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Histopathological Characteristics of Folliculo-stellate Cells in Pituitary Glands of Wild Type, Obese and High-fat Diet Induced Mice

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ARTICLE INFO

Article history

Received: 31 January 2022

Accepted: 3 March 2022

Published Online: 4 March 2022

Keywords:

Folliculo-stellate cells (FSC)

S-100 protein

Basal metabolism

Pituitary

ABSTRACT

The anterior pituitary gland regulates growth, metabolism, and reproduction by secreting hormones. Folliculo-stellate cells (FSC) are non-endocrine cells located among hormone-producing cells in the anterior pituitary glands, but little is known about the exact roles of those cells. Although, with their net organization, they seem to have an important role in the hormonal cells regulation and maintenance.

In this work, the first ever made in this area, 33 pituitaries of three groups of mice (18 wild type [WT], 11 genetically obese [OB] and 4 under a high-fat diet [HFD]) were studied in order to determine if there was any relation between the number of FSC and alterations of the basal metabolism in each group of mice. For that, immunohistochemical staining using the S-100 protein was used and also the Image-J software, to calculate the percentage of FSC present in each sample.

The authors found that, although there wasn't any significant difference between WT and OB mice, the group of HFD mice tend to have substantially higher percentage of FSC than the mice from other groups. This might suggest some yet unknown link between diet, precisely with a high-fat diet, and the presentation of FSC in the anterior pituitary.

1. Introduction

The pituitary gland (pituitary) is a highly complex endocrine structure that has the ability to respond to multiple signals from both central and peripheral locations^[1]. It regulates and acts on different systems of the body, being

in close relationship with the hypothalamus^[2].

Anatomically there are two lobes, an anterior or adeno-hypophysis and a posterior or neurohypophysis, of whose physiology and histology there are long and consensual descriptions^[3]. Regarding the roles of the anterior pituitary, its action on growth through GH, on lactation with

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DOI: <https://doi.org/10.30564/jer.v4i2.4426>

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prolactin, on gonadal activity through FSH and LH, as well as on adrenal and thyroid activity with ACTH and to TSH, respectively, are well known^[4]. As for the neurohypophysis, the effects of vasopressin, involved in water balance, and oxytocin, responsible for uterine contractions and lactation, stand out^[5]. Although much is still unknown^[6]. In the anterior pituitary there are also described cells traditionally reported as support, the folliculo-stellate cells (FSC)^[7]. These cells, characterized by their star-like appearance and ability to form follicles, do not appear to have the ability to produce hormones^[8,9]. However, due to their network organization, they seem to play an important role in the regulation and maintenance of the population of hormonal cells, as they conduct stimulatory or inhibitory factors from the hypothalamus and transport the secretory products of the gland^[10,11].

Even so, the pathophysiological role of the FSC network is still poorly understood. After a long period of research on the pituitary, several hypotheses have been raised about the role of FSC in the adenohypophyseal machine, functions as varied as paracrine regulation, cell turnover and neuroimmune crosstalk, but many problems still remain without solution^[12-14].

Other studies also suggest that these cells, which are positive for the S-100 protein, due to their heterogeneous and plural behavior, may have an extrapituitary origin^[15,16]. Its proliferation may result from the invasion of the anterior pituitary lobe by other structures during organogenesis^[17,18].

While some new studies tried to approach these cells from some different perspectives, none of the ones published focused on the relationship between FSC and alterations of the basal metabolism, although the known relationship between pituitary hormones and metabolism. With this work, the first of its kind, we seek to understand whether there is a relationship between FSC and changes in basal metabolism associated with obesity, using the study of wild type (WT), obese (OB) and induced high-fat diet (HFD) mice.

