




Senolytic treatment reverses obesity-mediated senescent cell accumulation in the ovary

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Abstract Senescent cells are in a cell cycle arrest state and accumulate with aging and obesity, contributing to a chronic inflammatory state. Treatment with senolytic drugs dasatinib and quercetin (D+Q) can reduce senescent cell burden in several tissues, increasing lifespan. Despite this, there are few reports about senescent cells accumulating in female reproductive tissues. Therefore, the aim of the study was

to characterize the ovarian reserve and its relationship with cellular senescence in genetically obese mice (ob/ob). In experiment 1, ob/ob ($n=5$) and wild-type (WT) mice ($n=5$) at 12 months of age were evaluated. In experiment 2, 2-month-old female ob/ob mice were treated with senolytics (D+Q, $n=6$) or placebo ($n=6$) during the 4 months. Obese mice had more senescent cells in ovaries, indicated by increased p21 and p16 and lipofuscin staining and macrophage infiltration. Treatment with D+Q significantly reduced senescent cell burden in ovaries of obese mice.

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Neither obesity nor treatment with D+Q affected the number of ovarian follicles. In conclusion, our data indicate that obesity due to leptin deficiency increases the load of senescent cells in the ovary, which is reduced by treatment by senolytics. However, neither obesity nor D+Q treatment affected the ovarian reserve.

Keywords Ovarian reserve · Obesity · Cell senescence · Dasatinib · Quercetin

Introduction

Aging is a physiological process of declining tissue function [1] due to cumulative damage resulting from genomic instability, telomere shortening, epigenetic changes, and loss of proteostasis [2]. As a response, compensatory mechanisms aimed at mitigating damage are initiated including the dysregulation of nutrient sensitivity, mitochondrial dysfunction, and cellular senescence [2]. In females, along with common somatic health-related complications of aging, there is a severe decline in fertility [3]. In this sense, ovarian aging refers to the reduction in the number of ovarian follicles with advancing age. The ovarian reserve is determined in utero and comprised primordial follicles that remain in a quiescent state until activated, at which point they are ovulated or undergo atresia [4]. When the ovarian reserve is severely reduced, menopause occurs in women [4]. In many strains of mice, menopause, or estropause, does not naturally occur, although the ovarian reserve does become severely reduced with age [5]. Therefore, the reproductive lifespan of a female depends on the initial size of the primordial follicle reserve, as well as its rate of depletion [6]. In addition to infertility, menopause considerably increases the risk for several metabolic diseases including cardiovascular disease, osteoporosis, hypertension, diabetes mellitus, and ovarian cancer [7, 8].

Interestingly, obesity can accelerate the development of age-related diseases [9]. Leptin-deficient mice (*ob/ob*) are widely studied as a model of human obesity [10]. The *ob/ob* mice are severely obese, insulin resistant, have impaired fertility, and display a shortened lifespan [11]. It is becoming well recognized that obesity reduces the primordial follicle ovarian reserve in young animals [12, 13]. Female

ob/ob mice are infertile due to leptin signaling being required in the hypothalamus for gonadotropin release and ovulation to occur [14]. However, fertility is restored when *ob/ob* mice are supplemented with exogenous leptin [14] or receive adipose tissue transplants from lean littermates [15].

The accumulation of senescent cells with advancing age is also surmised to play a causal role in decreasing health span and lifespan [16]. Obesity is associated with increased senescent cell burden in various tissues, thereby contributing to increased systemic inflammation [2, 13, 17]. Senescence is a cellular defense mechanism, inhibiting cell cycle progression and tumorigenesis. However, cell cycle arrest also inhibits apoptosis thereby promoting the accumulation of senescent cells during aging [18–20]. Senescent cells often accumulate lipofuscin [21] which is accompanied by high expression of p16 and p21 [22]. In addition, senescent cells are characterized by the secretion of pro-inflammatory factors that attract macrophages [23]. The senescence-associated secretory phenotype (SASP) contributes to the propagation of pro-inflammatory signals in the tissue microenvironment, which is believed to promote the progression of age-related diseases [2, 24]. We previously reported that several markers of cellular senescence increase with age in the ovary of mice [25]. However, the emergence of senescent cells in the ovary is not well defined, and perhaps more importantly, the role that obesity plays in promoting the premature accumulation of senescent cells in the ovary is critically understudied.

Therapeutics known as senolytics are a relatively new class of drugs that can selectively eliminate senescent cells from the body. These treatments have been reported to decrease aging-related disease burden and increase lifespan [16]. One of the earliest senolytic cocktails to be developed and studied is the combination of dasatinib and quercetin (D+Q), which have been shown to be effective at killing senescent cells [26]. Quercetin is a bioflavonoid highly present in the human diet, whereas dasatinib is a tyrosine kinase inhibitor used in cancer therapy [27]. Despite other reports demonstrating that senolytic treatment can reduce senescent cell accumulation in genetically- and diet-induced obese mice [28, 29], it remains unclear if obesity increases senescence cell burden in the ovary, or if senolytic treatment can attenuate senescent cell accumulation in the ovary

and/or improve follicular reserve. Thus, our study aimed to understand how obesity affects the ovarian reserve and accumulation of ovarian senescent cells and if senolytics can lessen/prevent this senescent cell burden within the ovary.

