

## RESEARCH

# Long-term increase of insulin secretion in mice subjected to pregnancy and lactation

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## Abstract

**Purpose:** Observational studies show that longer breastfeeding periods reduce maternal risk of type 2 diabetes mellitus. However, it is currently unknown if the long-term benefits of breastfeeding for maternal glucose homeostasis are linked to changes in the endocrine pancreas.

**Methods:** We presently evaluated functional, morphological and molecular aspects of the endocrine pancreas of mice subjected to two sequential cycles of pregnancy and lactation (L21). Age-matched mice not allowed to breastfeed (L0) and virgin mice were used as controls.

**Results:** L21 mice exhibited increased tolerance and increased glucose-stimulated insulin secretion (GSIS) by isolated islets. Pancreatic islets of L21 mice did not present evident morphological changes to justify the increased GSIS. On the other hand, islets of L21 mice exhibited a reduction in *Cavb3* and *Kir6.2* expression with concordant increased intracellular Ca<sup>2+</sup> levels after challenge with glucose.

**Conclusion:** Altogether, the present findings show the breastfeeding exerts long-term benefits for maternal endocrine pancreas by increasing intracellular Ca<sup>2+</sup> levels and GSIS.

## Key Words

- ▶ pregnancy
- ▶ lactation
- ▶ pancreatic islets
- ▶ insulin secretion
- ▶ type 2 diabetes mellitus

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## Introduction

The benefits of breastfeeding for infant health are solidly supported by a great number of studies. Since the first observational study showing reduced mortality rate in breastfed infants compared to bottle-fed infants (1), many investigations have further detailed the hazards arising from insufficient or absent breastfeeding to the newborn. Infants subjected to inadequate breastfeeding have higher rates of infectious morbidity, including otitis media, gastroenteritis and pneumonia (2). Adolescents exposed to shorter breastfeeding periods are also more prone to develop metabolic syndrome and type 2 diabetes mellitus (T2DM) (3, 4, 5).

More recently, epidemiological studies have also detailed a wide range of benefits that breastfeeding yields to mothers later in life (2). Retrospective cohort studies have demonstrated that short periods of breastfeeding are directly associated with higher risk for T2DM in young and middle-aged mothers with no history gestational diabetes (6, 7). Additional prospective data revealed that exclusive breastfeeding for at least 6 months presented increased insulin sensitivity as assessed by HOMA-IR index 3 years after delivery rather than increased pancreatic  $\beta$ -cell function as indicated by *in vivo* insulin secretion (8).

Experimental studies on this topic are rare but somehow concordant with the observational data. Female mice subjected to a history of pregnancy and lactation were described to maintain reduced glycaemia 6 months after delivery (9). On the other hand, rats exposed to subsequent cycles of pregnancy without lactation exhibited increased glucose levels at the 20th gestational day when compared to age-matched virgin rats (10).

Apart from these studies, no investigation has elucidated if the history of pregnancy with or without lactation can affect maternal pancreatic islet physiology later in life. To address this issue, we have assessed *in vivo* glucose homeostasis and morphological and functional aspects of islets isolated from mice subjected to a history of pregnancy with or without lactation. Other parameters such as intracellular calcium dynamics and the expression of genes that are pivotal for pancreatic islets physiology have also been evaluated.

## Materials and methods

### Experimental design

All experimental procedures were conducted with the approval of the Committee for Ethics in Animal Experimentation at the State University of Campinas (protocol n. 4385-1). Female and male C57BL/6J mice were purchased from Animal Breeding Center at the University of Campinas (CEMIB, Campinas, Sao Paulo, Brazil) and housed at  $22 \pm 2^\circ\text{C}$  under a 12-h light-dark cycle (lights 07:00–19:00h), with free access to food (Nutrilab, Colombo, Paraná, Brazil) and water.

The protocols for mating, assignment into the different groups and litter adjustment were performed as previously described (11). Briefly, 9-week-old female mice were continuously housed for 5 days with males of the same age (two females: one male). Age-matched mice were kept unmated to be assigned to the Virgin group. Pregnant mice were either allowed to breastfeed for 21 days (L21) or had the litter removed no longer than 24 h after delivery (L0). L21 and L0 mice were subjected to two cycles of pregnancy. The second mating occurred 5 weeks after the first delivery. The experiments were performed 3 months after the second delivery. The age-matched virgin mice were subjected to identical housing conditions. The mean success of pregnancy in the first mating was 70%, and the mean success of pregnancy in the second mating was 74%.

### Intraperitoneal glucose (ipGTT) and insulin tolerance test (ipITT)

Animals were submitted to overnight fasting and blood glucose was measured (time 0). For ipGTT, a glucose load of 2 g/kg of body weight was then administered by i.p. injection, and additional blood samples were collected at 15, 30, 60, 90 and 120 min to measure glycemia. For the ipITT, 1 U/kg of body weight of human insulin (Lilly) was administered by i.p. injection, and additional blood samples were collected at 5, 10, 15, 20, 25 and 30 min for blood glucose measurements. The area under the curve (AUC) of glycemia vs time was calculated above each individual baseline (basal glycemia) to estimate glucose tolerance at the ipGTT. The constant rate of glucose disappearance ( $K_{ITT}$ ) was calculated using the formula  $0.693/\text{half-life}$ . The glucose half-life was calculated from the slope of the least-squares analysis of the blood glucose concentrations during the linear phase of decay.

