REVISTA TÓPICOS
ISSN 2965-6672 | Qualis A2

TRANSGENIC FEMALE MICE FOR HUMAN APOCIII SHOW A MORPHOQUANTITATIVE REDUCTION OF NEURONS IN THE CARDIAC PLEXUS

CAMUNDONGAS TRANSGÊNICAS PARA APOCIII HUMANA APRESENTAM REDUÇÃO MORFOQUANTITATIVA DE NEURÔNIOS DO PLEXO CARDÍACO

Ciências Biológicas, Ciências da Saúde • 06/11/2026

REGISTRO DOI: 10.70773/revistatopicos/793689491

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ABSTRACT

Dyslipidemias, especially hypertriglyceridemia resulting from overexpression of apolipoprotein CIII (apoCIII), are associated with metabolic disorders that impact the liver, muscle, and heart. The cardiac plexus, an intrinsic nervous system responsible for autonomic regulation, and metabolically active epicardial adipose tissue may undergo remodeling in response to metabolic, hormonal and reproductive factors. This study aimed at evaluating the effects of apoCIII over the cardiac plexus and epicardial adipocytes of transgenic (CIII) and non-transgenic (NTG) female mice at different ages and reproductive conditions. Morphological and morphometric parameters were analyzed by histology and the data were submitted to statistical analysis by two-way ANOVA. Hypertriglyceridemia was the main factor associated with the reduction in the neuronal área of the cardiac plexus, especially at 20 months. In epicardial adipose tissue, age was related to adipocyte hypertrophy. It is concluded that metabolic, age and reproductive factors interact in neural and adipose remodeling, highlighting the importance of integrated study of these variables for a better understanding of the cardiovascular repercussions of chronic hypertriglyceridemia.

Keywords: Hypertriglyceridemia; Age; Neurons; Epicardial adipose tissue.

RESUMO

As dislipidemias, em especial a hipertrigliceridemia decorrente da superexpressão da apolipoproteína CIII (apoCIII), estão associadas a distúrbios metabólicos que impactam o fígado, o músculo e o coração. O plexo cardíaco, sistema nervoso intrínseco responsável pela regulação autonômica, e o tecido adiposo epicárdico, metabolicamente ativo, podem sofrer remodelações em resposta a fatores metabólicos, hormonais e reprodutivos. Este estudo

objetivou avaliar os efeitos da superexpressão da apoCIII sobre o plexo cardíaco e os adipócitos epicárdicos de camundongas fêmeas transgênicas (CIII) e não transgênicas (NTG), em diferentes idades e condições reprodutivas. Foram analisados parâmetros morfológicos e morfométricos por histologia, e os dados foram submetidos à análise estatística por two-way ANOVA. A hipertrigliceridemia foi o principal fator associado à redução da área neuronal do plexo cardíaco, principalmente aos 20 meses. No tecido adiposo epicárdico, a idade esteve relacionada à hipertrofia dos adipócitos. Conclui-se que fatores metabólicos, etários e reprodutivos interagem na remodelação neural e adiposa, destacando a importância do estudo integrado dessas variáveis para melhor compreensão das repercussões cardiovasculares da hipertrigliceridemia crônica.
Palavras-chave: Hipertrigliceridemia; Idade; Neurônios; Tecido adiposo epicárdico.

1. INTRODUCTION

Dyslipidemias are metabolic disorders characterized by changes in serum lipid levels, especially triglycerides and cholesterol, and are important risk factors for cardiovascular disease (CVD), (Abera et al., 2024). Hypertriglyceridemia, in particular, is associated with the development of atherosclerosis, insulin resistance, hepatic steatosis, and systemic inflammatory processes (Sarwar et al., 2007; Feingold et al., 2018; Peng et al., 2017; Packard; Boren; Taskinen, 2020).

In lipid metabolism, triglycerides, cholesterol, and other lipids are transported by lipoproteins, such as chylomicrons (CM) and very low-density lipoproteins (VLDL). Lipoprotein lipase (LPL), an endothelial enzyme responsible for triglyceride hydrolysis, is activated by apolipoprotein CII (apoCII) and inactivated by apolipoprotein CIII

(apoCIII). The latter acts as a negative regulator of LPL, compromising plasma triglyceride clearance and promoting hypertriglyceridemia (Curi, 2002). In animal models (such as C5TBl/6 mice), overexpression of the human apoCIII gene induces widespread metabolic disorders with morphophysiological repercussions (Ito et al., 1990; Salerno et al., 2007; Raposo et al., 2015; Godoi et al., 2018, Jimenes et al., 2020; Teixeira Júnior et al., 2024a, Teixeira Júnior et al., 2024b).

Aging is also a relevant risk factor for CVD, associated with structural and inflammatory changes in the cardiovascular system (Aha, 2017; Spitler & Davies, 2020). In women multiparity and cessation of reproductive activity add variables that can influence heart health (Okoth et al., 2020; Kan et al., 2024).

In this context, the cardiac plexus stands out, consisting of neuronal ganglia located at the base of the heart, between the myocardium and the epicardial adipose tissue. This set, also called the Intrinsic Cardiac Nervous System (ICNS), is capable of locally regulating heart rate, electrical conduction, and contractility, independently of the central nervous centers (Hou et al., 2007; Armour, 2008; Zarzoso et al., 2013). Structural changes in the plexus have been described in aging and inflammatory processes, which may impact cardiac autonomic regulation (Brack, 2015; Rajendran et al., 2016). However, the effects of chronic hypertriglyceridemia and apoCIII overexpression on this structure remain poorly explored (Jimenes et al., 2021; Teixeira Júnior et al., 2024).

