

## RESEARCH ARTICLE

# Omentin expression in the ovarian follicles of Large White and Meishan sows during the oestrous cycle and *in vitro* effect of gonadotropins and steroids on its level: Role of ERK1/2 and PI3K signaling pathways

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## Abstract

Omentin (ITLN1) is a novel adipokine mainly expressed in the white adipose tissue. It plays a crucial role in the metabolic homeostasis and insulin sensitivity. Our last study documented that ITLN1 levels in the adipose tissue and plasma are lower in fat Meishan (MS) compared to normal weight Large White (LW) pigs. The aim of this study was to investigate transcript and protein concentrations of ITLN1 as well as its immunolocalisation in the ovarian follicles and examine the molecular mechanism involved in the regulation of its expression in response to gonadotropins (FSH, LH) and steroids ( $P_4$ , T,  $E_2$ ). Ovarian follicles were collected from LW and MS sows on days 2–3, 10–12, and 14–16 of the oestrous. We found the elevated ITLN1 expression in the ovarian follicles and the increase of concentrations in follicular fluid (FF) of LW pigs vs MS pigs; in both breeds of pigs, the levels of ITLN1 increased with the oestrous progression. We noted ITLN1 signals in oocyte, granulosa and theca cells. Gonadotropins and steroids increased ITLN1 levels in the ovarian follicle cells of LW pigs, while in MS pigs, we observed only the stimulatory effect of LH and T. Both extra-cellular signal-regulated kinase (ERK1/2) and phosphatidylinositol 3'-kinase (PI3K) were involved in the regulation of ITLN1. Our study demonstrated the levels and regulation of ITLN1 in the porcine ovarian follicles through ERK1/2 and PI3K signaling pathways.

## Introduction

Omentin, also known as intelectin [1] and intestinal lactoferrin receptor [2], is a novel 34-kDa adipokine [3], first described in 1998 by Komiya *et al.* [4] in the intestinal Paneth cells of mice.

**Competing interests:** The authors have declared that no competing interests exist.

The omentin gene is localized on a chromosomal region of 1q22–q23 in humans and on chromosome 4 in pigs [5, 6]. A direct product of gene expression is a secretory protein consisting of 313 amino acids, including an 18-amino acid signal peptide [1], which occurs as two main structural forms in humans: omentin-1 (intelectin-1, ITLN1), a more active form presented in the circulatory system, and omentin-2, a homolog with 83% amino acid identity with ITLN1 [7], highly expressed in the small intestine and secreted into the lumen [5]. Moreover, in pigs, the complementary DNA sequence of this adipokine displays 82% and 79% similarity with the human and mouse homologs, respectively [8].

Gene and protein expression of ITLN1 has been noted in various tissues of humans, mice, and pigs. For example, in humans, ITLN1 was particularly confirmed in the visceral adipose tissue [9], thymus, spleen, heart [1], lungs [9], small and large intestines [5], and ovaries [10]. In mice, expression of ITLN1 was confirmed in the large intestine [1, 9], as well as in the lungs and brain [11], while in pigs, it is found in the peri renal adipose tissue [12, 13]. The specific receptors for ITLN1 have not yet been identified; nevertheless, some literature data report that ITLN1 increased the activity of insulin receptor substrate (IRS) by restraining the mammalian target of rapamycin [14]. Moreover, Cloix *et al.* [10] suggest that ITLN1 may modulate the phosphorylation of the insulin-like growth factor-1 (IGF-1) receptor beta subunit, as well as IRS-1, in the visfatin-knockdown human ovarian granulosa-like tumour (KGN) cells. Some studies indicate that dexamethasone, the agonist of the glucocorticoid receptor that promotes fat accumulation, stimulates the gene expression of ITLN1 in the human visceral adipose tissue [15]. Additionally, the gene and protein expression, as well as ITLN1 secretion are decreased by glucose and insulin in the human adipose tissue explants [16]. The studies also show that ITLN1 plasma levels are decreased in obese humans compared to normal weight controls [7], and ITLN1 gene expression in the peri renal adipose tissue is significantly lower in fat MS pigs compared to normal weight LW pigs [13]. Interestingly, the latest data suggest that ITLN1 enhances the sensitivity of adipose tissue to insulin and stimulates glucose metabolism, thus regulating the distribution of adipose tissue [16]. Moreover, ITLN1 increases glucose transport in the adipocytes *via* phosphatidylinositol 3'-kinase (PI3K) [7] and exhibits a proinflammatory role in the primary human adipocytes by the activation of extracellular signal-regulated kinase (ERK1/2) [17]. All of these data suggest that ITLN1 may regulate the distribution of adipose tissue and confirm its relationship with insulin resistance, which is often associated with obesity. Additionally, as suggested by the literature data, the metabolic status (negative energy balance as well as obesity) may impact on the reproductive functions of humans and animals by affecting the control of hypothalamus–pituitary–ovarian functions. These metabolic effects could lead to the hormonal homeostasis imbalance (e.g., increased luteinizing hormone [LH] and 17 $\beta$ -estradiol [E<sub>2</sub>] secretion as well as reducing circulating sex hormone binding protein concentrations) and, consequently, some disorders in the ovulation, implantation, oocyte and embryo development, or pregnancy loss [18].

The studies about the expression and role of ITLN1 in the reproduction system are limited. Cloix *et al.* [10] showed the gene and protein expression of ITLN1 in the human granulosa-lutein cells (hGLC) in the healthy and polycystic ovary syndrome (PCOS) patients and in KGN cells. Moreover, the authors assessed that PCOS women have decreased ITLN1 levels in the follicular fluid (FF) compared to the control, healthy patients [10], suggesting its dependence on the hormonal status; PCOS is associated with the elevated circulating levels of LH and hyperandrogenism [19]. Moreover, the concentrations of circulating ITLN1 in the blood negatively correlates with the concentrations of E<sub>2</sub> [16] and free testosterone (T) [20], suggesting a possible involvement of sex steroids in regulating ITLN1 production. Only one study shows that hormones like insulin, IGF-1, and FSH increase protein expression of ITLN1 in hGLC and KGN cell line [10].

Thus, we hypothesize that expression of ITLN1 in the porcine ovary is dependent on phase of the oestrous cycle, and its level is regulated by different reproduction-related hormones. This study aimed to investigate the gene and protein expression of ITLN1 in the ovarian follicles, its immunolocalization, and its concentrations in FF in normal weight LW and fat MS sows during the oestrous. In the next step, we assessed the *in vitro* effect of gonadotropins (FSH and LH) and steroids ( $P_4$ , T, and  $E_2$ ) on ITLN1 protein expression in the ovarian follicular cells of both LW and MS sows. Additionally, we investigated the involvement of ERK1/2 and PI3K on the regulation of ITLN1 levels in ovarian cells. As reported by literature data, MS sows reach puberty at an earlier age [21], and they are fatter than LW sows [22]. Also, MS pigs have a greater intramuscular fat content, backfat thickness and the muscles of MS pigs generally contain higher amount of lipids than that of LW pigs [23] and this is also observed in obese people [24]. MS pigs similar as obese people tend to have a higher triglyceride concentration as well as HOMA-IR score [25] or lower blood urea nitrogen level [26]. Our previously study confirmed that the adipokines profile in plasma and adipose tissue in fat MS pigs was similar as in obese human; concentration of leptin was increased, while adiponectin was decreased [13]. Interesting, MS pigs characterize some many reproductive diseases similar to note in obese female including fewer cells in the trophectoderm [27] or a reduction in embryo developmental rate [28]. However, obese women have fertility problems, while MS sows are very fertile. The LW breed name also Yorkshire is one of the most popular commercial pig breed for successful pig farming [29] as well as are a great model to study reproduction because of similarities in the reproductive track anatomy, cycle length (around 21 days in pigs and 28 days in women), hormonal fluctuations during oestrous and menstrual cycle as well as similarities in fertilization, *in vitro* development to the blastocyst stage, pregnancy and puberty [30–32].

## Materials and methods

### Ethical issue

The use of sows was in accordance with directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Ovarian follicles have been collected during meat processing as abattoir by-products by highly qualified and experienced laboratory staff. Thus, according to the ethical issues for the protection of animals, this project does not require the consent of the competent ethics committee for animal experiments.

### Animals and ovaries collection

The research was carried out on the ovarian follicles collected from normal weight LW and fat MS sexually mature gilts at a local abattoir under veterinarian control. LW gilts, with an average weight of  $91.76 \pm 8.2$  kg and age  $214.7 \pm 8.2$  days, and the same number of MS gilts, with an average body weight of  $30.62 \pm 5.8$  kg and age  $179.0 \pm 6.1$  days, were used in this study. The zootechnical parameters (body weight, backfat thickness, age at the puberty) were routinely collected by the UEPAO (doi: <https://doi.org/10.15454/1.5573896321728955E12>) and pig phenotyping as well as Innovative breeding facility (doi: <https://doi.org/10.15454/1.5572415481185847E12>) experimental units for the monitoring of the breeding. The MS pigs are genetically able to faster fattening than LW, and fattening does not depend on diet as well as slaughter time [33]. Moreover, as the literature data suggest obese pigs, are often marked by excessive backfat ( $24.3 \pm 0.4$  mm in MS compared to  $16.6 \pm 0.3$  mm in LW pigs) [13]. Additionally, the biochemical parameters as well as the pattern of adipokines concentration in plasma and expression in perirenal adipose tissues from MS pigs [13, 25] are similar to those reported in humans [34, 35]. Also, MS pigs reach puberty earlier ( $86.1 \pm 1.30$  days) than LW

pigs ( $171.96 \pm 2.8$  days) [13]. In this way, they are a good animal model for studying the effect of obesity on reproduction, because also childhood obesity has been associated with the earlier onset of puberty and menarche [36]. The reduced number of cells present in fat MS embryos results from a selective reduction in the number of trophoctoderm [37] and also blastocysts collected from obese women contain fewer cells notably in the trophoctoderm [27]. However, the prolificacy of the MS breed is about three to four piglets greater than in LW [38].

Within a few minutes after slaughter, the ovaries were removed and placed in 0.1 M ice-cold phosphate-buffered saline (PBS, S1 Table) supplemented with antibiotics and transported to the laboratory within 30 min of collection. Based on the morphological examination of the ovaries [39], and levels of  $P_4$  and  $E_2$  in plasma as well as  $E_2$  in the FF (S3 Table) during oestrous cycle in LW and MS pigs (S1 Fig) we collected small follicles (SF; 2–4 mm in diameter; days 2–3 of oestrous), medium follicles (MF; 4–6 mm in diameter; days 10–12 of oestrous), and large follicles (LF; 8–12 mm in diameter; days 14–16 of oestrous), as well as FF on days 2–3, 10–12, and 14–16 of the oestrous cycle from LW and MS pigs. In pigs, day 1 of estrous cycle refers to the first day after ovulation [40].