### Flow cytometry

Retroperitoneal and inguinal adipose fat pads were weighted, minced with scissors and incubated in medium containing collagenase (DMEM,  $\text{NaHCO}_3$ , HEPES, BSA and 0.8% of type IA collagenase from Sigma-Aldrich, pH adjusted to 7.5) at  $37^\circ\text{C}$ , under agitation (110 rpm) in an orbital shaker for 45 min. Macrophages were filtered in cell strainer (100  $\mu\text{m}$ ) and washed three times with DMEM medium without collagenase. Erythrocytes were removed using hemolysis buffer. After removal of the lysis buffer, cells were resuspended in 2 mL of FACS buffer and were counted in Neubauer chamber.

Cell suspension ( $1 \times 10^6$ ) was preincubated with Fc block (anti-CD16/CD32; Thermo Fisher) at  $4^\circ\text{C}$  for 5 min and then stained with the following antibodies: anti-CD11c-PE, anti-CD206-APC and anti-F4/80-APC/Cy7 (all from Biolegend, San Diego, CA, USA). Samples were incubated for 30–60 min at  $4^\circ\text{C}$  in the dark. Cells were then centrifuged (140 g, 5 min,  $4^\circ\text{C}$ ), the supernatant discarded, and the cells were washed with FACS buffer. Cells were suspended in 200  $\mu\text{L}$  of FACS buffer and then analyzed in flow cytometer (FACSVerse, BD Biosciences). The analyses were performed using FlowJo v. 9.0 with the gating strategy shown in Fig. 3A. Fat pad weights were expressed as the percentage of body weight (Table 1).

### Pancreatic islet isolation and insulin secretion

Islets were isolated after the perfusion and digestion of the pancreas with collagenase, as described before (12). After

**Table 1** Adiposity and pancreas morphometric data of Virgin, L0 and L21 mice.

	Virgin	L0	L21
Inguinal fat pad (% of body weight)	1.2 ± 0.2 (9)	1.5 ± 0.3 (10)	1.2 ± 0.3 (7)
Retroperitoneal fat pad (% of body weight)	0.6 ± 0.05 (9)	1.1 ± 0.3 (10)	0.7 ± 0.08 (7)
Pancreas weight (mg)	327 ± 38.5 (4)	397 ± 54.9 (4)	313 ± 22.5 (4)
Pancreatic islet mass (mg)	2.36 ± 0.9 (4)	4.04 ± 0.8 (4)	2.22 ± 0.3 (4)
Pancreatic β-cell mass (mg)	1.65 ± 0.7 (4)	3.07 ± 0.6 (4)	1.60 ± 0.2 (4)
Pancreatic non-β-cell mass (mg)	0.76 ± 0.3 (4)	1.04 ± 0.2 (4)	0.63 ± 0.04 (4)
Number of islets per pancreas section	55 ± 6 (4)	72 ± 11(4)	92 ± 9 (4)

Data are presented as the mean ± s.e.m.. Retroperitoneal adiposity, the masses of pancreatic islet, pancreatic β-cell and pancreatic non-β-cell, and the number of islets per pancreas section were compared using Kruskal-Wallis test. The pancreas weight and the inguinal adiposity were compared with one-way ANOVA. The numbers of independent measures are in parenthesis.

isolation and manual separation from the exocrine tissue, groups of five islets were incubated for 30 min at 37°C in Krebs–Ringer bicarbonate (KRB) buffer containing 115 mM NaCl, 5 mM KCl, 10 mM NaHCO<sub>3</sub>, 2.56 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 15 mM HEPES, pH 7.4, supplemented with 5.6 mM glucose plus 0.3 % BSA (Sigma Chemical). After this 30-min period of pre-incubation, the islets were incubated with a fresh KRB buffer with different glucose concentrations for 1 h at 37°C. The cumulative insulin release over 1 h was quantified in duplicate using commercial ELISA kit (Cat No. EZRMI-13K, Merck Millipore).

### Immunohistochemistry and pancreas morphology

The whole pancreas was removed, weighted, fixed in fixative solution and embedded in paraffin. The sections obtaining, insulin staining for immunohistochemistry and pancreatic morphometry were performed as previously described (13). Briefly, two sections of each pancreas were randomly selected for analysis of endocrine pancreas morphology. All islets present in the sections were covered systematically by capturing images with a digital camera (Olympus DP72) coupled to an Olympus BX51TF microscope. The pancreatic islet area was obtained by manual areas were obtained by manual tracing of insulin-positive and insulin-negative cells from all islets on the sections. Images analysis was performed with ImageJ software (<http://imagej.nih.gov/ij>) tracing of all islets on the sections. The islet, β-cell and non-β-cell masses (mg) were determined by multiplying their respective relative mass (obtained by the sum of the islet, β-cell or non-β-cell areas corrected by the total area of the pancreas section) by the pancreas mass.

### Cytoplasmatic Ca<sup>2+</sup> dynamics

Islets were processed for evaluation of cytoplasmatic Ca<sup>2+</sup> as previously described (14, 15). Briefly, after loading

with Fura-2 acetoxymethylester, the islets were perfused with albumin-free KRB-buffer (pH 7.4) containing either 5.6 mM or 11.1 mM glucose, as indicated in the figures. Changes in intracellular Ca<sup>2+</sup> concentrations were detected as a ratio image, using a dual filter wheel equipped with 340, 380 and 10 nm bandpass filters, and a range of neutral density filters (Photon Technology International, NJ, USA) every 3 s with a Cool One camera (Photon Technology International, Edison, NJ, USA). The data were acquired with Image Master software version 5.0 (Photon Technology International). The fluorescence ratio values were plotted vs time, and the AUC was calculated during the interval corresponding to the exposure to 11.1 mM glucose.