Epicardial adipose tissue, in turn, is metabolically active, secreting cytokines and adipokines capable of modulating the cardiac inflammatory environment (Iacobellis et al., 2021). Under

pathological conditions, it undergoes expansion and dysfunction, generating an inflammatory microenvironment associated with an increased risk of arrhythmia and coronary artery disease (Rajani et al., 2014; Gaborit et al., 2017).

Finally, this work represents a continuation of previously published studies that investigated these same analyses in transgenic animal models for human apolipoprotein CIII (Jimenes et al., 2021; Teixeira Júnior et al., 2024). Thus, considering previous findings that point to changes in the cardiac plexus and epicardial adipose tissue in transgenic male mice, it is plausible to assume that overexpression of this apolipoprotein in female mice at different reproductive ages may accentuate morphological and morphometric changes in the cardiac plexus, besides inducing changes in epicardial adipose tissue. Thus, this study is based on the hypothesis that age and reproductive status modulate these effects, potentiating or attenuating the repercussions of apoCIII on neuroadipose interaction in the heart. Considering this, the objective of this study was to evaluate the effects of apoCIII overexpression on the cardiac plexus and epicardial adipocytes of transgenic (CIII) and non-transgenic (NTG) female mice at different ages and reproductive conditions.

2. MATERIALS AND METHODS

2.1. Experimental Animals And Groups

The experiment was conducted with 36 mice, kept in the sectoral animal facility of the Department of Physiological Sciences (DFS-UEM), under temperature control (23 ± 1 °C), a 12/12-hour light/dark cycle, with free access to water and balanced feed (Nuvilab®,

Quintia, Brazil). The colony of transgenic animals (apoCIII-tgn) was maintained under the supervision of Prof. Jairo Augusto Berti, PhD, with permanent authorization from CTNBio (official letter no. 819/2013).

Female individuals were obtained through controlled crossbreeding and genotyped at 60 days of age by measuring plasma triglycerides via colorimetric method. Those with triglyceridemia greater than 300 mg/dL were considered transgenic, and those with levels below 100 mg/dL were considered non-transgenic (NTG) (Raposo et al., 2015). The mice were divided into six experimental groups (n = 6 per group) according to age, reproductive status, and presence of the human transgene (Table 1).

Multiparous females had at least three complete gestational cycles with weaning. The age criteria were: young (3 months), adult (8 months), and elderly (20 months). It is worth noting that all procedures were approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA-UEM), under protocol No. 6201190623, and by the Internal Biosafety Committee (CIBio-UEM).

Table 1. Experimental groups

Group	Genotype	Age (months)	Reproductive condition
CIII-3m	Transgenic apoCIII	3	Young, virgins
CIII-8m	Transgenic apoCIII	8	Adults, multiparous
CIII-20m	Transgenic apoCIII	20	Elderly, multiparous

NTG-3m	Non-Transgenic	3	Young, virgins
NTG-8m	Non-Transgenic	8	Adults, multiparous
NTG-20m	Non-Transgenic	20	Elderly, multiparous

CIII 3m, 8m and 20m (transgenic groups for apoCIII at 3, 8 and 20 months of age). NTG 3m, 8m and 20m (non-transgenic groups for apoCIII at 3, 8 and 20 months).

2.2. Euthanasia And Histological Processing

The female mice were weighed individually and anesthetized in a chamber with isoflurane (Isoforine®) at a continuous flow of 100% until they reached the surgical plane of anesthesia. The animals underwent thoracotomy for heart collection, which was removed, weighed, washed with PBS solution (0.1 M; pH 7.4), and fixed in 4% paraformaldehyde for 48 hours. They were then subjected to routine histological processing for embedding and sectioning. The organ was embedded in paraffin with the apex facing downwards, and all sections were made above the atrioventricular junction, in the area with the highest concentration of ganglia. Semi-serial 5 µm cross sections were made in the apex-base direction of the heart using a microtome (Leica RM 2255®) and placed on 12 sheets containing 5 sections each, totaling 60 sections per animal. The interval between sections was 60 µm (Jimenes et al., 2021).

2.3. Morphological, Morphometric And Quantitative Analysis Of Cardiac Plexus Neurons And Epicardial Adipose Tissue Adipocytes

For the analysis of the cardiac plexus, the histological sections were stained with hematoxylin-eosin (H.E.). In the H.E.-stained sections,

the area of 50 neurons/animal was identified and measured, and basic morphological aspects such as nucleus, nucleolus, and cytoplasm were also described. For quantitative analysis of cardiac plexus neurons, the neurons present in all ganglia found were counted, thus establishing an average number of neurons per ganglion in each animal (Jimenes et al., 2021).

The morphometric analysis of epicardial adipose tissue considered the area (µm?) of 50 adipocytes obtained from 5 non-consecutive images (10 random adipocytes per image), totaling 300 adipocytes per group. All images (cardiac plexus neurons and epicardial adipose tissue adipocytes) were captured using an optical microscope equipped with a video camera, coupled with a computerized image analysis system (Image Pro-Plus 4.0) and analyzed at 40x magnification.