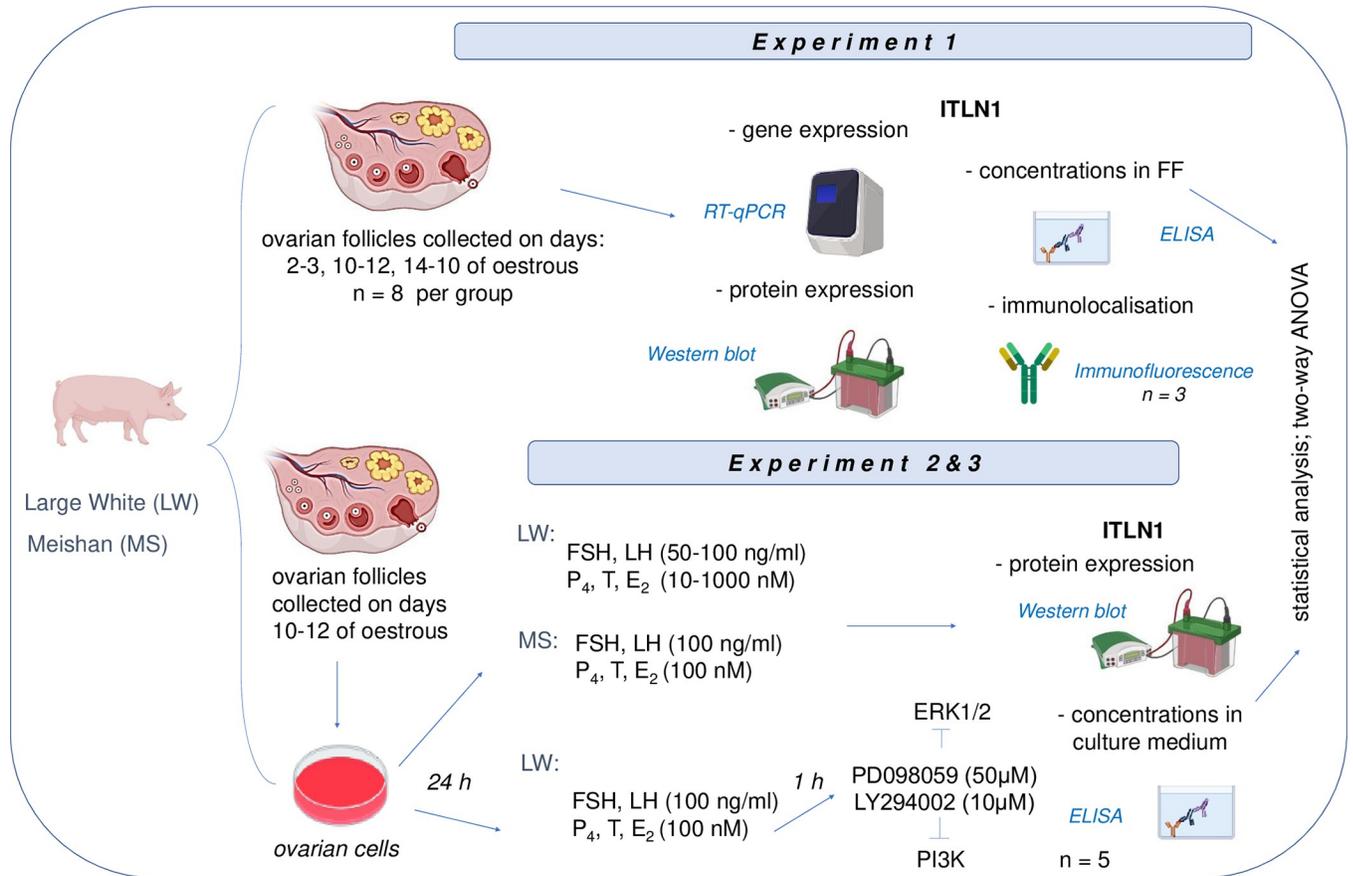
In *Experiment 1*, to determine ITLN1 gene and protein expression, whole healthy ovarian follicles with no atretic changes of LW and MS pigs were immediately frozen in liquid nitrogen and stored at  $-70^\circ\text{C}$  (the number of animals per each phase of the oestrous cycle were 8 for LW and 8 for MS,  $n = 8$ ). Additionally, the whole ovaries from LW pigs were collected during the oestrous (days 2–3, 10–12, and 14–16; the number of animals per each phase of the oestrous cycle were 3 for LW,  $n = 3$ ) and immersed in 4% buffered paraformaldehyde for future immunofluorescence analysis (see Experimental protocol, Fig 1). To assess ITLN1 concentrations in FF, the samples were prepared by low-speed centrifugation (2,000 g at  $4^\circ\text{C}$  for 10 min) and stored at  $-20^\circ\text{C}$  to determine the ITLN1 level.

### RNA isolation and real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using QIAzol Lysis Reagent (S1 Table), as previously described [13]. All methods were performed according to the manufacturer's protocols. RNA quantity and purity (A260/A280) were determined using a spectrophotometer (DeNovix, United States). The total RNA of follicles cells was reverse-transcribed for 1 h at  $37^\circ\text{C}$  in a final reaction volume of 20  $\mu\text{l}$  as previously [13]. cDNAs were amplified in the presence of specific primers for ITLN1 and reference genes (Cyclophilin A—PPIA;  $\beta$ -actin—ACTB; glyceraldehyde-3-phosphate dehydrogenase—GAPDH). The primers for the examined genes were designed based on the gene sequences deposited in GenBank. The reaction mixtures at the final volume of 20  $\mu\text{l}$  consisted of 10  $\mu\text{l}$  iQ SYBR Green Supermix, 0.25  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ), 4.5  $\mu\text{l}$  of water, and 5.0  $\mu\text{l}$  of the template. The cDNA templates were amplified and detected using the MYIQ Cycler real-time PCR system following the protocol previously described [10, 13]. The descriptions of the different primers are as follows: ITLN1 (F: 5'-GATTCTGCCTCCTGCTGTTC-3' and R: 5'-ATACAGGCCATCACCTGCTC-3'), PPIA (F: 5'-GCATACAGGTCCTGGCATCT-3' and R: 5'-TGTCCACATGCAGC AATGGT-3'), ACTB (F: 5'-ACGGAACCACAGTTTATCATC-3' and R: 5'-GTCCAGT CTTCAACTATACC-3'), and GAPDH (F: 5'-GCACCGTCAAGGCTGAGAAC-3' and R: 5'-ATGGTGGTGAAGACGCCAGT-3'). Relative gene expression levels of examined genes were determined using the comparative cycle threshold  $2^{-\Delta\Delta\text{Ct}}$  method [41], and normalization by the geometrical mean of the reference gene's expression levels was performed.

### Western blot

Western blot was performed as described by Rak *et al.* [42, 43]. Ovarian follicles were homogenised on ice with a cold buffer and then centrifugation at 15,000 g at  $4^\circ\text{C}$  for 30 min, and the



**Fig 1. Schematic diagram of materials and methods used for assessing the gene and protein expression of ITLN1, its immunolocalization in the ovarian follicle cells, concentrations in FF from LW and MS pigs during oestrous cycle as well as the effect of gonadotropins and steroids on ITLN1 level in ovarian follicle cells and its secretion to the culture medium in LW and MS pigs with involvement of ERK1/2 and PI3K in the regulation of ITLN1 expression in LW pigs.** ANOVA, analysis of variance; E<sub>2</sub>, 17β-estradiol; ELISA, enzyme-linked immunoassay; ERK1/2, extracellular signal-regulated kinase; FF, follicular fluid; FSH, follicle-stimulating hormone; Gc, granulosa cells; ITLN1, intelectin 1; LH, luteinising hormone; LW, Large White; MS, Meishan; P<sub>4</sub>, progesterone; PI3K, phosphatidylinositol 3'-kinase; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; T, testosterone; Th, theca interna cells.

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protein concentrations was determined by the Bradford method (BioRad) using bovine serum albumin (BSA) as a standard. Equal amounts of protein lysates (~30 μg of protein/lane) were separated by hand-casting 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The blots were blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% BSA for 1 h. Then, the membranes were incubated overnight at 4°C with the anti-ITLN antibody (sc-130923, Santa Cruz Biotechnology, United States; monoclonal; host species: mouse) diluted at 1:500 in 5% BSA/TBST and ACTB antibodies diluted at 1:1000 in 5% BSA/TBST. Next, the membranes were washed with TBST and incubated for 1 h with horseradish peroxidase (HRP)-conjugated antibody (7076S, Cell Signaling Technology, United States; monoclonal; host species: horse) diluted at 1:1000. An ACTB protein was used as a loading control and as a reference protein to determine relative amounts of analysed proteins. Immunolabeled bands were detected by chemiluminescence using a chemiluminescent HRP substrate reagent. Membranes were visualized using the ChemiDoc™ Imaging System (BioRad). All visible bands were quantified using ImageJ analysis software (National Institute of Health). The results were expressed as the intensity signal in arbitrary optical density units.

## Immunohistochemistry

Tissue preparation for fluorescent immunostaining was conducted as a standard procedure described by Rytelewska *et al.* [44]. In brief, prepared tissue slides of the ovaries from LW pigs were incubated overnight with rabbit polyclonal antibodies against ITLN1 (dilution 1:100), rinsed in PBS three times, and incubated with the secondary antibody (1:1000, A28175, Invitrogen, United States) for 90 min at room temperature. After that, tissue sections were rinsed in PBS three times and again dehydrated. Finally, the sections were covered with histology mounting medium Fluoroshield™ with DAPI for nuclear counterstaining. The labelled tissue sections were analyzed with an Olympus BX51 research microscope (Olympus, Japan) equipped with an EXFO x-Cite Series 120Q fluorescence illuminator (Excelitas Technologies Corp, United States) using appropriate filters set for DAPI and Alexa Fluor® 488. Images were acquired with an Olympus DP72 microscope digital camera and Cell F software (Olympus, Japan). For the negative control, the primary antibody was omitted and tissues were incubated in 0.1 M PBS.

## Enzyme-linked immunosorbent assay (ELISA)

The concentrations of ITLN1 in FF and culture medium from LW and MS pigs were determined using a commercial ELISA kit for porcine ITLN1 (E07O0010, Bluegene, China) according to the manufacturer's protocol. The range of the standard curve was 0–100 ng/ml. The sensitivity of the assay was defined as 0.1 ng/ml. Absorbance values were measured at 450 nm using a Varioskan™ LUX multimode microplate reader (ThermoFisher, United States). The intra-assay coefficient of variation of the assay was 5.2%.

## Cell isolation and *in vitro* cell culture

The procedure of porcine granulosa (Gc) and theca (Th) cells isolation and *in vitro* culture was conducted as described by Stoklosowa *et al.* [45] with modifications. To *in vitro* experiments we decided used MF on days 10–12 of the oestrous cycle from LW and MS pigs (the number of animals were 5 for LW and 5 for MS,  $n = 5$ ). Briefly, Gc was scrubbed mechanically, while Th layers from the same follicles were exposed three times to 0.25% trypsin in PBS for 10 min at 37°C. Following isolation, cells were counted using a Bürker chamber, and cell viability was determined by 0.4% trypan blue. The mean viability of Gc and Th was  $94 \pm 1.5\%$  and  $90 \pm 1\%$ , respectively. Cells were cultured in 96-well culture plates at the final concentrations of  $5 \times 10^4$  (Gc:  $4 \times 10^4$  and Th:  $1 \times 10^4$ , reflecting that observed *in vivo* in the ovary) in M199 medium supplemented with 10% fetal bovine serum (FBS). All cultures were maintained in a humidified incubator (terms: 5% CO<sub>2</sub>/95% O<sub>2</sub>; 37°C).

