Increased level of DNA damage in some organs of obese Zucker rats by γ-H2AX analysis.

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Abstract

In a recent study, we showed that lymphocytes of obese Italian children/adolescents displayed levels of double strand breaks (DSB), assayed as serine 139-phosphorylated histone H2AX (γ-H2AX), about eight-fold higher than normal weight controls, and that 30% of this damage generated micronuclei. These findings suggested that obese children could be at increased risk of obesity-mediated cancer later in life. We therefore aimed to assess the level of γ-H2AX in a genetic animal model of obesity (Zucker rat) to identify a genotoxic/carcinogenic risk in some organs. The DSB marker was studied in 3-4-week-old rats and in 9-13-week old rats. Paraffin-embedded sections of heart, thyroid, liver, pancreas, lung, kidney, oesophagus and gut from the fa-/fa- (obese) and the fa+/fa- (lean) control animals were processed for immunohistochemistry detection of γ-H2AX. Pancreas (0.0624±0.0195), lung (0.1197±0.0217), oesophagus (0.1230±0.0351), kidney (0.1546±0.0149) and gut (0.1724±0.0352) of 9-13-week-old obese rats showed a higher proportion of γ-H2AX positive nuclei, than their lean counterparts (0.0092±0.0033, 0.0416±0.0185, 0.0368±0.0088, 0.0686±0.0318 and 0.0703±0.0239, respectively). No difference was seen in the 3-4-week-old age group with regard to obesity, indicating that the DNA damage increased with older age of the rats. We hypothesize that the organs of the obese animals showing high levels of DSB, could represent target tissues for the development of obesity-related cancers.

1. Introduction

Obesity is a complex disease characterised by an excess of fat in the body in which genetic, physiological, environmental and socio-economic factors, interacting with each other, predispose and concur contribute to its pathogenesis as well as to the appearance of adverse effects later in life [Wright et al., 2012; Trésallet et al., 2014; Marcello et al., 2014; Brenner, 2014; Drew, 2012]. This pathological condition, in fact, can have severe outcomes for human health, causing chronic illnesses such as type 2 diabetes mellitus, asthma, cardiovascular diseases [Mitchell et al., 2013; Rosenkranz et al., 2005; Weiss et al., 2004; DeVallance et al., 2015] and also some types of cancers [Bardou et al., 2013; Calle et al., 2003; Karagozian et al., 2014; Renehan et al., 2008]. In this context, formation of double strand breaks (DSB) within the genome, if unrepaired, lead to mutations that, in turn, can initiate the carcinogenesis process. However, cells activate the DNA damage response (DDR) to reconstitute the integrity of DNA, in which phosphorylation at serine 139 of several histone H2AX molecules surrounding the site of DSB (γ-H2AX foci) represents an early and key event. Hence, it has been assumed that the number of γ-H2AX foci can reflect approximately the number of nuclear DSB formed, thus making γ-H2AX an excellent marker of early DNA damage [Rogakou et al., 1998; Watters et al., 2009; Scarpato et al., 2011; Redon et al 2011]. As a consequence, it was also possible to relate the presence of γ-H2AX in the nuclei to how and how much a given cell population responds to carcinogenesis stimuli [Bartkova et al., 2005; Nuciforo et al., 2007]. Recently, we have provided evidence that a group of obese Italian children/adolescents had about eight-fold higher levels of DSB, expressed as γ-H2AX nuclear foci, in their peripheral lymphocytes than normal weight subjects, and that 30% of this damage generated micronuclei [Scarpato et al., 2011; Azzarà et al., 2016]. These findings suggested that obese children could be at increased risk for developing obesity-mediated cancer later in life.

Genetic animal models have been developed in rodents to efficiently study obesity and/or the metabolic syndrome, and among these, the Zucker fatty rat offers a reliable *in vivo* genetic system.

The genome of this animal model carries the homozygous missense mutation that causes loss of function of the leptin receptor gene [Takaya et al., 1996]. As a consequence, the rats show hyperphagia, hypercholesterolaemia, hyperglycaemia, and hyperinsulinaemia [Cozzi et al., 2009; Geurts et al., 2009]. Leptin is encoded by the *lep* gene and produced by adipocytes [Zhang et al., 1994], regulating food intake in mammals via a cross-talk between the adipose tissue and central nervous system that maintains energy balance and normal body weight [Schwartz et al., 2000]. In addition, as described in the literature, these rats were also used to investigate some mechanisms connected to the obesity- or diabetes-mediated carcinogenesis [Koch et al., 2008; Ishii et al., 2011; Ishizaki et al., 2013; Imai et a., 2013]. Thus, we aimed to gain information on the obesity-mediated genotoxic risk by investigating, at tissue level, the expression and time-course of the γ-H2AX marker in eight organs of the Zucker rat model. We performed the study in the 3-4 week-old rats and in 9-13-week-old rats in order to mimic and cover, as much as possible, the human life period spanning from childhood and adolescence to maturity.

2. Materials and Methods

2.1. Zucker rat models and biochemistry determinations

Male Zucker fatty rats were purchased from Charles River Laboratories International, Inc., (Wilmington, MA, USA), housed in the animal facility under constant conditions of temperature (24–25 °C) and artificial lighting (12 h light–dark cycle), fed with standard rat chow (calories provided by: 27.0% protein 13.1% fat and 59.9% carbohydrates; Sandown Scientific, Middlesex, England) and water *ad libitum* until used for the study. The Zucker genome carries the homozygous missense mutation (C→A) at nucleotide 806 of the leptin receptor gene, with consequent Gln239Pro amino-acidic substitution in the extra-cellular domain of the receptor (fa^-/fa^- animals), loss of function and acquisition of an obese phenotype. The lean control group was represented by heterozygous counterparts (fa^+/fa^- animals). We

studied eight organs (heart, kidney, lung, oesophagus, gut, pancreas, thyroid and liver) in 44 rats subdivided into 23 animals aged 9-13 weeks and into 21 animals aged 3-4 weeks. According to their body weight and the phenotypic characteristic previously described, the 9-13-week-old rats were categorized as obese (n=13) and lean (control) rats (n=20). In the 3-4-week-old rats, 19 and 12 animals were classified as obese and normal weight (lean) animals, respectively. Due to specific difficulties in collecting the thyroid and pancreas from animals aged 3-4 weeks, we could not perform DNA damage analysis in these organs.