2.4. Statistical Analysis

An analysis of two factors variance (two-way ANOVA) was performed to identify possible differences between the factors age and hypertriglyceridemia (CIII) (GraphPad Prism 9®). The results were expressed as mean ± standard deviation, with $p<0.05$ considered significant. Cumulative distributions of neuronal size were obtained to demonstrate the frequency of cell sizes present in each group (GraphPad Prism 8®).

3. RESULTS

3.1. Body Mass And Biochemical Data

The data shown in Figure 1A show that age was the main factor in increasing body mass independently of hypertriglyceridemia

(p<0.0001). Regarding biochemical data, a significant difference was observed for hypertriglyceridemia in the increase in plasma triglyceride and cholesterol levels (Figures 1B and C, respectively; p<0.0001).

3.2. Morphological Characteristics And Location Of Cardiac Plexus

Hematoxylin and eosin (HE) staining showed that the ganglia of the cardiac plexus in all groups evaluated were located adjacent to the vessels at the base of the heart, between the myocardium and unilocular adipose tissue (Figure 2), presenting different sizes and morphologies. The neuronal bodies were oval in shape, with eccentric nuclei, and were surrounded peripherally by glial cells (Figure 3).

3.3. Morphometric And Quantitative Analysis Of Neurons Present In The Cardiac Plexus

Data showed that hypertriglyceridemia was the main factor associated with the reduction in the area of cardiac plexus neurons, regardless of age (p = 0.0088). Interestingly, the comparison between groups revealed a significant difference in 20-month-old animals, with a reduction in neuronal area in the 20-month-old CIII group (p = 0.0362; Figure 4A). In addition, age was also a significant factor for the increase in neuronal area, where 8- and 20-month-old NTG animals showed larger neurons compared to the 3-month-old NTG group (p = 0.0478).

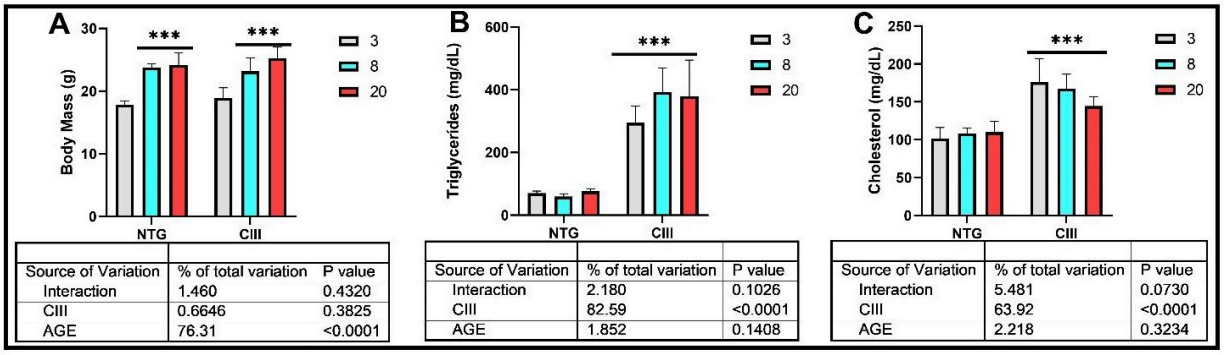
Regarding quantitative analysis (Figure 4B), different numbers of neurons were counted within the ganglia of the cardiac plexus. Although the data did not show a significant difference, the graph

shown in Figure 1b shows a gradual increase with age, with animals in the 20-month NTG group having 44% more neurons present in ganglia compared to the 3-month NTG group. Another highlight was in relation to the CIII 20m group, which showed a 64% reduction in neurons present in ganglia when compared to 20-month-old NTG.

Additionally, qualitative frequency distribution analyses were performed. These analyses not only confirmed the reduction in neuronal area associated with hypertriglyceridemia, but also showed that the 20-month-old NTG animals had neurons with areas of up to 500 μm^2, while the 3- and 8-month-old NTG animals had areas ranging from 300 to 350 μm^2 (Figure 5).

3.4. Morphometric Analysis Of Epicardial Adipose Tissue Adipocytes

The data indicate that age was the main factor associated with adipocyte area hypertrophy, with 8- and 20-month-old animals showing a significantly larger adipocyte area compared to 3-month-old animals ($p=0.0001$). Although hypertriglyceridemia did not show a significant difference, analysis between groups revealed that the 3-month CIII group had a larger adipocyte area than the 3-month NTG group ($p < 0.05$; Figure 6A).



Source of Variation	% of total variation	P value
Interaction	1.460	0.4320
CIII	0.6646	0.3825
AGE	76.31	<0.0001

Source of Variation	% of total variation	P value
Interaction	2.180	0.1026
CIII	82.59	<0.0001
AGE	1.852	0.1408

Source of Variation	% of total variation	P value
Interaction	5.481	0.0730
CIII	63.92	<0.0001
AGE	2.218	0.3234

Figure 1. Graphs showing mean ± standard deviation for body mass (g) (A), plasma triglyceride levels (TG; mg/dL) (B), and total cholesterol (TCH; mg/dL) (C).