## Experiment 2

To study the effect of hormones involved in the oestrous cycle progression on the ITLN1 level, ovarian cells were isolated from MF on days 10–12 of the oestrous (phase of the oestrous chosen based on ITLN1 expression results) from LW and MS pigs and incubated for 48 h in M199 supplemented with 1% FBS as a control medium or with FSH or LH at the doses of 50–150 ng/ml for LW or 100 ng/ml for MS and steroids P<sub>4</sub>, T, or E<sub>2</sub> at the doses of 10–1000 nM for LW or 100 nM for MS. Cell treatments for each tested factor and dose were performed in five wells of culture plate. Doses of hormones in LW pigs were chosen based on our previous study and according to the concentrations in FF [38, 42], while in MS pigs, they were based on the results obtained for LW pigs. After incubation, the medium was stored at -20°C to assess ITLN1 concentrations by ELISA, while the cells were boiled in Laemmli buffer for 5 min then stored at

-20°C for ITLN1 protein expression analysis by Western blot. Five independent *in vitro* cultures were performed (see Experimental protocol, Fig 1).

### Experiment 3

To investigate the involvement of ERK1/2 and PI3K on ITLN1 levels, cells isolated from the MF on days 10–12 of the oestrous cycle were plated in 96-well culture plates in M199 medium with 10% FBS for 24 h. Next, the medium was changed to 1% FBS, and the cells were pre-treated for 1 h with the ERK1/2 inhibitor (PD098059) the dose of 50  $\mu$ M and the PI3K inhibitor (LY294002) at a dose of 1  $\mu$ M. Then, gonadotropins, at the dose of 100 ng/ml, and steroids, at the dose of 100 nM, were added for an additional 48 h of culture. The concentrations of the inhibitors were chosen based on previous data [42, 46]. Previous data demonstrated inhibitory effect of PD098059 on phosphorylation of ERK1/2 signaling in rat granulosa cells [47, 48]. After incubation, the medium was stored at -20°C for ITLN1 concentrations, while the cells were boiled in Laemmli buffer for 5 min then stored at -20°C for ITLN1 protein expression analysis. Five independent *in vitro* cultures were performed (see Experimental protocol, Fig 1).

### Statistical analysis

Statistical analysis was performed in the GraphPad Software (PRISM software version 5). To determine the differences in ITLN1 gene and protein expression as well as its concentration in the FF during oestrous cycle between two tested types of breeds we performed two-way ANOVA test. Different lower case letters indicate statistical significance between days of oestrous cycle (2–3, 10–12, and 14–16) in one tested breed (LW or MS), whereas different capital letters indicate a significant difference between types of pig breeds.

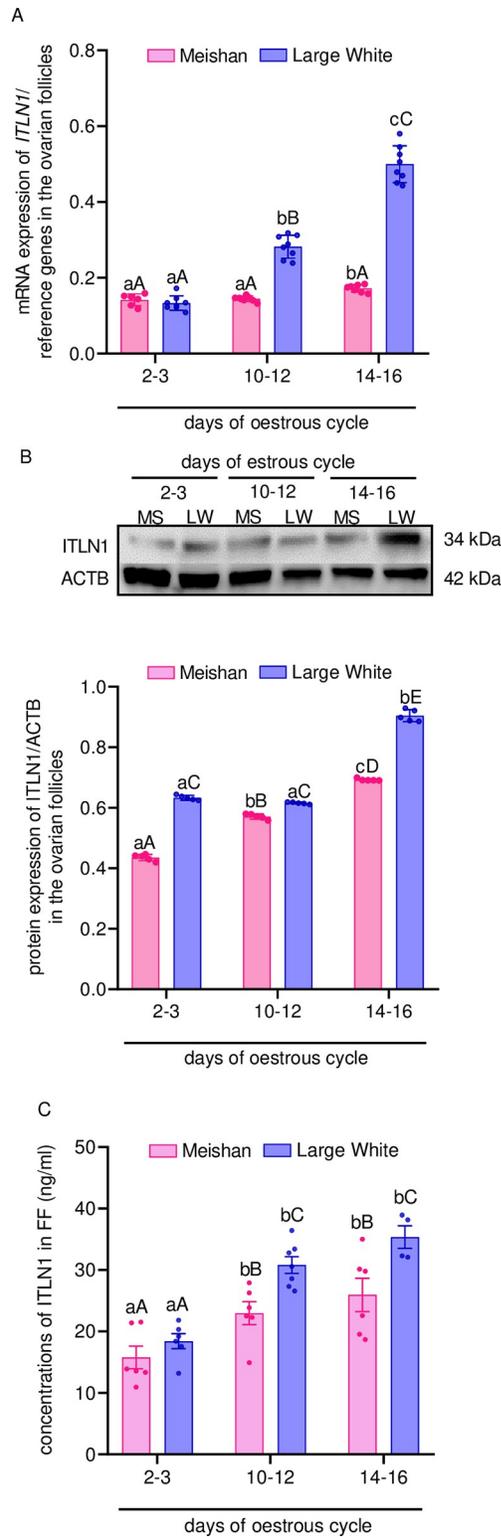
To demonstrate the differences in the effect of gonadotropins as well as steroid hormones on ITLN1 protein expression as well as its concentration in the culture medium in LW or MS pigs we performed one-way ANOVA test as well as two-way ANOVA to compare the effect of FSH (100 ng/ml), LH (100 ng/ml) as well as  $P_4$ , T, and  $E_2$  (100 nM) on ITLN1 levels between two tested breeds of pigs. Different lower case letters indicate statistical significance between studied hormones in LW or MS, while different capital letters indicate a significant difference between types of pig breeds. To indicate the involvement of ERK1/2 as well as PI3K signaling pathways in the regulation of protein expression of ITLN1 as well as its concentration in culture medium in LW pigs we performed one-way ANOVA test. Different lower case letters indicate statistically significant difference.

In each case, ANOVA analysis was followed by Tukey's honest significant post hoc test. All data were also tested for the assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Results were reported as mean  $\pm$  standard error of the mean. Statistical significance is indicated by different letters ( $p < 0.05$ ).

## Results

### Expression of ITLN1 in the ovarian follicles from LW and MS pigs and its concentrations in FF

We noted that gene and protein expression in the ovarian follicles from LW and MS pigs increased along with oestrous cycle progression; elevated gene and protein levels were observed on days 10–12, as well as days 14–16, of the oestrous of LW and MS. Moreover, comparing both gene and protein expression of ITLN1, we observed higher gene and protein expression of ITLN1 in the ovarian follicles from LW pigs compared with MS pigs in all studied days of the oestrous ( $p < 0.05$ ; Fig 2A and 2B). Additionally, as the oestrous progressed



**Fig 2.** Gene (A) and protein (B) expression of *ITLN1* in the ovarian follicles, as well as *ITLN1* concentrations (C) in the FF from LW and MS pigs during the oestrous cycle (days: 2–3, 10–12, 14–16). Relative gene expression was determined using RT-qPCR. Results were normalised with the geometric mean of reference gene (*GAPDH*, *PPIA*, *ACTB*) expression using comparative cycle threshold method. The protein expression was detected by western blot assay; each protein abundance was evaluated densitometrically and expressed as the ratio relative to *ACTB* abundance.

ITLN1 concentration was determined by ELISA assay. Results were reported as the mean  $\pm$  SEM of eight (gene and protein expression of ITLN1) or five (ITLN1 concentrations in the FF) independent determinations. The lower case letter denotes differences between days of oestrous cycle whereas an upper case letter denotes differences between types of pig breeds ( $p < 0.05$ ; two-way ANOVA followed by Tukey's HSD test). ACTB, actin beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FF, follicular fluid; SEM, standard error of the mean; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LW, Large White; MS, Meishan; PPIA, peptidylprolyl isomerase A; Tukey's HSD, Tukey's honest significant difference; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction.

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and follicles increased (SF  $\rightarrow$  MF  $\rightarrow$  LF), the concentrations of ITLN1 in FF from LW and MS pigs elevated. Comparing the two tested breeds of pigs, we observed that the mean of ITLN1 concentrations in FF on days 10–12, as well as days 14–16, of the oestrous was higher in LW pigs compared with MS pigs, whereas on days 2–3 of the oestrous, there were no significant differences in ITLN1 concentrations between LW and MS pigs ( $p < 0.05$ ; Fig 2C). The precise values (F-statistic and p-value) for the main effects (*phase of oestrous*: 2–3; 10–12; 14–16 and *breed of pigs*: LW; MS) and the interactions of examined factors (*phase\*breed*) are presented in S2 Table.

### Immunolocalization of ITLN1 in the ovarian follicles from LW pigs

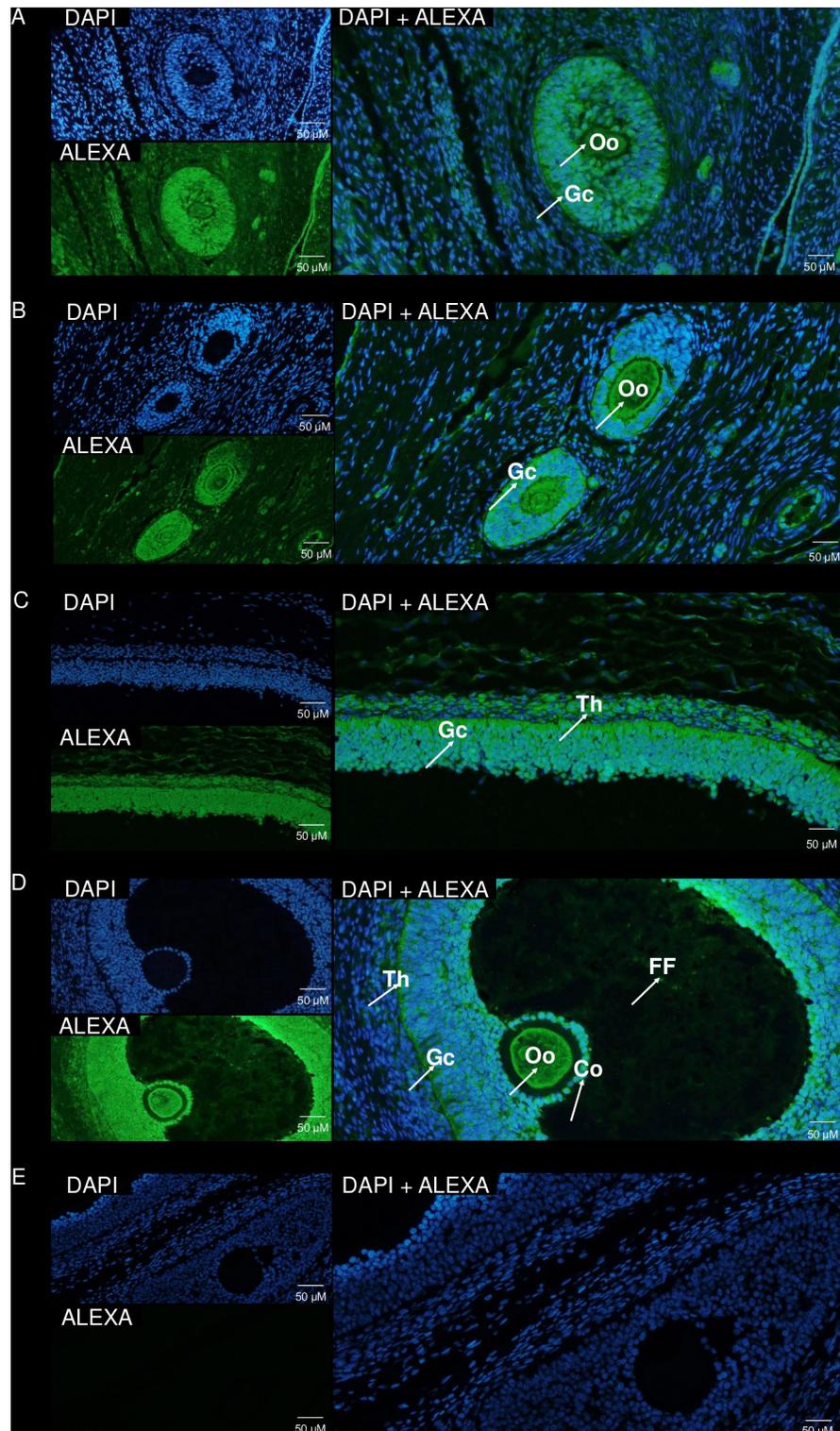
The immunolocalization of ITLN1 was confirmed in the cytoplasm as well as in the cell membrane of Gc, oocyte (Fig 3A–3D), and Th (Fig 3C and 3D) cells in antral follicles. Moreover, during ovarian follicles increasing, we noted ITLN1 expression in FF and cumulus cells (Fig 3D). No immunoreaction was observed for the negative controls (Fig 3E).

