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Resistin in endocrine pancreas of sheep: Presence and expression related to different diets

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ABSTRACT

Resistin (RETN), a recently discovered adipokine, is a cysteine-rich and secretory protein produced by adipocytes. RETN has been detected in several tissues, including human and laboratory animals' pancreas, wherein impairs glucose tolerance and insulin (INS) action and causes INS resistance. This study aims to evaluate the presence and expression of RETN in the pancreas of 15 adult female sheep reared on Apennine pastures, which show a decrease in their nutritional value due to the drought stress linked to the increasing summer aridity. The sheep were divided into 3 groups according to the diet they were subjected to: maximum pasture flowering (MxF) group, maximum pasture dryness (MxD) group, and experimental (Exp) group which received a feed supplementation in addition to the MxD group feeding. Immunohistochemistry and immunofluorescence were performed on formalin-fixed and paraffin-embedded sections of the pancreas to detect the RETN presence and to evaluate the co-localization of RETN with both glucagon (GCG)- and INS-producing cells. In addition, the expression of the three molecules was evaluated also in relation to different diets.

RETN was observed only in the endocrine pancreas, showing a wide distribution throughout the pancreatic islets with few negative cells and the RETN producing cells colocalized with both α cells and ß cells. No differences in distribution and immunostaining intensity of RETN, GCG and INS were observed among the three groups. Quantitative PCR showed the expression of RETN, GCG and INS in all tested samples. No significant differences were observed for RETN and GCG among all three groups of sheep. Instead, a high statistically significant expression of INS was detected in the MxF group with respect to the Exp and MxD groups.

These results highlight the localization of RETN in GCG- and INS-secreting cells involved in glucose homeostasis suggesting a modulatory role for RETN. Furthermore, the RETN expression is not influenced by food supplementation and thus is not affected by diet.

1. Introduction

The pancreas is a mixed gland composed of both exocrine and endocrine parenchyma representing the two functional components of the organ. The exocrine one, the most voluminous, is a compound acinar gland whose adenomers synthesize and secrete pancreatic juice including digestive enzymes such as lipase, amylase and trypsin which act on the gastric digestion's products once they reach the duodenum (Barone, 2014). The endocrine component has numerous small cell clusters, scattered among the acini, representing the pancreatic islets, once referred to as islets of Langerhans. The islets are formed by solid, anastomosed cellular cords, between which there is a rich capillary network, and they are composed of small and multifaceted cells, called *insulocytes* (Barone, 2014). Usually, the most abundant cell populations

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are the alpha (α) and beta (β) cells but the percentage and the position within the islet can vary among species (Tsuchitani et al., 2016). In ovine species, the α cells, the most voluminous, take up about 20 % of an islet and they are localized in the islet periphery (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). They secrete glucagon (GCG), which exerts a hyperglycemic effect by breaking down glycogen in the liver and increasing blood glucose (Quesada et al., 2008; Dunning and Gerich, 2007). The β cells are the most numerous, reaching up to 98 % and they are mostly in the islet center (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). They secrete insulin (INS) which has a hypoglycemic effect via the facilitation of glucose uptake by skeletal muscle and adipocytes and inhibiting its release from the liver (Marchetti et al., 2017; Bartolomé, 2023). A third type of cell population is the delta (δ) cells, much fewer in number and associated with the enterochromaffin cells of the digestive tract. They produce somatostatin, which has an inhibitory action on both GCG and INS production (Mahesh et al., 2017; Huising et al., 2018). A fourth type of cell population is represented by a small portion of pancreatic polypeptide producing cells (PP-cells) in sheep (Steiner et al., 2010). They secrete the pancreatic polypeptide that reduces gastric emptying, regulates intestinal motility, and inhibits the secretion of the exocrine pancreas (Brereton et al., 2015). In addition, the pancreatic islet population is characterized by other cell types, belonging to the gastro-entero-pancreatic (GEP) system, that influence the behavior of the other types just described (Sakata et al., 2019; Ehrhart et al., 1986).

It has been demonstrated that some adipokines, such as leptin, apelin, adiponectin and nesfatin, take place in the regulation of glucose homeostasis and pancreas' physiology (Tudurí et al., 2009; Morioka et al., 2007; Holland et al., 2011; Messaggio et al., 2017; Chaves-Almagro et al., 2022; Strutt et al., 2021; Gatta et al., 2018). Recently, the presence of another adipokine, the resistin (RETN), has been identified in human and rodent pancreas (Minn et al., 2003; Al-Salam et al., 2011; Sassek et al., 2016). The RETN was first characterized in 2001 by Steppan et al. in mice and described as a small circulating protein expressed and secreted primarily by white adipose tissue (adipose-tissue-specific secretory factor, ADSF) in proportion to the degree of adipocyte differentiation and the amount of adipose tissue present in the individual (Steppan et al., 2001; Filková et al., 2009). In humans, however, it remains unclear whether the main source of RETN secretion is adipocytes, preadipocytes or macrophages (McTernan et al., 2006). The physiology of the molecule is still very unclear, as well as the identification of its receptor or intracellular signaling pathways (Park and Ahima, 2013; Acquarone et al., 2019). Nevertheless, RETN was initially described, and is still considered, as an important risk factor for INS resistance and, in turn, leading to the development of type 2 diabetes mellitus (T2DM) (Steppan et al., 2001; Tripathi et al., 2020). Its action has been identified in the development of a pro-inflammatory state both "*in vitro*" and "*in vivo*", such as obesity, and in the development of cardiovascular disease (Filková et al., 2009). It has been reported to play a role in the regulation of glucose metabolism since its administration in healthy mice impairs glucose tolerance; the RETN affects glycogen metabolism by inhibiting the INS action in the liver and skeletal muscle in rats (Filková et al., 2009; Yang et al., 2009; Adeghate, 2004).

Furthermore, the active role of RETN on the onset of INS resistance is made consistent by the molecule localization and expression in the pancreatic islets of humans and rodents, exclusively at the level of α and β cells with a defined species-specific pancreatic distribution pattern (Al-Salam et al., 2011; Sassek et al., 2016). In fact, following *in vitro* treatment with this molecule, the INS release increased at lower glucose concentration and decreased at higher glucose concentration (Adeghate, 2004; Minn et al., 2003). In a non-obese mouse model, it was observed that an overexpression of RETN induced INS resistance in pancreatic islets and impaired glucose-stimulated INS secretion (Nakata et al., 2007). In INS resistance models, increased production of the molecule has been observed at the level of the pancreatic islets (Minn et al., 2003). In addition, at different glucose concentration, it was observed that RETN can modulate INS and GCG secretion from clonal α and β cell lines and isolated islets in rats (Sassek et al., 2016).

In vitro studies have also shown that at "physiological" levels, as in lean subjects, RETN acts as a ß-cell growth factor on which it exerts an anti-apoptotic and thus protective effect. However, when the concentration of RETN increases sharply, as in the condition of obesity, the proliferative and anti-apoptotic effects do not occur but rather are replaced by an apoptotic effect against β-cells, as in cases of insulinoma, probably leading to β-cell dysfunction (Brown et al., 2007). Elevated levels of RETN have also been found in pancreatic islet cells in the condition of acute pancreatitis, suggesting a role for the molecule in regulating the degree of inflammation (Xue et al., 2015).

RETN belongs to the adipokine group whose secretion is regulated by the distribution and quantity of adipose tissue which varies according to animal nutritional status. Previous studies reported differences in the expression of some adipokines in peripheral tissues and organs, such as RETN in the uterus (Dall'Aglio et al., 2019) and apelin in both the mammary gland (Mercati et al., 2018) and abomasum (Palmioli et al., 2021) of sheep subject to different nutritional conditions. This suggests that also the adipokine local expression may be affected by diet.