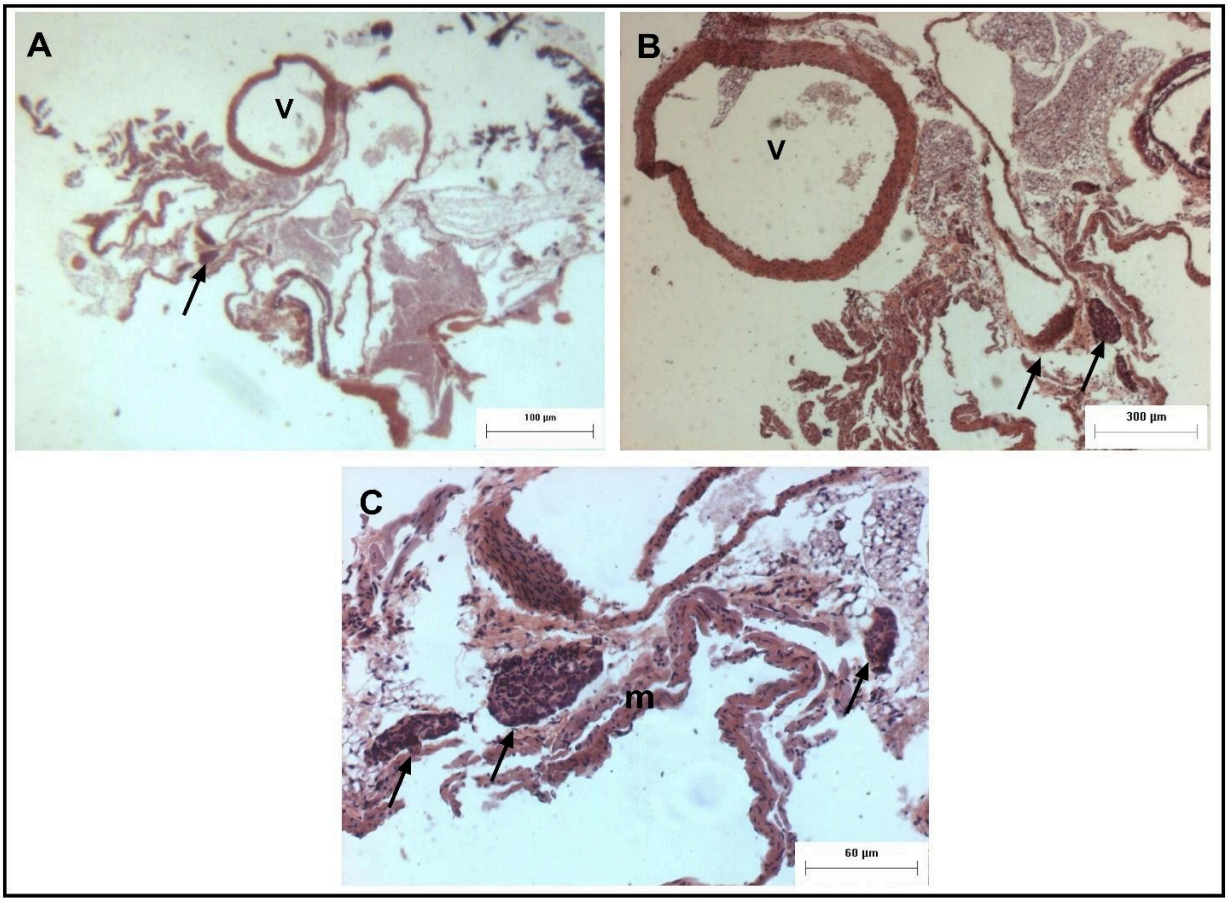


Figure 2. Photomicrographs of cross sections of the heart, at the level of the emergence of the large vessels at the base, stained using the Hematoxylin-eosin (H.E.) technique. (A) Image obtained with a 1x objective lens. (B) Image obtained with a 4x objective lens. (C) Image obtained with a 10x objective lens. Arrows indicate ganglia of the cardiac plexus. Abbreviations: m - myocardium; a – adipocytes; v – blood vessel.