Methods

Mice and experimental design

Experiments were approved by the Animal Experimentation Ethics Committee from Universidade Federal de Pelotas (Numbers 3715–2014 and 24,915–2020). All mice (C57BL/6 background) were maintained in ventilated cages with ad libitum access to food and water unless otherwise specified. The vivarium is maintained at 20 ± 2 °C at 40–70% humidity on a 12-h light cycle. In experiment 1, 12-month-old ob/ob ($n=5$) and C57Bl/6 wild-type (WT; $n=5$) females were euthanized, and ovarian tissue was collected. In a second experiment, 2-month-old ob/ob female mice were divided into control ($n=6$) and D+Q ($n=6$) treatment groups. Treated females received the senolytic drugs dasatinib and quercetin (D+Q) for 4 months beginning at 2 months of age. Treatment consisted of dasatinib (5 mg/kg) and quercetin (50 mg/kg) dissolved in 60% phosal, 30% PEG400, and 10% ethyl alcohol [16] for 3 consecutive days every 2 weeks. Control mice received the vehicle solution, composed of 60% phosal, 30% PEG400, and 10% ethyl alcohol. Females from experiment 2 were euthanized at 6 months of age to collect ovarian tissue. For both experiments, one of the ovaries was stored in 10% formaldehyde for histological and immunofluorescence assays, whereas the other was stored at -80 °C for RNA extraction.

Insulin tolerance test

An insulin tolerance test (ITT) was performed at 6 months of age in obese mice from experiment 2 receiving D+Q or vehicle. After 2-h fasting, basal glycemia was measured and, subsequently, human insulin (5 IU/kg body weight, Novolin R®, Novo Nordisk) was administered i.p. [30]. Glycemia was measured in a drop of blood from the tail at 5, 20, 35, and 60 min after insulin injections using a commercial glucometer (Accu-Chek Performa, ROCHE).

Follicle counting and classification

The ovaries were processed as previously described [25]. Nine ovarian sections from each mouse were used to count follicles at $40\times$ magnification and to calculate follicle density. Follicles were classified according previous descriptions [25, 31]. The number of follicles for each of the nine ovarian sections was then divided by the section area to calculate the follicle density (number of follicles/ mm^2) [25, 31]. The average density of the nine sections/mice was used for statistical analysis.

Lipofuscin staining by Sudan black

The lipofuscin staining was performed with the Sudan black dye on ovarian sections as an indicator of senescence. The protocol used was adjusted from a previous description [32]. The slides were dewaxed with xylol, washed in a gradient of ethyl alcohol until reaching 70% alcohol, and rehydrated with water. After diluting the Sudan black in 70% ethyl alcohol, avoiding its precipitation, a 10-ml syringe with disc filter was used to drip it onto a clean slide, and the slide containing the tissue sections was inverted over for approximately 2 min. After this procedure, the slide with the sections was separated and immediately washed with 50% ethyl alcohol and distilled water. The slides were assembled with glycerol and then observed under a light microscope at 4 and $10\times$ magnification. The area of lipofuscin staining in the images was calculated using the ImageJ software and expressed as the percentage of the total area of the section.

Immunofluorescence for macrophage, p21 and p16

Slides were deparaffinized and rehydrated with 3 washes of Safeclear (Fisher Scientific, Pittsburgh, PA, USA) for 10 min each, followed by 3 washes of each concentration in a graded series of ethanol (100%, 95%, 80%, 70%) for 5 min. After rinsing the slides 2 times for 5 min in distilled water, the slides were incubated in sodium citrate pH 6.0 during 40 min at 95 °C. Permeabilization was performed in 0.2% of Triton-X 100 in PBS for 1 h. Sections were blocked for 4 h in a blocking solution (2.52 mg/ml glycine, 10% goat serum, 3% BSA, 0.2% Triton-X in PBS-T) and then incubated with primary antibody

(diluted in blocking solution) overnight at RT (CD68 #ab955 Abcam Plc, UK; P16 #10,883–1-AP; and P21 #10,355–1-AP, Proteintech, USA) [33–37]. After 2 washes of 10 min with PBST, the slides were incubated with secondary antibodies for 2 h at RT (goat anti-rabbit Cy3 for p16 and p21, Jackson Immuno Research Laboratories, West Grove PA, USA; and Alexa Fluor® 488 #ab150113, Abcam). The slides were rinsed twice with PBST for 5 min. DNA was counterstained by applying an antifade solution (Vector laboratories, Burlingame, CA, USA) containing 0.1 µg/ml of DAPI (4',6'-diamidino-2-phenylindole) (Sigma). Image acquisition was performed using a Zeiss AxioImager M2 microscope (Carl Zeiss AG, Oberkochen, Germany). Images were processed using Zen 2 (Carl Zeiss AG, Oberkochen, Germany). Digital images were analyzed for relative fluorescence intensity using Fiji (ImageJ, NIH). Positive and negative controls for p16 (Suppl. Fig. 1) and p21 (Suppl. Fig. 2) antibodies are provided. Macrophage number was evaluated as a total number of macrophages per ovary section in 3 random ovary sections/mice.

RNA extraction and gene expression

Total RNA from ovarian samples was extracted and processed as previously described [38]. The genes Gapdh (glyceraldehyde-3-phosphate dehydrogenase), β 2m (beta-2 microglobulin), Actb (actin beta) and Ppia (peptidylprolyl isomerase A) were evaluated as endogenous controls. According to results from the

geNorm software [39], the β 2m gene was the most stable in both experiments. Relative gene expression was calculated using the comparative CT method [40]. Primer sequences used in these analyses are shown in Table 1.

Statistical analysis

All the data are presented as mean \pm SEM and a $P < 0.05$ was considered significantly different. All the statistical analyses were performed using student's *t*-test in GraphPad Prism 5.0.

