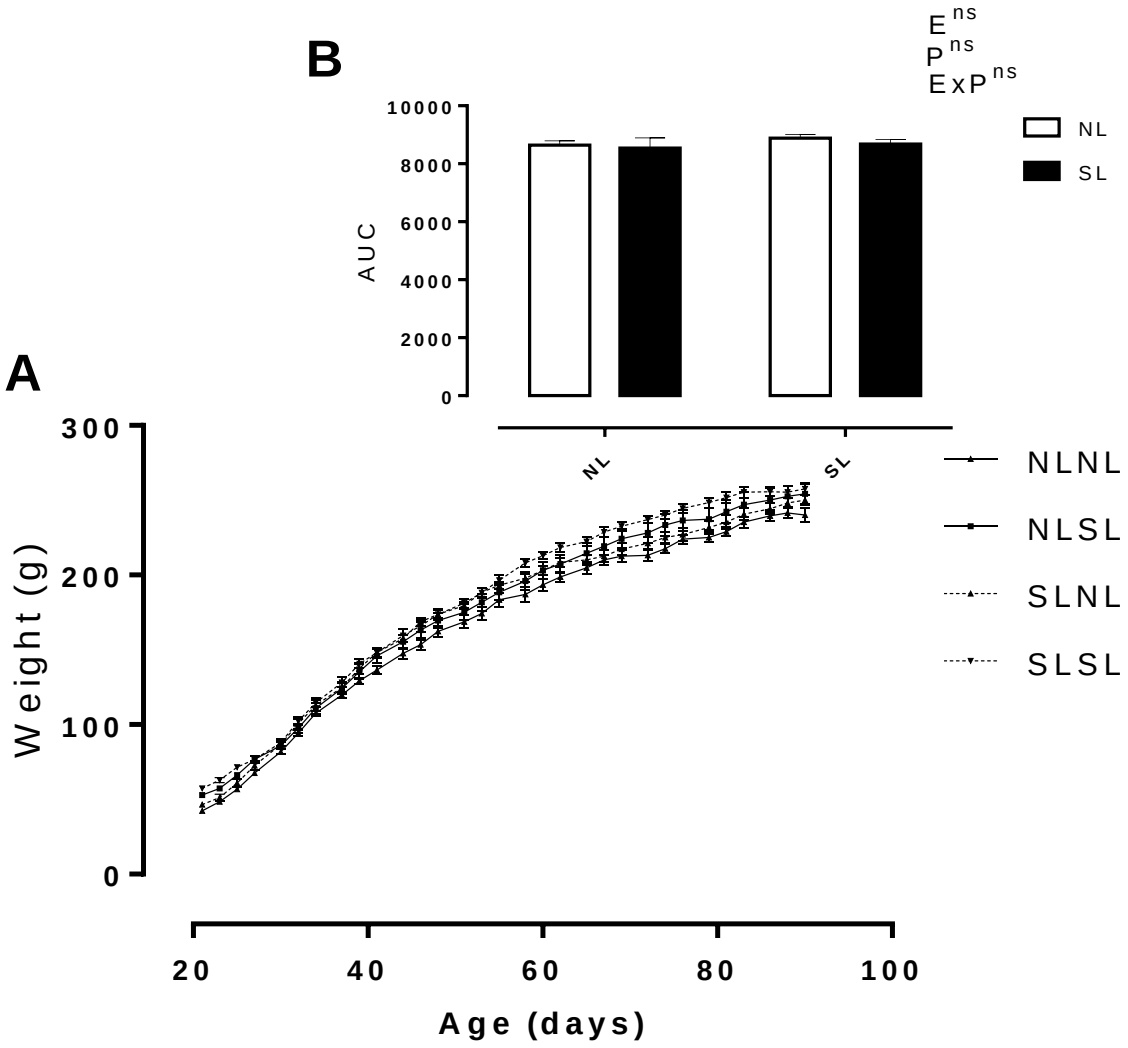
Figures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E ns
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8000
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4000
2000
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NL
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A
300
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100
0
Weight (g)
20
40
60
80
100
Age (days)
NLNL
NLSL
SLNL
SLSL

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Figure 1: Body weight growth from 21th days until 90th days old (**A**), Area under curve of body weight (**B**), n=12, 4 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

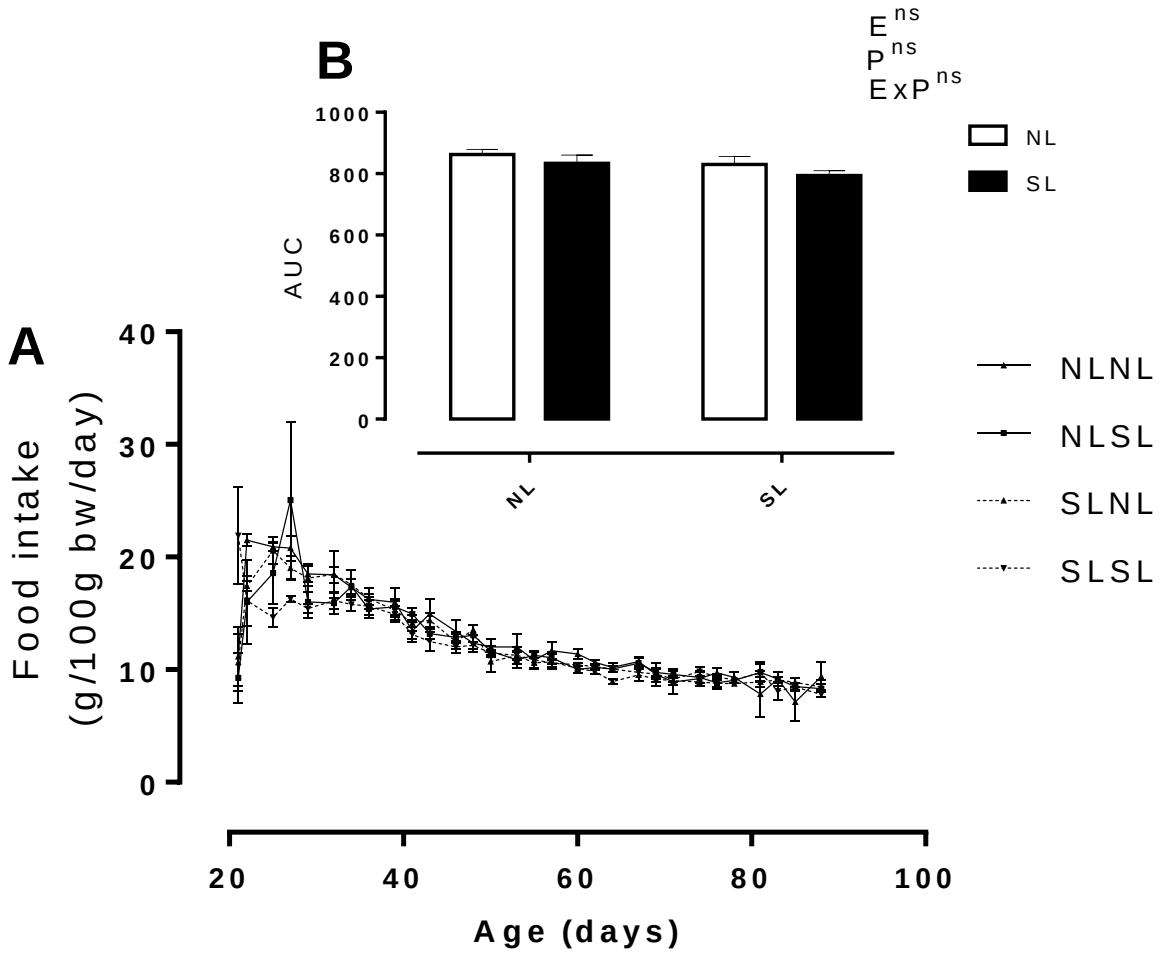

Figure 2: Relative food intake from 21th days until 90th days old (**A**), Area under curve of relative food intake (**B**), n= 4 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