Based on the above information, this study aimed to investigate the RETN presence and localization in the pancreas of sheep to point out a possible pancreatic secretion of RETN in ovine species. In addition, RETN expression was analyzed to detect variations related to diet.

2. Material and methods

2.1. Animal recruiting and sample collection

To conduct the study, a flock of 15 Comisana x Appenninica adult female sheep were used (Mercati et al., 2018). Once their housing period in the barn was over, the sheep were left to graze on the pasture feeding on fresh forage until maximum pasture flowering (MxF). Until this period, pasture has a high nutritional value due to its low fiber and high content of proteins and fats (Scocco et al., 2018).

At this point, five subjects were slaughtered (MxF group) while, the remaining sheep were divided into two homogeneous groups based on age, reproductive performance, and body condition score (BCS) and left on the pasture for the entire period between the MxF and the maximum pasture dryness (MxD; Mercati et al., 2018). With increasing dryness, the pasture protein content decreases and the fiber content increases, some of which, such as lignin, is indigestible, reducing the nutritional quality of the plant species (Scocco et al., 2018). Each group consisted of 5 sheep: the MxD group continued to feed only on fresh forage, instead, the experimental (Exp) group, also received a food supplementation of 600 g/day/head of barley and corn (1:1), particularly enhancing the protein intake (Palmioli et al., 2021). The composition of the feed supplementation is described in Table S1 (Mercati et al., 2018).

The animals, intended for human consumption, were slaughtered at the abattoir according to the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing under law n.333/98 (Council Directive 93/119/EC of 22 December 1993) as specified by Annex C of Section II. The experimental procedures were approved by the Ministry of Health (no. of approval 95/ 2018-PR).

Specimens of the pancreas were collected from the left lobe of the organs (Mahesh et al., 2017; Steiner et al., 2010) of all tested animals. Pancreas specimens, about 1 cm^2 wide, intended for immunohistochemical evaluation were fixed in 10 % formaldehyde in phosphate – buffered saline (PBS) (pH 7.4) for 36 h at room temperature and then processed using routine tissue processing techniques (formalin-fixed and paraffin-embedded, FFPE). Pancreatic samples intended to be used for investigating the gene expression were washed in an RNase free PBS solution and then frozen at − 80 °C (Mercati et al., 2019).

2.2. Immunohistochemistry

The collected pancreas samples of each group were treated as previously described (Dall'Aglio et al., 2021). The FFPE sections were dewaxed in xylene and hydrated through a series of ethanol concentrations until distilled water. All specimens were cut into 5-μm thick serial sections and, at first stained with hematoxylin-eosin solution to carry out a morphological evaluation and to exclude potential pathologies. For immunohistochemical investigation, sections were treated with 3 % hydrogen peroxide solution for 10 min to block endogenous peroxidase activity and microwaved for 3 cycles of 5 min at 750 W in citrate buffer (pH 6.0) to expose the epitopes to the antibodies. The slices were incubated with normal serum (Table 1) for 30 min to avoid non-specific bindings. For the immunohistochemical reaction, sections were incubated overnight (O.N.) with a monoclonal anti-RETN, polyclonal anti-GCG or polyclonal anti-INS antibodies (Table 1) and, on the second day, they were treated with a biotin-conjugated antibody (Table 1) for 30 min. The avidin–biotin complex solution (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) was used to detect the immunological binding sites, which were revealed with 3,3′-diaminobenzidine (DAB) chromogen (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with hematoxylin.

Sections were washed with PBS between every incubation step, except after normal serum. Every step was performed at RT and the slides were incubated in a humid chamber (H.C.). Negative control sections were incubated with normal IgG (Table 1), omitting the primary antibody. Sheep uterus sections were used as a positive control of the reaction (Dall'Aglio et al., 2019).

A photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera) was used to observe all sections. The staining intensity for RETN, GCG, and INS was assessed in arbitrary units, as follows: absent (0), weak (1), moderate (2), strong (3) and very strong (4) (Maranesi et al., 2020a). Three separate observers conducted the staining evaluation, with each observer assessing three randomly selected distinct islets on each tissue section to determine the mean intensity values. Hence, RETN, GCG, INS immunostaining intensity was examined in five individuals per group, with the mean intensity derived from nine scored observations.

2.3. Double-label immunohistochemistry

Double-label immunoperoxidase localization of GCG with INS was performed as previously described (Palmioli et al., 2021) to characterize the histological distribution of α and β cells in the pancreatic islets of sheep. After immunohistochemical detection of GCG cells, performed as described in the 2.2 paragraph, sections were treated again with 3 % hydrogen peroxide solution for 10 min and then incubated with avidin/biotin blocking system reagents (Avidin/Biotin blocking kit, Vector Laboratories, Burlingame, CA, USA) for a total time of 30 min to block all the biotin binding sites. The sections were incubated with normal serum for 30 min, incubated O.N. with a monoclonal anti-INS antibody (Table 1) and the next day with a biotin conjugated antibody (Table 1) for 30 min. After the incubation with avidin–biotin complex for 30 min, the immunological binding sites were revealed with SG chromogen (Vector SG substrate kit, Peroxidase, Vector Laboratories, Burlingame, CA, USA).

Table 1
Source and working dilutions of the antibodies used.

Antibody	Host	Sources	Dilution
Monoclonal anti – RETN	Mouse	sc-376336, Santa Cruz Biotechnology, CA, USA	IHC 1:100 IF 1:10
Monoclonal anti – INS	Mouse	sc-8033, Santa Cruz Biotechnology, CA, USA	IHC 1:800
Polyclonal anti – INS	Rabbit	20056, Immunostar, WI, USA	IHC 1:500 IF 1:400
Polyclonal anti – GCG	Rabbit	orb213971, SL, USA	IHC 1:200 IF 1:600
Anti-mouse Biotin conjugated	Horse	BA-2000–1.5, Vector Laboratories, CA, USA	1:200
Anti-rabbit Biotin conjugated	Goat	BA-1000, Vector Laboratories, CA, USA	1:200
Anti-mouse IgG Alexa Fluor 488	Donkey	ab150105, Abcam, Cambridge, CB2 0AX, UK	1:1000
Anti-rabbit IgG Alexa Fluor 555	Goat	A-21428, Invitrogen, Thermo Fisher Scientific, ORE, USA	1:1000
Mouse IgG	Mouse	I-2000–1, Vector Laboratories, CA, USA	1:100
Rabbit IgG	Rabbit	I-1000–5 Vector Laboratories, CA, USA	1:100

2.4. Double-label immunofluorescence and colocalization analysis

Double-label localization of RETN with GCG or INS was performed in the FFPE sections of the pancreas as previously described (Palmioli et al., 2023) to identify the possible secretion of RETN from α and β cells. After placing the slides at 60 °C for 2 h, the sections were treated with xylol for 30 min and hydrated through a series of ethanol concentrations until distilled water. The slices were incubated with an antigen retrieval solution (Antigen Unmasking Solution, Tris-based, Vector Laboratories, Burlingame, CA, USA) in a microwave, washed with acetone for 20 min at −20° and incubated with NH_4Cl for 30′ R.T. All subsequent steps were conducted in the H.C. The sections were permeabilized with triton solution (Triton X-100, BioChemika, Sigma-Aldrich, Darmstadt, DE) for 10 min in H.C. and incubated with fish serum blocking solution (Fish Serum Blocking Buffer, ThermoScientific, Rockford, USA) for 1 h 30 min. The sections were incubated with primary antibody O.N. and the next day with a fluorescent secondary antibody for 1 h 30 min. Before mounting the sections with DAPI solution (Fuoroshield with DAPI, SIGMA, St. Louis, USA), the sections were washed with Sudan black solution (Sudan Black B, Sigma-Aldrich, Darmstadt, DE) for 20 min.