Results

Experiment 1: ovarian senescence is increased in obese mice

As expected, body weight (Suppl. Fig. 3) was two-fold higher in ob/ob animals when compared to lean WT animals. Conversely, to our surprise ob/ob mice had an ovarian follicular reserve similar to age-matched WT lean mice. No difference was observed in the number of primordial, primary, secondary, tertiary, or total follicles between ob/ob and WT mice (Fig. 1). Interestingly, the percentage of lipofuscin positivity, a marker of senescent cells, was greater ($P = 0.005$; Fig. 2A) in the ovary of ob/ob females when compared to lean WT females. Furthermore, the number of macrophages

Table 1 Murine primer pairs (forward and reverse) used in the experiments

Gene	Primers	Size	Accession
β -2-Microglobulin (β 2m)	F: AAGTATACTCAGCCACCCA R: CAGGCGTATGTATCAGTCTC	217	NM_00935.3
Anti-Mullerian hormone (Amh)	F: TCCTACATCTGGCTGAAGTGATATG R: CAGGTGGAGGCTCTTGGAACT	66	XM_006513119.3
Growth/differentiation factor 15 (Gdf15)	F: GAGCTACGGGGTCGCTTC R: GGGACCCCAATCTCACCT	130	NM_001330687.1
Matrix metalloproteinase 12 (Mmp12)	F: GAGTCCAGCCACCAACATTAC R: CGGAAGTGGGTCAAAGACAG	232	NM_001320076.1
Chemokine ligand 2 (Ccl2)	F: GAAGCCAGCTCTCTTCTCCTC R: TTGCTGGTGAATGAGTAGCAG	150	NM_011333.3
Interleukin 1 alpha (Il1a)	F: GAGTCGGCAAAGAAATCAAGATG R: CAATGGCAGAACTGTAGTCTTCGT	96	NM_010554.4
Stanniocalcin-1 (Stc1)	F: CTACTTTCCAGAGGATGATCGC R: ACTTCAGTGATGGCTTCCGG	100	NM_009285.3