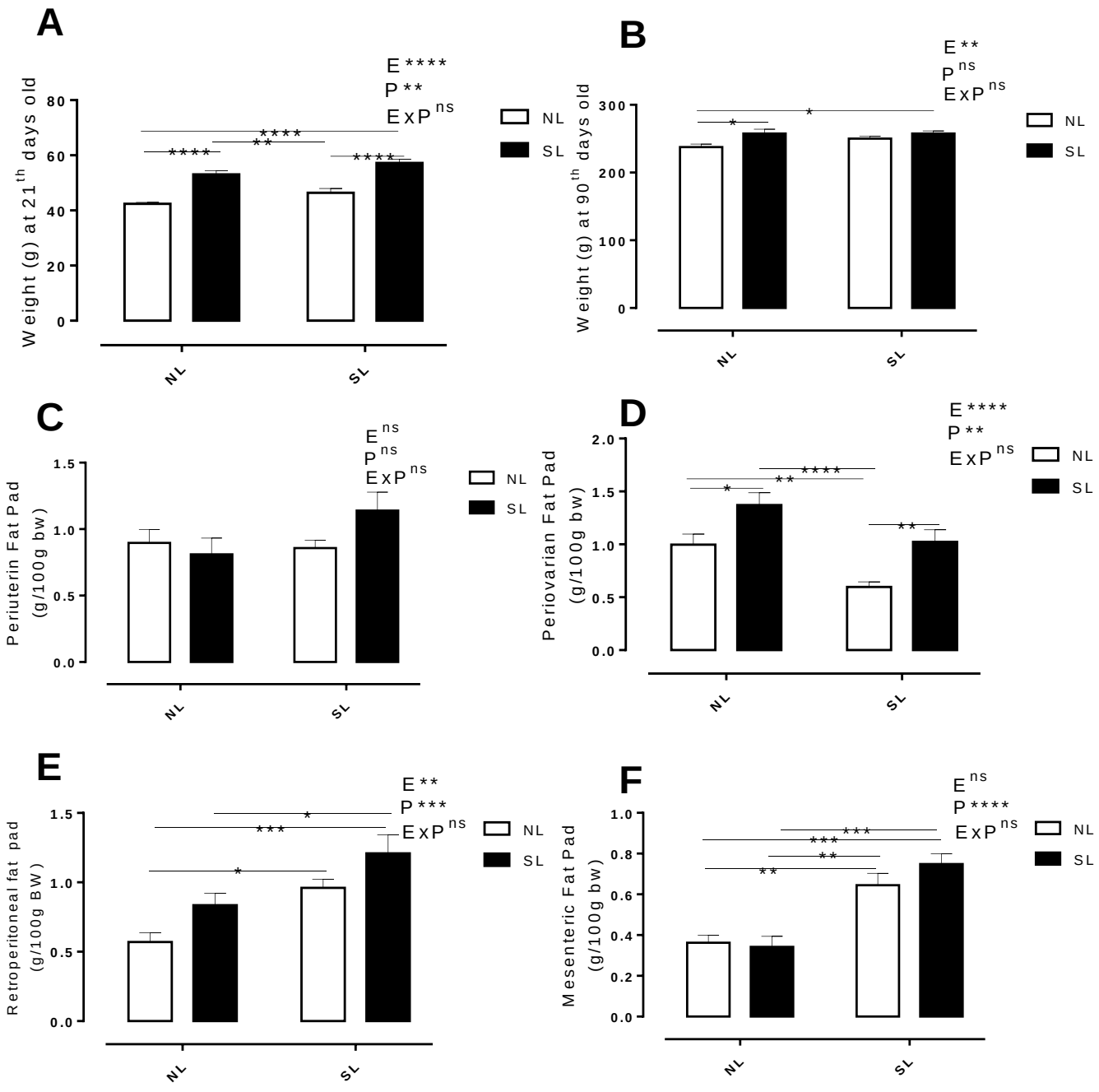

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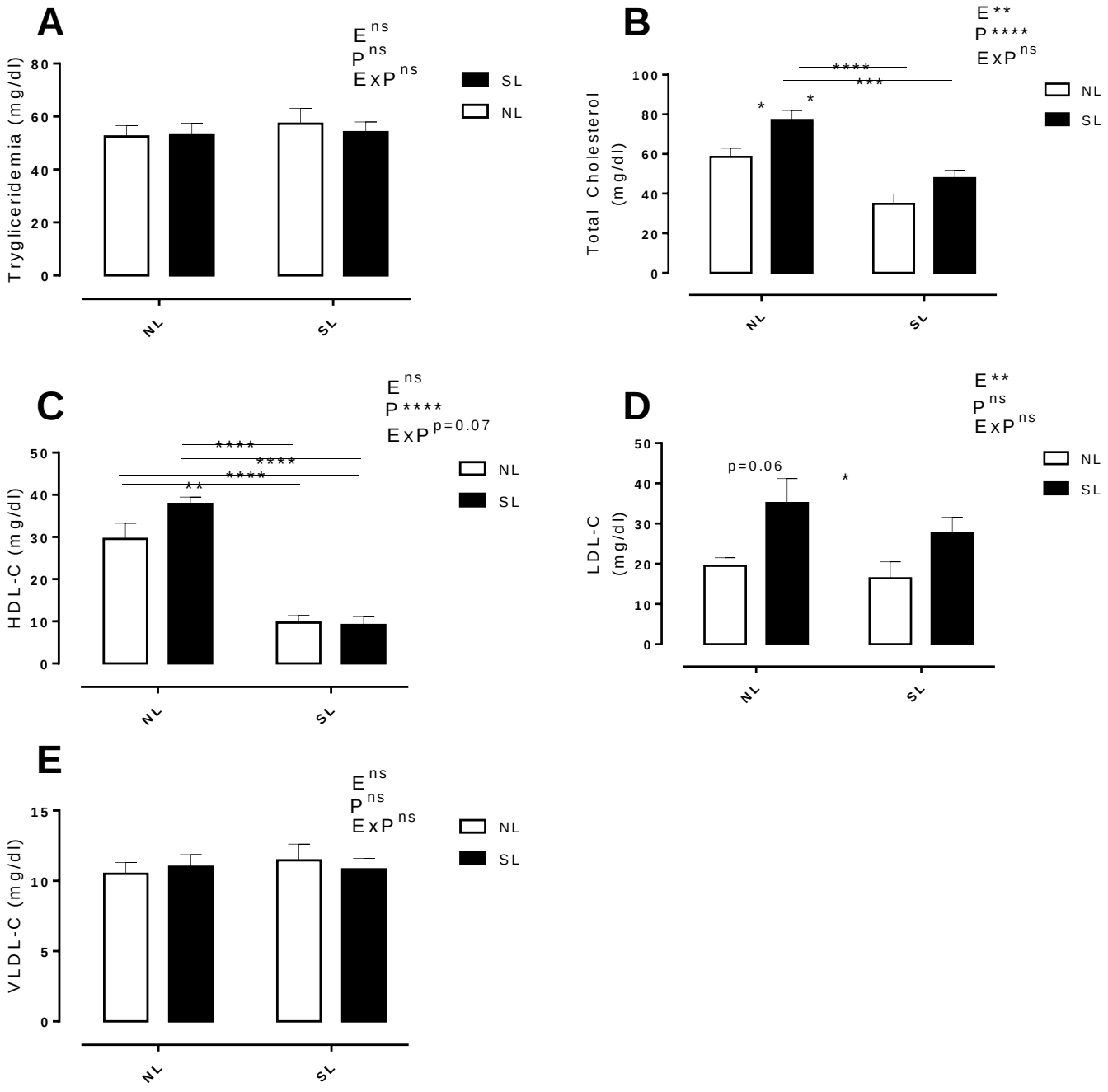
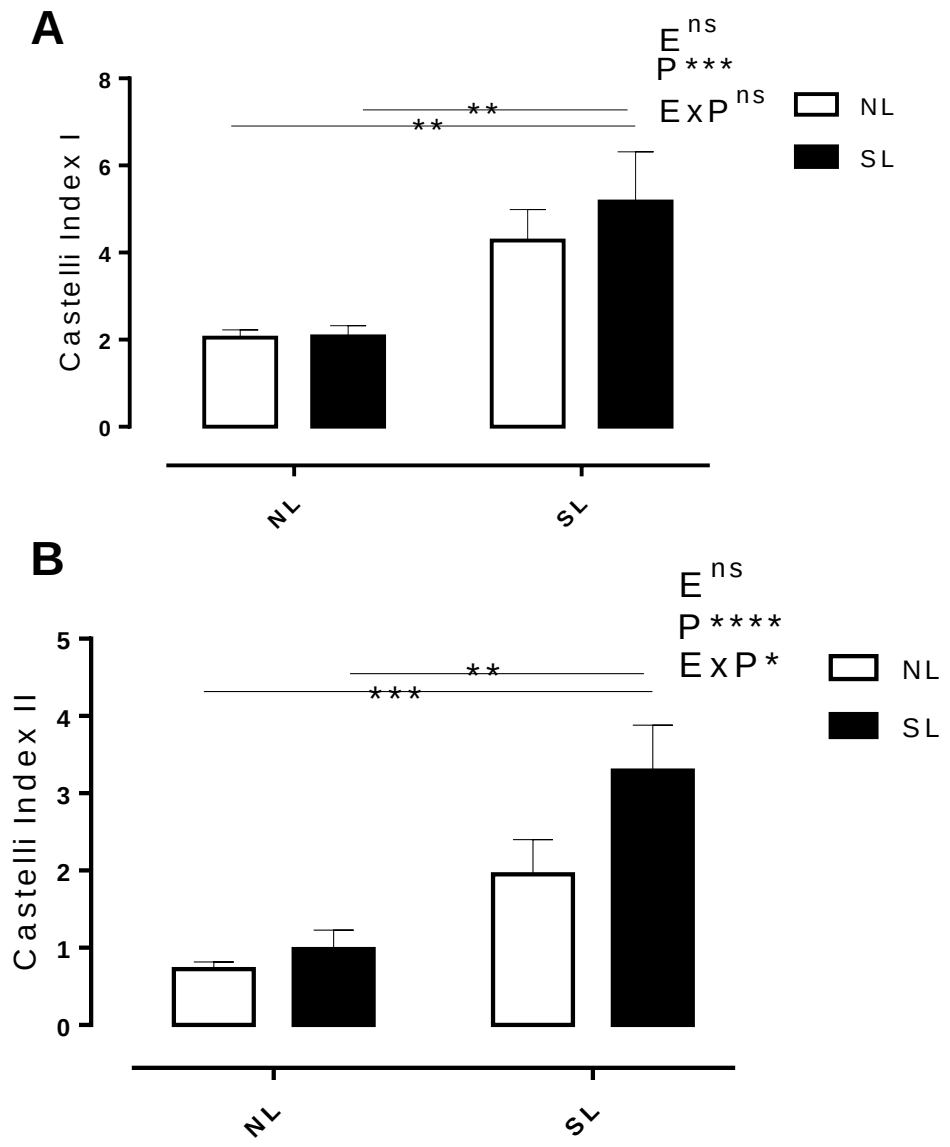
Figure 3: Body weight at 21t^{h} days old (**A**), Body weight at 90th days old (**B**), Periuterin fat pad deposition (**C**), Periovarian fat pad deposition (**D**), Retroperitoneal fat pad deposition (**E**), Mesenteric fat pad deposition (**F**). n= 9-12 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.



449
450
451 **Figure 4:** Trygliceridemia (**A**), Total Cholesterol (**B**), HDL-Cholesterol (**C**), LDL-
452 Cholesterol (**D**), VLDL-Cholesterol (**E**). n= 9-12 animals, at least 3 different litters per
453 group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from
454 Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter

offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.



464
465 **Figure 5:** Casteli Index I (**A**), Casteli Index II (**B**). n= 9-12 animals, at least 3 different
466 litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring
467 from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal
468 litter offspring from small litter parents, SLSL small litter offspring from small litter parents.
469 E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early
470 overfeeding and parental obesity. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns –
471 non significant based on a two way anova analysis plus Tukey multi comparisons test.

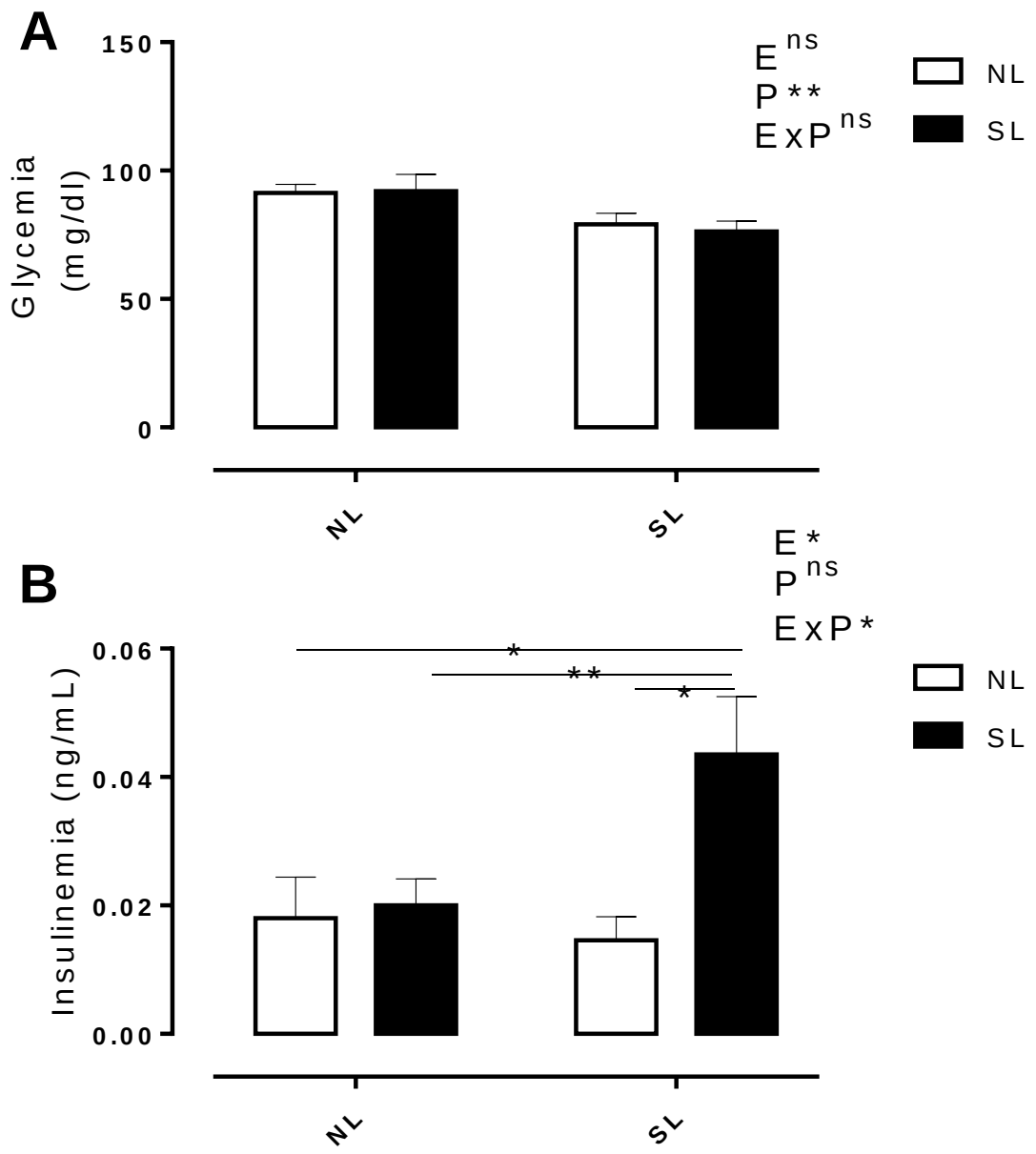


Figure 6: Fasting Glycemia (**A**), Fasting Insulinemia (**B**). n= 9-12 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction

between early overfeeding and parental obesity. * $p<0.05$, ** $p<0.01$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