### In vitro effect of gonadotropins on ITLN1 level in the ovarian follicle cells of LW and MS pigs

We next investigated the dose-dependent effect of gonadotropins on ITLN1 expression in the ovarian cells collected from MF. In LW pigs, FSH decreased ITLN1 protein expression at 50 ng/ml and increased at 150 ng/ml; on the other hand, no effect was noticed at a dose of 100 ng/ml. In MS pigs, FSH at the dose of 100 ng/ml elevated the protein expression of ITLN1. Additionally, in both breeds of pigs, LH had a stimulatory effect at 100 ng/ml. In LW pigs, we also noted that LH at the dose of 50 ng/ml increased ITLN1 protein expression, while inhibiting it at a dose of 150 ng/ml (Fig 4A,  $p < 0.05$ ). Moreover, in LW pigs, FSH and LH at the doses of 100 and 150 ng/ml increased the concentrations of ITLN1 in the culture medium; also in MS pigs, only LH upregulated ITLN1 level in the culture medium. Interestingly, when comparing both breeds of pigs, the higher ITLN1 concentrations were observed in LW pigs compared with MS pigs after FSH and LH treatment at the dose of 100 ng/ml (Fig 4B,  $p < 0.05$ ). The precise values (F-statistic and p-value) for the main effects and the interactions of examined factors are presented in S2 Table.

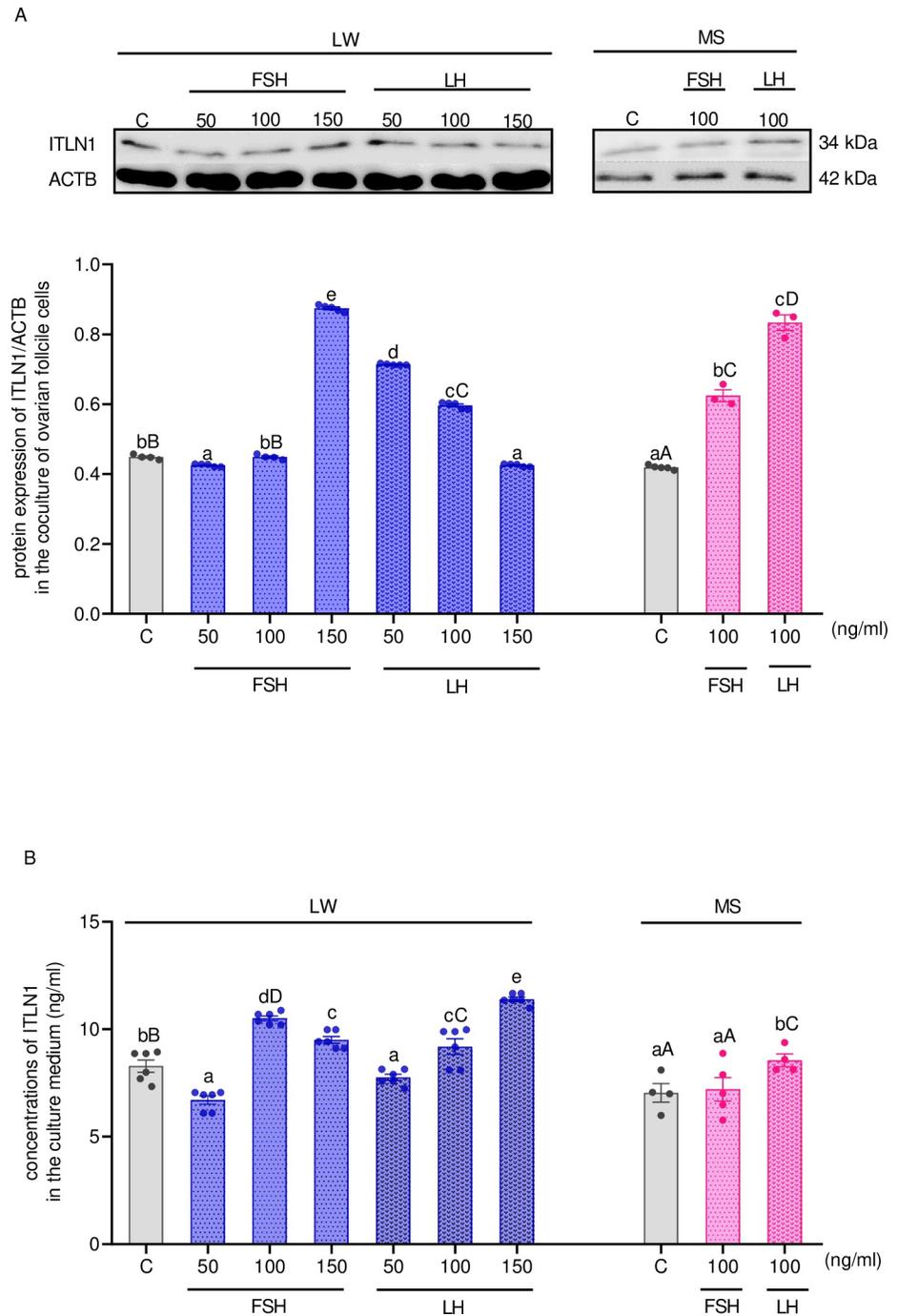
### In vitro effect of steroids on ITLN1 level in the ovarian follicle cells of LW and MS pigs

In ovarian cells of LW pigs,  $P_4$  at the doses of 100 and 1000 nM, T at all studied doses, and  $E_2$  at the dose of 10 nM increased ITLN1 protein expression, whereas in MS pigs, only T (100 nM) stimulated ITLN1 protein level (Fig 5A,  $p < 0.05$ ) in the ovarian follicular cells from MF. Additionally, we showed that ITLN1 concentrations in the culture medium from LW pigs were higher after stimulation with  $P_4$  at the doses of 100 and 1000 nM, as well as with  $E_2$  at the doses of 10–1000 nM. We also noted that T at a dose of 1000 nM significantly decreased



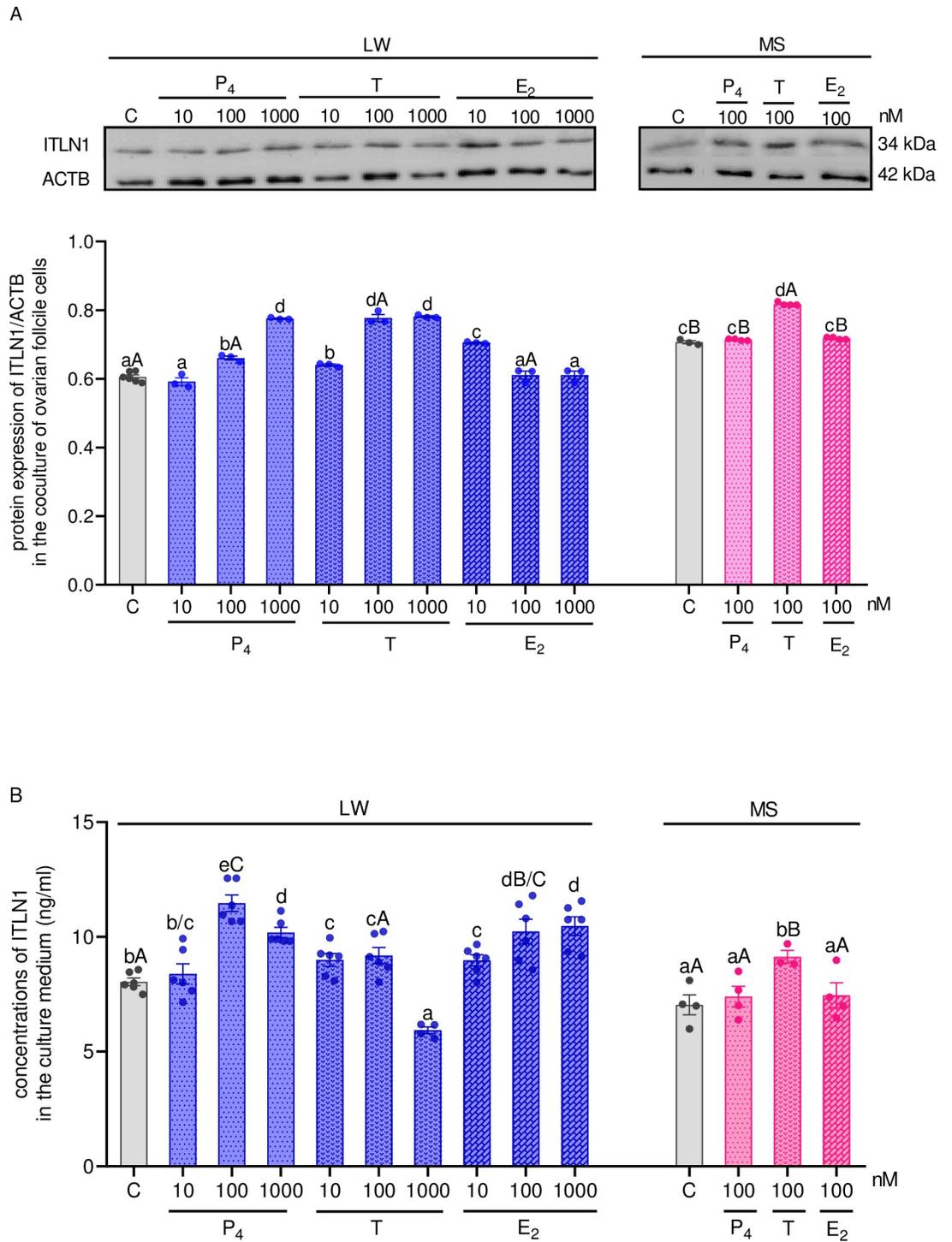
**Fig 3. The localization of ITLN1 in the ovarian structures from LW pigs.** The pictures show representative images of the immunolocalization of ITLN1 determined in the ovarian tissue slices ( $n = 3$  per group) obtained on days 2–3, 10–12, and 14–16 of the oestrous cycle. Nuclear staining with DAPI is shown in pictures marked as DAPI, and localization of ITLN1 protein detected by immunocomplexes labeled with Alexa Fluor® 488 is shown in pictures marked as ALEXA, while the merged images are marked as DAPI + ALEXA. Arrows indicate ITLN1 presence. C, cumulus cells; FF, follicular fluid; Gc, granulosa cells; LW, Large White; ITLN1, intelectin-1; Th, theca interna cells; Oo, oocyte.