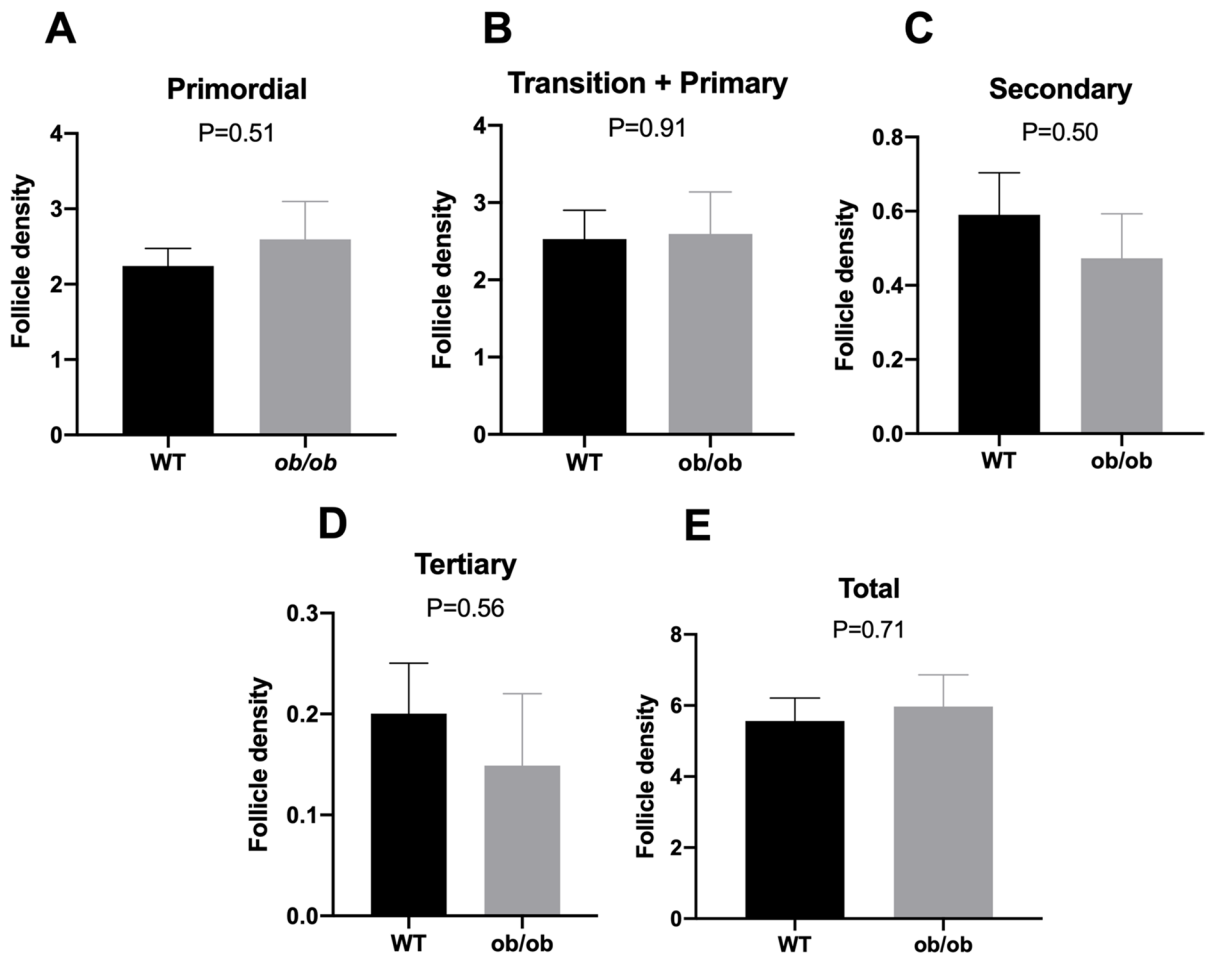


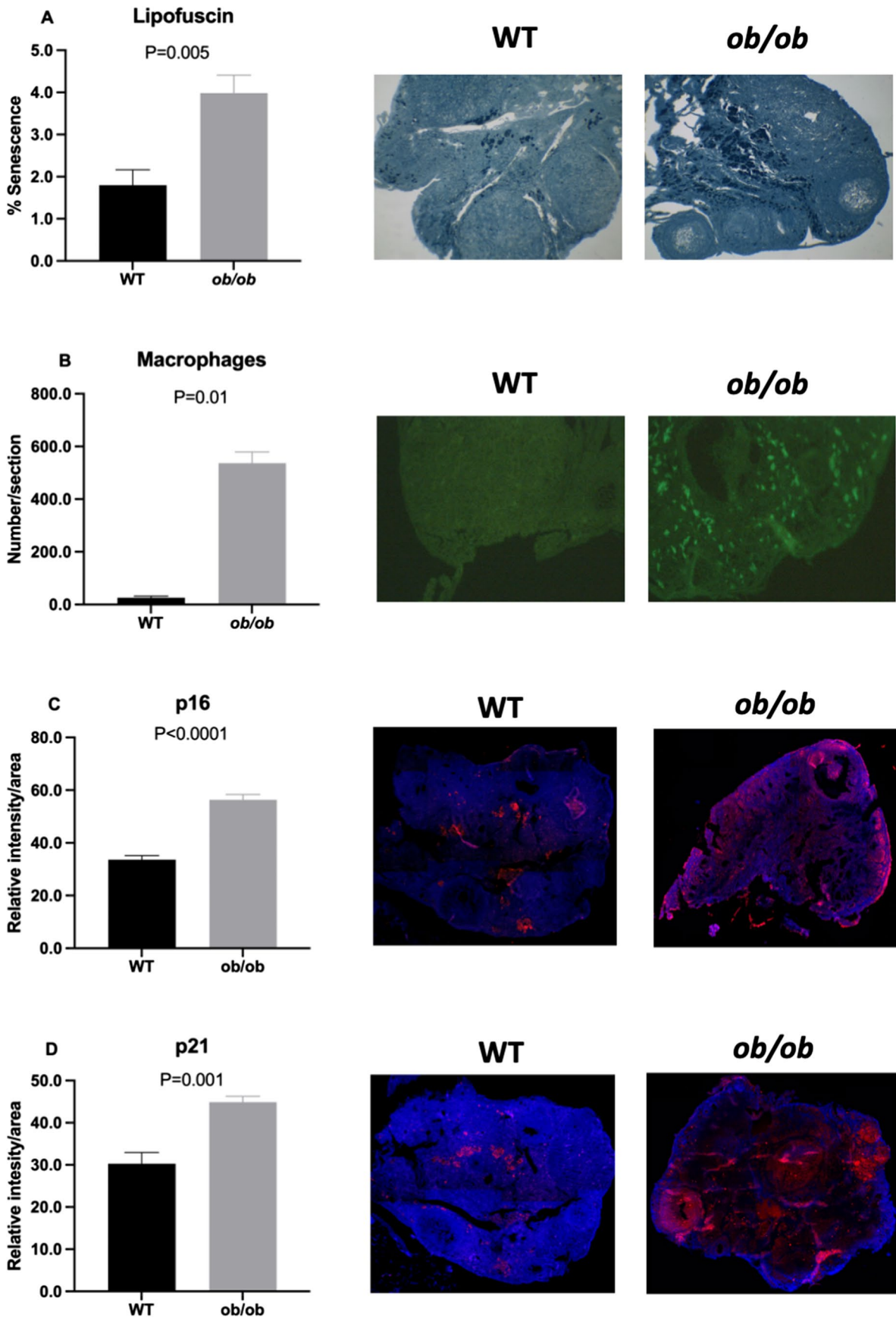
Fig. 1 Mean follicle density at different growth stages in obese (*ob/ob*, $n=5$) or lean wild-type (*WT*, $n=5$) mice at 12 months of age. Primordial (A), transition and primary (B),

secondary (C), tertiary (D), and total number of follicles (E). Values of $P < 0.05$ were considered significant

per ovarian section was higher in the ovary of *ob/ob* females when compared to lean *WT* females of the same age ($P=0.01$; Fig. 2B). p16 (Fig. 2C) and p21 (Fig. 2D) positivity was also found to be greater in *ob/ob* females ($P < 0.0001$ and $P=0.001$, respectively), suggesting that obesity does indeed promote a greater abundance of senescent cells in the ovary. There was no change in *Amh* gene expression ($P=0.29$; Fig. 3A), an ovarian reserve indicator, which supports our follicular reserve findings. Other transcriptional markers of senescence including ovarian expression of *Ccl2* ($P=0.20$), *Stc1* ($P=0.57$), *Il1-a* ($P=0.64$), *Gdf-15* ($P=0.24$), and *Mmp12* ($P=0.27$) were not different between *ob/ob* and *WT* mice (Fig. 3B–F).

Experiment 2: senolytics can reduce ovarian senescence in obese mice

In experiment 2, treatment with D+Q in *ob/ob* mice did not affect body weight or insulin sensitivity after 4 months of treatment (Suppl. Fig. 4). No changes were observed in follicle numbers after treatment with D+Q. No difference was found for the number of primordial (Fig. 4A), transitional and primary (Fig. 4B), secondary (Fig. 4C), tertiary (Fig. 4D), and total (Fig. 4E) follicles between the control and D+Q-treated obese mice. Treatment with senolytics (D+Q), however, decreased the percentage of senescent cells in the ovaries of *ob/ob* females treated with D+Q compared to controls, as indicated by



◀**Fig. 2** Lipofuscin staining (A), macrophage infiltration (B), and p16 (C) and p21 (D) staining in obese (ob/ob, $n=5$), and wild-type lean (WT, $n=5$) females at 12 months of age. Values of $P < 0.05$ were considered significant

lipofuscin staining ($P=0.002$; Fig. 5A). Similarly, the number of macrophages per ovarian section was lower in the ovary of D+Q-treated obese females ($P=0.01$; Fig. 5B). In addition, p16 (Fig. 5C) and p21 positivity (Fig. 5D) were lower in D+Q-treated females ($P=0.0002$ and $P=0.0002$, respectively), further suggesting the ability of D+Q to reduce ovarian senescence. There was also no change in *Amh* gene expression ($P=0.28$; Fig. 6A). Other markers of senescence, including *Ccl2* ($P=0.24$), *Stc1* ($P=0.48$), *Il1-a* ($P=0.84$), *Gdf-15* ($P=0.66$), and *Mmp12* ($P=0.62$) were not different between groups (Fig. 6B–F).