To run each colocalization, a mouse monoclonal anti-RETN was used respectively with a rabbit polyclonal anti-GCG and a rabbit polyclonal anti-INS. Alexa Fluor 488 donkey anti-mouse and Alexa Fluor 555 goat anti-rabbit were used as secondary antibodies (Table 1).

The sections were washed with PBS for 5 min after each incubation step. Negative control sections were incubated with normal IgG, omitting the primary antibodies. Sections were observed with a fluorescence microscope (Olympus BX51 Fluorescence Microscope) and images were acquired with Nikon DS-Qi2 microscope digital camera and NIS-Elements D software (Nikon Europe B.V.). Colocalization analysis for both RETN with GCG and RETN with INS was performed via the FIJI version of ImageJ software (version 1.53 t; Schindelin et al., 2012) using the JACoP plugin (Bolte and Cordelières, 2006). An intensity threshold (IT) was applied for each channel in every image to distinguish the signal from the background and minimize noise and artifacts. The following colocalization metrics were reported: Pearson's correlation coefficient (PC) represented by the "r" value; Manders' coefficients (MCs) using the above-mentioned ITs represented by M1 (fraction of the 519 nm wavelength [green channel – Alexa Fluor 488] overlapping the 565 nm wavelength [red channel – Alexa Fluor 555]) and M2 (fraction of the 565 nm wavelength overlapping the 519 nm wavelength); Overlap coefficient (OC) represented by the "r", "k1" and "k2" values.

2.5. RNA extraction and Real-Time PCR

The total RNA was extracted from the pancreas tissue of five sheep for each experimental group as previously described (Maranesi et al., 2020b). Genomic DNA contamination was prevented by treatment with deoxyribonuclease as previously described (Zerani et al., 2012).

Five micrograms of total RNA obtained were reverse-transcribed in 20 μl of Superscript III Reverse transcriptase cDNA synthesis mix using random hexamer according to the protocol provided by the

manufacturer. Genomic DNA contamination was checked by developing the PCR without reverse transcriptase. The multiplex RT-PCR amplification was performed using 1.0 μl of cDNA as a template for RETN, INS, GCG and β-Actin (ACTB) housekeeping primer (Table 2) (Mercati et al., 2018). Cycling conditions consisted of a denaturing cycle at 94 °C for 1 min and 15 s, followed by 30 cycles at 94 °C for 15 s, 62 °C for 30 s and 72 °C for 45 s and a final extension cycle at 72 °C for 10 min. For each PCR, a negative control without cDNA was included. The complete set of cDNA samples (five pancreas for each experimental group) was processed in a single PCR and each sample run in triplicate. The analysis of the amplified product was carried out as reported elsewhere (Maranesi et al., 2010; Maranesi et al., 2021).

Each sample was normalized to the geometric mean of one reference gene, ACTB (Mercati et al., 2018).

2.6. Statistical analysis

The overall mean ± standard deviation (SD) for RETN, GCG, and INS immunostaining intensity in each diet group was computed based on the mean intensity values obtained from individual assessments. To evaluate the statistical differences in mean immunostaining intensities among individuals across distinct groups, a one-way analysis of variance (ANOVA) test was employed. The essential assumptions required for this parametric test were verified using Levene's test for homogeneity and the Shapiro-Wilk test for normality.

Shapiro-Wilk test was used to check the normality of data of RETN, GCG, INS and ACTB gene expressions, which were analyzed by the non-parametric Kruskal-Wallis test followed by the Student-Newman Keulst test.

3. Results

3.1. Immunohistochemical analysis

The immunohistochemical investigation revealed the presence of RETN-positive cells in the pancreas of all three sheep groups. The molecule signal was exclusively detected in the endocrine pancreas, while both pancreatic acini and ducts were negative (Fig. 1).

The islands were irregularly distributed and had a highly variable shape (Fig. 1a). RETN-positive cells exhibited a broad distribution within the islets and only a few cells appeared negative (Fig. 1a-b). Immunohistochemical staining was localized in the cytoplasm of pancreatic islet cells (Fig. 1c).

Immunoperoxidase colocalization between GCG and INS showed that GCG-secreting cells were mainly found in the periphery while the INS-secreting ones were mainly in the center of the islets (Fig. 2).

ANOVA tests performed to assess differences within RETN, GCG, INS mean immunostaining intensities among different groups had no significant results ($p > 0.01$) in all cases (Fig. 3; Table 3).

The intensity of immunostaining was sorted on a scale ranging from 0 (negative) to 4 (very strong). *Significantly different values were considered at $p < 0.01$. The means ± SD of RETN, GCG, and INS immunostaining intensity were calculated for 5 animals/group. The right column represents results (F and p values) from the ANOVA tests performed for each molecule immunostaining reaction among different groups. RETN: Resistin; GCG: glucagon; INS: insulin.

Table 2
Primers for RETN, INS, GCG, and ACTB (used as internal standard) for Real-Time PCR quantification.

Gene	Forward sequence	Reverse sequence	bp
RETN	CCAGTCACTGTGCCCCATAG	AGGAACATTGGCCTGGACTG	99
GCG	CTGCTCTGTTCCACCTCCTG	TGAAGGGAATGTTGCCAGCT	110
INS	GAGAGCGCGGCTTCTTCTAC	ACTGCTCCACGATGCCAC	138
ACTB	CCTTAGCAACCATGCTGTGA	AAGCTGGTGCAGGTAGAGGA	130

Double-label immunofluorescence localization between RETN and GCG showed that all α cells secrete RETN while some of the RETN-positive cells were not colocalizing. The colocalizing cells were more abundant in the periphery of pancreatic islets (Fig. 4).

Double-label immunofluorescence localization between RETN and INS revealed that all ß cells secrete RETN while some of the RETN-positive cells were not colocalizing. The colocalizing cells were more abundant in the center of pancreatic islets (Fig. 5).

No positive signal was detected in the control sections of all samples where the primary antibodies were omitted.

Colocalization analysis for RETN with GCG using ITs of 24 (green channel, i.e. RETN) and 16 (red channel, i.e. GCG) resulted in $r = 0.791$ (PC), $M1 = 0.111$ (MC), $M2 = 0.202$ (MC), $r = 0.96$ with $k1 = 0.693$ and $k2 = 1.329$ (OC).

The same analysis for RETN with INS using ITs of 23 (green channel, i.e. RETN) and 21 (red channel, i.e. INS) resulted in $r = 0.746$ (PC), $M1 = 0.121$ (MC), $M2 = 0.388$ (MC), $r = 0.841$ with $k1 = 0.672$ and $k2 = 1.051$ (OC).

3.2. Real-Time PCR analysis

The Real-Time PCR analysis reported the expression of RETN, GCG and INS in the ovine pancreas of all tested sheep.

For both RETN and GCG analyzed genes there was no significant variation ($P > 0.01$) in the transcript levels among the groups fed with different diets. On the other hand, INS expression was significantly higher in MxF vs Exp and MxD while no significant differences were evidenced between Exp and MxD (Fig. 6).