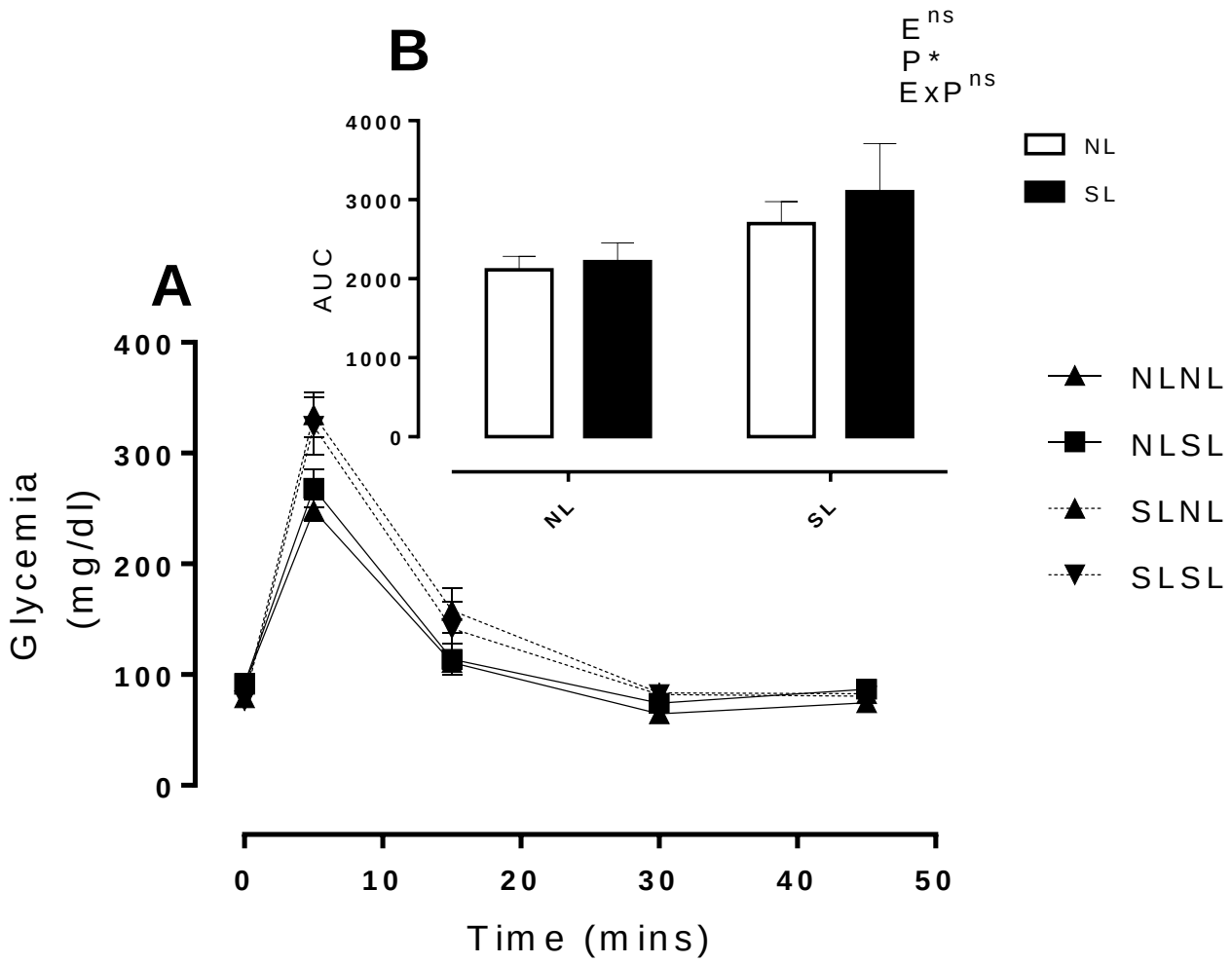


Figure 7: Intravenous Glucose Tolerance Test (**A**), Area under the curve of the Glucose Tolerance Test (**B**). n= 9-12 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. * $p<0.05$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

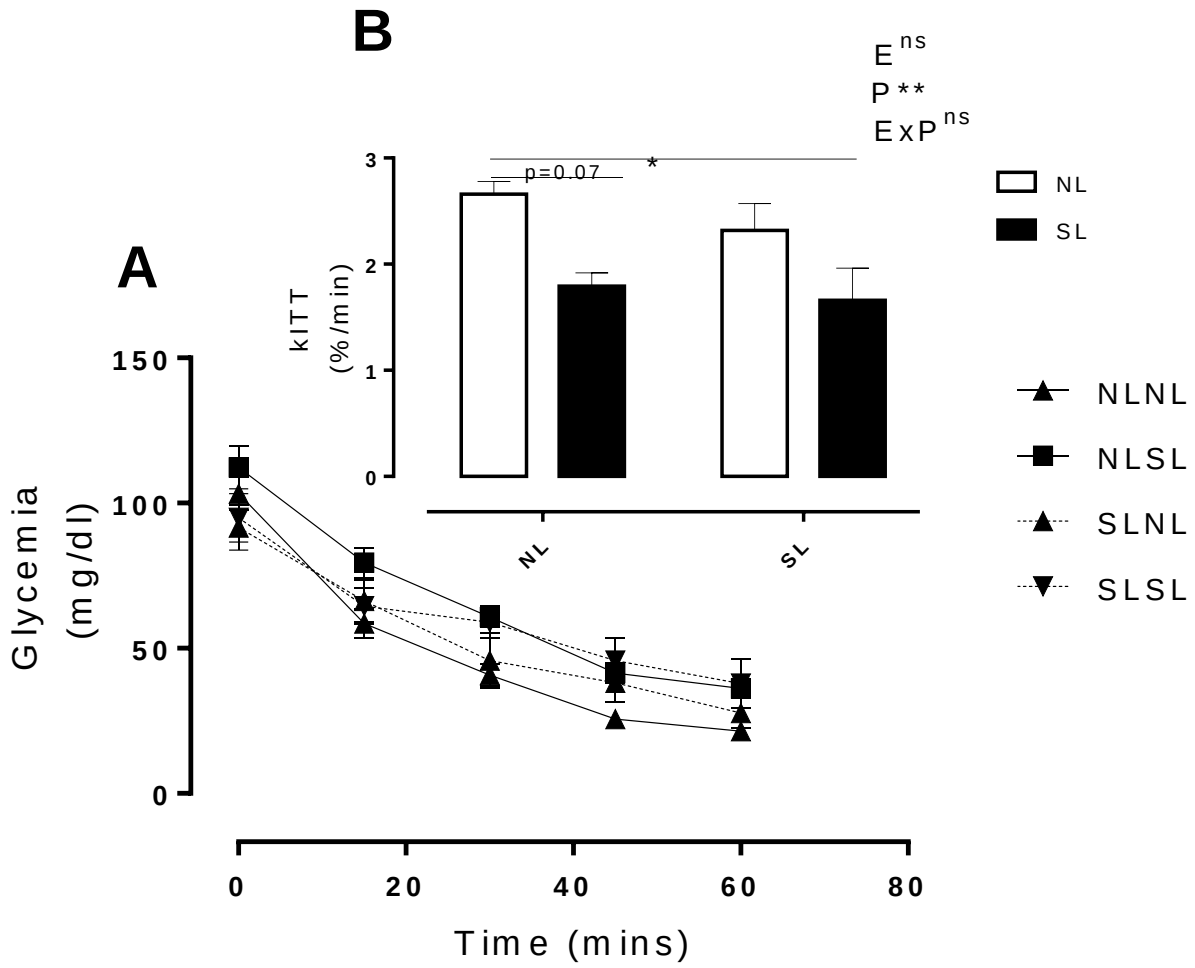
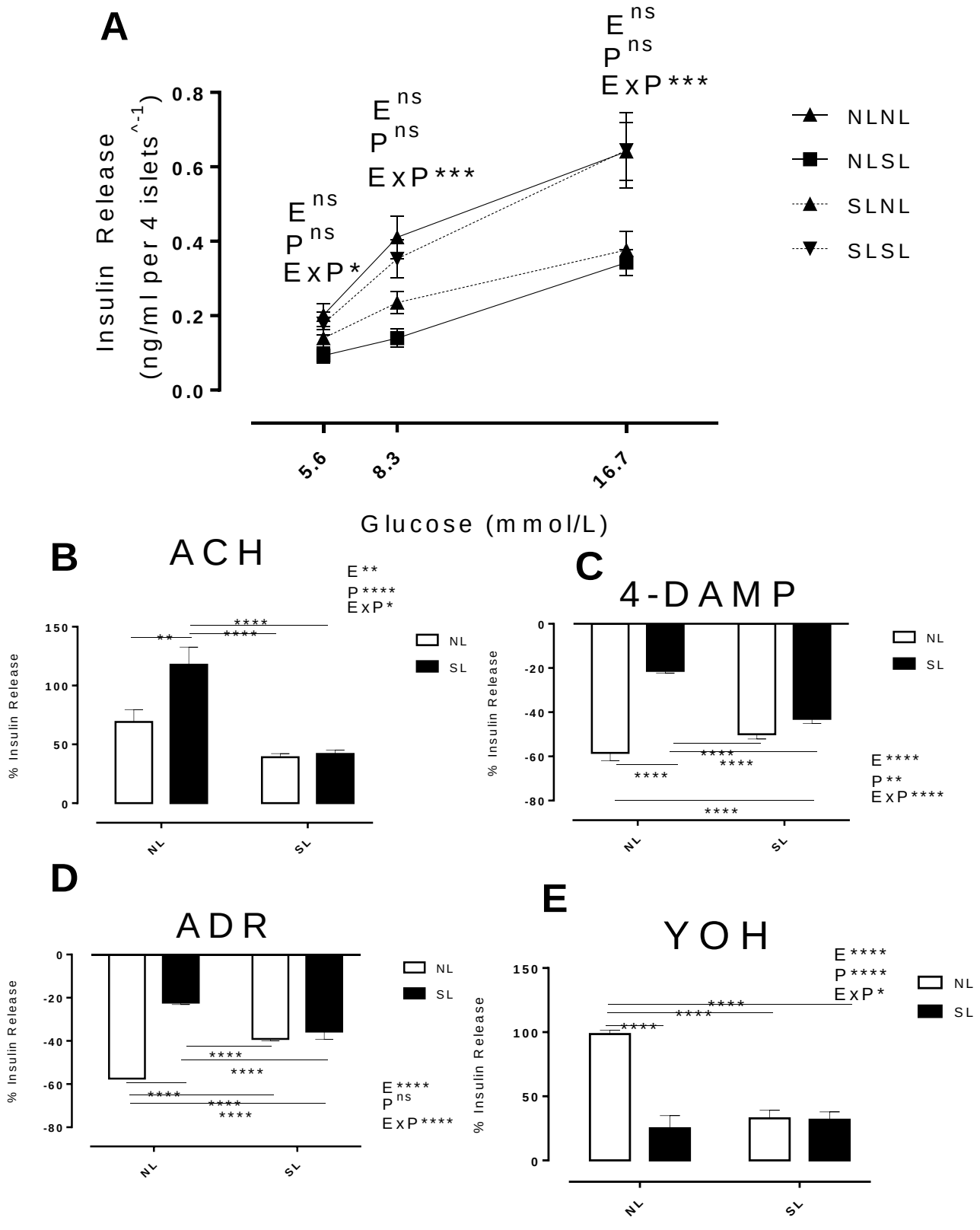


Figure 8: Intraperitoneal Insulin Tolerance Test (**A**), Area under the curve of the Insulin Tolerance Test (**B**). n= 9-12 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. * $p<0.05$, ** $p<0.01$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.