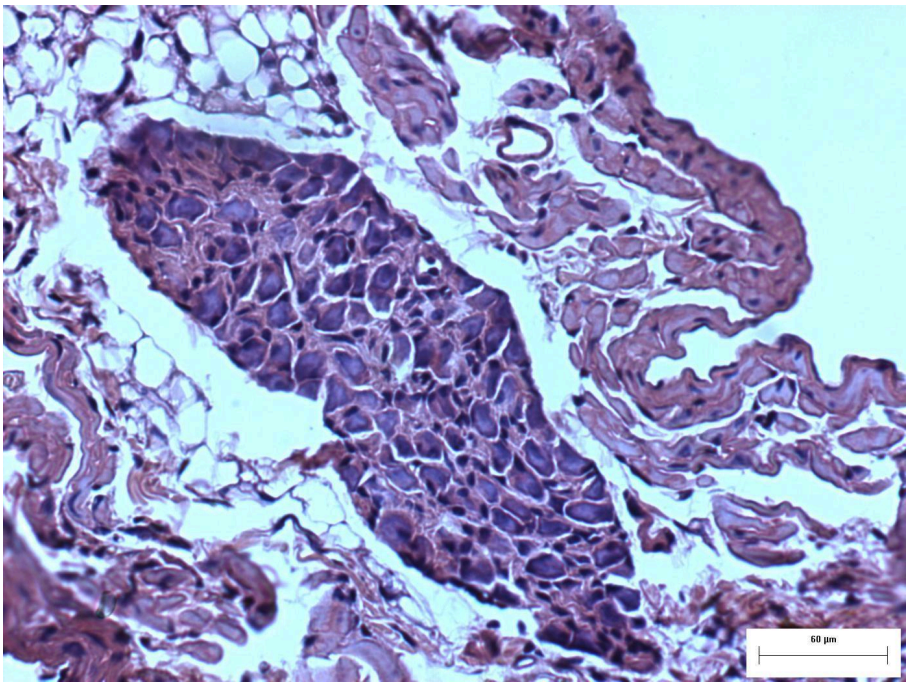
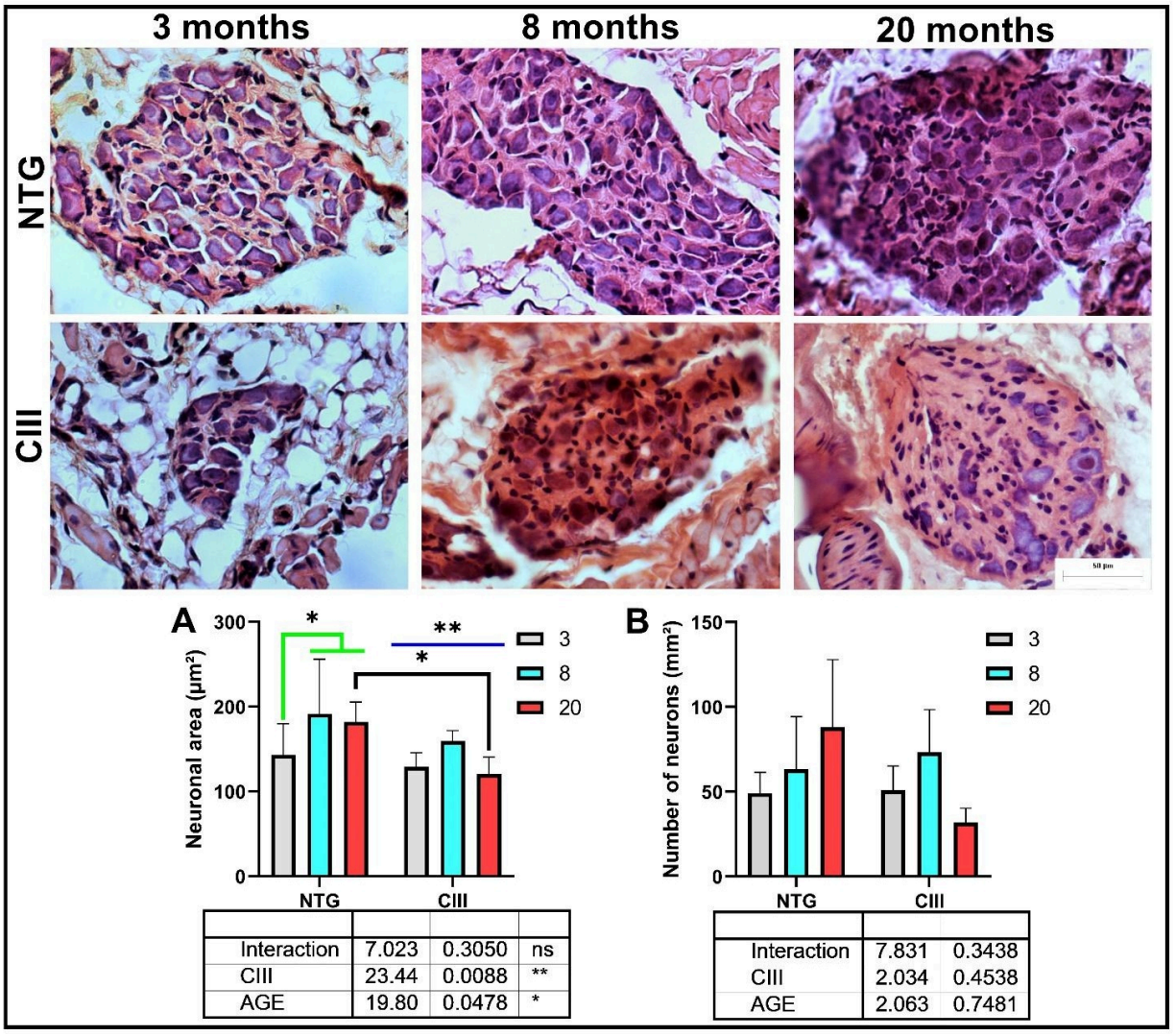


Figure 3. Photomicrograph of a cardiac plexus ganglion, showing oval-shaped neurons with eccentric nuclei, surrounded peripherally by glial cells. Black arrow: cardiac plexus neuron; yellow arrow: glial cell. Abbreviations: m – myocardium; a – adipocytes. Scale bar: 50 µm.



Interaction	7.023	0.3050	ns
CIII	23.44	0.0088	**
AGE	19.80	0.0478	*

Interaction	7.831	0.3438
CIII	2.034	0.4538
AGE	2.063	0.7481

Figure 4. Photomicrographs of cross sections of the heart showing cardiac plexus ganglia, stained with H.E. and observed with a 40x objective lens. Graphs showing mean ± standard deviation for neuronal area (µm²) (A) and number of neurons (mm²) (B). Green bar showing significant difference in relation to age; blue bar showing significant difference in relation to hypertriglyceridemia; black bar showing significant difference between the NTG-20m and CIII-20m groups. Arrows indicate cardiac plexus ganglia. Abbreviations: m – myocardium; a – adipocytes.