2. Materials and Methods

B6 (C57BL/B6J, own production of the *Gulbenkian Institute of Science*) and ob/ob (Jackson Laboratory [JAX], stock no. 000632) mice were used in this study. All mice used for the study were male and were kept at controlled temperature and humidity under a 12 hour light/dark cycle. The use of only male mice it was done to limit the hormonal variation that occurs in females and that could affect this study's results. Food and water were provided *without restrictions*. The diet-induced obesity (DIO) model was generated by placing the animals on an HFD at the eighth week and lasting 12 weeks. All animal procedures

were approved by the ethics committee of the *Gulbenkian Institute of Science* and by the *National Network of Entities Responsible for Animal Welfare*.

2.1 Histology and Immunohistochemistry

For the preparation of this work, 37 pituitary glands were obtained: 19 from WT mice (of which, due to lack of quality of the remaining samples, we were able to evaluate 18), 14 OB mice (of which, due to lack of quality of the remaining samples, we were able to evaluate 11) and 4 HFD (all evaluated). Sacrifice and sample collection were performed between 01/11/2017 e 01/12/2017.

The mice's pituitary glands were dissected and fixed with 4% buffered formaldehyde immediately after extraction. Subsequently, after performing the macroscopic examination of the glands, they were processed according to the usual technical procedures for obtaining paraffin blocks for complete histological evaluation.

The presence and distribution of FSC was studied by immunohistochemistry in deparaffinized 3-micron histological sections, subjected to antigen recovery and incubated with an individual antibody directed against the specific cellular protein S-100 β (polyclonal; provenance: Leica; dilution : 1/400)^[19-22].

All samples were evaluated by an experienced pathologist. The staining index for S-100 was calculated as the percentage of positive cells in at least 500 cells in the areas of highest immunostaining, analyzed under an optical microscope with 400x magnification. In all cases, the percentage of FSC was also calculated with the help of an immunohistochemical analysis image processing software (Image J 1.49. National Institutes of Health, United States).

2.2 Statistical Framework

A basic statistical and comparative analysis appropriate to the data distribution was performed. For this, parametric and non-parametric (Kruskal-Wallis) one-way ANOVA statistical tests were used. A value of $p < 0.05$ was considered statistically significant, also resorting to non-parametric multiple comparisons (Tukey-Kramer-Nemenyi, Conover's and Dunn's) between the different groups.

3. Results

After analyzing the pituitary glands, the data presented in Tables 1, 2 and 3 were obtained, which represent the percentage of FSC in each pituitary gland evaluated.

In WT mice, Table 1, there was a relative prevalence of FSC ranging from 3% to 60%. Percentage distributions are presented in Figures 1 and 2.

Table 1. Percentage of FSC in WT mice - Percentage of FSC in relation to the total tissue observed in the pituitary sample of each WT mouse.

WT 1	WT 2	WT 3	WT 4	WT 5	WT 6	WT 7	WT 8	WT 9	WT 10	WT 11	WT 12	WT 13	WT 14	WT 15	WT 16	WT 17	WT 18
17%	15%	35%	11%	25%	20%	3%	18%	20%	12%	17%	9%	60%	24%	46%	13%	16%	26%

