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Immunohistochemical analysis of caspase expression in the brains of individuals with obesity or overweight

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Abstract

Mechanisms underlying the negative effects of obesity on the brain are still unknown. Obesity is associated with oxidative stress in the brain and neuroinflammation that promotes neurodegenerative diseases. Chronic low-grade neuroinflammation in obesity could be associated with lower volumes of gray matter and lower neuronal density. If neuroinflammation mediated by the expression of cytokines and chemokines leads to apoptosis, this can be assessed by examining caspase expression. The aim of this study was to compare the expression of caspases in the 16 brains of donors with obesity/overweight ($n = 8$; Body Mass Index [BMI] = 31.6 ± 4.35 kg/m^2; 2 females; Age = 52.9 ± 4.76 years) and normal weight ($n = 8$; BMI = 21.8 ± 1.5 kg/m^2; 3 females; Age = 37.8 ± 19.2 years). Sixteen human brain samples were processed. Serial paraffin sections were examined by anti-caspase immunochemistry (caspase-3, caspase-4, caspase-6, caspase-1, caspase-8, and caspase-9 antibodies). Postmortem samples of cerebral cortex tissue were captured as photomicrographs and the images obtained were analyzed using ImageJ software to obtain the percentage of positive caspase expression. Nonparametric Mann–Whitney U tests were performed to compare caspase expression between samples from donors with obesity/overweight and normal weight. Taking into consideration the immunohistochemistry results, the Search Tool for the Retrieval of Interacting Genes was used to model molecular interactions. Results showed that brain samples from individuals with obesity/overweight exhibited significantly greater values of positive expression for Caspase-1 ($U = 16.5$, $p = 0.05$, Cohen $d = 0.89$) and −8 ($U = 15$, $p = 0.03$, Cohen $d = 0.99$) than those from donors with normal weight. This study contributes to the knowledge about the inflammatory effects of obesity/overweight on brain, suggesting the activation of the alternative inflammasome pathway in which interact caspase-1 and -8.

KEYWORDS
caspase-1, caspase-8, caspases, immunohistochemical, neuroinflammation

1 | INTRODUCTION

Although the biological mechanisms underlying the damaging effects of obesity on the brain are still not well understood, animal models and human studies have shown that obesity is associated with increased oxidative stress in the brain[1] and neuroinflammation[2]; both factors are involved in the pathogenesis of neurodegenerative diseases.[3]

Macrophages in adipose tissue promote an increase in cytokines and proinflammatory chemokines and initiate a mild chronic response[4] which increased expression of tumor necrosis factor alpha, plasminogen activator inhibitor-1, C-reactive protein, interleukin-1-beta (IL-1-β) and interleukin-6 (IL-6).[5] The inflammatory response mediated by M1 cells during obesity might be analogous to the necrotic clearance mechanisms.[6] The obesity-associated gut microbiota may also contribute to neurochemical and inflammatory alterations.[7,8]

Neuroinflammation can lead to apoptosis.[9] Dysregulation of apoptosis plays a key role in the etiology and/or progression of a variety of disorders.[9,10] The process of apoptosis involves the interplay of a wide array of proteins and signal transducers, as well as cascades of signaling pathways.[11] Apoptosis involves the activation of caspases, which orchestrate all morphological changes that characterize this cell death process.[9] Activation of initiator caspases (Caspase-2, -8 or -10) is the first step in a regulated, irreversible, and self-amplifying proteolytic pathway that leads to the activation of effector caspases (Caspase-3, -6 and -7).[12]

Caspase-1 plays an important role in the innate immune response.[13] Pathogens, stress, and damage signals activate caspase-1, and this process is typically mediated by proximity-induced autoproteolysis in multimeric protein complexes called the inflammasome.[14] In monocytes and macrophages, caspases cleave the proinflammatory cytokine pro-interleukin (IL)-1β to an active secreted molecule.[15] Active caspase-1 is the final step in the process of the nucleotide-binding domain and leucine-rich repeat containing gene NOD-like receptor (NLR) family pyrin domain containing 3 (NLRP3) inflammasome, a three-protein intracellular complex involved in inflammation and the induction of pyroptosis.[16] Although Caspase-4 activation alone is sufficient to induce pyroptosis, this process depends on NLRP3 inflammasome activation to drive IL-1β maturation.[17]