Discussion

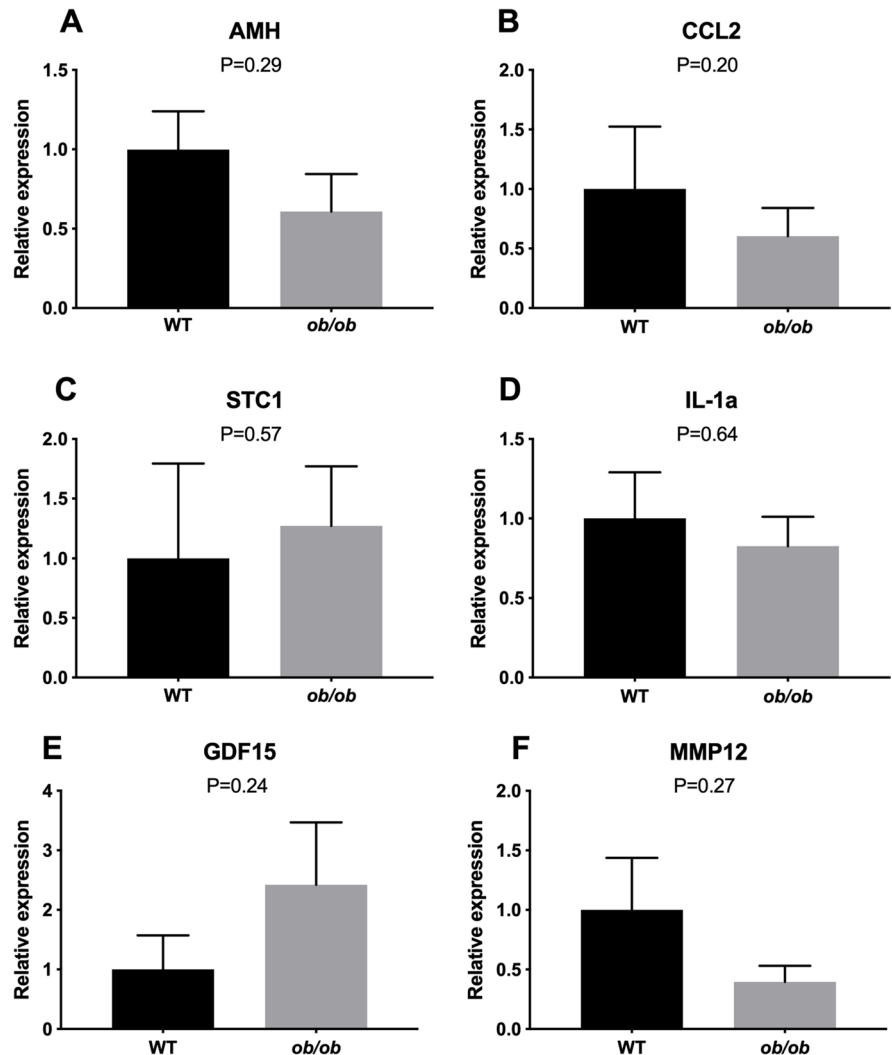
In this study, we characterized the ovarian reserve and accumulation of senescent cells in the ovaries of lean and obese mice. We also evaluated the effects of senolytic treatment on senescent cell burden in the ovary. We found that obese mice displayed a greater presence of senescent cells in the ovary when compared to lean littermates. This was demonstrated by greater lipofuscin accumulation and elevated p21 and p16 expression. To our knowledge, this is the first report demonstrating that obese mice accumulate more senescent cells within the ovary. We previously reported that lipofuscin accumulates in the ovary with advancing age, and that this occurs in conjunction with increased p21 and p16 expression [25]. Lipofuscin staining is a senescence marker highly correlated to β -galactosidase staining [21]. In addition, senescent cells have a high expression of p16 and p21, which result in cell cycle arrest [22]. It is well known that senescent cells are present in several tissues [41, 42]; however, data regarding the presence of senescent cells in female reproductive organs is scarce, especially in obese mice.

It is known that cellular senescence can be triggered by different types of stressors, such as oxidative stress, DNA damage, metabolic dysfunction, inflammation, and elevated glucose [43]. Interestingly, exposure of cells to high glucose concentration can induce

senescence, observed by higher β -galactosidase staining [44]. As insulin resistance and increased circulating levels of glucose are a common phenotype of diet-induced or genetic obesity, they can be linked to the increased presence of senescent cells. High glucose levels also trigger oxidative stress and inflammation [45]; therefore, obesity can stress the organism similarly to aging. Obesity has been implicated with the accelerated development of aging diseases, remodeling of tissues, acquisition of a pro-inflammatory phenotype, [42], which are changes commonly associated to senescence. There is an abundance of senescent cells in adipose tissue that increases with age [46]. Obesity has also been shown to increase senescent cell burden in adipose tissues [28], thereby supporting the idea that obesity may represent a mild progeria syndrome [42, 47–49]. In this sense, it was showed that both aging and obesity lead to accumulation of cellular senescence and SASP in a similar fashion [16, 28]. In fact, treatment of obese mice with senolytics reduced senescent cell burden and decreased insulin resistance [28], which suggests that these two conditions are closely interconnected. In this context, we can infer that obesity can accelerate the aging phenotype, resulting in accumulation of senescent cells. Our current findings support this idea by demonstrating that obesity also increases senescent cell burden in the ovary. Furthermore, senolytic treatment decreased the proportion of senescent cells in the ovaries of obese mice as shown by declines in lipofuscin accumulation and the expression of p16 and p21. This is the first study to evaluate the effects of senolytic treatment on ovarian senescence, and findings are analogous to previous reports indicating that senolytics can effectively reduce senescent cell burden in the context of obesity [28].