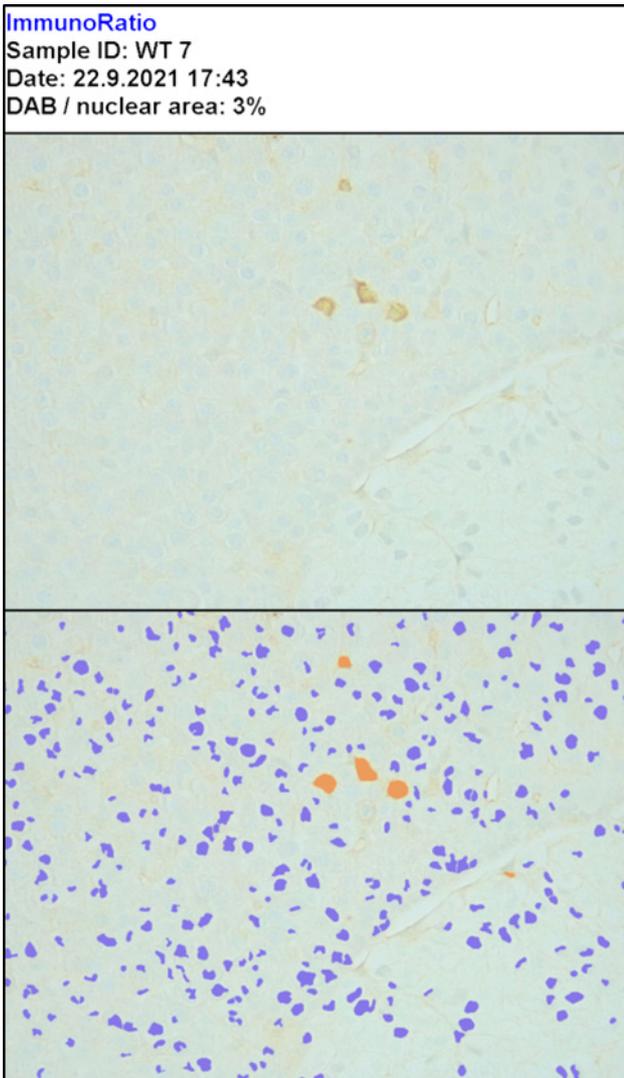


Figure 1. Immunohistochemical staining of S-100 positive cells in the WT7 mouse - Microscopic images of the pituitary gland of a mouse revealing a 3% prevalence of FSC: Above, the immunohistochemical technique (using an antibody against the S-100 protein); below, with an image processor program, the proportionality of these cells (in brown) in relation to the remaining cells (nuclei in blue) is shown (Image J, ImmunoRatio plugin).

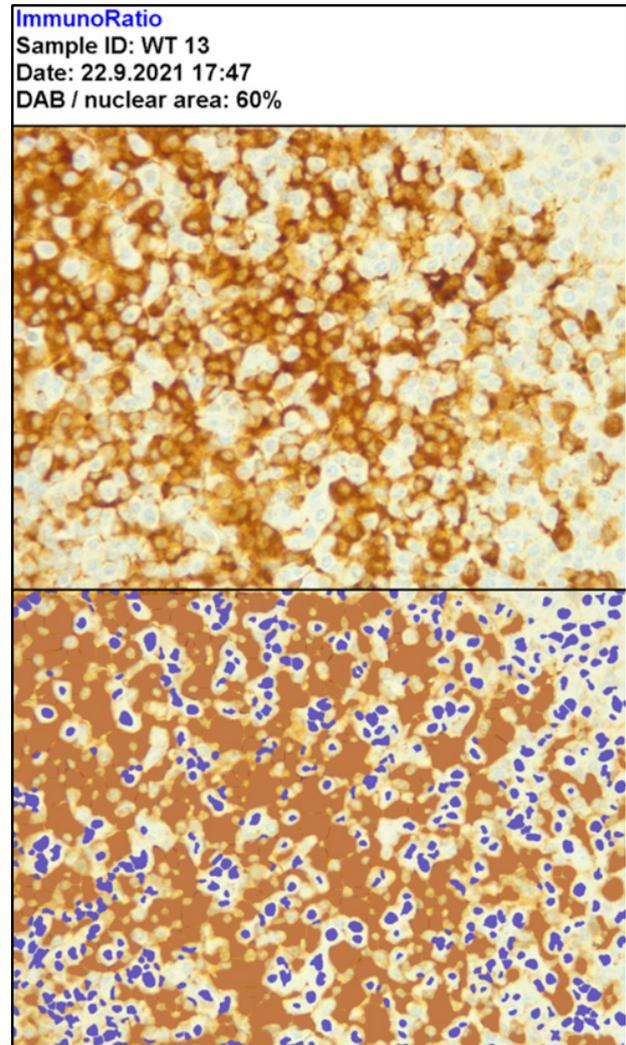


Figure 2. Immunohistochemical staining of S-100 positive cells in the WT13 mouse revealing a 60% prevalence of FSC.