4. Discussion

The RETN has initially been considered a link among obesity, INS resistance and T2DM (Nieva-Vazquez et al., 2014). However, further investigations have changed this view, as they show a broad tissue distribution of the molecule and, therefore, its diverse action and physiological properties, such as in the pancreas (Adeghate, 2004). To date, the molecule has been mainly studied in humans and lab animals and little information is present about RETN in Ruminants, especially in ovine species (Dall'Aglio et al., 2019; Reverchon et al., 2014; Zhang et al., 2021). This research represents the first study aimed at describing the RETN localization and mRNA expression in the pancreas of sheep. The animals used for the evaluation were grazing on semi-natural pasture and fed with different diets (Mercati et al., 2018).

The obtained results highlighted the molecule presence in the ovine pancreas, exclusively in the endocrine component of the organ. In sheep, the islets had a very heterogeneous morphology and appeared round, oval, or mostly irregular in shape, occurring randomly within the exocrine portion and in the interlobular connective tissue. The RETN positivity was observed throughout most of the islet cells even if some cells were negative. The observed wide distribution of RETN in pancreatic islets of sheep leads us to suppose the prevalent secretion of this molecule from GCG- and INS-secreting cells, as already assumed in other studies reporting the molecule localization only in α cells of lab animals and in β cells of human (Al-Salam et al., 2011; Sassek et al., 2016). Alpha and β cells turn out to be the most abundant cell populations in the pancreatic islets, from humans, rats, to Ruminants (Brissova et al., 2005; Tsuchitani et al., 2016; Mahesh et al., 2017). The immunoperoxidase colocalization confirmed the prevalence of these cell populations and evidenced the distribution of α cells in the periphery and β ones toward the center of the islets as already described in sheep and goat (Dellmann and Eurell, 2000; Mahesh et al., 2017; Al-Redah et al., 2021). A similar pattern has been observed in the bovine, dog, and human (Mahesh et al., 2017; Brissova et al., 2005; Hafez et al., 2015). In contrast, an inverse pattern was observed in the horse and Indian donkey, wherein the α cells were located towards the center, while the β cells were situated towards the periphery, showing

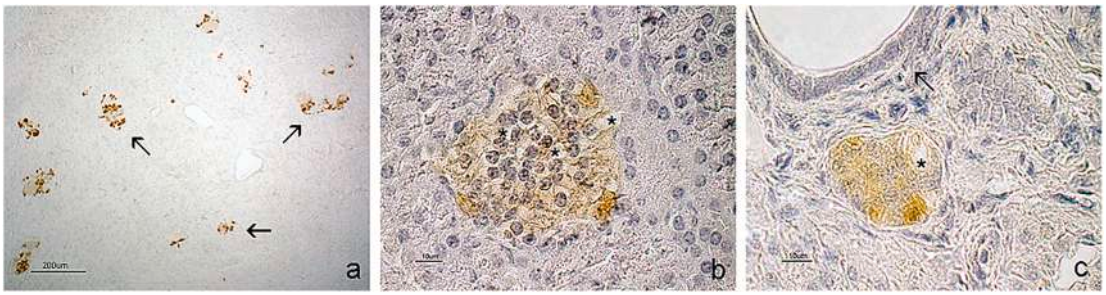

Fig. 1. RETN immunolocalization in pancreas. Pancreatic islets positive to RETN (arrows) are spread in the exocrine parenchyma where no signal detection can be observed (a). RETN shows a wide distribution within the islets in both the center and periphery of pancreatic islets; few cells are negative (asterisks) (b; c). Arrow points to a negative pancreatic duct (c).

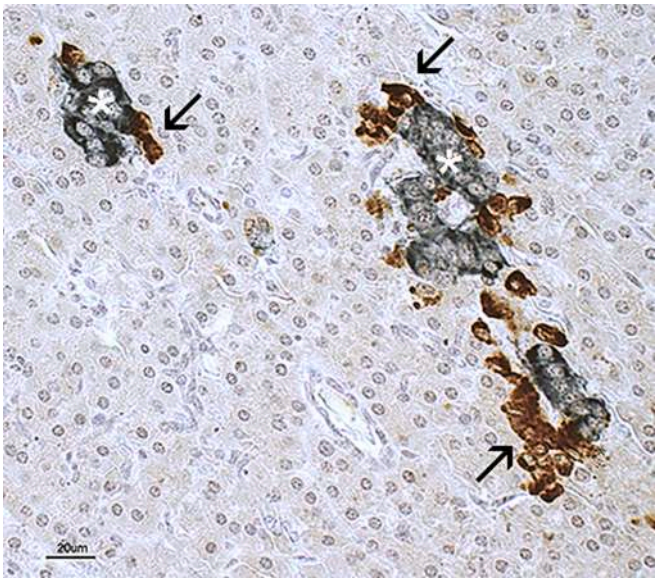

Fig. 2. Double-label immunohistochemistry of INS with GCG in pancreatic islet: positivity of INS (dark blue) and GCG (dark brown), respectively, in the center (asterisks) and periphery (arrows) of pancreatic islets.

interspecific variability (Mahesh et al., 2017).

The presence of RETN in both α and β cells, in ovine, was supported by colocalization analysis performed on RETN with GCG and INS double-label immunofluorescence results. An overlapping of all GCG and INS positive cells with RETN positive ones with the same distribution pattern outlined above was shown. Additionally, the MCs emphasized that a larger fraction of the signal from either GCG or INS overlaps with the RETN signal and not vice versa. These results point out the secretion of RETN from α and β cells.

Interestingly, the secretion of RETN in sheep by pancreatic endocrine cells is different from in rats and humans where the molecule is secreted by α or β cells, respectively, highlighting a species-specific pancreatic pattern of RETN secretion (Al-Salam et al., 2011; Xue et al., 2015; Sassek et al., 2016). δ cells, PP-cells and the gastro-entero-pancreatic (GEP) system secreting cells were not evaluated in this work. These cells represent a minor component of the endocrine islets (Mahesh et al., 2017); they could represent the negative cells observed in the immunohistochemical analysis, but it cannot be excluded RETN secretion by these cell types. Further investigations are needed to clarify this question in sheep.

The RETN receptor has not yet been identified; however, it is known that adipokines perform their function not only at the systemic level, but also at the local level through an autocrine and paracrine mechanism of action (Lago et al., 2007). The molecule detection at the source of both GCG and INS points out the RETN secretion by the investigated endocrine cell populations. This suggests that the RETN in sheep may play a modulatory role, likely in autocrine mode, in islet biology as hypothesized by other authors in humans and rats (Al-Salam et al., 2011; Sassek et al., 2016), the latter role could also reflect on glucose homeostasis. To date it is not possible to evaluate the RETN receptor since not yet clearly identified (Park and Ahima, 2013; Acquarone et al., 2019), hence we are not able to define the molecule action on a specific cell type. However, in this condition, a possible paracrine action toward other negative cells cannot be ruled out.

In normal-weight subjects with physiological RETN levels, the molecule plays a protective role against β cells by exerting an anti-apoptotic effect. This effect disappears in obese subjects in whom RETN levels increase significantly and an apoptotic effect of RETN on β cells is observed impairing their functions (Brown et al., 2007). The sheep in our trial are “normal weight” subjects. Although not aware of the serological levels of RETN since they have not yet been tested, one could likely speculate an anti-apoptotic effect of the molecule on INS-secreting cells and, thus, a protective effect even in this animal model under study.