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**Fig 4.** The effect of FSH and LH at doses of 50–150 ng/ml on ITLN1 protein expression (A) and its concentrations (B) in the culture medium of coculture of ovarian Gc and Th from LW and MS pigs on days 10–12 of the oestrous cycle. The protein expression was detected by western blot assay; each protein abundance was evaluated densitometrically and expressed as the ratio relative to ACTB abundance. ITLN1 concentration was determined by ELISA assay. Results were reported as the mean  $\pm$  SEM of five independent determinations. The lower case letter denotes differences between hormones doses whereas an upper case letter denotes differences between types of pig breeds ( $p < 0.05$ ; one-way ANOVA followed by Tukey post hoc test). ACTB, actin beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LW, Large White; MS, Meishan; SEM, standard error of the mean; Tukey's HSD, Tukey's honest significant difference.

<https://doi.org/10.1371/journal.pone.0297875.g004>



**Fig 5.** The effect of P<sub>4</sub>, T, and E<sub>2</sub> at doses of 10–1000 nM on ITLN1 protein expression (A) and its concentrations (B) in the culture medium of coculture of ovarian Gc and Th from LW and MS pigs on days 10–12 of the oestrous cycle. The protein expression was detected by western blot assay; each protein abundance was evaluated densitometrically and expressed as the ratio relative to ACTB abundance. ITLN1 concentration was determined by ELISA assay. Results were reported as the mean ± SEM of five independent determinations. The lower case letter denotes differences between hormones doses whereas an upper case letter denotes differences between types of pig breeds ( $p < 0.05$ ; one-way ANOVA followed by Tukey HSD test). ACTB, actin beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; E<sub>2</sub>, 17 $\beta$ -estradiol; LW, Large White; P<sub>4</sub>, progesterone; SEM, standard error of the mean; T, testosterone; Tukey's HSD, Tukey's honest significant difference.

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ITLN1 concentrations compared to control group in LW pigs; however, the concentrations of ITLN1 in the culture medium of MS pigs were significantly enhanced only after T stimulation compared to control group (Fig 5B,  $p < 0.05$ ).

### Involvement of ERK1/2 and PI3K signaling pathways in the regulatory action of gonadotropins on ITLN1 level in LW pigs

As shown in Fig 6A, ITLN1 protein expression was significantly higher in the ovarian cells treated with LH and PD098059. No significant differences were observed in ITLN1 protein levels after incubation with LY294004 in combination with FSH and LH ( $p < 0.05$ ). Such results were confirmed by ITLN1 concentrations in the culture medium; we noted that PD098059 with LH increased secretion of ITLN1 (Fig 6B,  $p < 0.05$ ) compared to cells treated only with PD098059.

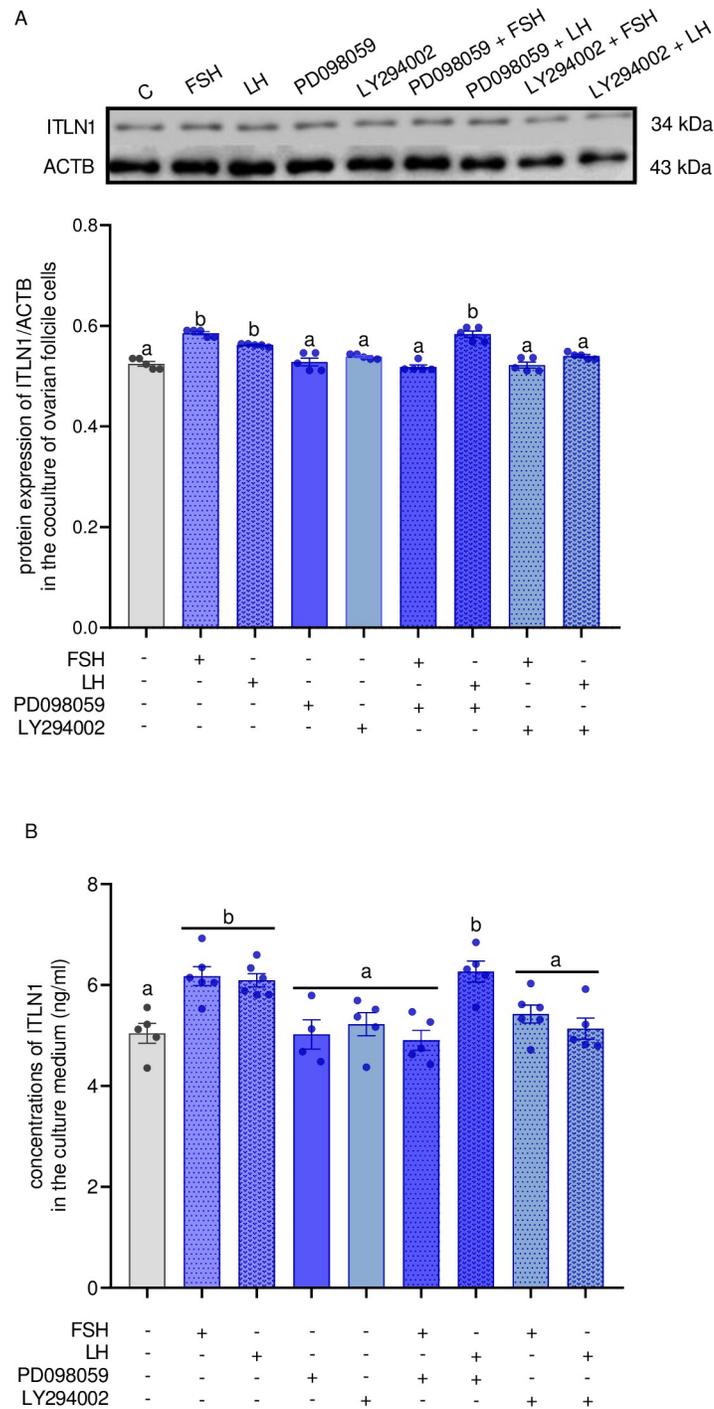
### Involvement of ERK1/2 and PI3K signaling pathways in the regulatory action of steroids on ITLN1 level in LW pigs

We noted that the protein contents of ITLN1 were elevated only in cells treated with  $E_2$  and LY294002. The opposite effect was observed in cells treated with PD098059 and all studied steroid hormones, as well as LY294002 in combination with T (Fig 7A,  $p < 0.05$ ). On the other hand, ITLN1 secretion to the culture medium increased after treatment with PD098059 and  $P_4$  compared to treatment only with PD098059, as well as with LY294002 in combination with  $P_4$ , T, and  $E_2$  compared to cells treated only with LY294002 (Fig 7B,  $p < 0.05$ ).

## Discussion

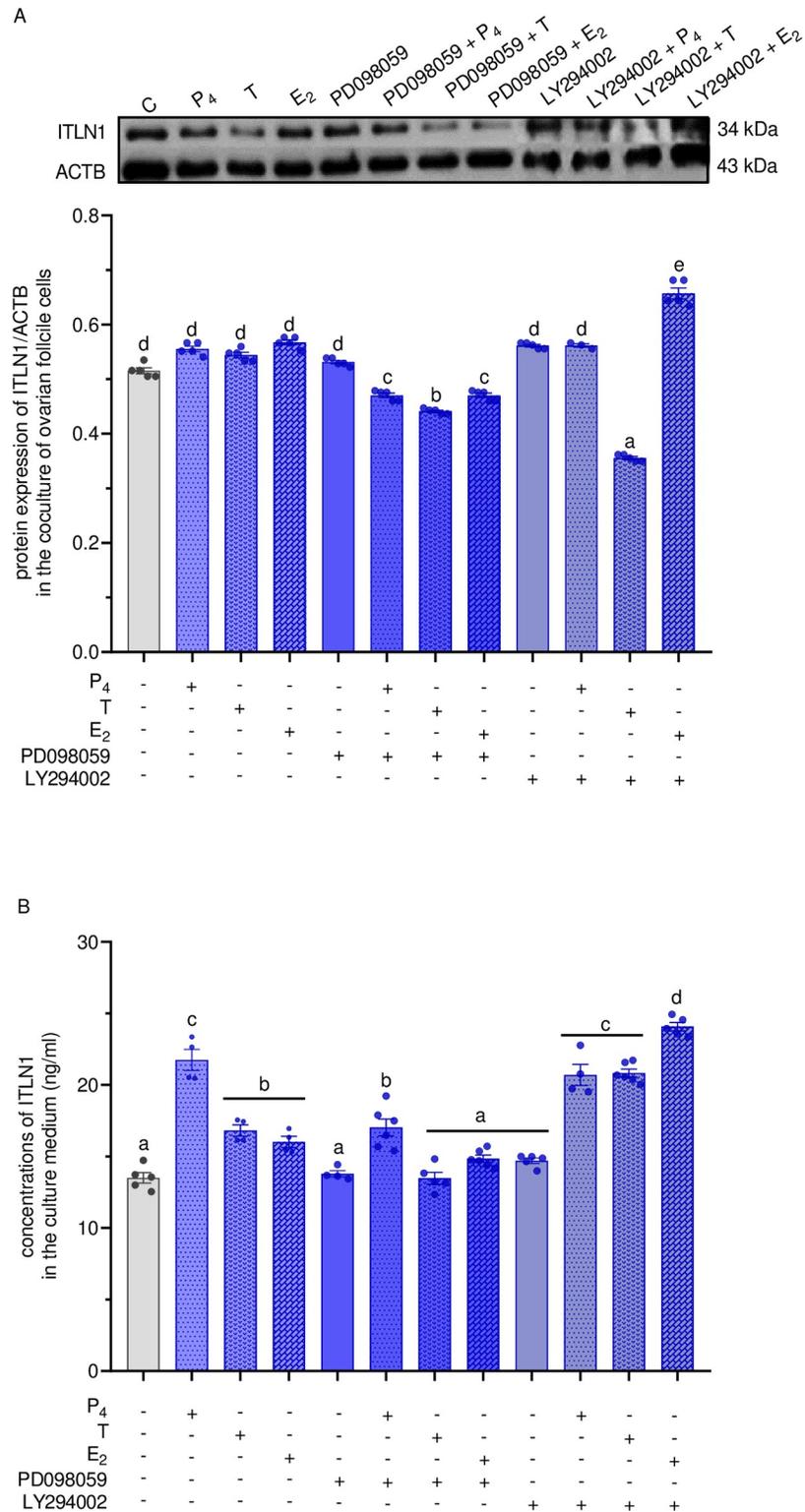
ITLN1 is a novel adipokine associated with the maintenance of body homeostasis, whose role in the reproduction is still unknown. In our study, we confirmed the hypothesis that ITLN1 expression (gene and protein), as well as its concentrations in FF, in pigs depends on fat quantification. For the first time, we noted that obese MS pigs have the lower ITLN1 levels in the ovary compared to normal weight LW pigs; a similar phenomenon has been shown by Barbe *et al.* [13] in the peri renal adipose tissue and serum. Also, the differences in adipokine ovarian expression are well described in the literature; Nakajima *et al.* [25] showed lowered adiponectin gene expression in MS pigs compared to normal weigh Landrace pigs and Kurowska *et al.* [46] noted the opposite pattern of vaspin expression in LW and MS pigs (LW > MS). The explanation of observed differences is that generally obese humans and rodents are characterized by an excessive mass of adipose tissue accompanied by an increase in circulating glucose, insulin, and triglyceride levels [49], which may interfere with adipokine content. For example, Tan *et al.* [16] showed that in the omental adipose tissue explants, insulin dose-dependently decreased the gene and protein expression of ITLN1, as well as its secretion into conditioned medium. In agreement with our observation, MS pigs have the higher homeostasis model assessment-estimated insulin resistance (HOMA-IR) score and triglyceride amount [33] compared to LW pigs, which negatively correlate with ITLN1 plasma concentrations [50]. Negative correlations between the ITLN1 expression and the percentage of body adipose tissue in obese people were noted [50], which is in good agreement with the results obtained in this study.