The OB mice, in Table 2, have a similar distribution of FSC, ranging from 10% to 54%. Percentage distributions are presented in Figures 3 and 4.

The group of HFD mice, in Table 3, only varied between 85% and 93% (Figure 5).

Table 2. Percentage of FSC in OB mice - Percentage of FSC in relation to the total tissue observed in the pituitary sample of each OB mouse.

OB1	OB2	OB3	OB4	OB5	OB6	OB7	OB8	OB9	OB10	OB11
22%	25%	54%	10%	17%	16%	12%	26%	25%	19%	29%

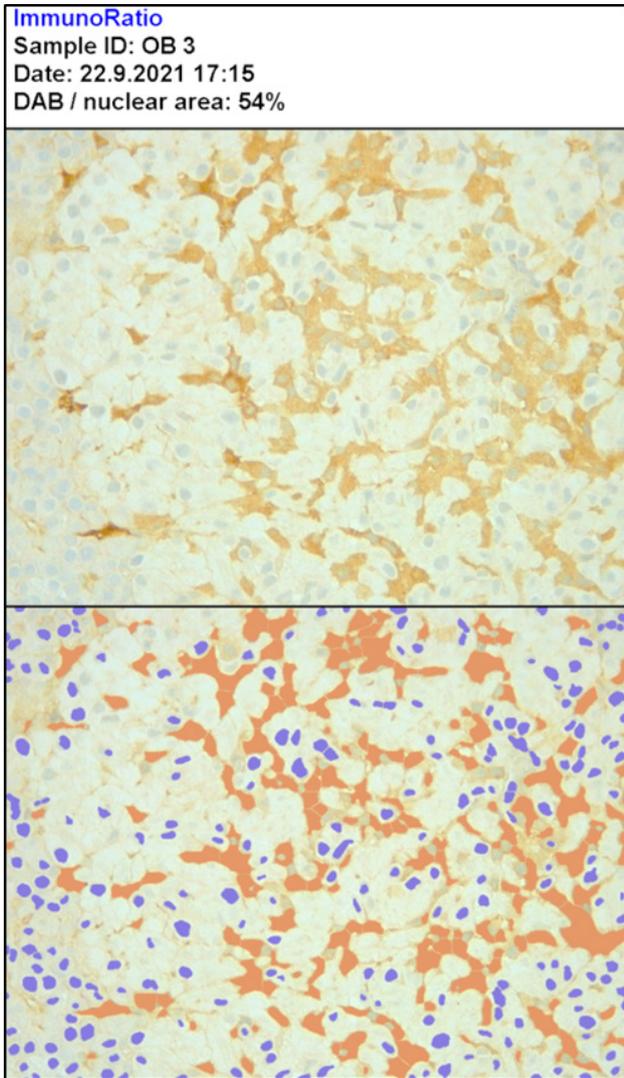


Figure 3. Immunohistochemical staining of S-100 positive cells in the OB3 revealing a 54% prevalence of FSC.