### RNA extraction and quantitative polymerase chain reaction (qPCR)

Analysis of mRNA expression was performed as previously described (16). Total RNA was extracted using Qiazol reagent from pools of 300 freshly isolated islets. Aliquots containing 2 µg of RNA were subjected to RT using a high-capacity cDNA RT kit (Applied Biosystems). PCR reactions were conducted using QuantiNova SYBR Green PCR Kit (Qiagen) in a StepOnePlus Real-Time PCR System (Applied Biosystems).

The primer sequences were as follows: *Gck* sense: 5'-GCACAAGTCGTACCAGCT-3'; *Gck* antisense: 5'-AAA GCTCAGCGAACCCCG-3'; *Slc2a2* sense: 5'-TCTTCA CGGCTGTCTCTG-3'; *Slc2a2* antisense: 5'-GCACAGAAA AACATGCCA-3'; *Kir6.2* sense: 5'-TGAGCGGTAGAG CACAAGC-3'; *Kir6.2* antisense: 5'-TTTATACTACTAGCC TCA-3'; *Sur1* sense: 5'-CAAGGTGTCCTCAACAACGG-3'; *Sur1* antisense: 5'-CCAGGTGCTATGGTGAATGTG-3'; *Cavb2* sense: 5'-CACTATTTCTCTGGCGTTCTGT-3'; *Cavb2* antisense: 5'-CTGAGAAGACACCAACTGCCT-3'; *Cavb3* sense: 5'-CGCTAACCCGGTCTATGTCT-3'; *Cavb3* antisense: 5'-GTGTAGGAGTCGGCTGAACC-3'; *Rpl37a*

sense: 5'-CAAGAAGGTCGGGATCGTCG-3'; and *Rpl37a* antisense: 5'-ACCAGGCAAGTCTCAGGAGGTG-3'. The mRNA expression values were normalized using the geometric mean calculated from the internal control gene *Rpl37a*. The fold changes were calculated via the 2- $\Delta\Delta$ CT method.

### Statistical analysis

The results are presented as the mean  $\pm$  standard error of the mean (S.E.M.). Kolmogorov–Smirnov and Shapiro–Wilk normality tests were used to check for parametric distribution. Comparisons of parametric data were made with one-way ANOVA. Parametric insulin secretion data were compared using two-way ANOVA considering (i) glucose concentrations and (ii) experimental groups. Tukey's and Sidak's multiple comparisons test were applied when appropriate. Non-parametric data were compared with Kruskal–Wallis test followed by Dunn's multiple comparison test (GraphPad Prism Software, version 8.0, Inc.). *P* values <0.05 indicated a significant difference.

## Results

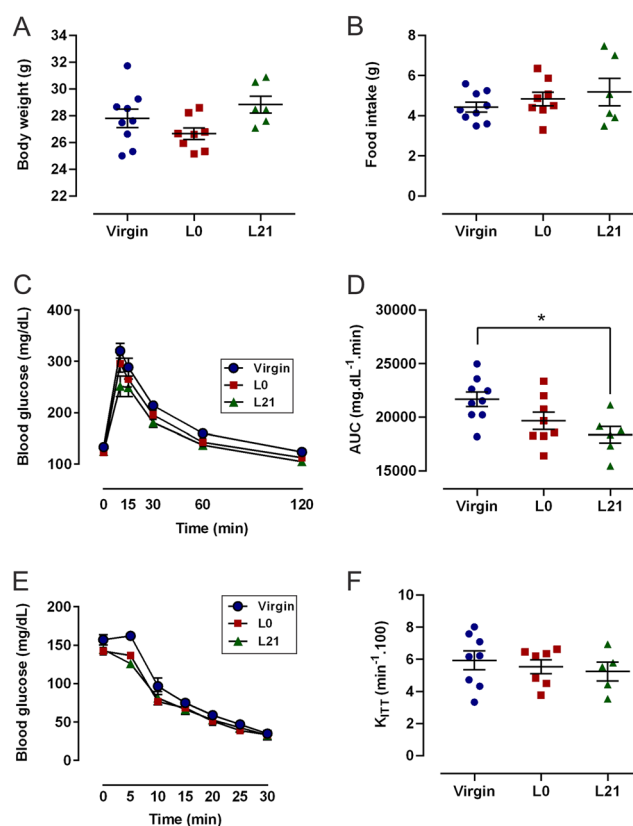
### History of pregnancy followed by lactation improves maternal glucose tolerance later in life

Mice belonging to the virgin, L0 and L21 groups had similar body weights and daily food intake one day before the metabolic assays (Fig. 1A and B). The mice belonging to the different groups also had similar adiposity when evaluating either the inguinal or the retroperitoneal fat pads (Table 1). On the other hand, the AUC values obtained from GTT were reduced in L21 but not in L0 mice (16% lower than in virgin mice; *P*=0.01) (Fig. 1C and D). The increased glucose tolerance seen in L21 mice was not accompanied by changes in whole-body insulin sensitivity. Such affirmation is sustained by the similar  $K_{ITT}$  values among the three groups (Fig. 1E and F).

### History of pregnancy followed by lactation increases glucose-stimulated insulin secretion by maternal pancreatic islets later in life

Our data demonstrating that increased glucose tolerance in L21 mice was not paralleled by changes in peripheral insulin sensitivity was an indicative that mice allowed to breastfeed after delivery were able to exhibit a long-term increased in glucose-stimulated insulin secretion (GSIS) by pancreatic islets.