It has already been reported the variation of adipokines expression, such as RETN in the uterus and apelin in both mammary gland and abomasum of sheep under study, in relation to different nutritional conditions (Dall’Aglio et al., 2019; Mercati et al., 2018; Palmioli et al., 2021). Accordingly, the RETN was analyzed in the same studied sheep to highlight variations related to feeding in the pancreas. Data obtained by quantitative PCR showed the expression of RETN and the other molecules tested in the ovine pancreas. Both RETN and GCG analyzed genes did not vary significantly ($P > 0.01$) in the transcript levels among the treatment groups. Regarding dietary supplementation, the molecular investigation findings showed that RETN expression does not vary with feed integration and is therefore not influenced by this diet. The RETN behavior also resembles the leptin system behaviour previously observed in the sheep abomasum of the same trial (Palmioli et al., 2023), where no statistical differences in leptin system expression were detected. Probably expression of RETN in the pancreas is not sensitive to the diet administered. Other concentrations of the integration may be tested otherwise it can be hypothesized that the molecule may have a primary role in an inflammatory state that may arise in the individual (Filková et al., 2009).

Finally, as the different pattern of INS expression observed indicates, it is important to consider that the sheep of this trial were in the dry period in which the MxF mammary gland parenchyma showed a morphological framework characteristic of the early involution following glandular activity during lactation (Mercati et al., 2018). The Ruminant mammary epithelial cells do not regress as quickly as found in rats after weaning (Tatarczuch et al., 1997) and the gland keeps its activity during the first dry period (Holst et al., 1987; Sordillo et al., 1988). One of the primary roles of INS is to facilitate glucose entry into INS-dependent tissues, including mammary glands. Recent *in vivo* studies

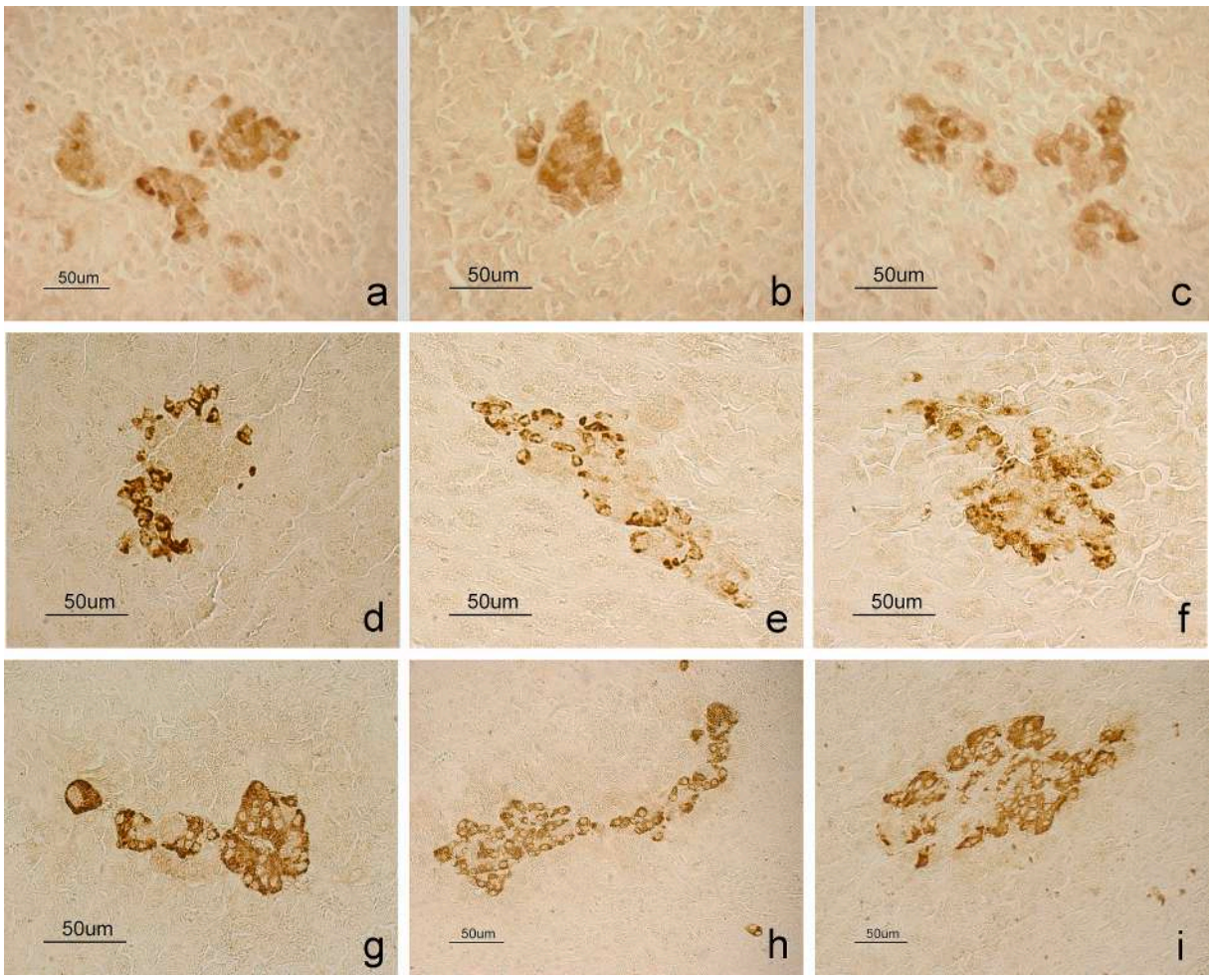

Fig. 3. Immunostaining for RETN (a, b, c), CGC (d, e, f) and INS (g, h, i) in the pancreatic islets of the MxF (a, d, g), Exp (b, e, h) and MxD sheep group (c, f, i).

Table 3
Intensity of RETN, GCG, INS immunostaining in MxF, Exp, and MxD groups expressed as mean ± SD.

	MxF		Exp		MxD		ANOVA
	Mean	SD	Mean	SD	Mean	SD	F & p Values
RETN	2.644	0.547	2.556	0.515	2.511	0.482	0.087; 0.917
GCG	3.533	0.372	3.244	0.493	3.333	0.283	0.711; 0.511
INS	3.867	0.122	3.467	0.346	3.533	0.595	1.409; 0.282

demonstrated that INS stimulates lipid synthesis in mammary tissue and takes place in the milk protein synthesis at multiple levels, including transcriptional regulation, stabilization of milk protein mRNA, and translation of milk protein mRNA, from rats to Ruminants through its normal signaling pathway in the lactating gland to maintain milk synthesis (Cohick, 2016; Neville and Picciano, 1997; Schmidt, 1966). Therefore, INS exerts a relevant role in maintaining milk secretion in the lactating animal (Neville et al., 2013). So, it can be hypothesized that the high expression of INS shown by the MxF group is due to the stimulatory effect of the molecule on milk fat synthesis, since the mammary gland

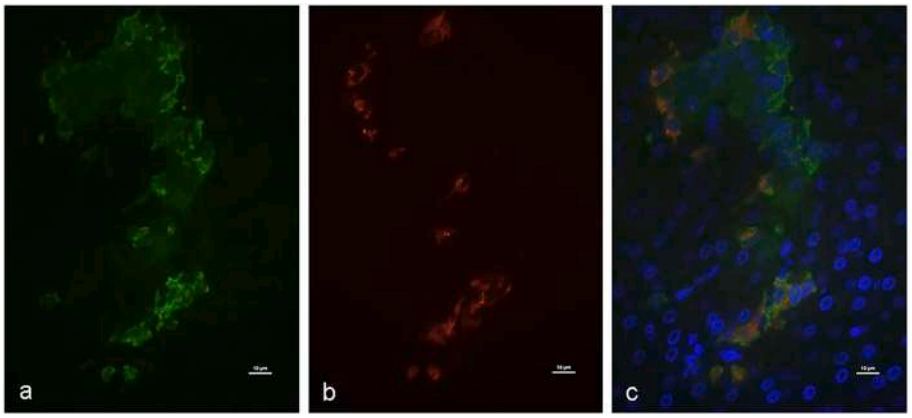

Fig. 4. Immunofluorescence staining of RETN and GCG in pancreatic islets: a) RETN (green), b) GCG (red), c) Merged (RETN, green; GCG, red; DAPI, blue).