Inflammation causes hypoperfusion and ischemia, which can lead to apoptosis via an intrinsic route.[10] This apoptosis pathway is characterized by nonreceptor-mediated initiation and mitochondrial regulation. In the intrinsic pathway, stimuli directly generate intracellular signals that lead to biochemical changes within the cell.[12] Apoptosomes cleave procaspase-9 to yield active caspase-9, which in turn activates the effector caspase caspase-3.[12] Thus, both extrinsic and intrinsic pathways converge at the execution phase. This phase refers to the final apoptosis pathway.[18]

Structural abnormalities in gray matter have been observed in patients with obesity.[19] Neuroimaging studies have shown consistent reductions in gray matter in individuals with obesity, which affects the inferior frontal gyri, right insula, the left and right precentral gyri, the left middle frontal gyrus, the left middle temporal gyrus, the left amygdala, and the left cerebellar hemisphere.[19–21] These structural changes in the brains of individuals with obesity could be associated with lower neuronal density[22] and are probably derived from neuroinflammation that induces apoptosis mechanisms. Animal model studies have shown that high-fat diet and diet-induced obesity are associated with neuroinflammation and apoptosis in central nervous system.[23–27]

In the present study, the apoptotic activity of brain tissue from donors with obesity was evaluated by immunohistochemistry. The mechanisms underlying the damage and therefore neuronal death in obesity are still unknown, but the evidence suggests that chronic systemic humoral inflammation may be associated with a reduced volume of gray matter.[26,27] Thus, the aim of this study was to compare the expression of caspases in the brains of donors with obesity or overweight and normal weight. Therefore, it is expected that initiator and effector caspases are more highly expressed in the brain tissues of donors with obesity than in those of lean donors.

2 | METHODS

2.1 | Brain cases

Brain samples from the right superior frontal gyrus were taken from the study previously published by Gómez-Apo et al.[22]

Eight donors with obesity or overweight (Body Mass Index [BMI]: mean = 31.6 kg/m^2; SD = 4.35; 2 females; Age: mean = 52.9 years, SD = 4.76) and eight donors with normal weight (BMI: mean = 21.8 kg/m^2; SD = 1.5; 3 female; Age: mean = 37.8 years.o., SD = 19.2) were included. Donors with obesity (n = 4; BMI: mean = 34.79 kg/m^2; SD = 3.81; 0 female; Age: mean = 56.5 years SD = 2.65; 2 cases with obesity Class I and 2 cases with obesity Class II) or overweight (n = 4; BMI: mean = 28.31 kg/m^2; SD = 1.29; 2 females; Age: mean = 49.3 years.o., SD = 3.30) were studied. In Gómez-Apo et al study,[22] the acquired brain samples from the subjects with obesity or overweight and normal weight showed no evidence of neuropathology. Clinical record files were reviewed to rule out the presence of diabetes mellitus or hypertension. Relatives of the deceased donors authorized and signed all legal documents to perform necropsy studies at the hospital.

2.2 | Immunohistochemical staining and immunoexpression evaluation

Serial paraffin sections were stained using primary polyclonal anti-rat antibodies against selected caspases (Caspase-3, Caspase-4, Caspase-6 antibodies [Sino Biologicals US, Inc, PA, USA]; Caspase-1, Caspase-8, Caspase-9 antibodies [Santa Cruz Biotechnology, Inc, CA, USA.]). The immunohistochemical procedure was developed using the manual immunoperoxidase technique with a BioSB ® electric pressure cooker, a Sequenza® rack and a BioSB® immunohistochemistry

kit. The 2-μm paraffin sections were deparaffinized, rehydrated, and treated with ImmunoDNA Background Blocker (BIOSD Co.) for 10 minutes. The sections were incubated for 60 minutes at room temperature with the primary antibody. The sections were subsequently incubated with a biotinylated secondary antibody at room temperature. After being rinsed with phosphate-buffered saline, the reaction products were visualized by immersing the sections in diaminobenzidine. Finally, the sections were counterstained with hematoxylin, dehydrated and cover-slipped using Ecomount (Biocare®).

2.3 | Image acquisition

Light microscopy was performed to examine the staining with different antibodies in the 16 cases. Sixty-four representative nonoverlapping fields of the cerebral cortex were captured as photomicrographs in a high-power field (400X) (Olympus ® microscope model CX-31; Olympus ® adapter E330-ADU 1.2X, Olympus ® camera Model E−620).