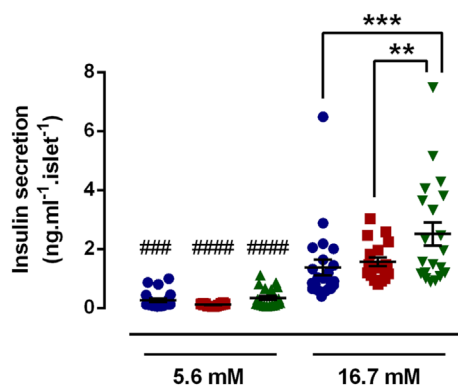
Attempting to clarify this issue, we evaluated GSIS by pancreatic islets isolated from virgin, L21 and L0 mice. Isolated pancreatic islets were incubated with glucose at a final concentration of either 5.6 mM or 16.7 mM. Islets isolated from both virgin, L0 and L21 mice secreted more insulin when incubated with 16.7 mM glucose (respectively 426, 1327 and 648% higher than islets of the same group incubated with 5.6 mM glucose; *P*<0.001 and *P*<0.0001). However, insulin secretion stimulated by 16.7 mM glucose was further elevated in islets of L21 mice (respectively 83 and 59% higher than insulin secretion by islets of virgin and L0 mice incubated 16.7 mM glucose; *P*<0.001 and *P*<0.01) (Fig. 2).



**Figure 1**

Glucose homeostasis in virgin, L0 and L21 mice. Body weight (A) and daily food intake (B) were recorded 3 months after the second delivery in L0 and L21 mice. Simultaneous measurements were made in age-matched virgin mice (Virgin). Glucose tolerance tests were performed and the glycaemia vs time curves (C) were used to calculate the area under the curve (AUC) (D). Insulin tolerance tests were performed and the glycaemia vs time curves (E) were used to calculate the  $K_{ITT}$  (F). Data are presented as the mean  $\pm$  S.E.M. Comparisons were made using one-way ANOVA. Virgin (*n* = 9 for body weight, food intake and GTT and *n* = 9 for ITT), L0 (*n* = 8 for body weight, food intake and GTT and *n* = 7 for ITT), L21 (*n* = 6 for body weight, food intake and GTT and *n* = 5 for ITT). \**P* = 0.01.



**Figure 2**

Insulin secretion by pancreatic islets isolated from Virgin, L0 and L21 mice. Pancreatic islets were isolated from L0 and L21 mice 3 months after the second delivery. Simultaneous isolations were made from age-matched virgin mice (Virgin). Isolated islets were incubated with 5.6 mM and 16.7 mM glucose. After incubations, the supernatants were collected and used for insulin concentration determinations with commercially available ELISA kits. Data are presented as the mean  $\pm$  s.e.m. Comparisons were made using two-way ANOVA. Virgin ( $n = 23$ ), L0 ( $n = 18$ ), L21 ( $n = 20$ ). ### $P < 0.001$  and #### $P < 0.0001$  vs islets from the same group incubated with 5.6 mM glucose; \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

### Increased insulin secretion by islets of mice with history of pregnancy followed by lactation is not associated with changes in macrophages infiltrated in adipose fat pads

Macrophages infiltrated in distinct adipose fat pads are recognized to produce several interleukins that are able to cause insulin resistance (17). Adipose tissue macrophages (ATM) can assume two distinct phenotypes, the M1-polarized macrophages (F4/80<sup>+</sup>/CD11c<sup>+</sup>) and the M2-polarized macrophages (F4/80<sup>+</sup>/CD206<sup>+</sup>). The M1 macrophages, also known as pro-inflammatory macrophages, produce several interleukins such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) that induce insulin resistance in peripheral insulin-sensitive tissues (17). Importantly, accumulated evidence also indicates that these cytokines chronically impair pancreatic  $\beta$ -cell function (18, 19, 20).

Aiming to elucidate if the increased insulin secretion capacity of pancreatic islets isolated of L21 mice would be due to changes in the pro-inflammatory status of the white adipose tissue, we evaluated the amount of F4/80<sup>+</sup>/CD11c<sup>+</sup> and F4/80<sup>+</sup>/CD206<sup>+</sup> cells in two distinct adipose fat pads. Our data demonstrate, however, similar percentages of F4/80<sup>+</sup>/CD11c<sup>+</sup> and F4/80<sup>+</sup>/CD206<sup>+</sup> cells in both retroperitoneal and inguinal fat pads of virgin, L0 and L21 mice (Fig. 3B, C, D and E, respectively).