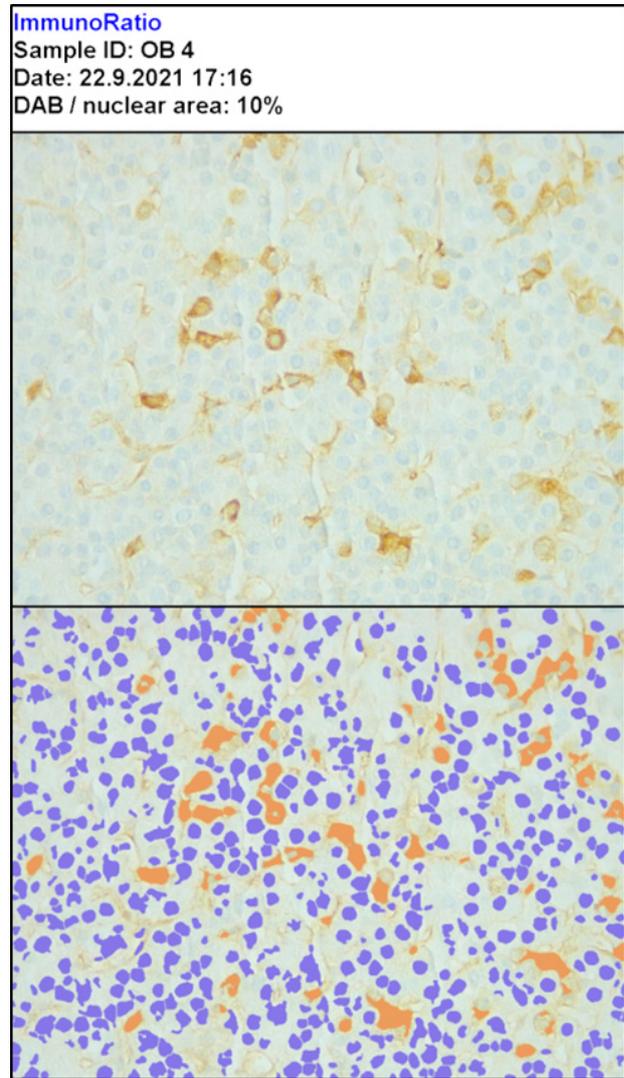


Figure 4. Immunohistochemical staining of S-100 positive cells in the OB4 mouse revealing a 10% prevalence of FSC.

Table 3. Percentage of FSC in HFD mice - Percentage of FSC in relation to the total tissue observed in the pituitary sample of each HFD mouse.

HFD1	HFD2	HFD3	HFD4
89%	93%	85%	93%

As seen in Table 4, an average of 22% FSC in WT mice, 23% FSC in OB mice and 90% FSC in HFD mice could be obtained. None of the data obtained was considered statistically as an outlier, having obtained a dispersion of data that could mean a tendency for the percentage of FSC to be higher when eating a diet rich in fatty acids.

In addition to what has already been described, visualizing the data in the scatter plot (Figure 6), it can be seen that the data referring to the HFD group are always higher than the data from the other two groups, which in turn are distributed very similar.

This difference is better visualized when the boxplots are analyzed in Figure 7. In these, there is a total overlap of the boxplots referring to the WT and OB groups, with no overlap of these to the boxplot of the HFD group.

Based on these descriptive data, as well as the values of the respective averages (22% [WT], 23% [OB] and 90% [HFD]) and standard deviations (0.14% [WT], 0.12% [OB] and 0.04% [HFD]), the data seem to point towards the hypothesis that the means of the observations of the groups are significantly different, more particularly between the HFD group and the rest.

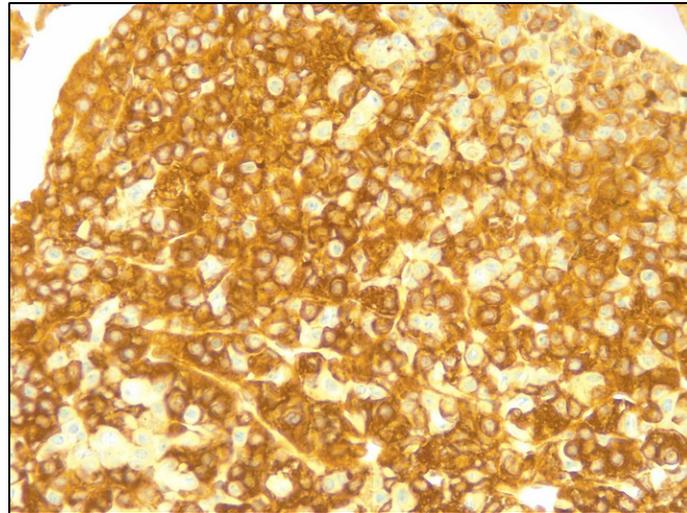


Figure 5. Immunohistochemical staining of S-100 positive cells in the HFD4 mouse - Microscopic images of the pituitary of a mouse revealing a 93% prevalence of CFE (immunohistochemical technique using an antibody against the S-100 protein).