The captured images were transferred to a computer for image analysis. The evaluation was performed using ImageJ (NIH) software, version 1.53a (National Institutes of Health, Bethesda, MD, USA). The white balance was adjusted for each photograph. The ImmunoRatio plugin was used, which is an ImageJ plugin, to evaluate immunohistochemically stained tissue sections. This plugin determines the percentage of cells with cytoplasmic positivity with respect to the total number of nuclei detected. The 64 photos were placed in the ImageJ toolbar. The software calculated the percentage of positive expression with respect to the number of nuclei. First, the percentage was computed per photograph, and then an average of the 64 photos was calculated (Figure 1). This result is the mean Caspase expression.

2.4 | Statistical analyses

Statistical Package for the Social Sciences statistics software (version 20.1; IBM Corp., Armonk, New York) was used for all statistical analyses. A series of nonparametric Mann–Whitney U tests was performed to compare the mean Caspase expression (Caspase-8, -9, -3, -6, -1, -4) between groups (obesity/overweight and normal weight). Cohen's *d* was also computed for each comparison.

Significant correlations between age and BMI were observed in the data from Gomez-Apo et al.[22] This bias was considered by performing partial correlation analyses between the mean Caspase expression and BMI by controlling for age. Nonparametric analyses were performed again, excluding the measurements of the two youngest individuals from the normal weight group.

2.5 | Caspase-1 and Caspase-8 interaction

Taking into consideration our Caspase expression results, the potential interactions between proteins were examined with Search Tool for the Retrieval of Interacting Genes (STRING, version 11.5; http://string-db.org). Search Tool for the Retrieval of Interacting Genes is a database of known and predicted protein interactions that includes functional and physical associations, which are taken from previous knowledge, experiments, genomic context, and coexpression studies. This software evaluates protein–protein interactions in 14,094 organisms, 67.6 million proteins, and more than 20 billion interactions. The protein interactions available in the STRING database are delivered with a confidence score.

Sample size with G*Power 3.1 software (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-arbeitspsychologie/gpower) was calculated using the size effect of the difference means of the number of neurons between donors with obesity/overweight and the donors with normal weight from the above-mentioned study. Based on 1.51 effect size Cohen d, 1:1 ratio between the sample sizes of the two groups, one side type I error rate of 0.05, and power (1-β error probability) of 0.85, 8 cases and 8 controls were obtained.

Data supporting the statistical analysis described above is fully available at https://figshare.com/s/676eb55d5153f5cc8602.

3 | RESULTS

Table 1 shows the statistical comparisons between the groups regarding the mean Caspase expression, as determined by using nonparametric statistics. People with obesity or overweight exhibited significantly higher mean values for Caspase-8 and Caspase-1 than subjects with normal weight. The effect of these differences (Cohen's *d* values) was large. There were no significant differences in the other caspases between groups.

The box-and-whisker plots in Figure 2 show the significant differences in Caspase-8 and Caspase-1 expression between groups. Although there was a large effect for Caspase-3 and -6 between groups, there was a significant dispersion of the values of the normal weight group, mainly for Caspase-3. This fact may explain why there were no significant differences between the groups.

Since subjects with obesity or overweight had significantly higher BMI and were older than subjects with normal weight, it was explored whether this bias was associated with Caspase expression. Partial correlation analyses between mean Caspase expression and BMI while controlling for age showed a significant positive correlation between Caspase-1 and BMI ($r = 0.47$, $p = 0.03$). BMI did not correlate with the mean expression of Caspase-8 ($r = 0.25$, $p = 0.17$), Caspase-9 ($r = 0.39$, $p = 0.07$), Caspase-3 ($r = 0.14$, $p = 0.30$), Caspase-6 ($r = -0.09$, $p = 0.36$), or Caspase-4 ($r = 0.35$, $p = 0.10$). Figure 3 shows the scatter plot of Caspase-1 expression and Age. There is an important change in the regression line when data points of the two youngest donors were removed.

