



International Conference on Sustainable and Intelligent Manufacturing, RESIM 2016, 14-17
December 2016, Leiria, Portugal

Micro computed tomography detects changes in liver density in control and in prediabetes rats

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Abstract

Fatty liver disease is an early event in the development of insulin resistance that predicts the presence and progression of the metabolic syndrome. In humans, fatty liver diagnosis is usually performed by imaging techniques based on ultrasound, computed tomography and magnetic resonance. Rodent models are often used in metabolic research allowing access to tissue biopsies however, studies describing *ex vivo* computed tomography of biological samples are scarce. X-ray Micro Computed Tomography (Micro-CT) is an imaging technique that reveals the internal structure of materials in great detail, also allowing a quantitative analysis of properties such as density measured as arbitrary Hounsfield Units (HU). Herein, we tested the hypothesis that Micro-CT detects changes in liver tomographic density induced by metabolic diseases and its reversal upon therapeutic surgical intervention. Two groups of male Wistar rats were used: a group submitted to a hypercaloric diet for 14 weeks to induce prediabetes and the control group submitted to a standard diet. The animals were randomly submitted to a surgical treatment and maintained on their respective diets after the procedure for 11 more weeks. Liver and adipose tissues samples were excised and samples were scanned using a compact X-ray micro-CT scanner. The projection images obtained were analyzed and reconstructed and values of HU density were calculated after calibration for all samples. Results showed that liver density was lower in prediabetes rats (74.8±5.87 HU) than in control animals (97.2±6.3 HU), $p < 0.05$. Liver density was not affected by surgical treatment in control animals however, in prediabetes animals, the surgical therapy restored liver density to control values. Visceral fat density was significantly lower than hepatic density, as expected and was affected, neither by the disease condition nor by the surgical treatment. We concluded that micro-CT detects metabolic disease-induced changes in liver density, but not in visceral adipose tissue density in biopsy samples *ex vivo*. Changes in hepatic density, assessed by micro-CT, correlate with disease state and with therapeutic interventions.

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Peer-review under responsibility of the scientific committee of the International Conference on Sustainable and Intelligent Manufacturing

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Keywords: metabolic syndrome; micro computed tomography; liver density; HU.

1. Introduction

As recently reported by the World Health Organization's World Health Statistics 2014 chronic non-communicable diseases, such as cardiovascular and metabolic diseases contribute significantly to mortality and morbidity in the world without significant advances in the field of therapeutics [1,2]. While investigating pathophysiological mechanisms of insulin-resistance associated conditions, our team unraveled an innovative role for a peripheral nerve, the carotid sinus nerve (CSN), in the pathogenesis of insulin resistance and dysglycemia. The therapeutic potential of this finding was demonstrated by prevention of metabolic disease progression achieved by CSN surgical resection in animal models of prediabetes [3]. The CSN is the sensitive nerve that connects the peripheral chemoreceptors, located at the carotid bodies (CB), with the central nervous system. These tiny organs are located bilaterally in the bifurcation of the common carotid arteries and respond to low oxygen levels in the blood by increasing chemosensory activity in the CSN to signal the sympathetic nervous system to increase ventilation and cardiac output [4, 5]. Besides its role in the control of ventilation, the CB has been proposed as a metabolic sensor, implicated in the control of energy homeostasis [3,6-9], playing a direct role in the etiology of insulin resistance, the core metabolic feature of the metabolic syndrome and type 2 diabetes [3, 6, 8-11] (Fig.1).