We noted that ITLN1 levels in the ovarian follicles and its concentrations in FF depends on the oestrous cycle phase. We observed that ITLN1 levels increase with the progression of the oestrous cycle in both breeds. The differences between gene and protein expression noted on days 2–3 of the oestrous are probably dependent on the posttranscriptional regulation: 5'-end capping to generate a 7-methylguanosine cap, splicing out of introns, and 3'-end cleavage/polyadenylation [51] because, as Schwanhäusser *et al.* [52] suggested, dissimilarity in gene



**Fig 6.** The effect of FSH and LH at a dose of 100 ng/ml on ITLN1 protein expression (A) and its concentrations (B) in medium of coculture of ovarian Gc and Th at days 10–12 of the oestrous cycle of LW pigs after using pharmacological inhibitors of ERK1/2 (PD098059, 50µM) and PI3K (LY294002, 10 µM). The protein expression was detected by western blot assay; each protein abundance was evaluated densitometrically and expressed as the ratio relative to ACTB abundance. ITLN1 concentration was determined by ELISA assay. Results were reported as the mean ± SEM of five independent determinations. Different letters denote statistically significant differences ( $p < 0.05$ ; one-way ANOVA followed by Tukey’s HSD test). ACTB, actin beta; ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LW, Large White; PI3K, phosphatidylinositol 3’-kinase; SEM, standard error of the mean; Tukey’s HSD, Tukey’s honest significant difference.

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**Fig 7.** The effect of P<sub>4</sub>, T, and E<sub>2</sub> at a dose of 100 nM on ITLN1 protein expression (A) and concentrations (B) in medium of coculture of ovarian Gc and Th at days 10–12 of the oestrous cycle of LW pigs after using pharmacological inhibitors of ERK1/2 (PD098059, 50µM) and PI3K (LY294002, 10µM). The protein expression was detected by western blot assay; each protein abundance was evaluated densitometrically and expressed as the ratio relative to ACTB abundance. ITLN1 concentration was determined by ELISA assay. Results were reported as the mean ± SEM of

five independent determinations. Different letters denote statistically significant differences ( $p < 0.05$ ; one-way ANOVA followed by Tukey's HSD test). ACTB, actin beta; ANOVA, analysis of variance;  $E_2$ , 17 $\beta$ -estradiol; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; LH, luteinizing hormone; LW, Large White; MS, Meishan;  $P_4$ , progesterone; PI3K, phosphatidylinositol 3'-kinase; SEM, standard error of the mean; T, testosterone; Tukey's HSD, Tukey's honest significant difference.

<https://doi.org/10.1371/journal.pone.0297875.g007>

expression explained approximately 40% of the variation in protein levels. The observed pattern of ITLN1 expression during the oestrous in pigs and higher levels in the LW pigs compared to MS pigs may be clarified by the fact that on day 20 of the oestrous, LW gilts have a higher number of Gc, FF volume, and Th mass [53] which, as we showed, express ITLN1. Additionally, to confirm our results, previous papers have documented that MS and LW gilts have elevated aromatase activity in Gc and Th [53] and plasma levels of  $E_2$  [54] during both the early and late follicular phases, which negatively correlates with ITLN1 concentrations [16]. In our study, we also noted that ITLN1 levels in FF of both tested breeds of pigs increased during the oestrous cycle; on the other hand, ITLN1 plasma concentrations in women was stable during the menstrual cycle [55]. Such differences may be explained by the fact that FF that fills the antrum of the growing follicle is derived from both the blood constituents that cross the blood-follicle barrier and products of the follicle structures [56]. We showed immunolocalization of ITLN1 in the cytoplasm and cell membrane in the ovarian follicles collected from a the different phases of the oestrous cycle in LW pigs. ITLN1 was present mainly in Gc, Th, oocyte, cumulus cells, and FF; the same pattern of ITLN1 has been described in the compartments of human ovaries by Cloix *et al.* [10]. Interestingly, we noted strong expression of ITLN1 in the zona pellucida of ovarian follicles before ovulation, suggesting the role of ITLN1 in the oocyte maturation.

Based on results indicating that the expression of ITLN1 was changing during the oestrous cycle, in the second aim, we derive novel observations about the regulation of ITLN1 in the ovarian follicle cells by gonadotropins and steroids, which participates in the oestrous cycle progression and regulation of ovarian follicle growth, ovulation and corpus luteum formation [46, 57]. In our study, we observed that gonadotropins and steroid hormones increased ITLN1 levels in LW pigs in a dose-dependent manner. Previous studies have reported that gonadotropins and steroids are involved in the regulation levels of another adipokine, vaspin; similarly, Kurowska *et al.* [46] documented that FSH, LH,  $P_4$ , T, and  $E_2$  increased vaspin levels in the ovarian follicle of LW pigs. These findings are consistent with the results obtained by us for LW pigs and suggest that ITLN1 levels during the oestrous may be regulated by hormones involved in its progression. Interestingly, Cloix *et al.* [10] documented that INTL1 expression was inhibited after 48 h of treatment with FSH at the dose of 10 nM in non-obese human Gc and the KGN cell line. In our study, after 48 h, we also observed the reduction of ITLN1 expression and its secretion to culture medium by ovarian cells in LW pigs after FSH treatment at the dose of 50 ng/ml (1.5 nM). At this point, it is worth adding that the higher dose of FSH used by Cloix *et al.* [10] may be connected with lowered gene expression of the FSH receptor in KGN cell line [58] than in the primary culture of ovarian follicle cells.

We also noted a different pattern in the regulation of ITLN1 levels in LW and MS pigs because we observed only the stimulatory effect of LH and T on ITLN1 levels in MS pigs. We suggest that such differences may be associated with the alternative endocrine relationship between LW and MS pigs. For example, Tilton *et al.* [59] noted increased levels of gonadotropins in normal weight pigs compared to fat MS pigs, which may be explained by differential binding of gonadotropin to FSH and LH receptors. We suggest that the stimulatory effect only by one gonadotropin (LH) in MS pigs on ITLN1 levels may be associated with elevated levels of plasma cortisol in MS pigs compared to lean breeds of pigs [54], which can intensify LH response in Gc of rats [60] and may also occur in MS pigs because they demonstrated earlier

estrus and shorter intervals between LH surge compared to LW pigs [53]. Moreover, MS pigs have higher inhibin levels which reduce the secretion of FSH from the pituitary gland and the expression of its receptor and may give an explanation for FSH having no effect on ITLN1 levels [54]. At this point, it is worth adding that no impact of  $E_2$  on ITLN1 levels in MS pigs may be connected with a higher concentrations of  $E_2$  in FF and plasma compared to LW pigs [61]. We propose that higher levels of  $E_2$  in both plasma and FF of MS pigs (S1 Fig) may be associated with  $E_2$  receptor desensitisation linked with the decreased responsiveness of  $E_2$  that occurs after repeated or chronic exposure. Moreover, we assess the stimulatory effect of only one steroid, T, on ITLN1 levels in MS pigs. Previous studies indicated only higher plasma levels of T in MS boars compared to normal weight Duroc pigs and white composite breed, and the increased free T level is accompanied by decreased plasma levels of ITLN1 levels in obese women [62]. Based on this interesting findings in MS pigs, we also suggest that different ITLN1 regulation between these two types of breeds may be linked with hypothesize that LW and MS pigs may exhibit a different pattern in the expression and sensitivity of LH, FSH,  $P_4$ , T, and  $E_2$  receptors in the ovarian follicle cells, which we plan to study in our future experiments.

Finally, we investigated the involvement of ERK1/2 and PI3K in the regulation of ITLN1 expression in response to gonadotropins and steroids in LW pigs. We focused on these kinases because signalling pathways, such as Janus kinase, ERK1/2, PI3K, adenosine 5'-monophosphate-activated protein kinase, and nuclear factor- $\kappa$ B, were activated in vaspin regulation in the cultured ovarian cells in LW pigs [46]. Moreover, available literature data suggest that ITLN1 may suppress tumour necrosis- $\alpha$ -induced expression of endothelial adhesion molecules and cyclooxygenase-2 in the primary human umbilical vein endothelial cells via ERK1/2 [63] and stimulate the human osteoblast proliferation through PI3K [64]. In our study, we observed that specific inhibitors of ERK1/2 and PI3K abolish the stimulating effect of both gonadotropins and steroids on ITLN1 levels, suggesting that the expression of this adipokine in the ovarian cells can be mediated via these kinases. It is worth adding that in this part, some obtained results showed the incoherence between ITLN1 protein expression and corresponding ITLN1 secretion to the culture medium; however, as Praznik *et al.* [65] suggested, not all proteins that enter the endoplasmic reticulum (ER) are secreted. Some proteins exert their function inside ER and need to be retained there or recycled between ER and post-ER compartments, and the processes of transcription and translation can require several hours before the protein is secreted to the functionally relevant concentrations.

In summary, our data showed that: *i*) ITLN1 expression was significantly higher in the ovarian follicles and FF from LW pigs compared to MS pigs; levels of ITLN1 in the ovarian follicles and its concentrations in FF increased in LW and MS pigs during the oestrous cycle; and ITLN1 signal is present in Gc, Th, and oocyte; *ii*) ITLN1 levels were increased by FSH, LH,  $P_4$ ,  $E_2$ , and T in LW pigs, while in MS pigs, we observed only the stimulatory effect of LH and T; and *iii*) ERK1/2 and PI3K are associated in the regulation of ITLN1 levels in the ovarian cells. The latest literature data noted that ITLN1 is associated with the pathology of ovaries, like ovarian cancer and PCOS linked with obesity; interestingly the ovarian cancer cell line treated with ITLN1 metastasis was inhibited [66]. For this reason, further studies are necessary to understand the role of ITLN1 as a new regulator in the ovarian physiology (for example, steroid secretion, cell proliferation, apoptosis, and oocyte maturation), thus providing new insights about their function in the reproduction of animals with different metabolic status.