Nonparametric analyses were performed after removing the measurements of the two youngest individuals from the normal weight group. Table 2 displays the nonparametric statistical analysis results, which showed the same pattern as the previous

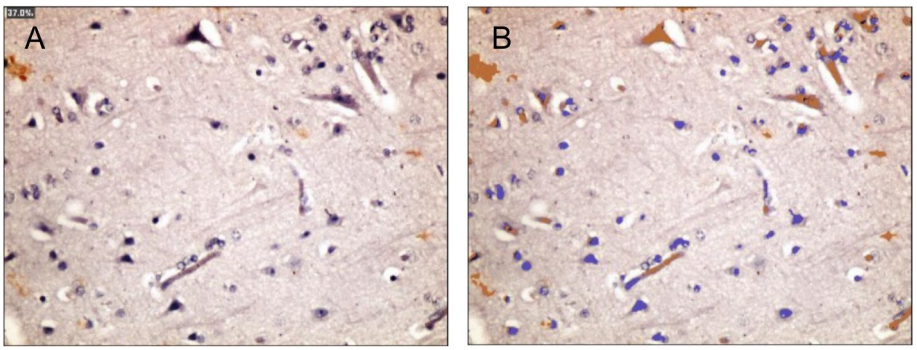

FIGURE 1 (A), A representative photograph of the original. (B), Images obtained from ImageJ software. This photograph is the image that resulted from the ImmunoRatio plugin (diaminobenzidine/nuclear area average). The pale blue background is identified, the nuclei were counted by the software and are shown in intense blue, and the immunohistochemical expression was determined by the software and is shown in brown. First, the percentage of expression was computed for each photograph; at the end of this process, the mean expression of the 64 photographs was obtained

TABLE 1 Comparisons of caspase expression between obesity/overweight versus control

Type	Enzyme	Normal weight $n = 8$	Obesity/Overweight $n = 8$	Mann-W U test	Cohen's d
		Mdn (CV)	Mdn (CV)		
Initiator	Caspase-8	9.30 (0.41)	13.95 (0.35)	15*	0.99[a]
Initiator	Caspase-9	5.50 (0.60)	6.75 (0.95)	27	0.26
Executor	Caspase-3	49.20 (0.39)	40.90 (0.34)	17	0.85
Executor	Caspase-6	44.15 (0.36)	38.55 (0.40)	19	0.72
Inflammatory	Caspase-1	12.10 (0.56)	18.10 (0.28)	16.5**	0.89[b]
Inflammatory	Caspase-4	43.70 (0.27)	43.95 (0.21)	30	0.10

Abbreviations: CV, Coefficient of Variation; Mdn, Median.

[a]Power $(1-\beta) = 0.58$.

[b]Power $(1-\beta) = 0.53$.

*Monte Carlo Significance (1-tailed) $p = 0.03$.

**Monte Carlo Significance (1-tailed) $p = 0.05$.

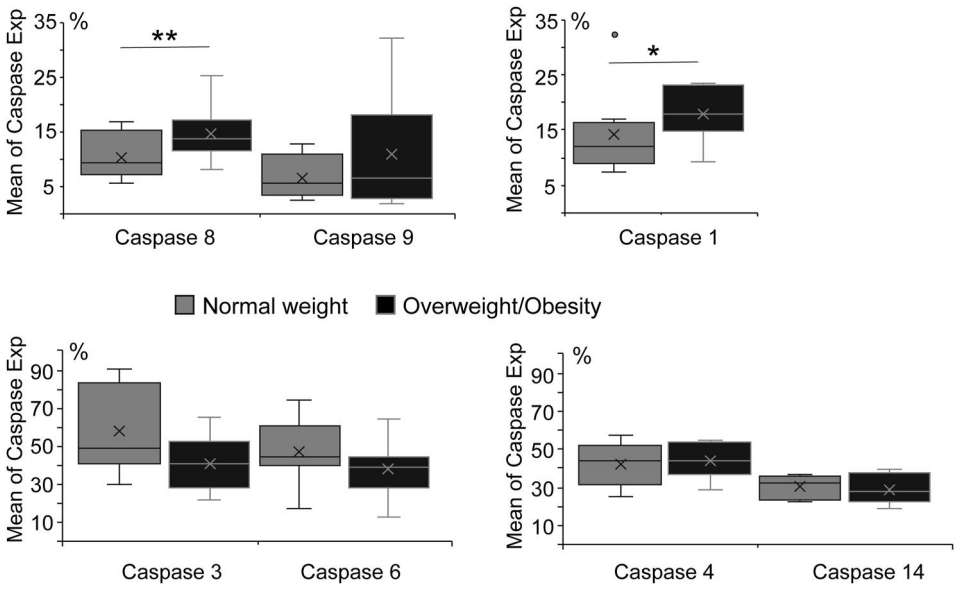

FIGURE 2 Percentage of the mean caspase expression in the obesity/overweight and normal weight groups. Note the significantly greater percentage of caspase-8 and -1 in the obesity/overweight group than the normal weight group. *$p = 0.05$, **$p = 0.03$