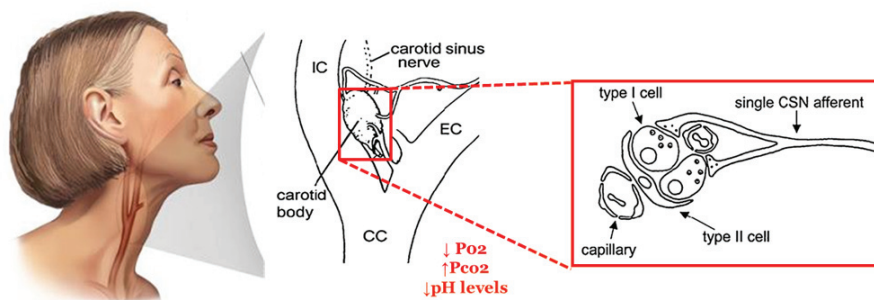


Fig. 1. The carotid bodies (CB) are peripheral chemoreceptors that sense changes in arterial blood O_2 , CO_2 and pH levels. Hypoxia, hypercapnia and acidosis activate the CB, which respond by increasing the action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). CSN activity is integrated in the brain stem to induce a panoply fan of cardiorespiratory reflexes aimed, primarily, aimed to normalize the altered blood gases, via hyperventilation, and to regulate blood pressure and cardiac performance, via sympathetic nervous system activation. Besides its role in the cardiorespiratory control, in the last decade the CB has been proposed as a metabolic sensor implicated in the control of energy homeostasis and, more recently, in the regulation of whole body insulin sensitivity. CC- common carotid artery; IC-internal carotid artery; EC- external carotid artery; CSN: carotid sinus nerve; type I cell: chemosensory glomus cell; type 2 cell: sustentacular glomus cell. Adapted with permission from Gonzalez et al., 2011 Respiratory Physiology & Neurobiology and Creative Commons License.

Knowing that fatty liver disease is an early event in the development of insulin resistance that predicts the presence and the progression of the metabolic syndrome [12], the work presented herein had two main aims: the first one was to test if X-ray Micro Computed Tomography (Micro-CT) could detect changes in liver and visceral adipose tissue *ex vivo* biopsy samples of control and metabolic syndrome rats; the second aim was to evaluate if the surgical resection

of the CSN caused changes in liver and adipose tissue composition susceptible of being detected by micro computed tomography.

2. Research Design and Methods

2.1. Animals and experimental procedure

Experiments were performed in Wistar rats (200–420 g) of both sexes, aged 8 weeks, and obtained from the animal house of NOVA Medical School/ Faculdade de Ciências Médicas. In order to obtain an animal model of the metabolic syndrome the rats were fed a combined high-sucrose-high fat diet consisting of 35% of sucrose administered in drinking water together with lipid enriched chow (60% fat; 20% carbohydrate and 20% protein, Mucedola, Italy). Age-matched control rats were also used and were fed a standard rat chow (7.4% fat+75% carbohydrate (4% sugar) + 17% protein, SDS diets RM1, Probiológica, Portugal). Following the diet protocol for 11 weeks, insulin resistance was confirmed by an insulin tolerance test (ITT), 14 weeks after beginning the diet protocol as previously described [11]. Ensuing insulin sensitivity evaluation, the animals were submitted to unilateral or bilateral CSN transection under ketamine (30mg/kg)/ xylazine(4mg/kg) anesthesia and brupenorphone (10µg/kg) analgesia, as previously described [11].The control groups were submitted to a sham procedure. Caloric and liquid intake were monitored daily, before and after the surgical procedures in all groups of animals. Body weight and animal behavioral changes were assessed twice per week. After the surgical procedure the animals were kept under the respective HF and HSu diets to guarantee access to hypercaloric diets during both the recovery period and the remaining experimental period for 11 more weeks. After that period, the animals were euthanized with a lethal dose of pentobarbital and liver and visceral adipose tissue samples were immediately collected and snap frozen in liquid nitrogen to be stored at -80°C until further analysis. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the Ethics Committee of the NOVA Medical School/Faculdade de Ciências Médicas.

2.2. Micro Computed Tomography

Ex vivo micro-CT assays were used to assess liver density of the samples making use of their CT attenuation coefficients or x-ray density. Density was determined using Hounsfield unit scale as a reference. HU are a standard unit of x-ray CT density, in which air and water are ascribed values of 0 and 1000 respectively. It is conventional to displace the HU value downwards by 1000 units, so that the values for water and air are 0 and -1000 respectively. An HU calibration procedure was performed at the point of image reconstruction, through the use of CT-analyzer software and using a tube of water as a reference scanned phantom.

For the images acquisition, a SkyScan scanner (1174v2) from Bruker, Belgium was used and the parameters adjusted as follows: Voltage: 40 kV; Current: 579 µA; Exposure time: 3500 ms; Rotation Step: 0.700 degrees; with 360° Rotation; frame averaging of 3 and a 0.25 Al filter. The scans had a duration of 1h 33 min.