A
Insulin Release (ng/ml per 4 islets^-1)
0.0
0.2
0.4
0.6
0.8
E ns
P ns
ExP*
E ns
P ns
ExP***
E ns
P ns
ExP***
NLNL
NLSL
SLNL
SLSL
5.6
8.3
16.7
Glucose (mmol/L)
B
ACH
E**
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ExP*
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NL
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4-DAMP
E****
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P****
ExP*

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Figure 9: Pancreatic Islet Insulin Secretion. Insulin secretion stimulated by different glucose concentrations (5.8, 8.3 and 16.7 mmol/l) (**A**); acetylcholine (**B**); acetylcholine receptor antagonist (4-Damp - 4-diphenylacetoxy-N-methylpiperidine methiodide) (**C**); epinephrine (**D**); adrenoceptor antagonist (Yoh – Yohimbine) (**E**). The line at 0 represents 100% of the glucose-induced insulin release under the effect of 8.3 mmol/l glucose (**B**), 8.3 mmol/l plus 10 µmol/l acetylcholine (**C**), 16.7 mmol/l glucose (**D**), 16.7 mmol/ glucose plus 1 µmol/l epinefrine (**E**). The bars above or below the line at 0 (B,C,D,E) represent the increased or decreased glucose-induced insulin release percentages that were altered by the treatments. The data was obtained from 6 animals from 3 different litters of each experimental group. Data are expressed as mean ±S.E.M. NLNL- Normal litter size offspring from Normal Parents, NLSL, small litter offspring from Normal Parents, SLNL- Normal litter offspring from small litter parents, SLSL small litter offspring from small litter parents. E – early overfeeding factor, P – parental obesity factor, ExP- interaction between early overfeeding and parental obesity. * $p<0.05$,** $p<0.01$, *** $p<0.001$, **** $P<0.0001$,ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

MANUSCRITO 2

Fecal microbiota transplantation during lactation promotes protection of pancreatic islet function in obese female rats

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Keywords: small litter, metabolic programming, fecal microbiota transplantation, female rats.

Abstract: Intestinal microbiota is involved in many physiological process. Recently, it has been implied that the microbiota is involved in obesity onset. The first contact happens during early life but the effects of microbiota in metabolic programming at adulthood are still not understood. The aim of this work was to evaluate the transplantation of fecal microbiota during lactation to female offspring rats from lean and obese mothers.Obese parents were induced by litter reduction during the lactational period. At 90th days old, NL and SL males and females (parents), from different litters, were mated, NL male vs NL female; SL male vs SL female. At birth, the litter was standardized in the 3rd day of life to NL or SL. From the 10th until the 25th day of life the offspring received gavage of a solution containing the diluted feces of the opposite dam. Four experimental groups were created using early life obesity condition and transplantation of fecal microbiota; normal litter offspring that received saline (NLS), normal litter offspring that received diluted fecal microbiota (NLM), small litter offspring that received saline (SLS), small litter offspring that received diluted fecal microbiota (SLM). Fecal microbiota transplantation caused decreased body weight gain during life and increased fat deposition in the SLM group. SLM animals had increased HDL-C and total cholesterol levels, reflecting in lower atherosclerosis risk assessment by the castelli index I and II. By the other hand, NLM animals showed the opposite effect. Early life obesity caused glucose intolerance in SLS and SLM groups, but fecal microbiota transplantation protected against insulin resistance in SLM animals. All groups had increased secretory response of insulin in 5.6 and 8.3 mmol/L of glucose compared with NLS, but fecal microbiota transplantation lowered this value in 16.7 mmol/L of glucose in NLM and SLM groups. Fecal microbiota transplantation lead to decreased cholinergic insulinotropic response. NLM animals showed increased

adrenergic insulinostatic response, SLM animals showed an opposite response to the same parameter. Fecal microbiota transplantation caused protection against pancreatic islet dysfunction caused by obesity in early life.

Introduction

Metabolic homeostasis is maintained by a myriad of different factors. A new interesting link in normal physiological functions is the influence of intestinal and fecal microbiota. The microbiota forms a complex community in symbiosis with their host, in this case, us. With an estimated quantity of $10^{\wedge 14}$ cells, its 10 times the number of human cells (Goulet 2015) . New research shows an increasing number of metabolic functions that are influenced by intestinal microbiota (Tojo, et al. 2014). In those functions are included the maturing and stimulation of the gastrointestinal tract, the immune system and also modulation of metabolic pathways, including a link between the hypothalamus and the pancreas (Tojo et al. 2014), that is probably regulated by short chain fatty acids, such as acetate (Perry, et al. 2016).

A very interesting question is if there is a role for the microbiota in obesity. One of the first studies addressing this question used germ free mice and colonized those mice with the microbiota of healthy mice and also of obese mice. This lead to increased fat pad deposition and the deposition was higher in the fat microbiota receiving mice, showing that the intestinal microbiota indeed has a role to play in obesity (Backhed, et al. 2004).

Obesity is a complex disease, and there are many factors involved in its genesis (Hruby and Hu 2015). A new prospect to understand how obesity develops is the Developmental Origins of Health and Disease (DOHaD) concept (Haugen, et al. 2015). The early life environment is a very important part of development according with this concept, and the environment surrounding this phase can predicts the future health of the individual, be it for health or for disease. A nutritional insult during those phases, such as pregnancy,

lactation and also adolescence, can programs the metabolism of the individual to metabolic diseases later in life (Barella, et al. 2014).

Many animal models were developed to better understand this relationship, one of those models are the small litter rat. This model becomes obese by the increased milk consumption during early life and shows increased body weight, fat pad accumulation, hyperglycemia, hyperinsulinemia and hyperleptinemia at adulthood (de Almeida, et al. 2013; Plagemann, et al. 2009; Plagemann, et al. 1992).

The first contact with fecal microbiota happens during delivery by the mother and the metabolic health of the mother influences the composition of the microbiota transmitted (Galley, et al. 2014). Maternal vaginal fluids have a microbiota which colonizes the newborn (Collado, et al. 2015). The microbiota of newborns by C-section compared with normal delivery shows a less diverse microbiota, with higher concentration of genera associated with obesity and immune diseases such as asthma (Jakobsson, et al. 2014). The sterile conditions involved during C-section can explain those conditions, and a hypothesis called “Hygiene Hypothesis” postulates that the increase of sanitation and antibiotics lead to a decreased contact with the environment, causing increased immune diseases (Stiemsma, et al. 2015). The microbiota is probably the mediator of those changes.

New therapies are using a method of fecal microbiota transplantation for gastrointestinal diseases (Smits, et al. 2013). The effect of the method is by the normalizing the altered environment, leading to a decreased prevalence of the problem species. Still, it is not known how the microbiota shapes the metabolism in early life and its programming effects during adulthood. The aim of this work was to evaluate the effect of fecal microbiota

transplantation during early life in a model of lean and obese female rats and if those changes can program health or disease during adulthood.

Methods

Ethical approval

All experiments were undertaken according to the norms established by the Brazilian Association for Animal Experimentation (COBEA) and were approved by the Ethics Committee in Animal Research of the State University of Maringá (protocol number 9648231014).

Animal model and experimental design

Obtainment of maternal obesity

Obese parents were induced by litter reduction during the lactational period. For this, control Wistar rats were mated (three 70 days-old females for one 80 days-old male). Pregnant females were separated and placed in individual cages with free access to water and food. At birth, the litter was standardized in the 3rd day of life to 9 pups per dam (normal litter, NL) or 3 pups per dam (small litter, SL).

Offspring

At 90th days old, NL and SL males and females (parents), from different litters, were mated, NL male vs NL female ; SL male vs SL female. Pregnant female were separated and placed in individual cages with free access to water and food. At birth, the litter was

standardized in the 3rd day of life to NL or SL. A mix of male and female offspring was utilized. After that, female offspring were weaned at 21th days of life (3 animals per cage) and were kept under controlled temperature (22±2 C°), photoperiod (12 hours dark and 12h hours of light – 07:00 a.m. – 07:00 p.m.) and had *ad libitum* access to water and the standard chow (Nuvital®, Curitiba/PR, Brazil).

Transplant of fecal microbiota

From the 10th until the 25th day of life the offspring received gavage of a solution containing the diluted feces of the opposite dam. The methods used to prepare the microbiota solution were as according with (Aas, et al. 2003). The feces were collected from the dams, diluted in sterile saline, filtered and after that administered by gavage at a dose of 1g/kg, the saline groups received 0,9% saline in the same dose as the microbiota animals, coprophagy was not prevented. Thus, four experimental groups were created using early life obesity condition and transplantation of fecal microbiota; normal litter offspring that received saline (NLS), normal litter offspring that received diluted fecal microbiota (NLM), small litter offspring that received saline (SLS), small litter offspring that received diluted fecal microbiota (SLM).

Body weight and food intake evaluation

After weaning, offspring from all groups were weighed and food intake was determined tree times per week. Food intake was calculated for each cage as $\frac{\frac{(Chow\ placed - chow\ eaten)}{number\ of\ animals}}{number\ of\ days}$ for the absolute food intake (expressed in g) and the relative food intake was calculated by

$\left(\frac{(absolute\ food\ intake*100)}{mean\ of body\ weight}\right)$ and expressed as g/100g of bw. The total area under the curve (AUC) of food consumption throughout experimental protocol was calculated.

Obesity evaluation

At 90th days old, animals from all groups were weighted, anaesthetized by an intraperitoneal injection of pentobarbital sodium (thiopental 45 mg/kg bw) and euthanized by decapitation to had blood samples collected,centrifuged and the plasma was stored at -20 C° for biochemical analyses. Insulin concentration was determined by radioimmunoassay (RIA). The limit of detection was 0.006 ng/ml. The measurements were performed in a single assay. The retroperitoneal, perieuterin, periovarian and mesenteric fat pads were removed and weighed, and the results were expressed in relation to the bw of each animal (g/100 g bw).

Intravenous glucose tolerance test (ivGTT)

At 91thdays old, rats from all groups underwent a surgery for the silicone cannula implantation into the right jugular vein, as previously described (de Oliveira, et al. 2011). At 24 hours after the surgery, and an overnight fasting, the rats received a glucose infusion (1 g/kg bw). Blood samples were collected in heparinized syringes at 0 (before glucose administration), 5, 15, 30 and 45 min after the glucose administration. Plasma samples were stored at -20°C for determination of glucose concentration. The glucose concentration was evaluated by the glucose oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The AUC of glycemia and throughout the ivGTT was calculated.

Intraperitoneal insulin tolerance test (ipITT)

The animals were fasted for 6h prior to an intraperitoneal insulin tolerance test (1 U/kg bw), as previously described (Miranda et al., 2014). The blood was collected in the tail of the animals with capillary tubes. After the test, the blood was centrifuged and plasma samples were samples were stored at -20°C for determination of glucose concentration. The absorption rate of glucose by the tissue (rate constant for the disappearance of plasma glucose, K_{itt}) was calculated as already described (Lundbaek 1962). The plasma glucose t1/2 was calculated from the slope of the least squares analysis of the plasma glucose concentrations during the linear phase of decline.

Lipid Profile

The plasma samples were used to determine the concentrations of the Total Cholesterol, HDL-Cholesterol and Triglycerides. Triglyceridemia was determined by the lipase lipoprotein – glycerol-3 phosphate oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The Total Cholesterol was determined by the cholesterol oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil) and HDL-Cholesterol was precipitated and determined by the cholesterol oxidase method (Gold Analisa®; Belo Horizonte/MG, Brazil). The VLDL-Cholesterol and LDL-Cholesterol was evaluated using the Friedwald equation (Fukuyama, et al. 2008), whereas the VLDL concentration was $trygliceridemia/5$ and LDL was $total\ cholesterol - (HDL - VLDL)$. The Castelli Index was determined as showed by (Millan, et al. 2009), with the Castelli Index I equal to $total\ cholesterol/HDL$ and Castelli Index II equal to LDL/HDL.