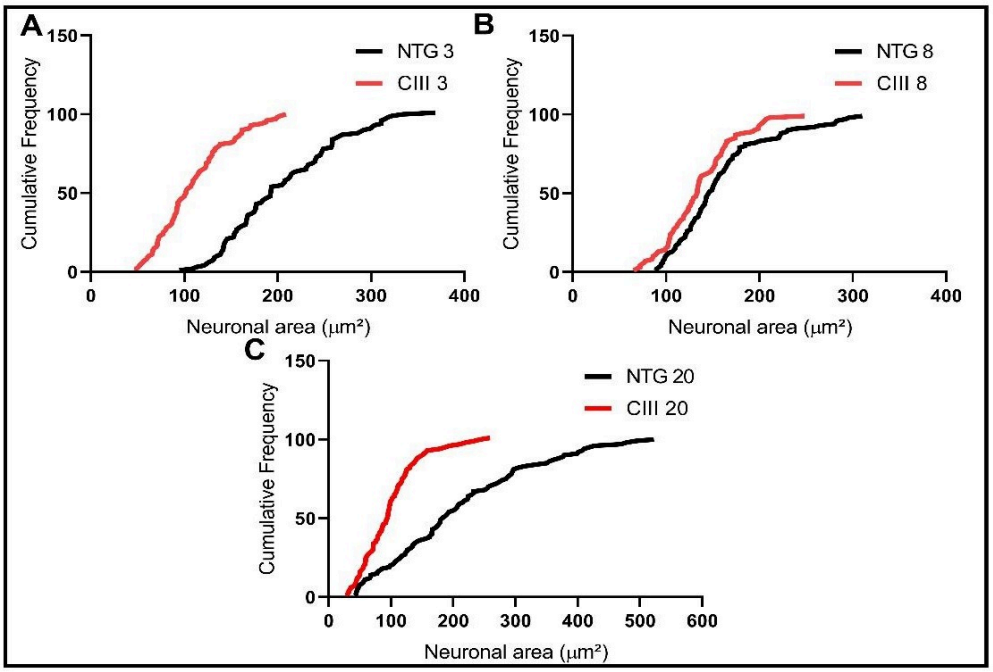


Figure 5. Cumulative distributions of the neuronal area of the cardiac plexus, which were compared using the unpaired t-test.