Despite displaying a reduction in senescent cell burden, we did not observe reduced expression of several SASP factors in ovarian tissue following senolytic treatment. It remains unclear if ovarian cells establish a unique SASP following the conversion to senescence, which could explain our inability to detect changes in known SASP factors. This is supported by a recent report demonstrating that different mouse tissues develop unique SASP profiles [50], although the ovary was not included in these analyses. To our knowledge, there has never been a characterization of the ovarian SASP, or an analysis to determine which ovarian cells become senescent.

Fig. 3 Analysis of relative gene expression in ovaries from obese (*ob/ob*, $n=5$) and wild-type lean (WT, $n=5$) females at 12 months of age. **A** Anti-Müllerian hormone (Amh), **(B)** chemokine ligand 2 (CCL2), **(C)** Stanniocalcin-1 (Stc1), **(D)** Interleukin 1 alpha (IL1a), **(E)** growth/differentiation factor 15 (Gdf15), **(F)** matrix metalloproteinase 12 (Mmp12). Values of $P < 0.05$ were considered significant



Given that tissue remodeling and pro-inflammatory processes are part of a normal functioning ovary, further studies will be needed to firmly establish the best markers for ovarian senescence and the SASP. Despite the fact that no changes were observed in SASP factors in our studies, ovaries from obese females did display a substantial increase in macrophage infiltration when compared to lean mice, and this was prevented by senolytic treatment. This finding supports previous work indicating that senescent cells attract macrophages as a means of removing damaged cells [23]. These findings also do not appear to be isolated to mice, since a report found that senolytic treatment decreased senescent cell burden, p21 and p16 expression, and macrophage infiltration in adipose tissue of humans [51].

In the current study, the ovarian follicular reserve was not affected by obesity. It is well established that diet-induced obesity accelerates activation of primordial follicles leading to premature exhaustion of the follicular reserve [12]. Calorie restriction (CR) has the opposite effect and preserves ovarian reserve [38]. Previous studies have shown that 3-month-old *ob/ob* mice have fewer preantral follicles when compared to WT mice [13]. Similarly, fewer primordial and total follicles were found in 7-week-old *ob/ob* mice compared to lean WT mice [52]. Both of these studies used very young mice; therefore, it is possible that differences in follicle counts disappear with aging as the ovarian reserve becomes severely reduced. It remains unclear if this is the reason; we did not observe differences between 12-month-old WT and

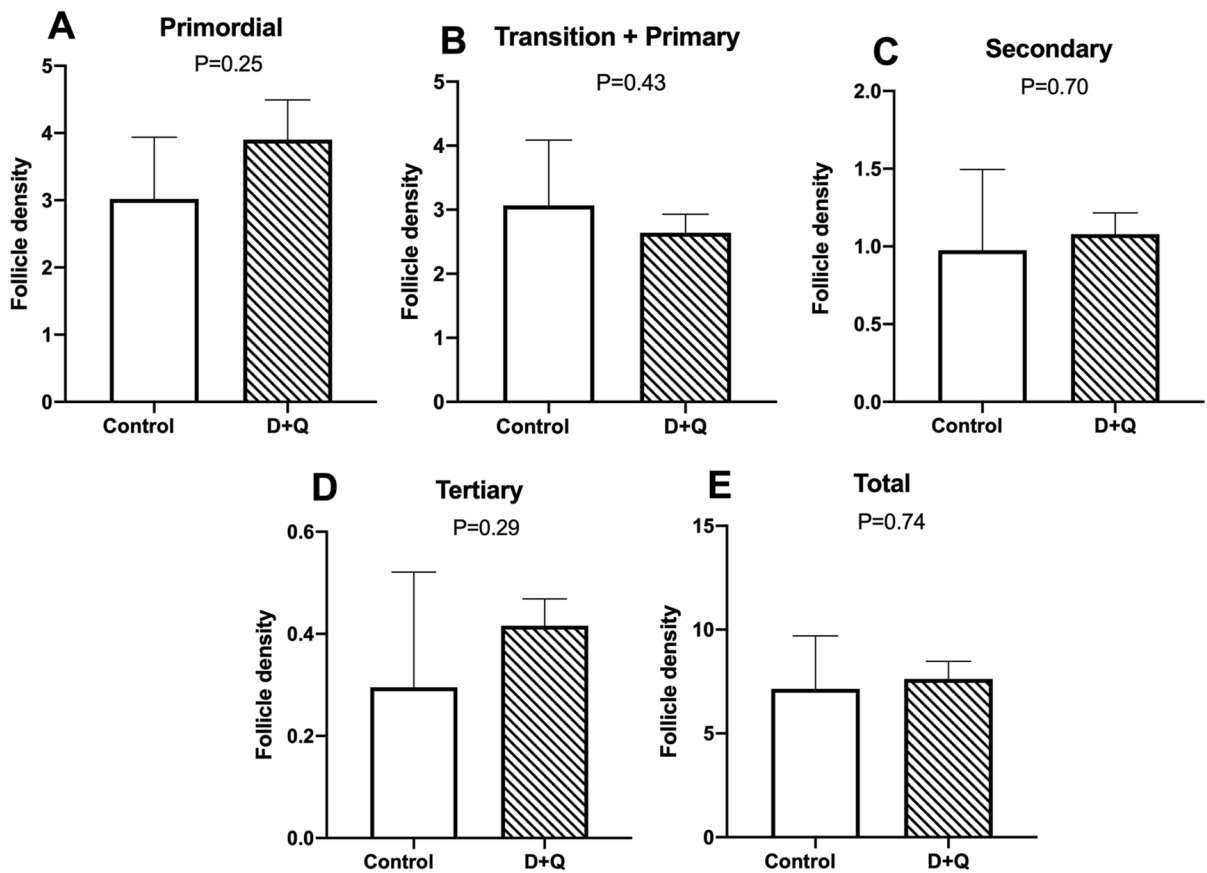


Fig. 4 Analysis of the follicle density (number/mm²) at different growth stages in 6-month-old obese (ob/ob) females, control ($n=6$) or treated with dasatinib plus quercetin ($n=6$).