Beam hardening was corrected and sufficient smoothing was applied to remove the excess of background noise. Reconstruction was made with the help of NRecon software, using the same region of interest (ROI) for all samples.

The steps to calibrate density in the SkyScan micro-CT instrument included performing a water phantom, scanning the sample and reconstruction. The phantom was a polypropylene micro-tube filled with distilled water. All the parameters of the scan and all the reconstruction settings were identical to the ones used for the samples.

For density evaluation, CTAn software was used and all the calculations (phantom and samples) done within a Volume of Interest (VOI) with the same dimensions (3071.0 µm of diameter and 12 043 µm of width).

2.3. Statistical analysis

Data were evaluated using Graph Pad Prism Software, version 6 (GraphPad Software Inc., San Diego, CA, USA) and presented as mean values with their standard errors. The significance of the differences between the mean values

was calculated by two-way ANOVA with Tukey multiple comparison test, respectively. Differences were considered significant at $p < 0.05$.

3. Results

Administration of hypercaloric diets to Wistar rats produced changes in body weight, blood pressure and insulin sensitivity similar to the ones observed in humans with metabolic syndrome. Liquid and food intake were similar in all animals tested. Insulin resistance was confirmed by measurement of insulin sensitivity in HFHSu rats (data not shown). As previously reported by our group [11], insulin resistance induced by hypercaloric diets was totally reversed by CSN resection.

3.1. Micro-CT of liver and adipose tissue samples

As depicted in Fig. 2, HFHSu diet caused a significant reduction in liver density, from 97.2 ± 6.3 HU, in animals fed a standard chow, to 74.8 ± 5.87 HU in the metabolic syndrome group ($p < 0.05$, $n = 6$) (Fig. 2). Liver density was not affected by surgical treatment in control animals where the hepatic density assessed by micro-CT was 99.3 ± 5.04 HU, a value that did not differ significantly from control. However, in prediabetes animals, the surgical therapy restored liver density to control values (102 ± 4.53 HU; $p < 0.05$ compared to non-treated prediabetes rats, $n = 6$) (Fig. 2).

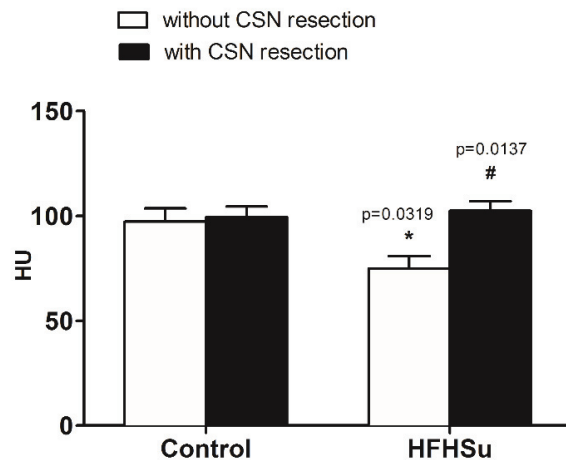


Fig. 2. Effect of carotid sinus nerve (CSN) transection on liver density assessed by micro computed tomography, expressed as Hounsfield units (HU) in control and metabolic syndrome animals (HFHSu). White bars represent values of liver density sensitivity in animals without CSN denervation. Black bars represent values of liver density sensitivity in animals after CSN denervation. Bars represent mean \pm SEM. Two-Way ANOVA with Tukey multicomparison tests; * $p < 0.01$ vs control without CSN resection; # $p < 0.01$ vs HFHSu without CSN resection.

Visceral fat density was significantly lower than hepatic density, as observable in Fig. 3; (Control visceral fat: -321.3 ± 34.3 HU, $n = 7$ and HFHSu visceral fat: -410.3 ± 18.7 , $n = 4$). Denervation of the carotid sinus nerve did not impact visceral fat density in the control-denervated animals (-337.0 ± 37.3 , $n = 7$) or in the HFHSu-denervated animals (-405.2 ± 26.6 , $n = 6$).