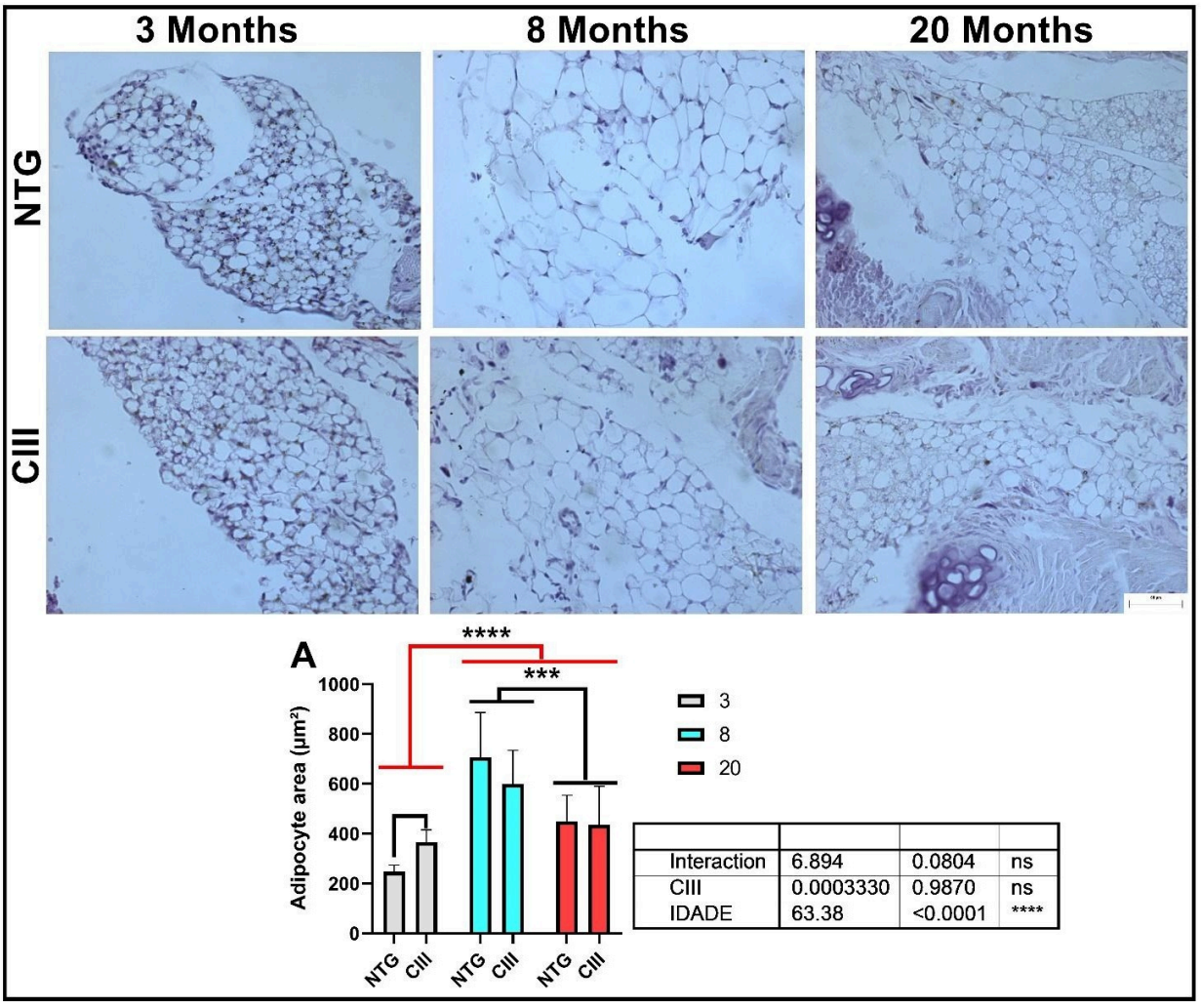


Figure 6. Photomicrographs of cross sections of epicardial adipose tissue stained with H.E., obtained with a 20x objective lens. (A) Graphs showing mean ± standard deviation of adipocyte area (µm²). Abbreviation: a – adipocytes.

4. DISCUSSION

The main objective of the present study was to analyze the effects of human apolipoprotein CIII overexpression on cardiac plexus morphology in female mice, considering different ages and reproductive conditions. In addition, a morphometric analysis of the adipocyte area of epicardial adipose tissue was performed, which has been extensively investigated due to its significant ability to influence cardiac function (Gaborit et al., 2017; Cristiakov et al., 2017). Furthermore, this work represents a continuation of previously published studies that investigated these same analyses in transgenic animal models for human apolipoprotein CIII (Jimenes et al., 2021; Teixeira Júnior et al., 2024).

Jimenes et al. (2021), for example, investigated the morphology, morphometric and quantitative profile of the cardiac plexus, as well as the morphometric and histopathological aspects of epicardial adipose tissue in 8- and 12-month-old male mice transgenic for human apolipoprotein CIII. More recently, Teixeira Júnior et al. (2024) analyzed the cardiac plexus and epicardial adipose tissue of transgenic and non-transgenic mice subjected to treadmill training (3 times a week, 40 minutes per session, at 60% of maximum speed) for 8 weeks.

4.1. Body Mass And Biochemical Data

Initial data showed that age was the main factor in increasing body mass, which was already expected due to the somatic growth of the animals. However, the condition of hypertriglyceridemia in the animal models used in this study promoted a significant increase in serum triglyceride and cholesterol levels. In the case of triglycerides,

this increase can be explained by the overexpression of human apoCIII gene and protein in this animal model, which impairs the hydrolysis of TG-carrying lipoproteins (chylomicrons and VLDL). Furthermore, this apolipoprotein is known to compete for binding to the hepatic LDL receptor (LDL-R), and is therefore a determining factor in the delay of plasma triglyceride clearance (Curi, 2002; Raposo et al., 2015; Godoi et al., 2018). Thus, the persistence of triglyceride-rich particles in the circulation is directly related to the observed phenotype. As for total cholesterol, part of the increase may be associated with the delay in plasma removal of LDL (rich in cholesterol), as well as the higher concentration of apoCIII-rich lipoproteins, which interfere not only with triglyceride metabolism but also with cholesterol remodeling and reverse transport (Aalto-Setälä et al., 1992; Raposo et al., 2015; Teixeira-Júnior et al., 2024).

4.2. Location And Morphological Description Of The Cardiac Plexus

Initial data from this study regarding the location and morphology of the cardiac plexus corroborate other studies (Rysevaite et al., 2011; Saburkina et al., 2014; Jimenes, 2021; Teixeira Júnior et al., 2024), which highlight that the ganglia of the cardiac plexus can have varied shapes and are always close to the vessels at the base of the heart, between the myocardium and the epicardial adipose tissue, with oval neurons, centralized nuclei, and surrounded by glial cells.

4.3. Neuronal Area

Our data showed that hypertriglyceridemia caused a reduction in the neuronal area of the cardiac plexus of the mice used in this study and, interestingly, our data showed that this reduction was

more pronounced at 20 months of age. Corroborating this, Teixeira Júnior et al. (2024) also observed this same reduction in male mice transgenic for human apoCIII at 15 months of age. Additionally, data contrary to ours were found by Jimenes et al. (2021), where hypertriglyceridemia did not cause a reduction in neuronal area, but in younger animals, at 8 and 12 months of age. Therefore, we believe that aging associated with hypertriglyceridemia may accelerate the process of neuronal degeneration in the cardiac plexus, potentiating the loss of neuronal area observed in elderly multiparous mice.

Additionally, as widely demonstrated in the reviewed literature, studies suggest that morphological changes in the cardiac plexus can modify cardiac function, since neurons belonging to this plexus exert significant control over cardiac activity (Jurgaitiene et al., 2004; Brack, 2015; Menard et al., 2014; Rajendran et al., 2016). Therefore, the combined effect of aging and hypertriglyceridemia suggests a compromise in the structural integrity of the cardiac plexus, negatively impacting the autonomic regulation of the heart.