Table 4. Descriptive analysis of the data obtained - Qualitative statistics of each group of mice including Extremes, Quartiles, Interquartile Range, Limit of Upper and Lower Outliers and Mean.

	WT	OB	HFD
Minimum	3%	10%	85%
1 ^o Quartile	13.50%	16.50%	88%
3 ^o Quartile	24.75%	25.50%	93%
Maximum	60%	54%	93%
IQR	11%	9%	5%
Outliers Lower Limit	-3%	3%	81%
Outliers Upper Limit	77%	68%	101%
Mean	22%	23%	90%

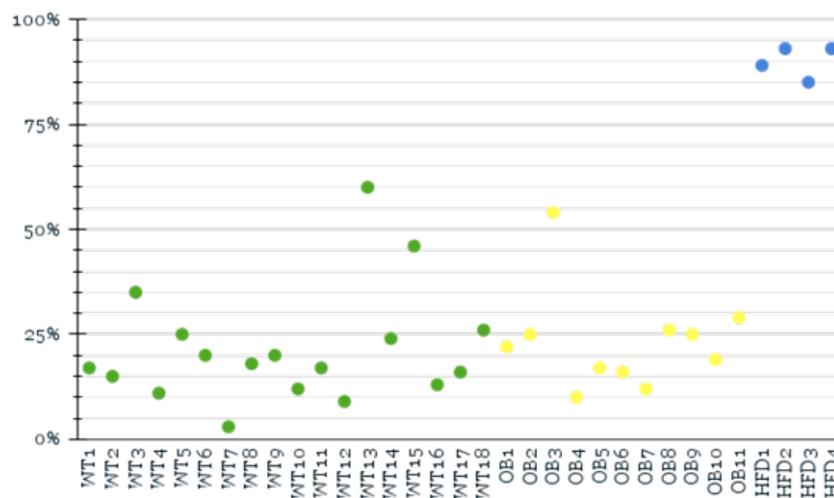


Figure 6. Dispersion of the percentage of FSC in each mouse - Graphic representation of the percentage of FSC in the pituitary of each mouse (WT - green; OB - yellow and HFD - blue).

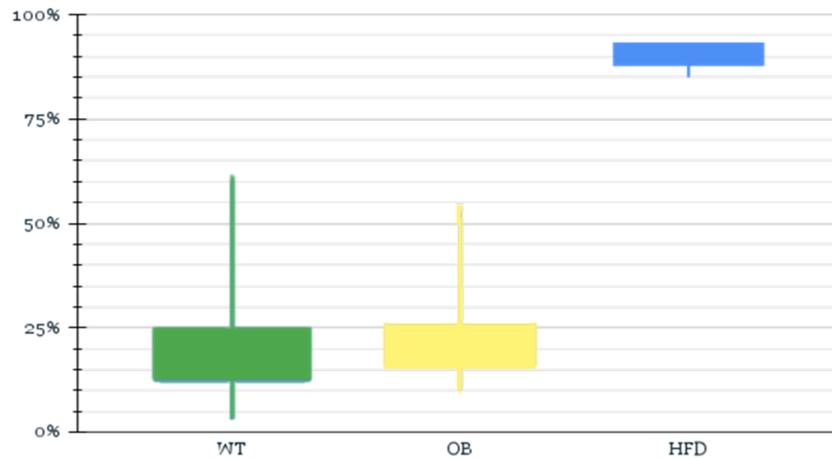


Figure 7. Boxplots distribution of the percentage of FSC in the different groups of mice - Graphic representation of the distribution of the variation of the numerical data obtained through quartiles.

Using the parametric and non-parametric one-way ANOVA (Kruskal-Wallis) statistical tests, we found that the hypothesis of equality of means/medians is rejected by both when they present p-value <0.05 (<<0.001 and 0.005, respectively), with significant differences between the observation groups.