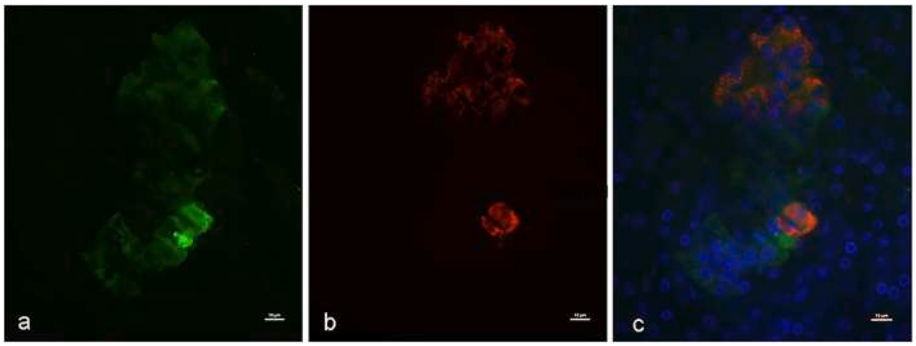

Fig. 5. Immunofluorescence staining of RETN and INS in pancreas: a) RETN (green), b) INS (red), c) Merged (RETN, green; INS, red; DAPI, blue).

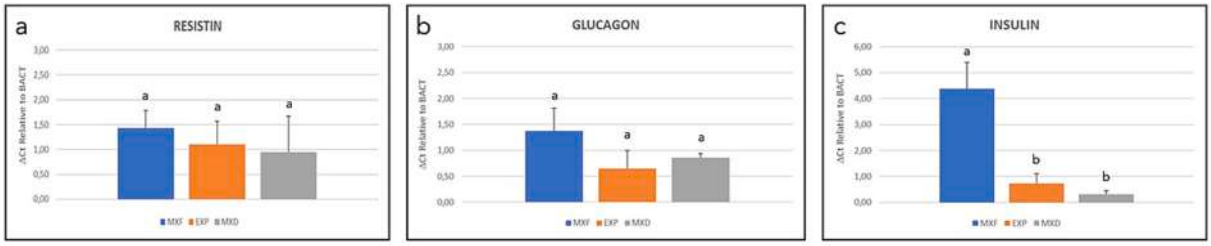

Fig. 6. Relative gene expression of RETN (a), GCG (b) and INS (c) in the pancreas of MxF, Exp and MxD sheep groups. The results are expressed as mean mRNA levels $2^{-\Delta Ct}$ relative to ACTB and standard deviation values of RETN, GCG and INS in pancreas tissue of sheep. Different letters above the bars indicate significantly different values ($P < 0.01$).

maintains synthetic and secretory activity during the early non-lactating phase.

5. Conclusion

This study, representing the first investigation aimed at evaluating RETN in the sheep pancreas, demonstrated the presence of RETN highlighting its specific localization. The RETN production and expression in ovine α and β cells of the pancreatic islets were observed, suggesting that this protein may play a role in islet cell functionality that, likely, reflects on glucose homeostasis in sheep. Expression of the molecule does not appear to be affected by diet and feed supplementation administered in this study, differently from previous investigations performed in the uterus and highlighting a different role of the molecules according to tissues. This study may contribute to further general knowledge about adipokines in sheep as an animal model in relation to food integration.

CRediT authorship contribution statement

Margherita Maranesi: Formal analysis, Investigation, Resources, Writing – review & editing. **Elisa Palmioli:** Methodology, Validation, Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Cecilia Dall'Aglio:** Project administration, Resources, Writing – review & editing. **Daniele Marini:** Formal analysis, Writing – review & editing. **Polina Anipchenko:** Writing – review & editing. **Elena De Felice:** Writing – review & editing. **Paola Scocco:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing. **Francesca Mercati:** Conceptualization, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2024.114452.

References

Acquarone, E., Monacelli, F., Borghi, R., Nencioni, A., Odetti, P., 2019. Resistin: A reappraisal. Mech. Ageing Dev. 178, 46–63. https://doi.org/10.1016/j.mad.2019.01.004.

Adeghate, E., 2004. An update on the biology and physiology of resistin. Cell. Mol. Life Sci. 61 (19–20), 2485–2496. https://doi.org/10.1007/s00018-004-4083-2.

Al-Redah, S.A.A., Fatimah, S.Z., Eman, I.D.A., 2021. Pancreas in Sheep Histochemical and Immunohistochemical Analysis. Ann. Romanian Soc. Cell Biol. 229–240. http://annalsofrscb.ro/index.php/journal/article/view/1501.

Al-Salam, S., Rashed, H., Adeghate, E., 2011. Diabetes mellitus is associated with an increased expression of resistin in human pancreatic islet cells. Islets 3 (5), 246–249. https://doi.org/10.4161/isl.3.5.16427.

Barone, R. 2014. Anatomia comparata dei Mammiferi domestici edizione. Edagricole. Vol. III.

Bartolomé A. The Pancreatic Beta Cell: Editorial. Biomolecules. 2023, 13(3),495. https://doi.org/10.3390/biom13030495.

Bolte, S., Cordelières, F.P., 2006. A guided tour into subcellular colocalization analysis in light microscopy. J. Microsc. 224 (Pt 3), 213–232. https://doi.org/10.1111/j.1365-2818.2006.01706.x.

Brereton, M.F., Vergari, E., Zhang, Q., Clark, A., 2015. Alpha-, Delta- and PP-cells: Are They the Architectural Cornerstones of Islet Structure and Co-ordination? J. Histochem. Cytochem. 63 (8), 575–591. https://doi.org/10.1369/0022155415583535.

Brissova, M., Fowler, M.J., Nicholson, W.E., Chu, A., Hirshberg, B., Harlan, D.M., Powers, A.C., 2005. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J. Histochem. Cytochem. 53 (9), 1087–1097. https://doi.org/10.1369/jhc.5C6684.2005.

Brown, J.E.P., Onyango, D.J., Dunmore, S.J. 2007. Resistin down-regulates insulin receptor expression and modulates cell viability in rodent pancreatic beta-cells. FEBS Lett. 10;581(17):3273-3276. https://doi.org/10.1016/j.febslet.2007.06.031.

Chaves-Almagro, C., Auriau, J., Dortignac, A., Clerc, P., et al., 2022. Upregulated Apelin Signaling in Pancreatic Cancer Activates Oncogenic Signaling Pathways to Promote Tumor Development. Int. J. Mol. Sci. 23 (18), 10600. https://doi.org/10.3390/ijms231810600.

Cohick, W.S., 2016. PHYSIOLOGY AND ENDOCRINOLOGY SYMPOSIUM: Effects of insulin on mammary gland differentiation during pregnancy and lactation. J. Anim. Sci. 94 (5), 1812–1820. https://doi.org/10.2527/jas.2015-0085.

Dall'Aglio, C., Scocco, P., Maranesi, M., Petrucci, L., Acuti, G., De Felice, E., Mercati, F., 2019. Immunohistochemical identification of resistin in the uterus of ewes subjected to different diets: Preliminary results. Eur. J. Histochem. 63 (2), 3020. https://doi.org/10.4081/ejh.2019.3020.