Pancreatic islets isolation and insulin secretion measurements

Pancreatic islets were isolated by collagenase digestion and washed with Hank's solution as previously described (Gravena, et al. 2002). Groups of four isolated islets were pre-incubated for 60 min at 37 °C in Krebs solution containing 5.6 mmol/l glucose in a mixture of 95% O_2: 5% CO_2 at pH 7.4 to stabilize insulin secretion. After that, these islets were used in one of the experimental condition to assess the responsivity to glucose as well as autonomic agents. To study the glucose-insulinotropic response, a batch of islets were incubated for 60 min more under the different glucose concentrations (5.6, 8.3 and 16.7 mmol/l). To study the muscarinic acetylcholine receptor (mAChR) function, another batch of the islets were incubated with a solution containing either 8.3 mmol/l glucose or 8.3 mmol/l glucose plus, 10 µmol /l acetylcholine in the presence of 10 µmol/l neostigmine to avoid acetylcholinesterase action in the islets. To study the insulinotrpic fucntion of the mAChR subtype M3, 100 µmol/l of the selective antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) were used. To study adrenergic receptor function in the islets, another batch of the islets were incubated in the presence of 16.7 mmol/l of glucose plus 1 µmol/l of adrenaline. To block α2 adrenergic receptor, we used an antagonist, Yohimbine (10 µmol/l) with 16.7 mmol/l of glucose plus 1 µmol/l of adrenaline. The supernatants from the incubations were collected and stored at –20°C for further insulin measurements by RIA.

Statistical analysis

The results are expressed as the mean ± SEM. Data were submitted to Student t-test or variance analysis (two-way ANOVA) with the factors: early obesity (O), fecal microbiota

(M) and early obesity x fecal microbiota (i). In the case of analyses with a significant F, the differences between the means were evaluated by Tukey's test; p<0,05) were considered statistically significant. Tests were performed using GraphPad Prism version 7 for Windows (GraphPad Software Inc., San Diego/CA, USA).

Results

Biometric Parameters

Weight gain and food intake throughout life

Fecal microbiota transplantation (p^m<0.01) lead to decreased body weight gain in the NLM (p<0.05) and SLM (p<0.05) groups when compared with NLS. The same was not observed regarding the relative food intake, with decreased food intake in the SLM group (p^o<0.05) (**Figure 1 and figure 2**).

Body weight and fat pad deposition

Early obesity lead to increased body weight at 21th days old (p^o<0.01). At 90th days old no differences were observed between the groups (**figure 3A and 3B**). The periuterin fat pad had increased fat deposition in the SLM group (p<0.01 vs NLS, p<0.05 vs NLM; p^o<0.05,p^m=0.06). The periovarian fat pad had a similar pattern, with increased deposition in SLM compared with all groups (p^o<0.0001; p<0.0001 vs NLS, p<0.0001 vs NLM, p<0.05 vs SLS). The same was observed in the retroperitoneal fat pad (p^o<0.001, p^m<0.001; p<0.0001 vs NLS, p<0.0001 vs NLM, p<0.01 vs SLS) and in the mesenteric fat pad (p<0.0001 vs NLS, p<0.0001 vs NLM; p^o<0.0001) (**figure 3C,3D,3E and 3F**).

Biochemical Parameters

Lipid Profile

No differences between the groups were observed in the trygliceridemia, LDL-cholesterol and VLDL-cholesterol (**figure 4A,4D and 4E**). Early obesity in the SLS group lead to decreased total and HDL-cholesterol ($p^o<0.05$ and $p^o<0.05$, respectively). Fecal microbiota transplantation corrected those value to normal levels ($p^m=0.08$ and $p^i=0.06$) (**figure 4B and 4C**).

Atherosclerosis risk indexes

Both the NLM and SLS groups had increased atherosclerosis risk was evaluated by the the castelli index I ($p^i<0.05$) and castelli index II ($p^i<0.05$). Fecal microbiota transplantation in the SLM groups corrected those values to normal levels (**figure 5A and 5B**).

Glycemic Profile

No differences were observed in the fasting glycemia between groups. Regarding the fasting insulinemia, the SLS group had it increased compared with NLS ($p^o<0.05$; $p<0.05$). Fecal microbiota transplantation in the SLM group lowered this value to control levels (**figure 6A and 6B**). Early obesity caused glucose intolerance in the SLS and SLM groups ($p^o<0.001$), with higher values compared with NLS ($p<0.01$ vs SLS, $p<0.01$ vs SLM) (**figure 7**). When the insulin sensitivity was assessed, the SLS group showed insulin resistance compared with NLS ($p<0.05$). Fecal microbiota transplantation in the SLM group lead to higher insulin sensitivity, similar to NLS (**figure 8**).

Pancreatic islet insulin secretion

All groups had increased insulin secretion at basal glucose levels (5.6 mmol/L) compared with NLS ($p^i<0.01$). The same pattern was observed at 8.3 mmol/L of glucose ($p^i<0.01$). At 16.7 mmol/L of glucose, fecal microbiota transplantation in the NLM and SLM groups decreased insulin secretion to control levels ($p^m<0.01$) (**figure 9A**). When it was added acetylcholine in 8.3 mmol/L of glucose, the cholinergic insulinotropic response was decreased in all groups compared with NLS ($p^i<0.05$; $p<0.0001$ vs NLM, $p<0.0001$ vs SLS; $p<0.0001$ vs SLM). The decreased response was higher in SLM, which had a decreased stimulation compared with SLS ($p<0.001$). The response to a muscarinic M3 receptor antagonist (4-DAMP) in the same conditions as before was decreased by microbiota transplantation ($p^m<0.01$) (**figure 9B and 9C**). The insulinostatic secretory response, measured by adding adrenaline in 16.7 mmol/L of glucose was increased in NLM compared with NLS ($p<0.001$) and decreased in SLM ($p^i<0.0001$). The response to an α2 antagonist (Yohimbine) in the same conditions described previously was increased in both microbiota transplantation groups ($p^m<0.0001$) with increased stimulation in NLM compared with NLS ($p<0.05$) and SLM compared with SLS ($p<0.01$) (**figure 9D and 9E**).

Discussion

Fecal microbiota transplantation programmed restoration of pancreatic islet function in obese animals. Contrary to this effect, transplantation of fecal microbiota to lean animals lead to pancreatic islet dysfunction. The control of insulin secretion is complex, and many factors are involved in the final quantity of insulin secreted during the postprandial period (Osundiji and Evans 2013). One of the most important regulators is the autonomic

nervous system. With its two branches, parasympathetic and sympathetic, it controls the modulation of insulin secretion by the liberation of neurotransmitters (Seoane-Collazo, et al. 2015). Parasympathetic stimulation occurs by acetylcholine, which has an insulinotropic effect, mediated by the class of muscarinic receptors (mACHr), the most influential receptor is the M3 receptor, that is a G protein coupled receptor that potentiates insulin secretion (Ruiz de Azua, et al. 2011). Sympathetic stimulus is mainly inhibitory and is mediated by adrenalin via the α2 receptor (Langer 1987). The biggest effect in on high glucose concentrations, by reducing insulin secretion it protects the body from too high levels of insulin. The interplay of those branches can explain the observed insulin secretion. SLS animals had increased insulin secretion, this effect can be explained by increased M3 receptor activation even if the total response to acetylcholine is lower. This suggests a decreased M2 receptor activation, because this receptor has an insulinostatic effect, the total effect is of increased insulin secretion on all concentrations of glucose. Transplantation of fecal microbiota corrects this dysfunction on high levels of glucose with increased α2 activation.

Autonomic stimulus is controlled by the hypothalamus, there is a complex feedback that involves neural and hormonal stimulation to determine how the branches should act to specific physiological conditions (Kalsbeek, et al. 2014). The microbiota has a direct effect on the enteric nervous system, considered a third branch of the autonomic nervous system (Obata and Pachnis 2016). This branch controls the motility of the gastrointestinal tract, but also has effect on the hormonal secretion of many hormones secreted by the tract, like GLP-1 and ghrelin (Cani, et al. 2013). The effect of the microbiota can shape the physiology the tract and a promissory lead is by short chain fatty acids, also by

shaping the levels of inflammatory response in the host (Belkaid and Hand 2014; Kasubuchi, et al. 2015).

There is evidence that short chain fatty acids such as acetate can indirectly modulate insulin secretion via the vagus nerve (Perry et al. 2016). Others short chain fatty acids such as proprionate and butyrate are also implied to have an effect on metabolic homeostasis (Soldavini and Kaunitz 2013). The effect of microbiota programming is not necessarily linked to long term changes of its composition (Weingarden, et al. 2015), by introducing different microbiota phyla, the relative proportions of the principal phyla, such as *Bacteroidetes* and *Phyrmicutes* changes temporally, those changes during early life alter the products and the interaction with the host, programming the phenotype expressed during adulthood. Those could be a possible mechanism explaining the pancreatic changes observed in microbiota transplanted animals.

Increased adipose tissue levels are observed as an effect of the transplantation, this could be linked to increased tissue insulin sensitivity, that is linked with the modulation of the pancreatic islet. Increased response to α2 receptor could be linked with a protective effect against the development of diabetes type 2 and obesity, in this hand, a decreased cholinergic response may be a feedback mechanism to lower insulin levels, both having a protective effect on metabolism.

Conclusion

Fecal microbiota transplantation protects obese female rats from pancreatic islet dysfunction with increased adrenergic and decrease cholinergic response.