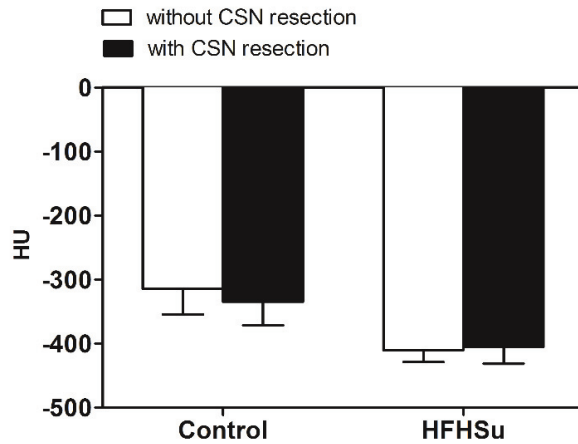


Fig. 3. Effect of carotid sinus nerve (CSN) transection on visceral adipose tissue density assessed by micro computed tomography, expressed as Hounsfield units (HU) in control and metabolic syndrome animals (HFHSu). White bars represent values of liver density sensitivity in animals without CSN denervation. Black bars represent values of liver density sensitivity in animals after CSN denervation. Bars represent mean \pm SEM. Two-Way ANOVA with Tukey multicomparison tests; * $p < 0.01$ vs control without CSN resection; # $p < 0.01$ vs HFHSu without CSN resection.

4. Discussion

Herein we describe that micro computed tomography detects changes in liver density in ex vivo biopsy samples of control and prediabetes rats. Also we showed that surgical ablation of the carotid sinus nerve restores liver density to control values, as detected by micro-CT.

4.1. Technical considerations

The ability to accurately measure density from a micro-CT scan of an object is not something that can be taken for granted. Certain artefacts of micro-CT imaging can compromise density measurement. An exact correlation between apparent grey level in the reconstructed image and object density cannot be assumed. To minimize and avoid this problem it was necessary to correct beam hardening and to apply sufficient smoothing to remove background noise. Also, reconstruction was always performed using the same region of interest (ROI) for all samples. Distinguish between adipose and hepatic cells was the main objective at the first place. However, due to sampling procedures and equipment characteristics, this aim was not achievable. The fat content was dispersed through the whole sample turning it into a quite homogeneous medium. For this reason, samples were compared based on sample lipid content, with consequences in x-ray density calculated in HU. During pilot experiments, optimal scanning conditions were tested. The method optimization involved choosing the best scanning parameters in order to obtain the best signal to noise ratio in the acquisition images. Several trials were performed to optimize voltage, current intensity, the favorable frame averaging, exposure time, the convenient rotation step, the relevance of doing 360° rotation and the need of aluminium filter. The subsequent flat field performed after each set of parameters tried, conducted and supported the choices, allowing us for the final validation of the method. NRecon reconstruction had to be made inside a ROI (in Methods), having in mind the need of a high beam hardening correction and applying enough smoothing. Prior to the HU density determination with CTAn software a ROI was selected in the water tube phantom and converted into a VOI over a number of layers of water (in Methods), as illustrated in Fig. 4.

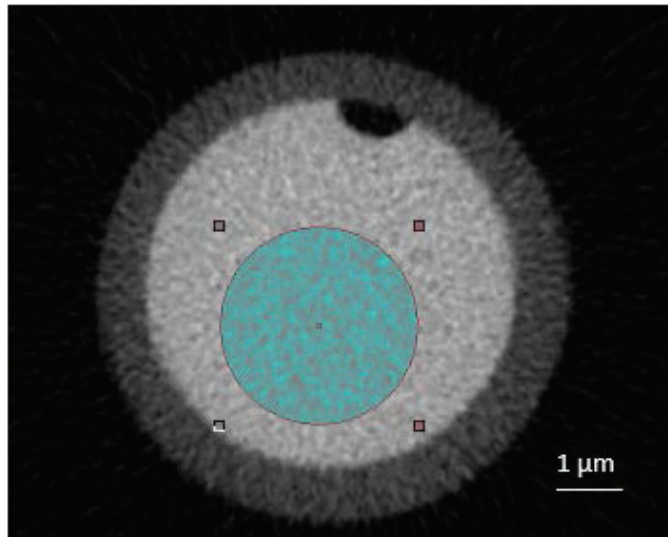


Fig. 4. Selected ROI inside a water tube phantom crosssection (3071.0 μm of diameter) done within a VOI with 12 043 μm of width.