Dall'Aglio, C., Maranesi, M., Di Loria, A., Piantedosi, D., Ciaramella, P., Alterisio, M.C., Lepri, E., Mercati, F. 2021. Effects of Obesity on Adiponectin System Skin Expression in Dogs: A Comparative Study. Animals (Basel). 11(8):2308. https://doi.org/10.3390/ani11082308.

Dellmann, H.D., Eurell, J.A. 2000. Istologia e anatomia microscopica veterinaria. Casa editrice ambrosiana, Ed. 2^. Milano.

Dunning, B.E., Gerich, J.E., 2007. The role of α-cell dysregulation in fasting and postprandial hyperglycemia in type 2 diabetes and therapeutic implications. Endocr Rev. 28 (3), 253–283. https://doi.org/10.1210/er.2006-0026.

Ehrhart, M., Grube, D., Bader, M.F., Aunis, D., Gratzl, M., 1986. Chromogranin A in the pancreatic islet: cellular and subcellular distribution. J. Histochem. Cytochem. 34 (12), 1673–1682. https://doi.org/10.1177/34.12.2878021.

Filková, M., Haluzík, M., Gay, S., Senolt, L., 2009. The role of resistin as a regulator of inflammation: Implications for various human pathologies. Clin Immunol. 133 (2), 157–170. https://doi.org/10.1016/j.clim.2009.07.013.

Gatta, C., De Felice, E., D'Angelo, L., Maruccio, L., Leggieri, A., Lucini, C., Palladino, A., Paolucci, M., Scocco, P., Varricchio, E., De Girolamo, P., 2018. The Case Study of Nesfatin-1in the Pancreas of Tursiops truncatus. Front. Physiol. 9, 1845. https://doi.org/10.3389/fphys.2018.01845.

Hafez, S.A., Zaghloul, D.M., Caceci, T., 2015. Immunohistochemical identification of the endocrine cells in the pancreatic islets of the camel, horse and cattle. Eur. J. Anat. 19 (1), 27–35.

Holland, W.L., Miller, R.A., Wang, Z.V., Sun, K., Barth, B.M., Bui, H.H., Davis, K.E., Bikman, B.T., Halberg, N., Rutkowski, J.M., Wade, M.R., Tenorio, V.M., Kuo, M.S., Brozinick, J.T., Zhang, B.B., Birnbaum, M.J., Summers, S.A., Scherer, P.E., 2011. Receptor-mediated activation of ceramidase activity initiates the pleiotropic actions of adiponectin. Nat. Med. 17 (1), 55–63. https://doi.org/10.1038/nm.2277.

Holst, B.D., Hurley, W.L., Nelson, D.R., 1987. Involution of the bovine mammary gland: Histological and ultrastructural changes. J. Dairy Sci. 70 (5), 935–944. https://doi.org/10.3168/jds.S0022-0302(87)80097-8.

Huising, M.O., van der Meulen, T., Huang, J.L., Pourhosseinzadeh, M.S., Noguchi, G.M., 2018. The Difference δ-Cells Make in Glucose Control. Physiology 33 (6), 403–411. https://doi.org/10.1152/physiol.00029.2018.

Lago, F., Dieguez, C., Gómez-Reino, J., Gualillo, O., 2007. The emerging role of adipokines as mediators of inflammation and immune responses. Cytokine Growth Factor Rev. 18 (3–4), 313–325. https://doi.org/10.1016/j.cytogfr.2007.04.007.

Mahesh, G.KM., Ashok, P., et al., 2017. Comparative histomorphology of endocrine pancreas in deccani sheep and bidri goat. MOJ Anat Physiol. 3 (1), 17–19. https://api.semanticscholar.org/CorpusID:10241924.

Maranesi, M., Zerani, M., Lilli, L., Dall'Aglio, C., Brecchia, G., Gobbetti, A., Boiti, C., 2010. Expression of luteal estrogen receptor, interleukin-1, and apoptosis-associated genes after PGF2alpha administration in rabbits at different stages of pseudopregnancy. Domest. Anim. Endocrinol. 39 (2), 116–130. https://doi.org/10.1016/j. domaniend.2010.03.001.

Maranesi, M., Di Loria, A., Dall'Aglio, C., Piantedosi, D., Lepri, E., Ciaramella, P., Mercati, F., 2020a. Leptin System in Obese Dog Skin: A Pilot Study. Animals. 10 (12), 2338. https://doi.org/10.3390/ani10122338.

Maranesi, M., Palermo, F.A., Bufalari, A., Mercati, F., Paoloni, D., Cocci, P., Moretti, G., Crotti, S., Zerani, M., Dall'Aglio, C., 2020b. Seasonal Expression of NGF and Its Cognate Receptors in the Ovaries of Grey Squirrels (Sciurus carolinensis). Animals 10 (9), 1558. https://doi.org/10.3390/ani10091558.

Maranesi, M., Dall'Aglio, C., Acuti, G., Cappelli, K., Trabalza Marinucci, M., Galarini, R., Suvieri, C., Zerani, M., 2021. Effects of Dietary Polyphenols from Olive Mill Waste Waters on Inflammatory and Apoptotic Effectors in Rabbit Ovary. Animals. 11 (6), 1727. https://doi.org/10.3390/ani11061727.

Marchetti, P., Bugliani, M., De Tata, V., Suleiman, M., Marselli, L., 2017. Pancreatic Beta Cell Identity in Humans and the Role of Type 2 Diabetes. Front. Cell Dev. Biol. 5, 55. https://doi.org/10.3389/fcell.2017.00055.

McTernan, P.G., Kusminski, C.M., Kumar, S., 2006. Resistin. Current opinion in lipidology 17 (2), 170–175. https://doi.org/10.1097/01.mol.0000217899.59820.9a.

Mercati, F., Maranesi, M., Dall'Aglio, C., Petrucci, L., Pasquariello, R., Tardella, F.M., De Felice, E., Scocco, P., 2018. Apelin system in mammary gland of sheep reared in semi-natural pastures of the central apennines. Animals. 8 (12), 223. https://doi.org/10.3390/ani8120223.

Mercati, F., Scocco, P., Maranesi, M., Acuti, G., Petrucci, L., Cocci, P., Renzi, A., De Felice, E., Dall'Aglio, C., 2019. Apelin system detection in the reproductive apparatus of ewes grazing on semi-natural pasture. Theriogenology. 139, 156–166. https://doi.org/10.1016/j.theriogenology.2019.08.012.

Messaggio, F., Mendonsa, A.M., Castellanos, J., Nagathihalli, N.S., Gorden, L., Merchant, N.B., VanSaun, M.N., 2017. Adiponectin receptor agonists inhibit leptin induced pSTAT3 and in vivo pancreatic tumor growth. Oncotarget. 8 (49), 85378–85391. https://doi.org/10.18632/oncotarget.19905.

Minn, A.H., Patterson, N.B., Pack, S., Hoffmann, S.C., Gavrilova, O., Vinson, C., Harlan, D.M., Shalev, A., 2003. Resistin is expressed in pancreatic islets. Biochem. Biophys. Res. Commun. 310 (2), 641–645. https://doi.org/10.1016/j.bbrc.2003.09.061.

Morioka, T., Asilmaz, E., Hu, J., Dishinger, J.F., Kurpad, A.J., Elias, C.F., Li, H., Elmquist, J.K., Kennedy, R.T., Kulkarni, R.N., 2007. Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. J. Clin. Investig. 117 (10), 2860–2868. https://doi.org/10.1172/JCI30910.