Besides that, a recent study showed that hypertriglyceridemic hamsters demonstrated functional impairments in pyramidal neurons of the frontal cortex, but without evidence of morphometric changes (Shen et al., 2024). Our results suggest that cardiac neurons, under similar conditions, show a significant reduction in area. This divergence may reflect differences in the vulnerability of specific neuronal types, as well as in the metabolic demands imposed by different tissue microenvironments (brain – heart). Together, these findings reinforce the idea that hypertriglyceridemia may exert a systemic neuronal effect, whose expression can occur at both the functional and structural levels.

When age was considered, our frequency analysis data showed that 20-month-old NTG mice had a higher prevalence of larger neurons compared to other ages. Corroborating this finding, Jimenes et al. (2021) also observed a larger neuronal area in 12-month-old male mice compared to 8-month-old mice, independently of hypertriglyceridemia. These authors believe that the thickening of the collagen capsule of the ganglion around the neurons with advancing age may have influenced the increase in neuronal area. In addition, Akamatsu et al. (1999) suggest that the increase in neuronal size of the cardiac plexus results from a compensatory effect associated with the loss of neurons with age in rats. However, our findings showed a reduction in the number of neurons only in elderly hypertriglyceridemic female mice.

4.4. Number Of Neurons

Our data showed a gradual increase in cardiac plexus neurons with advancing age. Teixeira Júnior et al. (2024) also reported this pattern in transgenic and non-transgenic mice for apoCIII between 8 and 12 months of age, and the opposite was observed in rats (Akamatsu et al., 1999). However, our study showed that hypertriglyceridemia can reduce the number of neurons in the ganglia in elderly multiparous female mice, differing from the findings of Teixeira Júnior et al. (2024) and Jimenes et al. (2021) in males. We therefore believe that aging associated with reproductive status in females, especially in elderly multiparous females, may accentuate such changes in the cardiac plexus.

4.5. Area Of Adipocytes In Epicardial Adipose Tissue

This analysis mainly revealed an influence on age, where 8- and 20-month-old animals had larger adipocytes than younger 3-month-old animals. In fact, research shows that aging can cause hypertrophy in adipocytes in various fat deposits (Gonçalves et al., 2017), and especially in epicardial adipose tissue (Jimenes et al., 2021). Interestingly, 20-month-old animals had a reduced adipocyte area compared to 8-month-old animals. Iacobellis et al. (2021) highlight in a systematic review that aging can affect the function and morphology of epicardial fat, more likely in women than in men, and that aging can likely influence and affect the genes and function of epicardial fat. Thus, the increase in body mass observed with age accompanies the hypertrophy of adipocytes in epicardial adipose tissue, reinforcing the idea that aging promotes not only systemic fat accumulation but also local remodeling in this specific deposit.

In relation to hypertriglyceridemia, this condition increased the area of adipocytes, but only at 3 months of age. Jimenes et al. (2021) also found that hypertriglyceridemia caused by apoCIII overexpression promoted hypertrophy of epicardial adipose tissue adipocytes in older male mice at 8 and 12 months of age. Other studies, such as Salerno et al. (2007), found similar results in perigonadal adipose tissue, and Raposo et al. (2015) correlated these findings with the fact that apoCIII overexpression reduces basal lipolysis of adipocytes, thus favoring increased adiposity in different fat deposits.

Finally, the exclusive use of females—young, multiparous adults, and elderly multiparous—represents an important variable, since multiparity and hormonal changes associated with aging can influence lipid metabolism and neural plasticity (Pataky et al., 2021; Been et al., 2022). In addition, the post-reproductive decline in estrogen levels is associated with increased visceral fat and

cardiovascular risk (Okoth et al., 2020; Kan et al., 2024), which may potentially enhance the effects observed in this study in relation to the morphological changes observed in cardiac plexus neurons and epicardial adipose tissue adipocytes. It should be noted that the use of females, especially in advanced age groups, is still uncommon in experimental studies, which makes this work a relevant contribution to the understanding of the effects of aging in this context.

5. CONCLUSION

The overexpression of human apolipoprotein CIII in female mice led to a significant reduction in the neuronal area of the cardiac plexus, an effect that was more pronounced in older multiparous females, suggesting that the combination of chronic hypertriglyceridemia, hormonal changes associated with aging, and reproductive history maximizes the impairment of this autonomic structure. In epicardial adipose tissue, adipocyte hypertrophy was observed, mainly associated with aging, reinforced by dyslipidemia in young animals, indicating tissue remodeling influenced by metabolic and hormonal mechanisms. These findings show that reproductive, metabolic, and age factors interact in complex ways on the cardiac plexus and epicardial adipose tissue, highlighting the importance of their integrated study to understand the cardiovascular repercussions of chronic hypertriglyceridemia. A limitation of the present study was the absence of measurement of sex hormones, such as estrogen and progesterone, which could assist in more accurately characterizing the reproductive status of females at different ages. However, future investigations that include this hormonal characterization may deepen the understanding of the effects of age and multiparity on epicardial fat and cardiac innervation. Therefore, understanding this interaction may pave the way for

preventive and therapeutic strategies more targeted to autonomic and metabolic changes in populations at high cardiovascular risk.

Ethics approval

We declare that all procedures were approved by the Ethics Committee on Animal Use of the State University of Maringá (CEUA-UEM), under protocol No. 6201190623.

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.

Funding Statement

This work was supported by grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES.

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