### Pancreatic islets of mice with history of pregnancy followed by lactation do not exhibit long-lasting changes in pancreatic islet morphology

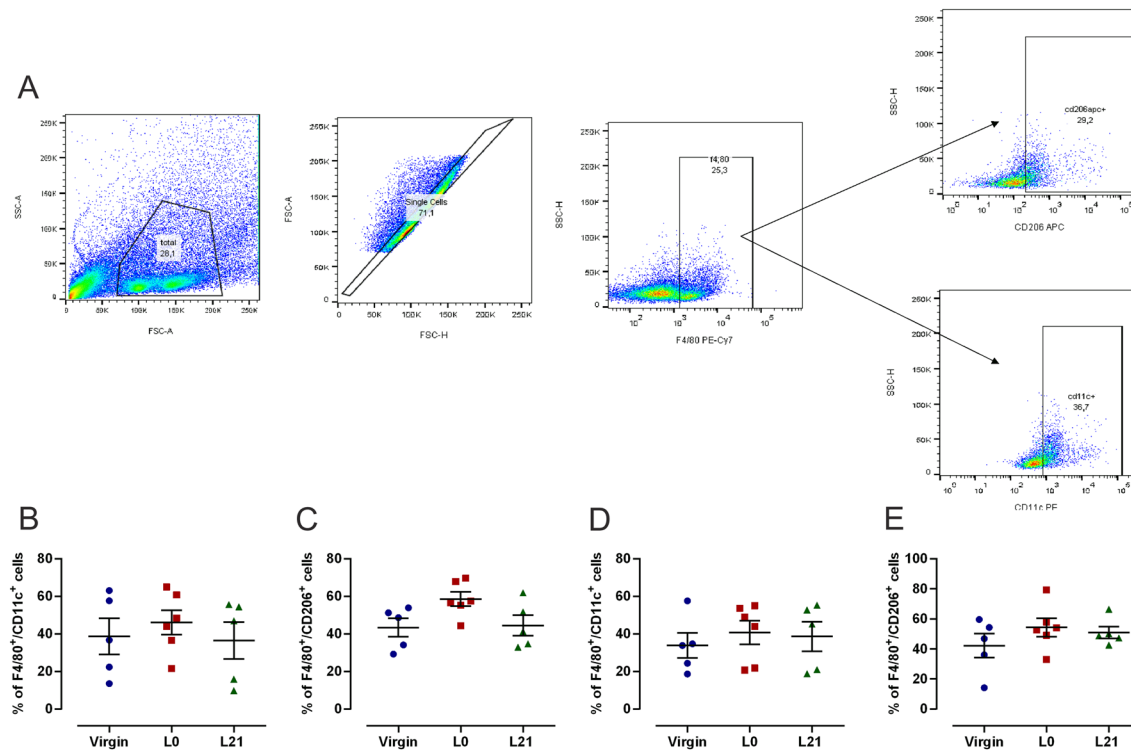
As our data have ruled out an extrinsic factor influencing insulin secretion in L21 mice, we decided to assess key morphological parameters that would impact pancreatic islet physiology. However, we found that mean pancreatic islet area, pancreatic islet  $\beta$ -cell area and pancreatic islet non- $\beta$ -cell area were similar among L21, L0 and virgin mice (Fig. 4B, C and D, respectively). Representative images of pancreata sections are shown in Fig. 4A. The masses of pancreatic islet, pancreatic  $\beta$ -cell and pancreatic non- $\beta$ -cell, and the number of islets per pancreas section were also similar among the different groups (Table 1).

### History of pregnancy followed by lactation leads to long-lasting changes in the expression of genes associated to pancreatic $\beta$ -cell insulin secretion

We next evaluated the expression of key genes that are pivotal for GSIS. The expression of *Gck*, *Slc2a2* and *Sur1* were equally expressed in islets of virgin, L0 and L21 mice (Fig. 5A, B and C, respectively). The expression of *Kir6.2*, however, was reduced in islets of L21 mice (36% lower than in islets of L0 mice;  $P = 0.02$ ) (Fig. 5D). The genes codifying two different subunits of voltage-operated calcium channels, *Cavb2* and *Cavb3*, were also evaluated. No changes were found in the expression of *Cavb2* (Fig. 5E), but the expression of *Cavb3* was reduced in islets of L21 mice (respectively 30 and 28% lower than in islets of virgin and L0 mice;  $P < 0.05$ ) (Fig. 5F).

### History of pregnancy followed by lactation modifies the dynamics in intracellular calcium levels induced by glucose in pancreatic islets

In order to verify if the changes in genes expression detected in islets of L21 were generating relevant biological outcomes, we next assessed the dynamics in intracellular calcium in isolated pancreatic islets after stimulus with glucose. The analysis of the fluorescence ratio vs time spectra evidenced that the 16.7 mM glucose-induced increase of cytoplasmic calcium was upregulated in islets of L21 mice. This finding was evidenced by both the AUC (49% higher than virgin and 66% higher than L0;  $P < 0.05$ ) (Fig. 6D) and the maximal fluorescence amplitude (66% higher than virgin and 59% higher than