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Figures

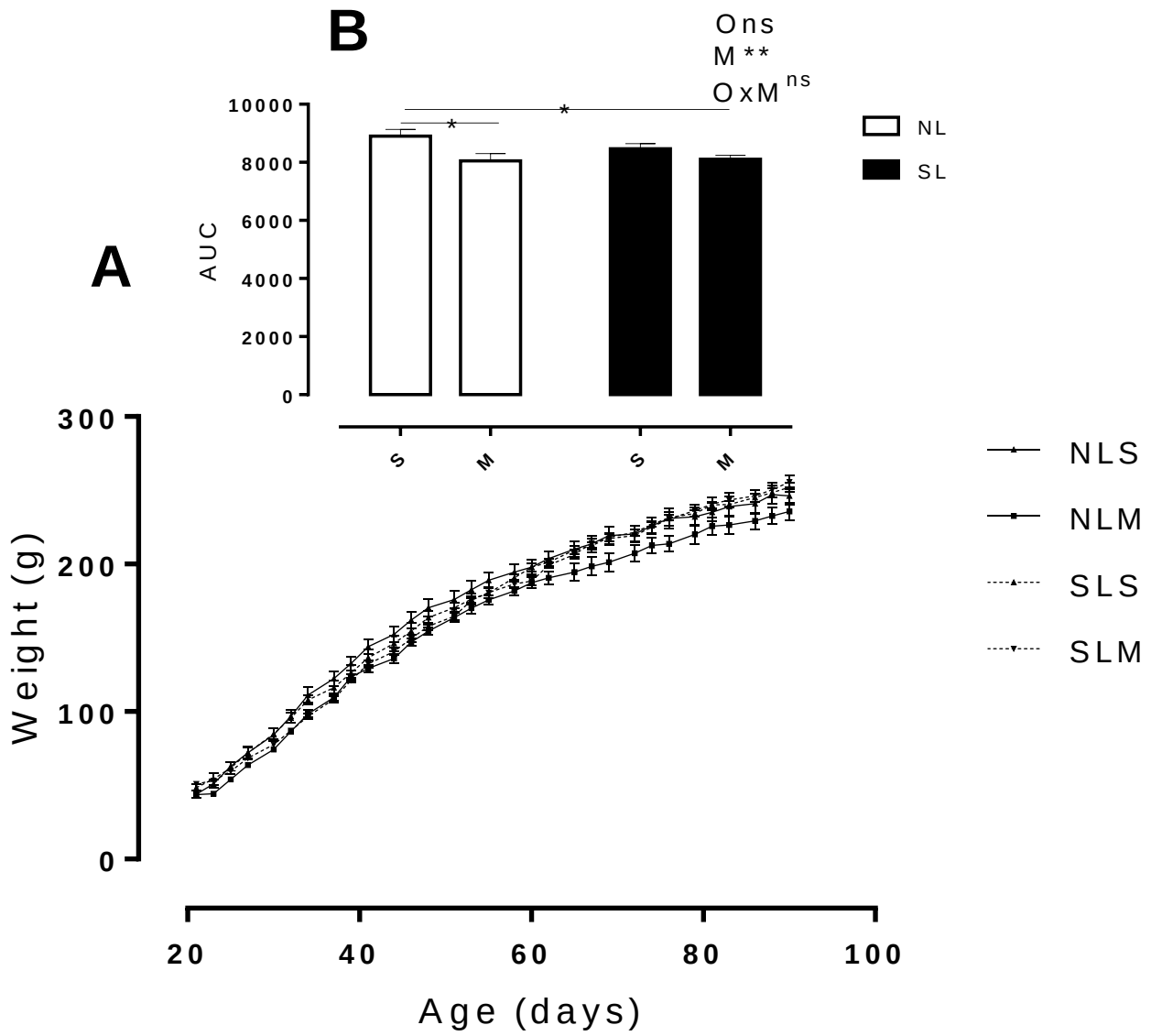


Figure 1: Body weight growth from 21th days until 90th days old (**A**), Area under curve of body weight (**B**), n=9, 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$, ** $p<0.01$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

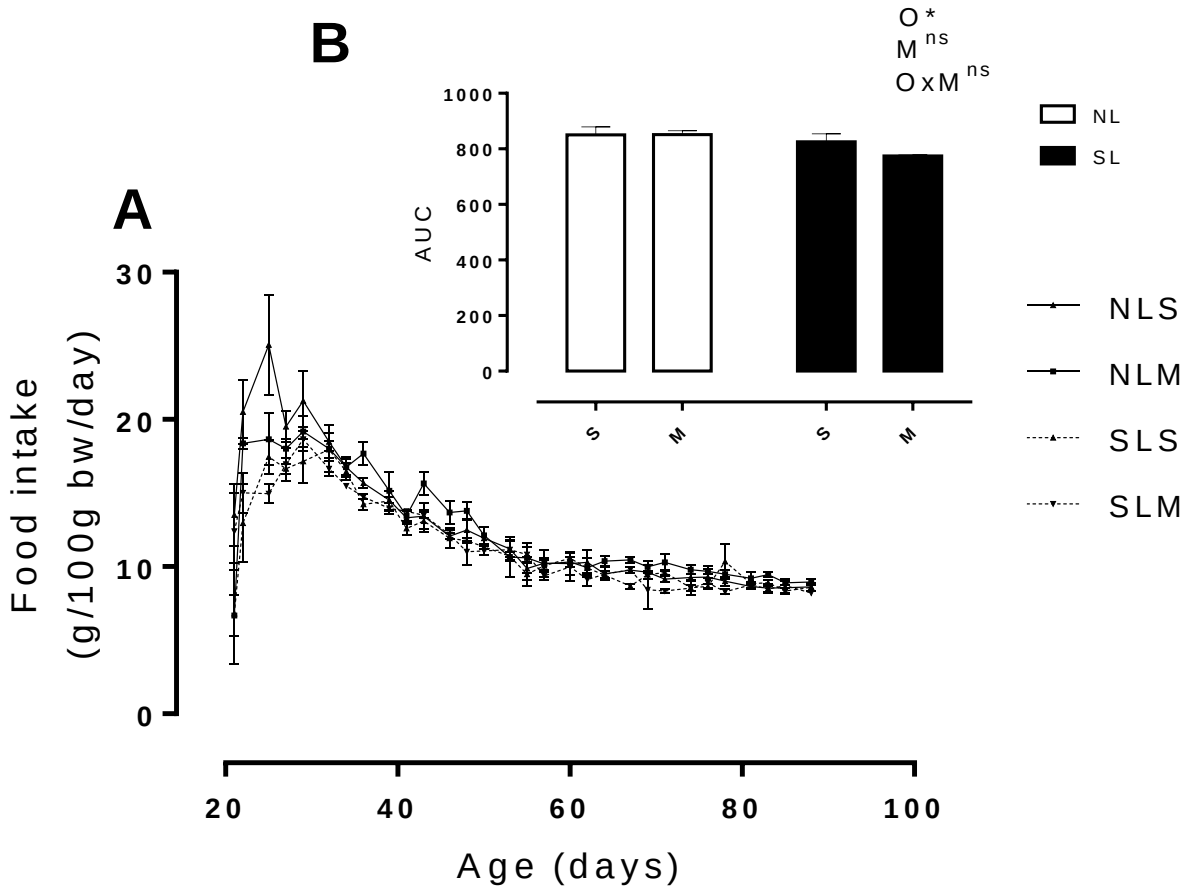


Figure 2: Relative food intake from 21th days until 90th days old (**A**), Area under curve of relative food intake (**B**), n= 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

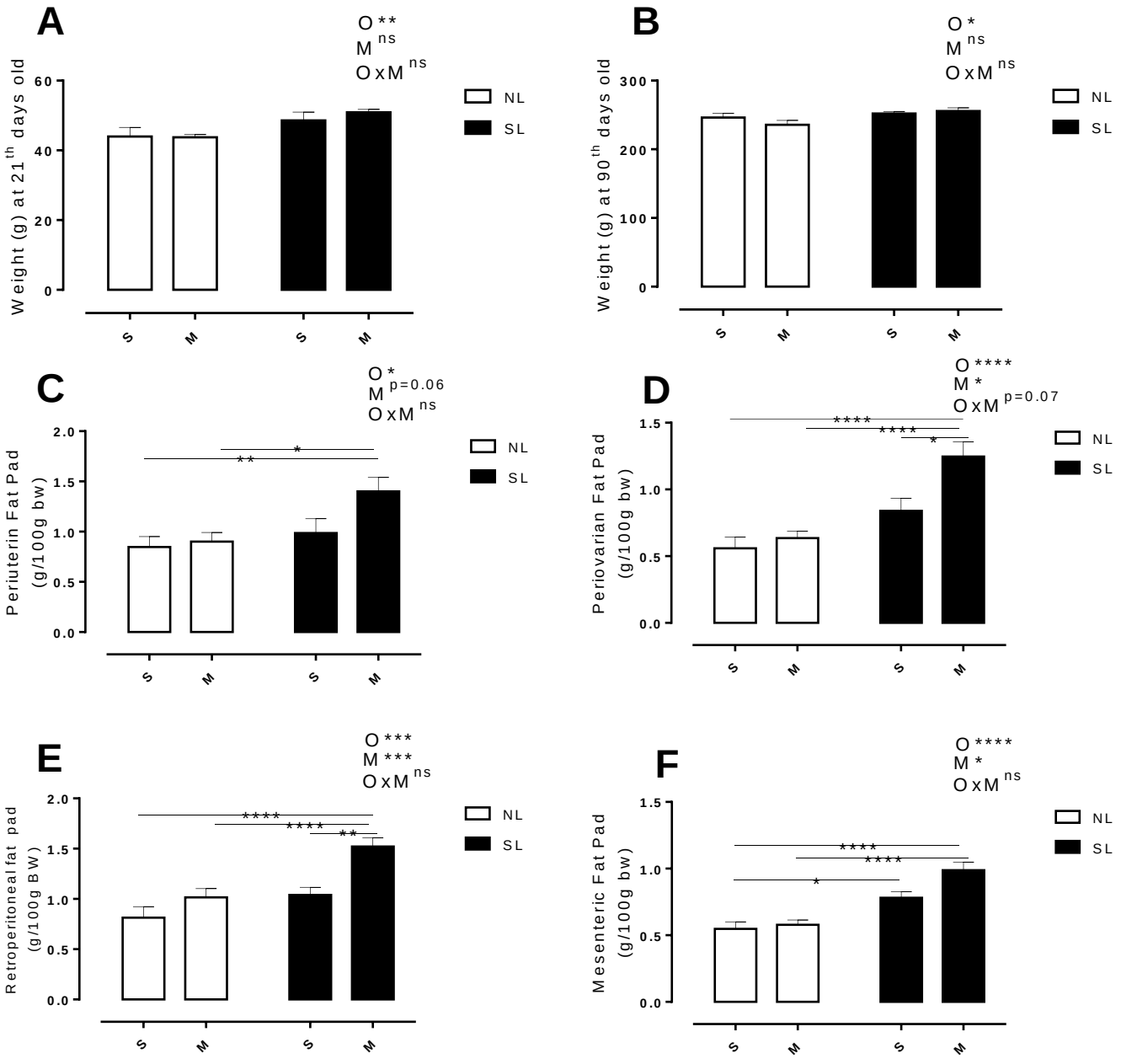


Figure 3: Body weight at 21th days old (**A**), Body weight at 90th days old (**B**), Periuterin fat pad deposition (**C**), Periovarian fat pad deposition (**D**), Retroperitoneal fat pad deposition (**E**), Mesenteric fat pad deposition (**F**). n= 9 animals, 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota

solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

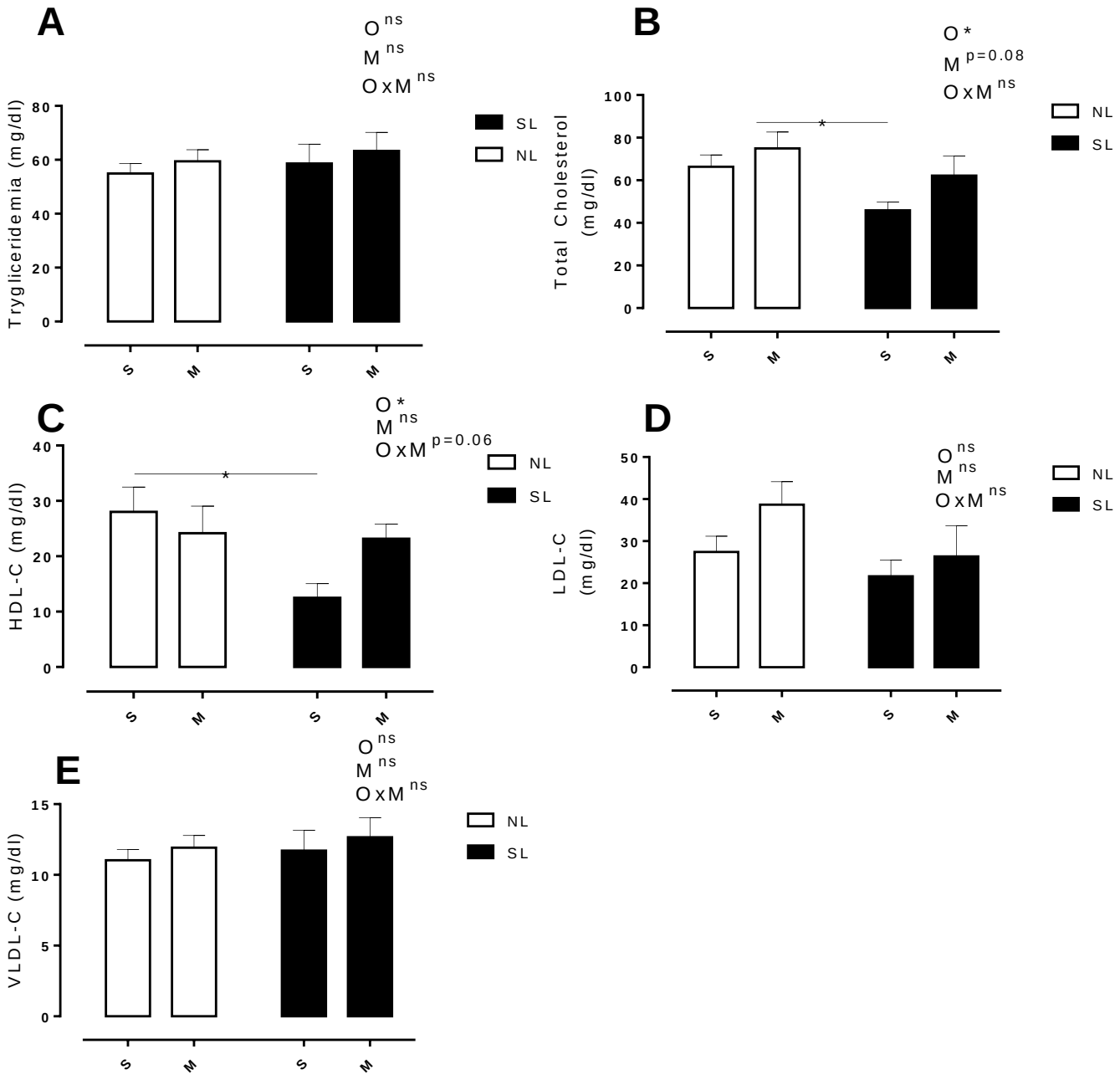


Figure 4: Trygliceridemia (**A**), Total Cholesterol (**B**), HDL-Cholesterol (**C**), LDL-Cholesterol (**D**), VLDL-Cholesterol (**E**). n= 9 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. . NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early

obesity and fecal microbiota factors. * $p<0.05$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