## Supporting information

**S1 Fig. Original blots.** Abbreviation: LW, Large White; MS, Meishan, C, control; FSH, follicle stimulating hormone; LH, luteinizing hormone;  $P_4$ , progesterone; T, testosterone;  $E_2$ , 17 $\beta$ -

estradiol.  
(DOCX)

**S1 Table. Sources of reagents used in the study.** Abbreviation: BSA, bovine serum albumin; HRP, horseradish peroxidase; E<sub>2</sub>, 17β-estradiol; FBS, fetal bovine serum; FSH, follicle stimulating hormone; LH, luteinizing hormone; P<sub>4</sub>, progesterone; PBS, phosphate buffered saline; PVDF, polyvinylidene fluoride; T, testosterone.  
(DOCX)

**S2 Table. The effect of main factors and their interaction on mRNA and protein expression of ITLN1 in the ovarian follicles as well as its concentration in FF.** Abbreviation: FF, follicular fluid; ITLN1, intelectin; phase, 2-3/10-12/14-16 of the oestrous cycle; breed, Large White/Meishan; \*, interaction.  
(DOCX)

**S3 Table. Concentration of P<sub>4</sub>, E<sub>2</sub> in plasma and E<sub>2</sub> in follicular fluid of Large White and Meishan pigs during the oestrous cycle.** Abbreviation: E<sub>2</sub>, 17β-estradiol; P<sub>4</sub>, progesterone. The hormone concentrations were evaluated using ELISA. Results are presented as at least eight independent replicates as means ± SEM for each group. Statistical significance is indicated by different letters (p < 0.05).  
(DOCX)

**S1 Graphical abstract. E<sub>2</sub>, 17β-estradiol; ERK1/2, extracellular signal-regulated kinase; FF, follicular fluid; FSH, follicle-stimulating hormone; ITLN1, intelectin 1; LH, luteinizing hormone; LW, Large White; MS, Meishan; P<sub>4</sub>, progesterone; PI3K, phosphatidylinositol 3'-kinase; T, testosterone.**  
(PDF)

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**Writing – original draft:** Karolina Pich, Patrycja Kurowska, Agnieszka Rak.

**Writing – review & editing:** Karolina Pich, Nina Smolińska, Joëlle Dupont, Agnieszka Rak.

## References

1. Tsuji S, Uehori J, Matsumoto M, Suzuki Y, Matsuhisa A, Toyoshima K, et al. Human Intelectin Is a Novel Soluble Lectin That Recognizes Galactofuranose in Carbohydrate Chains of Bacterial Cell Wall. *Journal of Biological Chemistry*. 2001 Jun; 276(26):23456–63. <https://doi.org/10.1074/jbc.M103162200> PMID: 11313366
2. Suzuki YA, Shin K, Lönnerdal B. Molecular Cloning and Functional Expression of a Human Intestinal Lactoferrin Receptor. *Biochemistry*. 2001 Dec 1; 40(51):15771–9. <https://doi.org/10.1021/bi0155899> PMID: 11747454
3. Schäffler A, Neumeier M, Herfarth H, Fürst A, Schölmerich J, Büchler C. Genomic structure of human omentin, a new adipocytokine expressed in omental adipose tissue. *Biochimica et Biophysica Acta (BBA)—Gene Structure and Expression*. 2005 Dec; 1732(1–3):96–102. <https://doi.org/10.1016/j.bbaexp.2005.11.005> PMID: 16386808
4. Komiya T, Tanigawa Y, Hirohashi S. Cloning of the Novel Gene Intelectin, Which Is Expressed in Intestinal Paneth Cells in Mice. *Biochemical and Biophysical Research Communications*. 1998 Oct; 251(3):759–62. <https://doi.org/10.1006/bbrc.1998.9513> PMID: 9790983
5. Nonnecke EB, Castillo PA, Akahoshi DT, Goley SM, Bevins CL, Lönnerdal B. Characterization of an intelectin-1 (Itln1) knockout mouse model. *Front Immunol*. 2022 Aug 22; 13:894649. <https://doi.org/10.3389/fimmu.2022.894649> PMID: 36072603
6. Dawson HD, Sang Y, Lunney JK. Porcine cytokines, chemokines and growth factors: 2019 update. *Res Vet Sci*. 2020 Aug; 131:266–300. <https://doi.org/10.1016/j.rvsc.2020.04.022> PMID: 32442727
7. de Souza Batista CM, Yang RZ, Lee MJ, Glynn NM, Yu DZ, Pray J, et al. Omentin Plasma Levels and Gene Expression Are Decreased in Obesity. *Diabetes*. 2007 Jun 1; 56(6):1655–61. <https://doi.org/10.2337/db06-1506> PMID: 17329619
8. Liao Y, Lopez V, Shafizadeh TB, Halsted CH, Lönnerdal B. Cloning of a pig homologue of the human lactoferrin receptor: Expression and localization during intestinal maturation in piglets. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 2007 Nov; 148(3):584–90. <https://doi.org/10.1016/j.cbpa.2007.08.001> PMID: 17766154
9. Yang RZ, Lee MJ, Hu H, Pray J, Wu HB, Hansen BC, et al. Identification of omentin as a novel depot-specific adipokine in human adipose tissue: possible role in modulating insulin action. *American Journal of Physiology-Endocrinology and Metabolism*. 2006 Jun; 290(6):E1253–61. <https://doi.org/10.1152/ajpendo.00572.2004> PMID: 16531507
10. Cloix L, Reverchon M, Cornuau M, Froment P, Ramé C, Costa C, et al. Expression and Regulation of INTELECTIN1 in Human Granulosa-Lutein Cells: Role in IGF-1-Induced Steroidogenesis Through NAMPT1. *Biology of Reproduction*. 2014 Aug 1; 91(2).
11. Gu N, Kang G, Jin C, Xu Y, Zhang Z, Erle DJ, et al. Intelectin is required for IL-13-induced monocyte chemotactic protein-1 and -3 expression in lung epithelial cells and promotes allergic airway inflammation. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2010 Mar; 298(3):L290–6. <https://doi.org/10.1152/ajplung.90612.2008> PMID: 19965981
12. Thomsen MK, Hansen GH, Danielsen EM. Galectin-2 at the enterocyte brush border of the small intestine. *Molecular Membrane Biology*. 2009 Jan; 26(5–7):347–55. <https://doi.org/10.1080/09687680903167781> PMID: 19657968
13. Barbe A, Kurowska P, Mlyczyńska E, Ramé C, Staub C, Venturi E, et al. Adipokines expression profiles in both plasma and peri renal adipose tissue in Large White and Meishan sows: A possible involvement in the fattening and the onset of puberty. *General and Comparative Endocrinology*. 2020 Dec; 299:113584. <https://doi.org/10.1016/j.ygcen.2020.113584> PMID: 32827511
14. Hernández-Díaz A, Arana-Martínez JC, Carbó R, Espinosa-Cervantes R, Sánchez-Muñoz F. Omentina: papel en la resistencia a la insulina, inflamación y protección cardiovascular. *Archivos de Cardiología de México*. 2016 Jul; 86(3):233–43.
15. Pickering RT, Lee MJ, Karastergiou K, Gower A, Fried SK. Depot Dependent Effects of Dexamethasone on Gene Expression in Human Omental and Abdominal Subcutaneous Adipose Tissues from Obese Women. *Aleman M, editor. PLoS ONE*. 2016 Dec 22; 11(12):e0167337. <https://doi.org/10.1371/journal.pone.0167337> PMID: 28005982

16. Tan BK, Adya R, Farhatullah S, Lewandowski KC, O'Hare P, Lehnert H, et al. Omentin-1, a Novel Adipokine, Is Decreased in Overweight Insulin-Resistant Women With Polycystic Ovary Syndrome. *Diabetes*. 2008 Apr 1; 57(4):801–8.
17. Niersmann C, Röhrig K, Blüher M, Roden M, Herder C, Carstensen-Kirberg M. Increased Release of Proinflammatory Proteins in Primary Human Adipocytes and Activation of the Inflammatory NFκB, p38, and ERK Pathways upon Omentin Treatment. *Obes Facts*. 2020; 13(2):221–36.
18. Westerman R, Kuhnt AK. Metabolic risk factors and fertility disorders: A narrative review of the female perspective. *Reproductive Biomedicine & Society Online*. 2022 Mar; 14:66–74. <https://doi.org/10.1016/j.rbms.2021.09.002> PMID: 34765754
19. De Leo V, Musacchio MC, Cappelli V, Massaro MG, Morgante G, Petraglia F. Genetic, hormonal and metabolic aspects of PCOS: an update. *Reprod Biol Endocrinol*. 2016 Dec; 14(1):38. <https://doi.org/10.1186/s12958-016-0173-x> PMID: 27423183
20. Halabis M, Dziedzic M, Warchulinska J, Kaznowska-Bystryk I, Solski J. Omentin—a new adipokine with many roles to play. *Current Issues in Pharmacy and Medical Sciences*. 2015 Sep 1; 28(3):176–80.
21. Miyano T, Akamatsu J, Kato S, Nanjo I, Kanda S. Ovarian development in Meishan pigs. *Theriogenology*. 1990 Apr; 33(4):769–75. [https://doi.org/10.1016/0093-691x\(90\)90812-8](https://doi.org/10.1016/0093-691x(90)90812-8) PMID: 16726773
22. Le Dividich J, Mormède P, Catheline M, Caritez JC. Body Composition and Cold Resistance of the Neonatal Pig from European (Large White) and Chinese (Meishan) Breeds. *Neonatology*. 1991; 59(5):268–77. <https://doi.org/10.1159/000243360> PMID: 1873362
23. Zhao J, Li K, Yang Q, Du M, Liu X, Cao G. Enhanced adipogenesis in Mashen pigs compared with Large White pigs. *Italian Journal of Animal Science*. 2017; 16: 217–225.
24. Straight CR, Toth MJ, Miller MS. Current perspectives on obesity and skeletal muscle contractile function in older adults. *Journal of Applied Physiology*. 2021; 130: 10–16. <https://doi.org/10.1152/jappphysiol.00739.2020> PMID: 33211593
25. Nakajima I, Kojima M, Oe M, Ojima K, Muroya S, Chikuni K. Comparing pig breeds with genetically low and high backfat thickness: differences in expression of adiponectin, its receptor, and blood metabolites. *Domestic Animal Endocrinology*. 2019; 68: 54–63. <https://doi.org/10.1016/j.domaniend.2019.01.002> PMID: 30851697
26. Li S, Cui Y, Luo Z, Lou Y, Liao M, Chen H, et al. Association between blood urea nitrogen and incidence of type 2 diabetes mellitus in a Chinese population: a cohort study. *Endocr J*. 2021; 68: 1057–1065. <https://doi.org/10.1507/endocrj.EJ20-0794> PMID: 33907068
27. Leary C, Leese HJ, Sturmey RG. Human embryos from overweight and obese women display phenotypic and metabolic abnormalities. *Human Reproduction*. 2015; 30: 122–132. <https://doi.org/10.1093/humrep/deu276> PMID: 25391239
28. Youngs CR, Christenson LK, Ford SP. Investigations into the control of litter size in swine: III. A reciprocal embryo transfer study of early conceptus development. *Journal of Animal Science*. 1994; 72: 725–731.
29. Houpt KA, Houpt TR, Pond WG. The pig as a model for the study of obesity and of control of food intake: a review. *Yale J Biol Med*. 1979; 52(3):307–29. PMID: 380187
30. Lorenzen E, Follmann F, Jungersen G, Agerholm JS. A review of the human vs. porcine female genital tract and associated immune system in the perspective of using minipigs as a model of human genital Chlamydia infection. *Vet Res*. 2015; 46: 116. <https://doi.org/10.1186/s13567-015-0241-9> PMID: 26411309
31. Bazer FW, Thatcher WW, Martinat-Butte F, Terqui M. Sexual maturation and morphological development of the reproductive tract in Large White and prolific Chinese Meishan pigs. *Reproduction*. 1988; 83: 723–728. <https://doi.org/10.1530/jrf.0.0830723> PMID: 3411562
32. Mordhorst BR, Prather RS. Pig Models of Reproduction. 1st ed. In: Constantinescu G, Schatten H, editors. *Animal Models and Human Reproduction*. 1st ed. Wiley; 2017. pp. 213–234.
33. Nakajima I, Oe M, Ojima K, Muroya S, Shibata M, Chikuni K. Cellularity of developing subcutaneous adipose tissue in Landrace and Meishan pigs: Adipocyte size differences between two breeds: ADIPOCYTE CELLULARITY OF LEAN AND OBESE PIGS. *Animal Science Journal*. 2011 Feb; 82(1):144–9.
34. Després JP, Moorjani S, Tremblay A, Ferland M, Lupien PJ, Nadeau A, et al. Relation of high plasma triglyceride levels associated with obesity and regional adipose tissue distribution to plasma lipoprotein-lipid composition in premenopausal women. *Clin Invest Med*. 1989; 12: 374–380. PMID: 2612090
35. Shashaj B, Luciano R, Contoli B, Morino GS, Spreghini MR, Rustico C, et al. Reference ranges of HOMA-IR in normal-weight and obese young Caucasians. *Acta Diabetol*. 2016; 53: 251–260. <https://doi.org/10.1007/s00592-015-0782-4> PMID: 26070771
36. Itriyeva K. The effects of obesity on the menstrual cycle. *Current Problems in Pediatric and Adolescent Health Care*. 2022; 52: 101241. <https://doi.org/10.1016/j.cppeds.2022.101241> PMID: 35871162