**Figure 3**

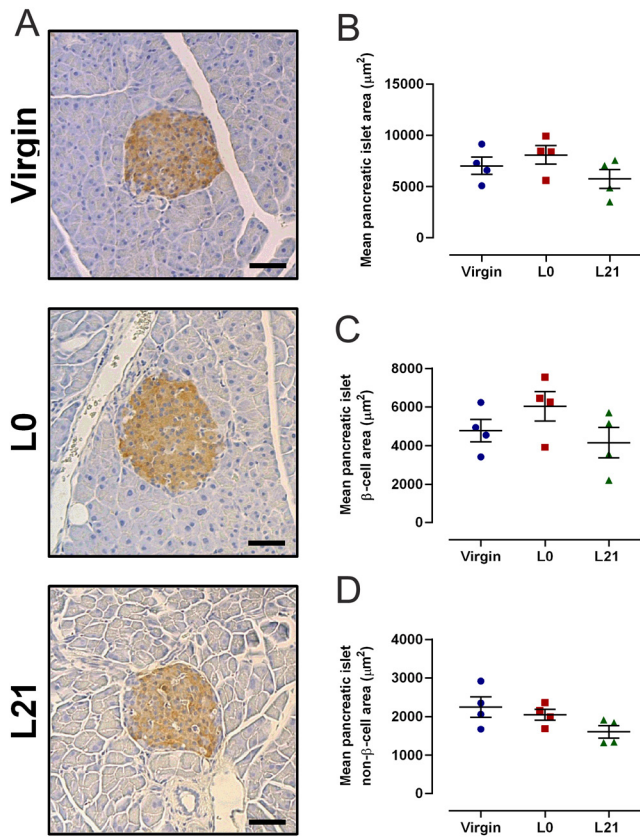
Macrophage infiltration in adipose tissues from virgin, L0 and L21 mice. Retroperitoneal and inguinal fat pads were isolated from L0 and L21 mice three months after the second delivery. Simultaneous isolations were made from age-matched virgin mice (virgin). Fat pads were digested and adipose tissue macrophages were processed for flow cytometry analyses. A gating strategy was adopted to identify F4/80<sup>+</sup>/CD11c<sup>+</sup> and F4/80<sup>+</sup>/CD206<sup>+</sup> macrophages (A). The number of F4/80<sup>+</sup>/CD11c<sup>+</sup> (B) and F4/80<sup>+</sup>/CD206<sup>+</sup> (C) macrophages in retroperitoneal fat pads and the F4/80<sup>+</sup>/CD11c<sup>+</sup> (D) and F4/80<sup>+</sup>/CD206<sup>+</sup> (E) in inguinal fat pads were expressed as the percentage of F4/80<sup>+</sup> cells. Data are presented as the mean ± s.e.m. Comparisons were made using one-way ANOVA. Virgin (*n* = 5), L0 (*n* = 6), L21 (*n* = 5).

L0; *P* < 0.05 and *P* < 0.01, respectively) values (Fig. 6E). No changes in the number of calcium oscillations were found when comparing islets of virgin, L0 and L21 mice (Fig. 6F). Representative histograms showing changes in islet fluorescence vs time are shown in Fig. 6A, B and C.

## Discussion

Observational studies consistently show that the duration of the breastfeeding is inversely associated with the risk of mothers developing T2DM later in life (6, 7). In spite of these epidemiological evidence, no investigation had applied an experimental approach to detailed examine how lactation exerts a long-term modulation on maternal glucose homeostasis. To the best of our knowledge, the present data are the first to report that the long-term impacts of breastfeeding in glucose tolerance of mice are associated with sustained modulations of the maternal pancreatic islets physiology.

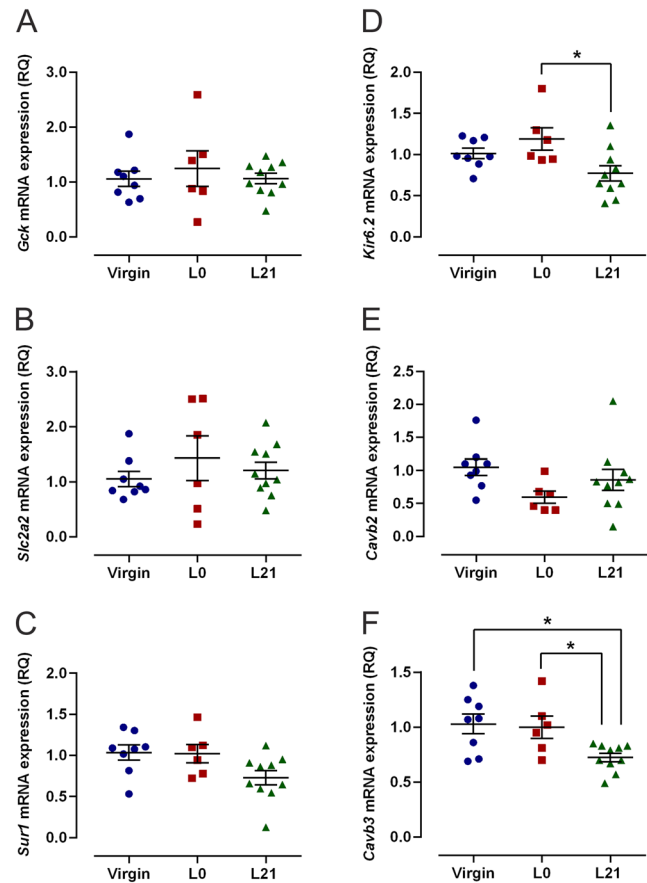
On the other hand, the present experiments with C57BL/6J mice demonstrated that repeated cycles of pregnancy with or without lactation do not interfere with maternal whole-body insulin sensitivity and pancreatic islet architecture later in life. Accordingly, we have also found that either pregnancy or pregnancy with lactation did not promote changes in M1 and M2 macrophages infiltration in maternal inguinal and retroperitoneal adipose tissue depots. Besides modulating peripheral insulin sensitivity through a mechanism dependent on pro-inflammatory cytokines (17), M1 and M2 infiltrated macrophages were also described to modulate adipocyte differentiation and adiposity (21). In general, central obesity resultant of increased visceral intra-abdominal adiposity is commonly associated to insulin resistance and increased risk for type 2 diabetes (22, 23). Recent human studies revealed, instead, that the amount of macrophages in s.c. adipose tissue correlates with visceral adiposity (24). Hence, our present data on unaltered M1 and M2 macrophages infiltration in both inguinal and retroperitoneal adipose tissue depots are also in



**Figure 4** Morphological features of pancreatic islet from Virgin, L0 and L21 mice. Panels show paraplasm-embedded pancreas sections (5 µm thick) from age-matched virgins, and L0 and L21 (A) 3 months after the second delivery, which were immunolabelled for insulin. Bars: 50 µm. All islets were used for morphometric analysis of pancreatic islet area (B), pancreatic islet β-cell area (C) and pancreatic islet non-β-cell area (D). Data are presented as the mean ± s.e.m. Comparisons were made using one-way ANOVA. Virgin (n = 4), L0 (n = 4), L21 (n = 4).

accordance with our data demonstrating that the history of pregnancy with or without lactation does not modulate adiposity in C57BL/6J.