It was important to make sure that image levels with water only were used, without including heterogeneous slices, since the changes in density due, for instance to air bubbles presence, could affect the results. This procedure was repeated for all the samples tested. The sample scan and reconstruction was repeated five times to guarantee the method accuracy and average values were used for the calculations.

4.2. Conclusion

The results showed that Micro-CT is an efficient imaging technique to assess ex vivo liver biopsies density since the results obtained herein correlate with the disease phenotype. This brings about new opportunities of assessing the density of liver and adipose tissue samples that have been collected and stored at -80°C without being processed for histopathological analysis. Future works would be extremely important to determine if the different densities detected in liver samples of control, prediabetes and CSN denervated prediabetes animals are related with the hepatic lipid content.

Acknowledgements

Joana Sacramento is supported by a PhD Grant from Portuguese Foundation for Science and Technology (Reference PD/BD/105890/2014).

The work was supported by Portuguese Foundation for Science and Technology, through the project UID/Multi/04044/2013.

References

- [1] World Health Statistics 2014- World Health Organization. ISBN 978 92 4 069267 1
- [2] Chaudhury, A., Duvoor, C., Dendi, R., Sena, V., Kraleti, S., Chada, A & Sasapu, A. (2017). Clinical Review of Antidiabetic Drugs: Implications for Type 2 Diabetes Mellitus Management. *Frontiers in Endocrinology*, 8, 6.
- [3] Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteiro EC, Conde SV. Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. *Diabetes* 2013; 62:2905-291

- [4] Gonzalez C, Almaraz L, Obeso A, Rigual R.. Carotid body chemoreceptors: From natural stimuli to sensory discharges. *Physiol Rev* 1994; 74: 829-98.
- [5] Marshall JM. Peripheral chemoreceptors and cardiovascular regulation, *Physiol Rev* 1994; 74:543-94.
- [6] Wehrwein, E. A., Basu, R., Basu, A., Curry, T. B., Rizza, R. A., & Joyner, M. J. (2010). Hyperoxia blunts counterregulation during hypoglycaemia in humans: possible role for the carotid bodies? *The Journal of physiology*, 588(22), 4593-4601.
- [7] Koyama Y, Coker RH, Stone EE et al..Evidence that carotid bodies play an important role in gluoregulation in vivo. *Diabetes* 2000; 49:1434-42.
- [8] Conde, S. V., Sacramento, J. F., Ribeiro, M. J., Melo, B. F., & Guarino, M. P. (2015). Insulin resistance: A new consequence of altered carotid body chemoreflex?.*Autonomic Neuroscience: Basic and Clinical*, 192, 16.
- [9] Conde, S. V., Ribeiro, M. J., Melo, B. F., Guarino, M. P., & Sacramento, J. F. (2017). Insulin resistance: a new consequence of altered carotid body chemoreflex? *The Journal of physiology*, 595(1), 31-41.
- [10] Vera-Cruz, P., Guerreiro, F., Ribeiro, M. J., Guarino, M. P., & Conde, S. V. (2015). Hyperbaric oxygen therapy improves glucose homeostasis in type 2 diabetes patients: a likely involvement of the carotid bodies. In *Arterial Chemoreceptors in Physiology and Pathophysiology* (pp. 221-225). Springer International Publishing.
- [11] Sacramento JF, Ribeiro MJ, Rodrigues T, Olea E, Melo BF, Guarino MP, Fonseca-Pinto R, Ferreira CR, Coelho J, Obeso A, Seïça R, Matafome P, Conde SV. (2017) Functional abolition of carotid body activity restores insulin action and glucose homeostasis in rats: key roles for visceral adipose tissue and the liver. *Diabetologia*. 2017 Jan; 60 (1):158-168.
- [12] Lubura M, Hesse D, Neumann N, Scherneck S, Wiedmer P, et al. (2012) Non-Invasive Quantification of White and Brown Adipose Tissues and Liver Fat Content by Computed Tomography in Mice. *PLoS ONE* 7(5): e37026. doi:10.1371/journal.pone.0037026