P values <0.05 were considered significant. Values were represented as mean \pm standard error

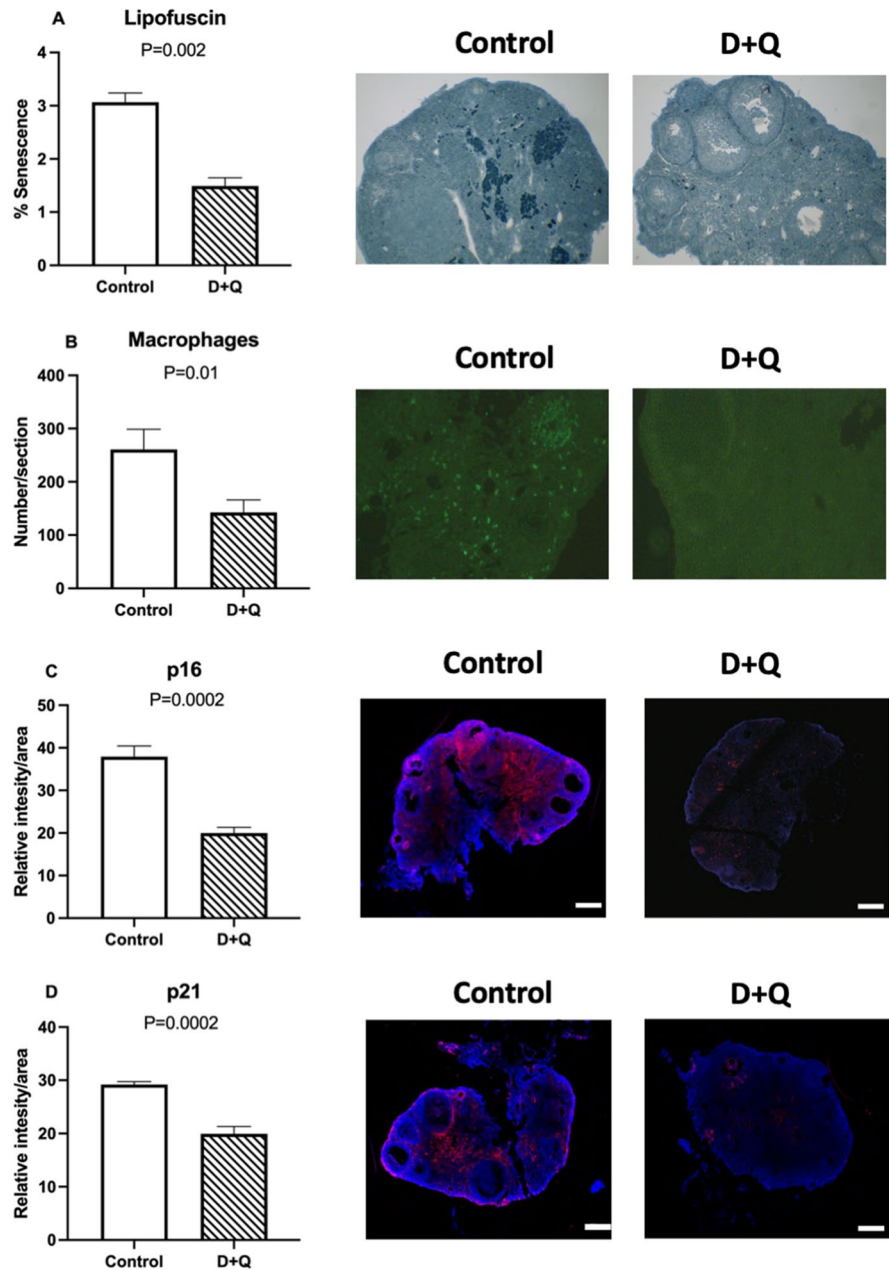
ob/ob mice in our study. It is possible that long-term leptin deficiency has beneficial effects on the ovarian reserve, as the follicular density we observed is similar to previous reports for normal mice using similar methods [53–55]. The ovarian follicular reserve in ob/ob mice was also not affected by treatment with senolytics (D+Q). This suggests that the reduction in ovarian senescent cell burden is uncoupled from the ovarian reserve, at least in leptin-deficient mice. Our previous study indicated that an age-related increase in senescent cell burden in the ovaries of WT mice does indeed correlate with ovarian reserve [25]; therefore, additional studies will be required to provide greatly clarity.

We observed that the D+Q treatment did not affect body weight. Others also found that the reduction in senescent cells did not affect body weight gain using leptin receptor-deficient mice (db/db) [28]. An

additional study using dietary-induced obese-male mice treated with senolytics (D+Q) also failed to modulate body mass following the elimination of senescent cells [29]. We also found that senolytic treatment did not affect insulin sensitivity, which is in contrast to previous reports [28]. Obese mice induced by a high-fat diet treated with D+Q also had an improvement in insulin sensitivity in a short period of time (3 treatment cycles), but these beneficial effects disappeared after 16 weeks of treatment [29]. These results suggest that chronic treatments with senolytics exert a transient effect on insulin sensitivity, which peaks after several weeks of treatment but decreases over time, as in the study mentioned before.