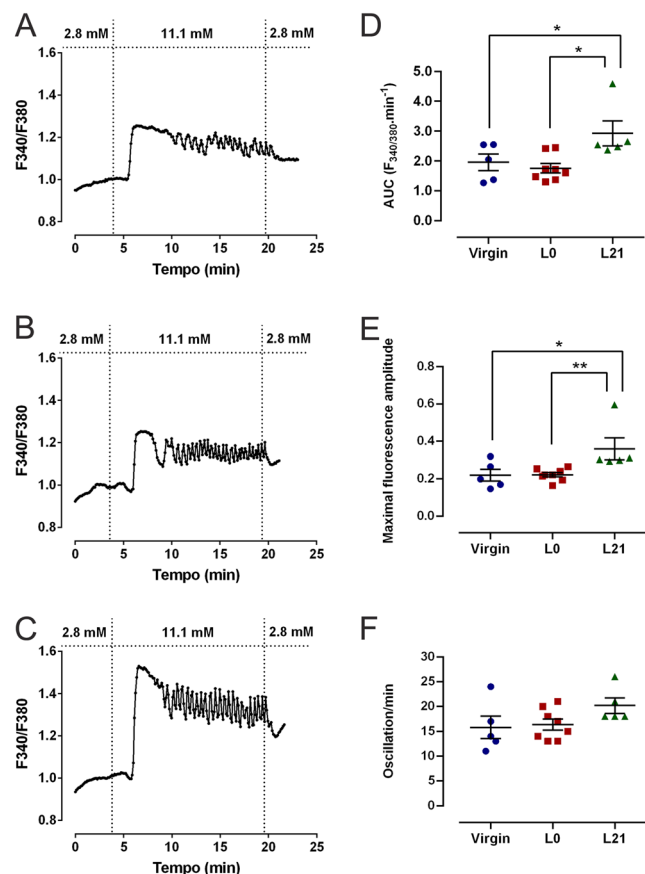
Important aspects differ the present data obtained in C57BL/6J mouse with those already published with women. Some important aspects, however, must be taken into account in order to explain such differences. Bajaj and colleagues have previously demonstrated that woman who had long periods of breastfeeding have increased insulin sensitivity and lower risk for pre-diabetes 3 years after delivery (8). The positive correlation between insulin sensitivity and breastfeeding duration seen in Bajaj's study was not accompanied by changes in Insulin Secretion Sensitivity Index-2 (ISSI-2) values (8). The ISSI-2 is a reliable index to assess the dynamics of *in vivo* insulin secretion during a glucose tolerance test (25). The current findings, instead, were based on *in vitro* assays to measure



**Figure 5** Gene expression in pancreatic islet from Virgin, L0 and L21 mice. Pancreatic islets were isolated from L0 and L21 mice 3 months after the second delivery. Simultaneous isolations were made from age-matched virgin mice (Virgin). Isolated islets were processed for mRNA quantification of *Gck* (A), *Slc2a2* (B), *Sur1* (C), *Kir6.2* (D), *Cavb2* (E) and *Cavb3* (F). Data are presented as the mean ± s.e.m. Comparisons were made using one-way ANOVA. Virgin (n = 8), L0 (n = 6), L21 (n = 10). \*P < 0.05.

insulin secretion from mouse isolated pancreatic islets. In this *in vitro* context, islets isolated from mice allowed to lactate secreted more insulin in response to glucose than those isolated from mice not allowed to lactate.

It is noteworthy that signals other than glucose act as primary stimulators of insulin secretion *in vivo*. Such signals can be both neurotransmitters released during the cephalic phase of insulin release or incretins such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) released by enteroendocrine cells in response to food transit (26). Human islets are particularly sensitive to GLP-1 and GIP effect on insulin secretion (27). Such features of human islets may serve to explain the greater insulin secretion in response to oral glucose when compared to *i.v.* glucose (26). Hence, the influence of these enteroendocrine signals on *in vivo* insulin secretion



**Figure 6** Intracellular calcium dynamics in pancreatic islet from Virgin, L0 and L21 mice. Pancreatic islets were isolated from L0 and L21 mice 3 months after the second delivery. Simultaneous isolations were made from age-matched virgin mice (Virgin). Representative curves of 11.1 mM glucose-induced intracellular  $Ca^{2+}$  oscillations in islets from Virgin (A), L0 (B) and L21 (C). The AUC (D), the maximal fluorescence amplitude (E) and the frequency of oscillations (F) were calculated within the interval of exposure to 11.1 mM glucose. The experiments were performed in a perfusion system in a medium that contained 2.8 mM or 11.1 mM glucose. Data are presented as the mean  $\pm$  s.e.m. AUC and maximal fluorescence amplitude were compared with Kruskal–Wallis test and numbers of oscillations per minute were compared with one-way ANOVA. Virgin ( $n = 5$ ), L0 ( $n = 8$ ), L21 ( $n = 5$ ). \* $P < 0.05$  and \*\* $P < 0.01$ .

may overcome those arising from breastfeeding period in women. Such influences might be absent when insulin secretion is measured from isolated pancreatic islets stimulated with glucose.