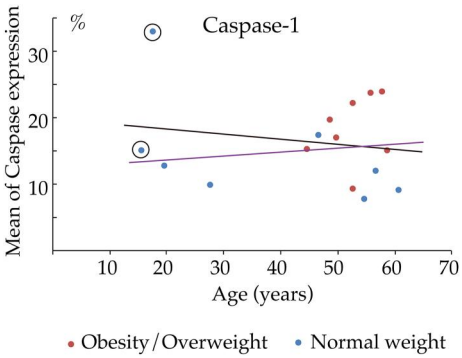

FIGURE 3 Relationship between Caspase-1 expression and age. Red points represent the data of the group of donors with obesity/overweight and the blue points the donors of the group with normal weight. The black line is the regression line when all points are included ($R^2 = 0.03$). The purple line is the regression line ($R^2 = 0.04$) when the data for the youngeSt donors were removed (blue dots surrounded by a black circle)

analysis. Mann–Whitney *U* test analyses without the data from the youngest individuals showed that brain samples from the obesity/overweight group exhibited significantly greater mean expression of Caspase-8 and Caspase-1 than samples from the normal weight group.

3.1 | Search Tool for the Retrieval of Interacting Genes analysis

Taking into consideration our immunohistochemical results, the interaction of Caspase-1 and Caspase-8 with some other element (not evaluated by immunohistochemistry) were explored using the STRING program. Canonical and noncanonical pathways associated with caspase-mediated apoptosis were evaluated. Additionally, the alternative inflammasome pathway was examined.[28] For this ASC- and caspase-8-dependent apoptotic pathway, interleukin 1A was selected to interact with Caspase -1 and -8.

3.2 | Alternative inflammasome pathway

The interaction confidence score between caspase-1 and caspase-8 was 0.91; between caspase-1 and interleukin 1A was 0.98; between caspase-1 and NLRP3 was 0.99; between caspase-8 and interleukin-1A was 0.47; between caspase-8 and NLRP3 was 0.91; and between interleukin-1A and NLRP3 was 0.69. The interactions between these molecules are shown in Figure 4.

4 | DISCUSSION

The purpose of the present study was to explore the association between caspase expression and obesity/overweight. The expression of caspases (caspase-8, -9, -3, -6, -1, and 4) in a collection of brains from donors who were identified as having either obesity/overweight or normal weight were compared. It was hypothesized that brain tissue samples from donors with BMI >25 kg/m^2 would show increased expression of caspase-8 (extrinsic pathway) or caspase-9 (intrinsic pathway) and caspase-3 for the final execution phase. Our results partially confirmed our hypothesis because subjects with obesity or overweight had higher expression of caspase-8 and caspase-1 than individuals who had normal weight, but no evidence of an increase in caspase-3 or caspase-6 (execution phase) in the group with obesity/overweight was found. However, it is important to highlight that the observed variability in caspase-3 expression in the normal weight group could have blurred a potential significant effect on this variable, and the effect sizes remained large.

The interaction between caspase-1 and caspase-8 was evaluated in the canonical pathways of caspase-mediated apoptosis and did not seem to be supported by our results. Instead, our results suggest interactions between these caspases in nonclassical pathways. It is important to emphasize that the alternative inflammasome pathway is primarily activated through an inflammatory stimulus. With the present study, it was impossible to determine if any of these pathways were active since both types of stimuli (inflammatory cytokines or ischemia) were present in individuals with obesity. It is also possible that both pathways are active during apoptosis.

An alternative pathway was observed to be functional, unlike the canonical or noncanonical pathways. This alternative NLRP3 inflammasome activation occurs independent of pyroptosis.[29] NLR family pyrin domain-containing 3 (NLRP3) represents the most-studied inflammasome sensor,[28,30] and it is present in microglia and astrocytes in the central nervous system. Activation of the NLRP3 inflammasome by a wide range of exogenous or endogenous stimuli[31] is associated with many neurological diseases and cognitive impairment via neuroinflammation.[32,33]

The NLRP3 inflammasome is considered a key contributor to the development of neuroinflammation.[34] NLRP3 inflammasome activation triggers caspase-1 activation and IL-1β maturation through priming and activation signals. Damage-associated molecular pattern molecules and/or pathogen-associated molecular pattern molecules may stimulate TLR4, leading to the activation of caspase-8 and its receptor.[35]