Nakata, M., Okada, T., Ozawa, K., Yada, T. 2007. Resistin induces insulin resistance in pancreatic islets to impair glucose induced insulin release. Biochem. Biophys. Res. Comm. 2007. 353(4): 1046–1051. https://doi.org/10.1016/j.bbrc.2006.12.134.

Neville, M.C., Picciano, M.F., 1997. Regulation of milk lipid secretion and composition. Annu. Rev. Nutr. 17, 159–183. https://doi.org/10.1146/annurev.nutr.17.1.159.

Neville, M.C., Webb, P., Ramanathan, P., Mannino, M.P., Pecorini, C., Monks, J., Anderson, S.M., MacLean, P., 2013. The insulin receptor plays an important role in secretory differentiation in the mammary gland. Am. J. Physiol. Endocrinol. Metab. 305 (9), E1103–E1114. https://doi.org/10.1152/ajpendo.00337.2013.

Nieva-Vazquez, A., Pérez-Fuentes, R., Torres-Rasgado, E., López-López, J.G., Romero, J. R., 2014. Serum resistin levels are associated with adiposity and insulin sensitivity in obese Hispanic subjects. Metab. Syndr. Relat. Disord. 12 (2), 143–148. https://doi.org/10.1089/met.2013.0118.

Palmioli, E., Dall'Aglio, C., Bellesi, M., Tardella, F.M., Moscatelli, S., Scocco, P., Mercati, F., 2021. The Apelinergic System Immuno-Detection in the Abomasum and Duodenum of Sheep Grazing on Semi-Natural Pasture. Animals (Basel) 11 (11), 3173. https://doi.org/10.3390/ani11113173.

Palmioli, E., Dall'Aglio, C., Fagotti, A., Simoncelli, F., Dobrzyn, K., Di Rosa, I., Maranesi, M., De Felice, E., Scocco, P., Mercati, F., 2023. Leptin system is not affected by different diets in the abomasum of the sheep reared in semi-natural pastures of the Central Apennines. Ann Anat. https://doi.org/10.1016/j.aanat.2023.152069.

Park, H.K., Ahima, R.S., 2013. Resistin in rodents and humans. Diabetes. Metab. J. 37 (6), 404–414. https://doi.org/10.4093/dmj.2013.37.6.404.

Quesada, I., Tuduri, E., Ripoll, C., Nadal, A., 2008. Physiology of the pancreatic alpha-cell and glucagon secretion: role in glucose homeostasis and diabetes. J. Endocrinol. 199, 5–19. https://doi.org/10.1677/JOE-08-0290.

Reverchon, M., Ramé, C., Cognié, J., Briant, E., Elis, S., Guillaume, D., Dupont, J., 2014. Resistin in dairy cows: plasma concentrations during early lactation, expression and potential role in adipose tissue. PloS One. 9 (3), e93198.

Sakata, N., Yoshimatsu, G., Kodama, S., 2019. Development and Characteristics of Pancreatic Epsilon Cells. Int. J. Mol. Sci. 20 (8), 1867. https://doi.org/10.3390/ijms20081867.

Sassek, M., Pruszynska-Oszmalek, E., Kołodziejski, P.A., Szczepankiewicz, D., Kaczmarek, P., Wieloch, M., Kurto, K., Nogowski, L., Nowak, K.W., Strowski, M.Z., Mackowiak, P., 2016. Resistin is produced by rat pancreatic islets and regulates insulin and glucagon in vitro secretion. Islets. 8 (6), 177–185. https://doi.org/10.1080/19382014.2016.1251538.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods. 9 (7), 676–682. https://doi.org/10.1038/nmeth.2019.

Schmidt, G.H., 1966. Effect of insulin on yield and composition of milk of dairy cows. J. Dairy Sci. 49 (4), 381–385. https://doi.org/10.3168/jds.S0022-0302(66)87878-5.

Scocco, P., Rivaroli, S., Mercati, F., Tardella, F.M., Malfatti, A., De Felice, E., Catorci, A., 2018. Anatomy for economy: Starting from the rumen keratinization degree to enhance the farm income. Econ. Agro-Aliment. 20, 261–272. https://doi.org/10.3280/ECAG2018-002010.

Sordillo, L.M., Nickerson, S.C., 1988. Morphologic changes in the bovine mammary gland during involution and lactogenesis. Am. J. Vet. Res. 49, 1112–1120. https://europepmc.org/article/med/3421535.

Steiner, D.J., Kim, A., Miller, K., Hara, M., 2010. Pancreatic islet plasticity: interspecies comparison of islet architecture and composition. Islets. 2 (3), 135–145. https://doi.org/10.4161/isl.2.3.11815.

Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H. R., Ahima, R.S., Lazar, M.A., 2001. The hormone resistin links obesity to diabetes. Nature. 409 (6818), 307–312. https://doi.org/10.1038/35053000.

Strutt, B., Szlapinski, S., Gnaneswaran, T., Donegan, S., Hill, J., Bennett, J., Hill, D.J., 2021. Ontology of the apelinergic system in mouse pancreas during pregnancy and relationship with β-cell mass. Sci Rep. 1 (1), 15475. https://doi.org/10.1038/s41598-021-94725-0.

Tatarczuch, L., Philip, C., Lee, C.S., 1997. Involution of the sheep mammary gland. J. Anat. 190 (Pt 3), 405–416. https://doi.org/10.1046/j.1469-7580.1997.19030405.x.

Tripathi, D., Kant, S., Pandey, S., Ehtesham, N.Z., 2020. Resistin in metabolism, inflammation, and disease. FEBS J. 287 (15), 3141–3149. https://doi.org/10.1111/febs.15322.

Tsuchitani, M., Sato, J., Kokoshima, H., 2016. A comparison of the anatomical structure of the pancreas in experimental animals. J. Toxicol. Pathol. 29 (3), 147–154.

Tudurí, E., Marroquí, L., Soriano, S., Ropero, A.B., Batista, T.M., Piquer, S., López-Boado, M.A., Carneiro, E.M., Gomis, R., Nadal, A., Quesada, I., 2009. Inhibitory effects of leptin on pancreatic alpha-cell function. Diabetol. Metab. Syndr. 6 (1), 91. https://doi.org/10.1186/1758-5996-6-91.

Xue, L.N., Wang, X.Y., Tan, Y., Lin, M., Zhang, W., Xu, K.Q., 2015. Significance of resistin expression in acute pancreatitis. Exp. Ther. Med. 9 (4), 1438–1442. https://doi.org/10.3892/etm.2015.2270.

Yang, Y., Xiao, M., Mao, Y., Li, H., Zhao, S., Gu, Y., Wang, R., Yu, J., Zhang, X., Irwin, D. M., Niu, G., Tan, H., 2009. Resistin and insulin resistance in hepatocytes: resistin disturbs glycogen metabolism at the protein level. Biomed. Pharmacother. 63 (5), 366–374. https://doi.org/10.1016/j.biopha.2008.06.033.

Zerani, M., Catone, G., Maranesi, M., Gobbetti, A., Boiti, C., Parillo, F., 2012. Gonadotropin-releasing hormone 1 directly affects corpora lutea lifespan in Mediterranean buffalo (Bubalus bubalis) during diestrus: presence and in vitro effects on enzymatic and hormonal activities. Biol. Reprod. 87 (2), 45. https://doi.org/10.1095/biolreprod.112.099598.

Zhang, Y., Wang, Y., Xu, Q., Zhu, J., Lin, Y., 2021. Molecular and expression characteristics of resistin (RETN) and its effects on the differentiation of intramuscular preadipocyte in goat. Anim. Biotechnol. 32 (1), 113–120. https://doi.org/10.1080/10495398.2019.1664564.