With the attempt to clarify the mechanism for increased GSIS in L21 islets, we investigated the expression of pivotal genes for insulin secretion. Our data revealed that the expression of the genes that codify Glut2 and glucokinase, two main glucose sensors of the pancreatic  $\beta$ -cell, were not differentially expressed in islets of mice subjected to cycles of pregnancy followed by lactation. Similarly, no modulations were found in the expression

of *Sur1*, the gene that codifies the ATP-sensitive regulatory subunit of the  $K_{ATP}$  channel that couples glucose metabolism to  $\beta$ -cell depolarization. Interestingly, our experiments revealed that the expression of *Kir6.2*, the pore forming subunit of  $K_{ATP}$ , was reduced in islets of mice allowed to lactate after delivery.

The phenotype of the mice expressing a dominant-negative form of *Kir6.2* in the pancreatic  $\beta$ -cell changes through the course of its life. These mice have hypoglycemia with hyperinsulinemia during neonatal life with higher basal intracellular calcium in pancreatic  $\beta$ -cells. The permanent increase in intracellular calcium, however, increases the apoptosis and decreases pancreatic  $\beta$ -cell mass during adulthood, leading these transgenic mice to a switch to a hyperglycemic phenotype later in life (28). This complex and dynamic phenotype limits the parallels that can be traced between the mice that completely lack *Kir6.2* in the  $\beta$ -cells and the mice belonging to the L21 group of the present study.

In our view, more appropriate is the interpretation of the results obtained with islets of L21 mice taking into account data previously reported with the transgenic AAA-TG line of mice. The AAA-TG mice have approximately 70% of pancreatic  $\beta$ -cells with nonfunctional *Kir6.2*. These  $\beta$ -cells showed absent hyperpolarizing  $K_{ATP}$  current and increased intracellular calcium levels and GSIS (29). *In vivo*, AAA-TG mice demonstrated improved glucose tolerance as they reached the age of 24 months (30). Interestingly, transcriptome analysis revealed that treatment with toxic concentrations of saturated fatty acids is able to increase *Kir6.2* expression in human pancreatic islets (31).

Another relevant finding in islets of mice belonging to the L21 group was the reduction in the expression of the *Cavb3*. While  $\alpha_1$  subunits of the voltage-gated L  $Ca^{2+}$  channel form the pore, the  $\beta$  subunits modulate  $Ca^{2+}$  currents (26). Mice knockout for *Cavb3*, the gene that encodes the  $\beta_3$  subunit of the voltage-gated L  $Ca^{2+}$  channel, have increased glucose tolerance and higher GSIS by isolated islets (30). Islets of mice knockout for *Cavb3* also presented a higher frequency and increased maximal amplitude in intracellular  $Ca^{2+}$  oscillation induced by glucose (30, 32).

Hence, our data indicating increased maximal amplitude of intracellular calcium and increased calcium concentration in L21 pancreatic islets incubated with stimulatory concentration of glucose points to a functional role of reduced *Cavb3* and *Kir6.2* for the long-lasting increased insulin secretion seen in this group of mice.

The endocrine and neuronal changes that occur in a lactating dam are complex and several factors can possibly



exert long-term effects on pancreatic islets. It is reasonable to point, however, to a putative role of the frequent bursts in pituitary prolactin (PRL) production presumably experienced by L21, but not by L0 and Virgin mice. Although both pregnant and lactating mice are known to have high levels of PRL, the production of this hormone during lactation is highly dependent on the suckling stimulus (33). PRL, in turns, is classically recognized to exert positive effects over insulin secretion. PRL-knockout mice display reduced insulin secretion during pregnancy and PRL *per se* increases GSIS *in vitro* (34, 35).

One limitation of our study is that although the present experiments clearly demonstrate that breastfeeding yields long-term benefits for the maternal endocrine pancreas of mice, we cannot assure that such changes also occur in women. Much more studies with isolated human islets are necessary to establish if the changes found in mice are replicated in women. Thus, it still premature to affirm that breastfeeding itself would exert long-term effect on human islet insulin secretion and calcium handling.

In conclusion, our results demonstrate that mice subjected to sequential cycles of pregnancy and lactation exhibits long-term improvement of glucose tolerance that is linked to increased GSIS. Conciliatory and long-lasting changes in *Cavb3* and *Kir6.2* expression and changes in calcium dynamics were found to explain such changes. No similar modulations were found in mice not allowed to breastfeed after delivery. Altogether, the present findings show that the sustained benefits elicited by breastfeeding for maternal glucose homeostasis in mice are mediated by changes in pancreatic  $\beta$ -cells. These results might serve to comprehend the mechanism related to the inverse relationship between breastfeeding duration and maternal risk for T2DM later in life.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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#### Ethics approval

The present experiments were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation after the authorization of the State University of Campinas Committee for Ethics in Animal Experimentation (protocols No. 4496-1, No. 3711-1 and No. 4863-1).

#### Author contribution statement

G F A and J M V designed the research. J M V, J C S-S, C J T, D N de S, J F V, F S F, I G A, F T S and M A R V performed the experiments and analyzed the data. E M C, M A R V, S B and G F A contributed to the discussions. G F A and S B wrote the paper.

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