As the assumptions of homogeneity of variances and normality were not verified and in order to better assess the differences between groups, non-parametric multiple comparisons were used (Tukey-Kramer-Nemenyi, Conover's and Dunn's - Tables 5, 6 and 7, respectively) having always verified the existence from two homogeneous groups: one formed by the WT and OB observation groups and the other formed by the HFD observations (p <0.05).

Table 5. Tukey-Kramer-Nemenyi.

	HFD	OB
OB	0,0215	-
WT	0,0032	0,7906

Table 6. Conover's.

	HFD	OB
OB	0,0099	-
WT	0,0015	0,7206

Table 7. Dunn's.

	HFD	OB
OB	0,0156	-
WT	0,0033	0,5133

4. Discussion

As mentioned above, there is a tendency for the percentage of FSC in HFD mice to be higher than in WT and OB mice. This could mean two different things, as we are looking at percentages: an increase in the number of FSC in HFD mice; or that is a decrease in other cell populations in these mice, consequently increasing the relative prevalence of FSC in the pituitary glands of these mice.

As for WT and OB mice, there does not seem to be a significant difference between the two groups of samples, so it can be inferred that FSC are not affected by genetically induced obesity.

In the light of current knowledge, we can try to explain the results obtained through a range of aspects.

It is now known that *in vitro* the Hedgehog signaling pathway leads FSC to stimulate the production and release of GH by the anterior pituitary, but the production of CXCL12 by FSC can have the same effect [23,24]. By establishing a communication network between the FSC and with the hormone-producing cells, this may correspond *in vivo* to a physiological reaction mechanism to the increased intake of fatty acids, evident in HFD mice, influencing the expression of FSC [25,26].

However, recent studies show that S-100 positive cells in the anterior pituitary may not be just FSC, but may even mark pituitary stem/progenitor cells [27,28]. Previously, it was hypothesized that the FSC themselves could be a type of pluripotent adult stem cell [29-31]. This question is still open [32].

It is also suggested by Higuchi, M., *et al.* (2014) [33], that these S-100 positive cells will have the ability, albeit infrequently, to become hormone-producing cells, or the fact of recognizing in folliculo-stellate-like cells an intermediate stage in the differentiation to hormone-producing

cells^[34,35]. Through the expression of SOX2 in progenitor cells, the variation in the prevalence of FSC can be influenced^[36]. Hence, the increase in FSC in HFD mice may be due to this physiological response of cell differentiation in the attempt to adapt to a diet rich in fatty acids.

On the other hand, the plurality of cells that showed positive S-100 staining may influence the results in the sense that the differences observed may be due not to changes in FSC, but in other populations of S-100 positive cells.

For this exclusion, in future studies, perhaps another specific marker for FSC should be used, or a panel of markers that would make the marking more specific. These could include, among others, Claudin-9, a marker of tight junctions of FSC^[37], Aldolase C^[38] or IL-6 itself, which is stimulated by Adenosine, produced by FSC^[39,40].

FSC are also defined as the major intrapituitary source of cytokines and growth factors^[41]. Therefore, there is a need, given the existence of several subtypes of FSC, to characterize them individually and arrange specific markers that allow their study^[42,43]. As well as their characterization depending on the age of the individual^[44,45].

5. Conclusions

In the vast world that can be a small organ like the pituitary, there are still many questions to be clarified regarding FSC. With this work, we intend to pave the way for what can be followed in further investigations.

Despite the discrepancy between the number of samples within each group, which will need confirmation in studies with a more significant sample, we can intuit that a diet rich in fatty acids will lead to an increase in the relative prevalence of FSC in mice, or at least, to an increase in S-100 positive cell populations, whose physiological mechanisms, both the origin of the alterations and the consequences thereof, should be studied later.

Conflict of Interest

The authors declare no conflict of interest.

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