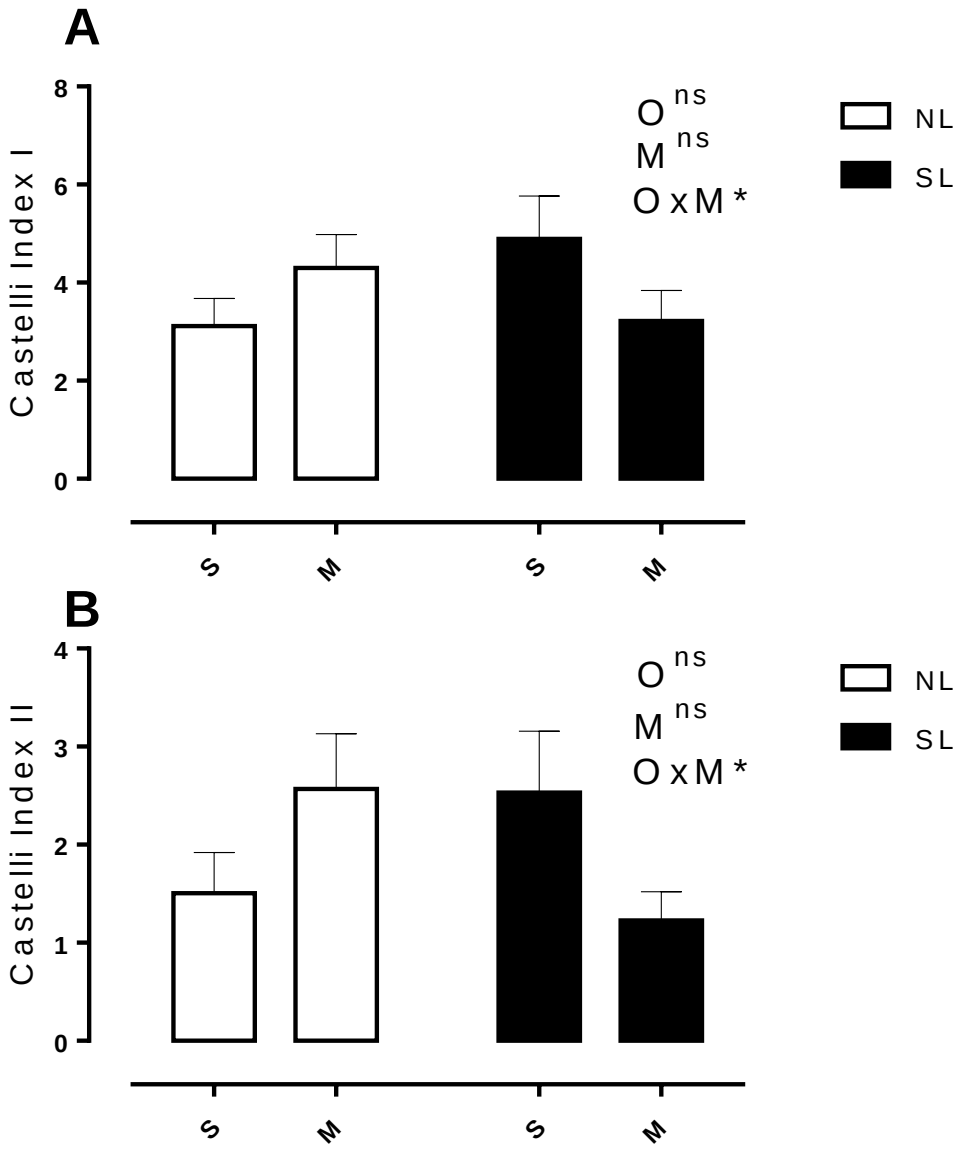


Figure 5: Casteli Index I (**A**), Casteli Index II (**B**). n= 9 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

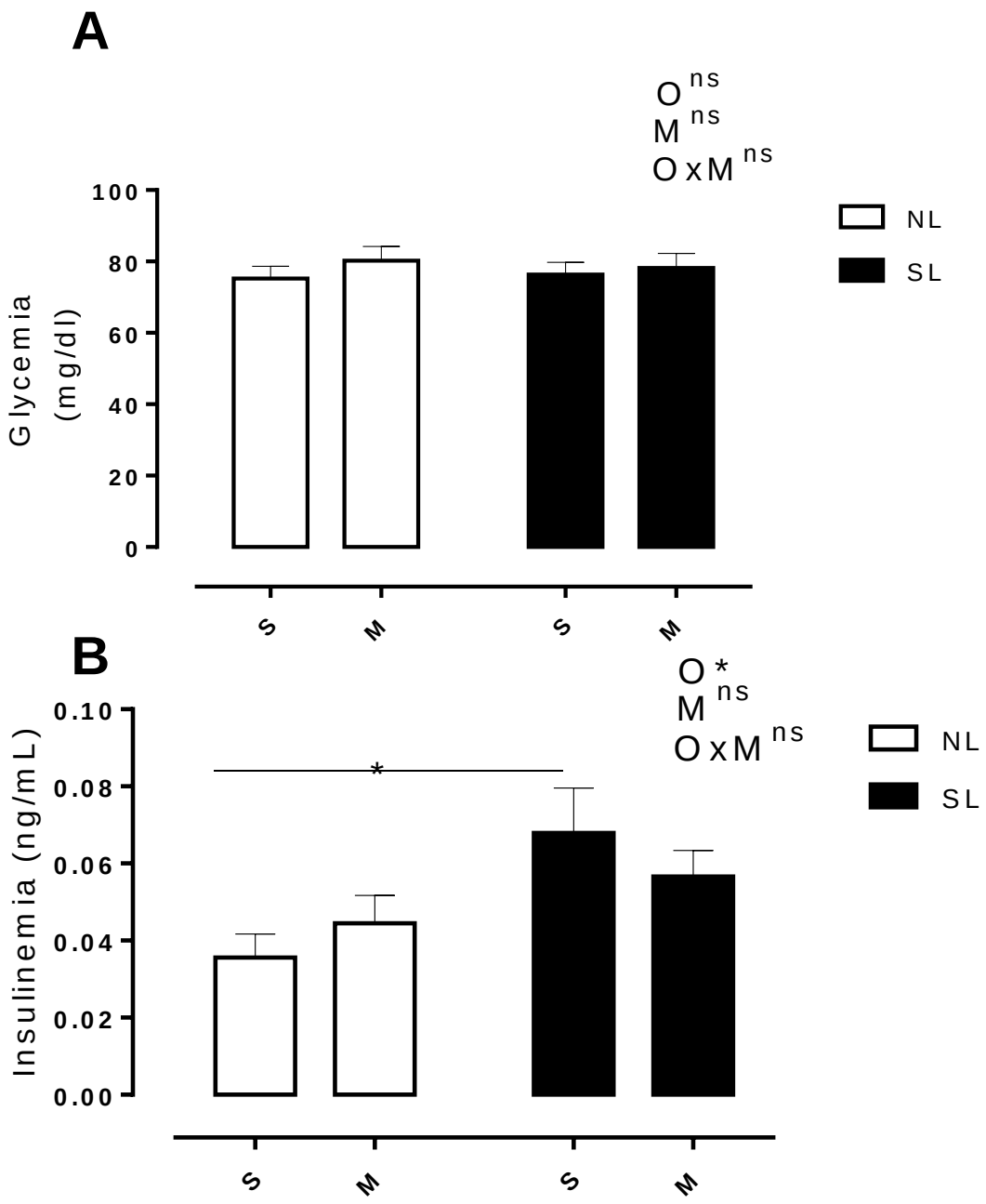


Figure 6: Fasting Glycemia (**A**), Fasting Insulinemia (**B**). n= 9 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction

between early obesity and fecal microbiota factors. * $p<0.05$, ** $p<0.01$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

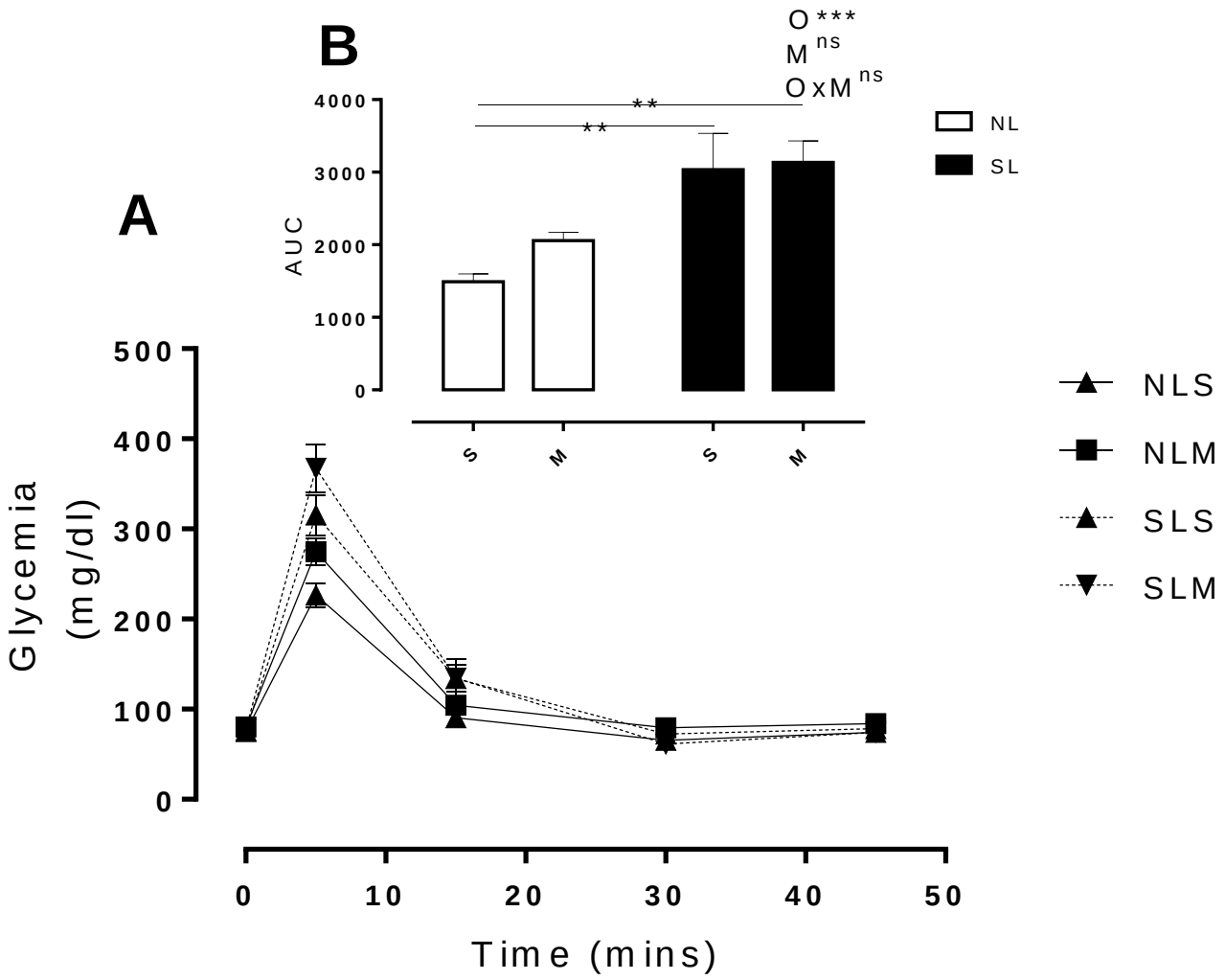


Figure 7: Intravenous Glucose Tolerance Test (**A**), Area under the curve of the Glucose Tolerance Test (**B**). n= 9 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. ** p<0.01, **** p<0.0001 ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.