The present study has limitations, which should be acknowledged. First, leptin-deficient mice are anovulatory; therefore, we could not evaluate the impact of senescent cells and its clearance on ovulation rate

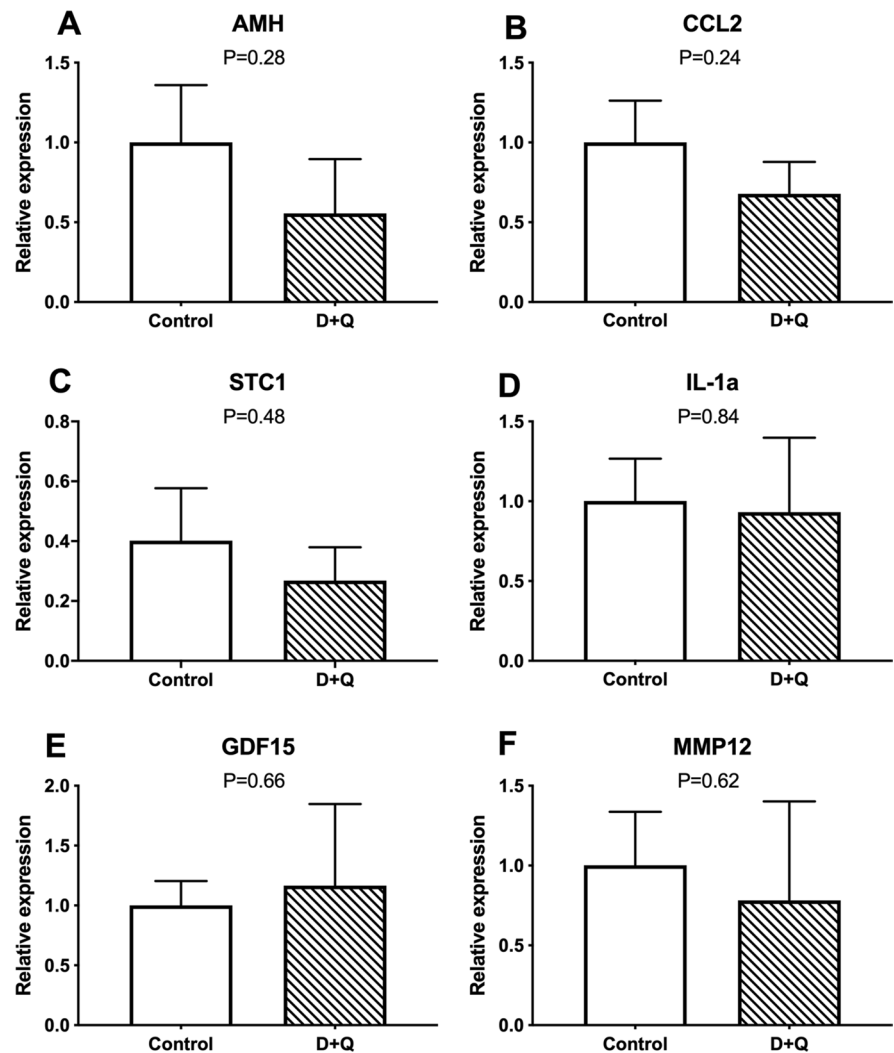
Fig. 5 Lipofuscin staining (A), macrophage infiltration (B), and p16 (C) and p21 (D) staining in 6-month-old obese (*ob/ob*) females, control ($n=6$) or treated with dasatinib plus quercetin ($n=6$). Values of $P < 0.05$ were considered significant



and fertility. Future studies using diet-induced obesity should provide evidence if senescent cell clearance will positively impact fertility of obese mice. It is known that obesity negatively affects fertility of female mice; therefore, treating obese females with senolytics can elucidate if senescent cells are involved in this process. Using a diet-induced obesity model will also help to establish the role of leptin

in the findings of the current study. Although previous studies had shown that both diet-induced obesity and leptin-receptor deficiency obesity resulted in increased senescent cell burden in adipose tissue [28], the same remains to be determined in the ovary. Another limitation of the current study is that despite demonstrating the presence of senescent cells in the ovary, we were not able to determine which

Fig. 6 Analysis of relative gene expression in ovaries from 6-month-old obese (ob/ob) females, control ($n=6$) or treated with dasatinib plus quercetin ($n=6$). **A** Anti-Mullerian hormone (Amh), **B** chemokine ligand 2 (CCL2), **C** Stanniocalcin-1 (Stc1), **D** Interleukin 1 alpha (IL-1a), **E** growth/differentiation factor 15 (Gdf15), **F** matrix metalloproteinase 12 (Mmp12). Values of $P < 0.05$ were considered significant



type of ovarian cells become senescent. Further studies using single-cell RNA sequencing and spatial transcriptomics will help to uncover the type of ovarian cells that become senescent. This can have important implications for the use of senolytic treatments to recover fertility or prevent age and obesity-associated infertility. The type of senescent cell will also have an impact in the choice of senolytics. To date, other drugs, such as fisetin and navitoclax have been also shown as effective for removing senescent cells. Future studies should compare the effectiveness of different drugs to target and remove ovarian-senescent cells.

In conclusion, female ob/ob mice accumulate more senescent cells in the ovary, and treatment with D+Q senolytic drugs can reduce this burden.

Despite this, no effect of obesity or D+Q treatment was observed on ovarian reserve size, suggesting that senescence has no direct effect on ovarian reserve in leptin-deficient obese mice. It must be noted that D+Q treatment is unable to kill all types of senescent cells; therefore, combination therapies including alternative senolytics and senomorphics must be tested to determine the efficacy of these treatments for ovarian health.

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Declarations

Competing interests The authors declare no competing interests.

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