37. Rivera RM, Youngs CR, Ford SP. A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages. *Reproduction*. 1996; 106: 111–116. <https://doi.org/10.1530/jrf.0.1060111> PMID: 8667335
38. Haley CS, Lee GJ. Genetic basis of prolificacy in Meishan pigs. *J Reprod Fertil Suppl*. 1993; 48: 247–259. PMID: 8145208
39. Akins EL, Morrissette MC. Gross ovarian changes during estrous cycle of swine. *Am J Vet Res*. 1968 Oct; 29(10):1953–7. PMID: 5692889
40. Senger PL. *Pathways to pregnancy & parturition*. 3rd edition. Redmond, OR: Current Conceptions; 2012.
41. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods*. 2001 Dec; 25(4):402–8.
42. Rak A, Drwal E, Wróbel A, Gregoraszczyk EŁ. Resistin is a survival factor for porcine ovarian follicular cells. *REPRODUCTION*. 2015 Oct; 150(4):343–55. <https://doi.org/10.1530/REP-15-0255> PMID: 26159832
43. Rak A, Drwal E, Karpeta A, Gregoraszczyk EŁ. Regulatory Role of Gonadotropins and Local Factors Produced by Ovarian Follicles on In Vitro Resistin Expression and Action on Porcine Follicular Steroidogenesis1. *Biology of Reproduction [Internet]*. 2015 Jun 1; 92(6).
44. Rytelawska E, Kisielewska K, Kiezun M, Dobrzyn K, Gudelska M, Rak A, et al. Expression of chemerin and its receptors in the ovaries of prepubertal and mature gilts. *Mol Reprod Dev*. 2020 Jul; 87(7):739–62. <https://doi.org/10.1002/mrd.23391> PMID: 32557981
45. Stoklosowa S, Bahr J, Gregoraszczyk E. Some Morphological and Functional Characteristics of Cells of the Porcine Theca Interna in Tissue Culture1. *Biology of Reproduction*. 1978 Nov 1; 19(4):712–9.
46. Kurowska P, Mlyczyńska E, Barbe A, Staub C, Gregoraszczyk E, Dupont J, et al. Vaspin in the pig ovarian follicles: expression and regulation by different hormones. *Reproduction*. 2019 Aug; 158(2):137–48. <https://doi.org/10.1530/REP-19-0034> PMID: 31063973
47. Seger R, Hanoch T, Rosenberg R, Dantes A, Merz WE, Strauss JF, et al. The ERK Signaling Cascade Inhibits Gonadotropin-stimulated Steroidogenesis. *Journal of Biological Chemistry*. 2001; 276: 13957–13964. <https://doi.org/10.1074/jbc.M006852200> PMID: 11278318
48. Tajima K, Dantes A, Yao Z, Sorokina K, Kotsuji F, Seger R, et al. Down-Regulation of Steroidogenic Response to Gonadotropins in Human and Rat Preovulatory Granulosa Cells Involves Mitogen-Activated Protein Kinase Activation and Modulation of DAX-1 and Steroidogenic Factor-1. *The Journal of Clinical Endocrinology & Metabolism*. 2003; 88: 2288–2299.
49. Jung U, Choi MS. Obesity and Its Metabolic Complications: The Role of Adipokines and the Relationship between Obesity, Inflammation, Insulin Resistance, Dyslipidemia and Nonalcoholic Fatty Liver Disease. *IJMS*. 2014 Apr 11; 15(4):6184–223. <https://doi.org/10.3390/ijms15046184> PMID: 24733068
50. Lis I, Pelczyńska M, Miller-Kasprzak E, Kujawska-Luczak M, Mądry E, Bogdański P. Association of serum omentin concentration with anthropometric, physiological, and biochemical parameters in obese individuals. *Nutrition*. 2020 Nov; 79–80:110866. <https://doi.org/10.1016/j.nut.2020.110866> PMID: 32640371
51. Corbett AH. Post-transcriptional regulation of gene expression and human disease. *Current Opinion in Cell Biology*. 2018 Jun; 52:96–104. <https://doi.org/10.1016/j.ceb.2018.02.011> PMID: 29518673
52. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. *Nature*. 2011 May 19; 473(7347):337–42. <https://doi.org/10.1038/nature10098> PMID: 21593866
53. Hunter MG, Biggs C, Pickard AR, Faillace LS. Differences in follicular aromatase activity between Meishan and Large-White hybrid gilts. *Reproduction*. 1994 May 1; 101(1):139–44. <https://doi.org/10.1530/jrf.0.1010139> PMID: 8064672
54. Wise T, Klindt J, Howard HJ, Conley AJ, Ford JJ. Endocrine relationships of Meishan and White composite females after weaning and during the luteal phase of the estrous cycle. *Journal of Animal Science*. 2001; 79(1):176. <https://doi.org/10.2527/2001.791176x> PMID: 11204698
55. Wyskida K, Franik G, Wikarek T, Owczarek A, Delroba A, Chudek J, et al. The levels of adipokines in relation to hormonal changes during the menstrual cycle in young, normal-weight women. *Endocrine Connections*. 2017 Nov; 6(8):892–900. <https://doi.org/10.1530/EC-17-0186> PMID: 29079670
56. Dufour JJ, Mariana JC. Comparative Follicular Development in Meishan and Large White Gilts during Prepubertal Periods and its Relation to Hormonal Stimulation1. *Biology of Reproduction*. 1993 May 1; 48(5):1020–5.
57. Kurowska P, Mlyczyńska E, Dawid M, Grzesiak M, Dupont J, Rak A. The role of vaspin in porcine corpus luteum. *Journal of Endocrinology*. 2020 Dec; 247(3):283–94. <https://doi.org/10.1530/JOE-20-0332> PMID: 33108345

58. Haltia UM, Pihlajoki M, Andersson N, Mäkinen L, Tapper J, Cervera A, et al. Functional Profiling of FSH and Estradiol in Ovarian Granulosa Cell Tumors. *Journal of the Endocrine Society*. 2020 Apr 1; 4(4):bvaa034. <https://doi.org/10.1210/jendso/bvaa034> PMID: 32309755
59. Tilton JE, Biggs C, Hunter MG, Foxcroft GR. Gonadotropin Secretion in Ovariectomized Chinese Meishan and Hybrid Large White Gilts; Responses to Challenges with Estradiol Benzoate, Gonadotropin-Releasing Hormone, or Porcine Follicular Fluid1. *Biology of Reproduction*. 1994 Nov 1; 51(5):963–70.
60. Keren-Tal I, Dantes A, Sprengel R, Amsterdam A. Establishment of steroidogenic granulosa cell lines expressing follicle stimulating hormone receptors. *Molecular and Cellular Endocrinology*. 1993 Sep; 95(1–2):R1–10. [https://doi.org/10.1016/0303-7207\(93\)90042-i](https://doi.org/10.1016/0303-7207(93)90042-i) PMID: 8243796
61. Biggs C, Tilton JE, Craigon J, Foxcroft GR, Ashworth CJ, Hunter MG. Comparison of follicular heterogeneity and ovarian characteristics in Meishan and Large-White hybrid pigs. *Reproduction*. 1993 Jan 1; 97(1):263–9. <https://doi.org/10.1530/jrf.0.0970263> PMID: 8464019
62. jung Choi Y, jung Bae H, young Lee J, jung Cho E, hwa Lee Y, suk Lee H, et al. Analysis of risk factors for lipid intolerance of intravenous fat emulsion in very low birth weight infants. *Arch Pharm Res*. 2015 May; 38(5):914–20. <https://doi.org/10.1007/s12272-014-0405-6> PMID: 24849035
63. Yamawaki H, Kuramoto J, Kameshima S, Usui T, Okada M, Hara Y. Omentin, a novel adipocytokine inhibits TNF-induced vascular inflammation in human endothelial cells. *Biochemical and Biophysical Research Communications*. 2011 May; 408(2):339–43. <https://doi.org/10.1016/j.bbrc.2011.04.039> PMID: 21514279
64. Wu SS, Liang QH, Liu Y, Cui RR, Yuan LQ, Liao EY. Omentin-1 Stimulates Human Osteoblast Proliferation through PI3K/Akt Signal Pathway. *International Journal of Endocrinology*. 2013; 2013:1–6. <https://doi.org/10.1155/2013/368970> PMID: 23606838
65. Praznik A, Fink T, Franko N, Lonžarić J, Benčina M, Jerala N, et al. Regulation of protein secretion through chemical regulation of endoplasmic reticulum retention signal cleavage. *Nat Commun*. 2022 Mar 14; 13(1):1323. <https://doi.org/10.1038/s41467-022-28971-9> PMID: 35260576
66. Au-Yeung CL, Yeung TL, Achreja A, Zhao H, Yip KP, Kwan SY, et al. ITLN1 modulates invasive potential and metabolic reprogramming of ovarian cancer cells in omental microenvironment. *Nat Commun*. 2020 Jul 15; 11(1):3546. <https://doi.org/10.1038/s41467-020-17383-2> PMID: 32669559