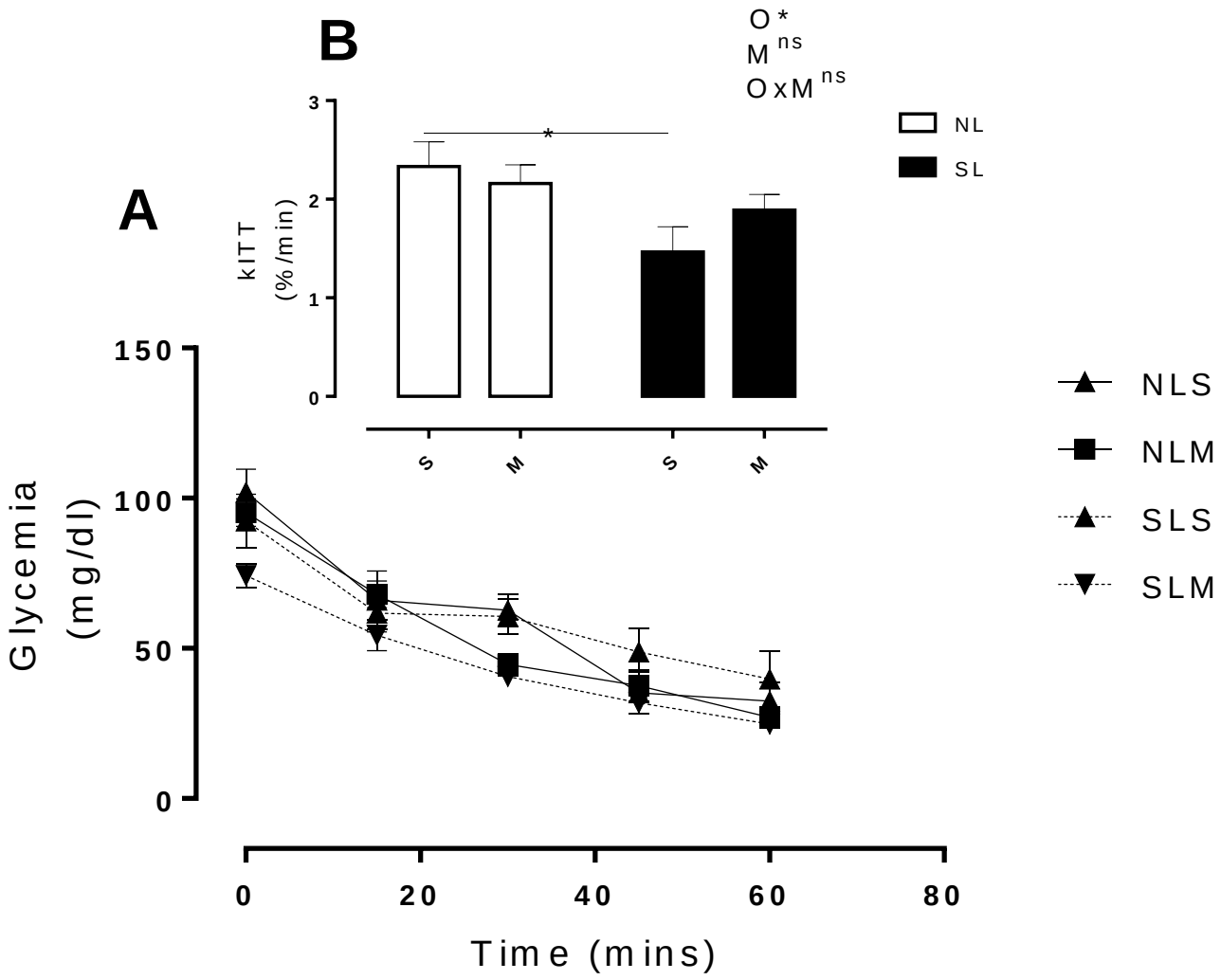
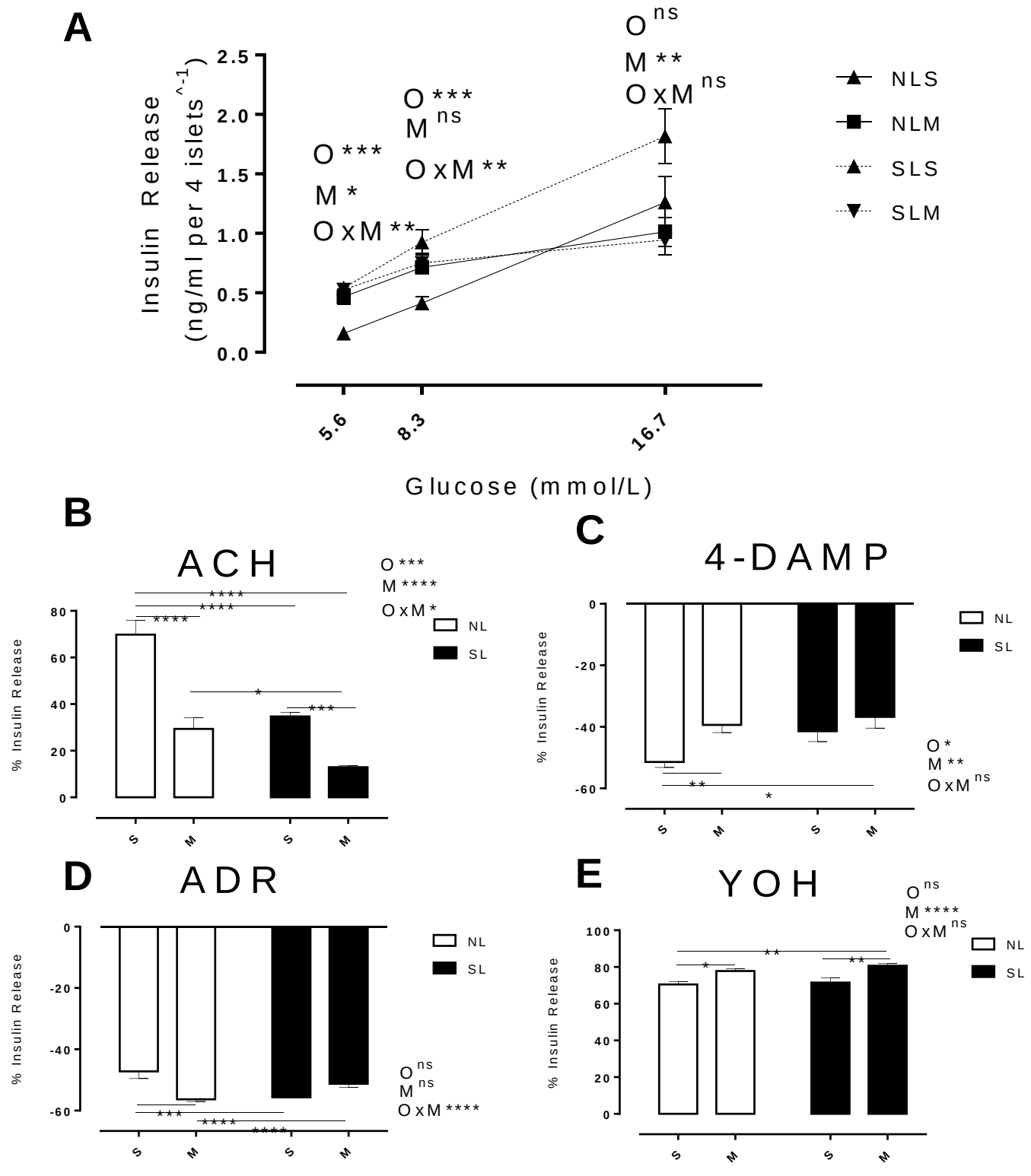


Figure 8: Intraperitoneal Insulin Tolerance Test (**A**), Area under the curve of the Insulin Tolerance Test (**B**). n= 9 animals, at least 3 different litters per group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$, ** $p<0.01$, ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.


A
Insulin Release (ng/ml per 4 islets^-1)
2.5
2.0
1.5
1.0
0.5
0.0
O ***
M *
OxM **
O ***
M ns
OxM **
O ns
M **
OxM ns
NLS
NLM
SLS
SLM
5.6
8.3
16.7
Glucose (mmol/L)
B
ACH
O ***
M ****
OxM *
NL
SL
% Insulin Release
80
60
40
20
0
****
****
****
*
***
S
M
S
M
C
4-DAMP
% Insulin Release
0
-20
-40
-60
NL
SL
O *
M **
OxM ns
**
*
S
M
S
M
D
ADR
% Insulin Release
0
-20
-40
-60
NL
SL
O ns
M ns
OxM ****
***
****
****
S
M
S
M
E
YOH
% Insulin Release
100
80
60
40
20
0
O ns
M ****
OxM ns
NL
SL
*
**
**
S
M
S
M

1098
1099

Figure 9: Pancreatic Islet Insulin Secretion. Insulin secretion stimulated by different glucose concentrations (5.8, 8.3 and 16.7 mmol/l) (**A**); acetylcholine (**B**); acetylcholine receptor antagonist (4-Damp - 4-diphenylacetoxy-N-methylpiperidine methiodide) (**C**); epinephrine (**D**); adrenoceptor antagonist (Yoh – Yohimbine) (**E**). The line at 0 represents 100% of the glucose-induced insulin release under the effect of 8.3 mmol/l glucose (**B**), 8.3 mmol/l plus 10 µmol/l acetylcholine (**C**), 16.7 mmol/l glucose (**D**), 16.7 mmol/ glucose plus 1 µmol/l epinefrine (**E**). The bars above or below the line at 0 (B,C,D,E) represent the increased or decreased glucose-induced insulin release percentages that were altered by the treatments. The data was obtained from 6 animals from 3 different litters of each experimental group. Data are expressed as mean ±S.E.M. NLS- Normal litter offspring that received saline, NLM- normal litter offspring that received a fecal microbiota solution, SLS- small litter offspring that received saline, SLM- small litter that received a fecal microbiota solution. O – Early Obesity factor, M – fecal microbiota factor, OxM- interaction between early obesity and fecal microbiota factors. * $p<0.05$,** $p<0.01$, *** $p<0.001$, ****-$P<0.0001$,ns – non significant based on a two way anova analysis plus Tukey multi comparisons test.