Receptor-interacting protein 1 (RIP1)–fatty acid synthase (FAS)-associated death domain (FADD) may induce both canonical NLRP3 activation and transcription. Furthermore, studies revealed that alternative inflammasome activation in monocytes (microglia and glia in the Central Nervous System) is mediated by the Toll-like receptors (TLR) adapter protein C-terminal Src Kinase-interacting membrane protein, which activates caspase-8 by triggering tyrosine kinase LYN/SYK-dependent calcium influx and the production of Reactive Oxygen Species. This specific activation pathway induced by the TLR2 or TLR4 signaling pathway without involving other secondary activators is referred to as alternative NLRP3 inflammasome activation.[36–40]

Among the limitations of this study, cross-sectional evaluation of caspases in tissue represents a snapshot of a process; therefore, an

TABLE 2 Comparisons of caspase expression between obesity/overweight versus control

Type	Enzyme	Normal weight $n = 6$	Obesity/Overweight $n = 8$	Mann-W U test	Cohen's d
		Mdn (CV)	Mdn		
Initiator	Caspase-8	9.30 (0.39)	13.95	9*	1.21[a]
Initiator	Caspase-9	5.50 (0.57)	6.75	20	0.27
Executor	Caspase-3	49.20 (0.42)	40.90	15	0.65
Executor	Caspase-6	44.15 (0.41)	38.55	16	0.57
Inflammatory	Caspase-1	10.60 (0.31)	18.10	7**	1.44[b]
Inflammatory	Caspase-4	39.40 (0.25)	43.95	16	0.57

Abbreviations: CV, Coefficient of Variation; Mdn, Median.

[a]Power $(1-\beta) = 0.67$.

[b]Power $(1-\beta) = 0.76$.

*Monte Carlo Significance (1-tailed) $p = 0.02$.

**Monte Carlo Significance (1-tailed) $p = 0.01$.

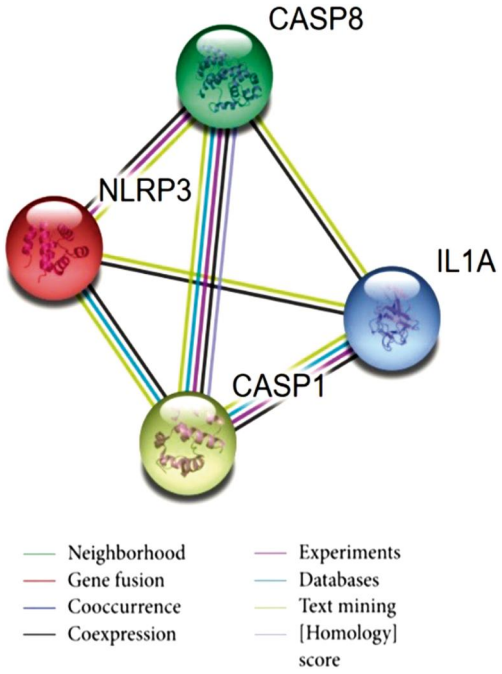

FIGURE 4 Protein interaction network of CASP1 with CASP8, NLRP3 and IL1A using Search Tool for the Retrieval of Interacting Genes (STRING) (https://string-db.org/). The interactions between input proteins are color coded and represent the type of interaction

increase in caspase-1 and caspase-8 without an increase in caspase-3 may be observed due to the dynamics of apoptosis. Another limitation is undersampling. Because this type of study involves a large amount of data, it takes relatively manageable data samples. In this case, a small portion of the crest of the second right frontal gyrus was taken for analysis.

The strength of this study is that it analyzed the expression of caspases in human tissue. Most of studies are done in animals, in which allows for greater control of variables; however, it is complicated to extrapolate the results to humans.

5 | CONCLUSIONS

Our results contribute to disentangle the mechanisms underlying the inflammatory effects of obesity on brain by suggesting an association between the expression of caspase-1 and -8 and obesity/overweight, likely due to the alternative inflammasome pathway.

AUTHOR CONTRIBUTIONS

Erick Gómez-Apo designed the project, collected and analyzed the data, and wrote the manuscript; Juan Silva-Pereyra designed the project, requested for funding, analyzed, interpreted and discussed the data; Virgilia Soto-Abraham, Alejandra Mondragón-Maya and Javier Sanchez-Lopez, analyzed, interpreted and discussed the data; Javier Sanchez-Lopez wrote and edited the manuscript. All the authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST

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

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