Standard laboratory methods were used for glycaemia, insulin and total cholesterol level determinations. A scil Vet ABC ™ Haematology Analyzer (scil animal care company S.r.l., Milan, Italy) was used to measure blood glucose and total cholesterol, while insulin plasma levels were measured by a commercial immunoassay kit (Crystal Chem Zaandam, Netherlands, sensitivity = 1.5 µIU/mL, CV < 10%). On the day of the study, the animals were sacrificed by inhalation of an overdose of isofluorane after collection of blood samples for clinical biochemistry measurements. In order to minimize experimental variability, groups of four to-five obese and lean animals were processed at once. We followed the recommendations of Italian legislation (DL No. 116, 1992), which implements the EEC directive 609/86 for the care and use of laboratory animals.

Body weight, genetic and phenotypic characteristics and clinical biochemistry measurements (glycaemia, insulin and total cholesterol levels) of all the Zucker rats are reported in Table 1.

2.2. Tissue histology and immunohistochemistry protocol

After sacrifice, a piece of tissue was randomly collected from each organ of each animal, washed of blood residues, cut into two small pieces and fixed in formalin until processed for γ-H2AX analysis by the immunohistochemistry protocol described elsewhere [Del Ry et al., 2015]. Briefly, the formalin fixed tissues were washed in tap water to remove fixative, dehydrated in an ethanol series, immersed two times in Isoparaffin for 1 h each (Panreac, Nova

Chimica, Milan, Italy), and included in paraffin. The paraffin-embedded organs were then sectioned into 5-µm slices using a Leica RM 2155 microtome (Leica instruments, Wetzlar, Germany). The slices were then placed on Superfrost Ultra Plus® slides (Manzel-Glaser, Thermo-Fisher, Milan, Italy) to guarantee firm electrostatic adhesion of the sections. Two coded slides, containing two sections of each organ, were set up, processed and scored blindly by three experienced persons. Following deparaffination/rehydration done in xylene/ethanol series and heat-mediated antigen unmasking, the sections were incubated in 3% hydrogen peroxide (Sigma-Aldrich, Milan, Italy) for 10 min, and then for 1 h in blocking solution (10% FBS (Life Technologies, Monza, Italy), 0.3% Triton-X, in 1X PBS). Then the slides were incubated overnight at 4°C with an 1:200 diluted primary polyclonal rabbit anti- γ-H2AX antibody (Abcam, Prodotti Gianni, Milan, Italy), followed by incubation for two hours at RT with an 1:100 diluted anti-rabbit horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, Milan, Italy) , subsequently revealed by application of the 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB; Sigma-Aldrich, Milan, Italy). Finally, the slides were counterstained in haematoxylin, dehydrated and mounted in glass coverslips for microscopy observation.

The presence of γ-H2AX positive nuclei within a haematoxylin-counterstained cell population was recognised by the formation of a homogeneous brown precipitate covering partially or entirely the DSB-damaged nuclei, which were easily distinguished by their undamaged, blue-coloured nuclei. Figures 1-2 show a panel of positive and negative sections for each organ. Slides incubated without the primary or the secondary antibodies, as well as with 1000 U/ml of alkaline phosphatase (Sigma-Aldrich, Milan, Italy), were also made to ensure on the proper recognition of histone H2AX molecules phosphorylated at ser 139.

A (semi)-quantitative analysis was made with the aid of Image J software (downloaded at hppt://imagej.nih.gov/ij/). Briefly, four areas in each section showing the signal for γ-H2AX were randomly identified, and the ratio between γ-H2AX positive (brown-stained) to negative

(blue-stained) nuclei was calculated. The average value from the four areas, adjusted for the degree of γ-H2AX positivity of the section expresses the DNA DSB marker in each section. This was achieved by scanning the entire section with the 10X objective.

To delineate the time-course of nuclear phosphorylation in each organ from 3-4-week-old rats to 9-13-week-old rats, γ-H2AX levels were expressed as the difference or the ratio between the average values obtained in the two age groups. Thus, for the lean or obese rats, two parameters were calculated subtracting or dividing the average values of the 9-13-week-old rats to the corresponding values of the 3-4-week-old group.

2.3. Statistical analysis

To detect differences in the level of DNA damage (γ-H2AX values) of the organs or in the values of the metabolic parameters between lean (fa+/fa+) and obese (fa+/fa-) rats, data were statistically analysed, in each age group (3-4-week-old rats and 9-13-week-old rats) by the Student's t-test using the Statgraphics Centurion XV software package (Statistical Graphics Corp., Rockville, MD, USA). *P*-value was considered significant when < 0.05.

3. Results

The 9-13-week-old obese rats weighted (353.4 ±19.3 g) approximately 20% more than the lean counterparts (289.3 ± 21.4 g), and compared to this group, they showed significantly higher levels of basal glycaemia (305.2 ± 28.1 g *vs*. 209.4 ± 35.6 g, $p < 0.05$), insulin (2944 ± 341 pg/ml *vs*. 750 ±199 pg/ml, $p < 0.001$) and total cholesterol (162.5 ± 12.8 mg/dl *vs*. 110.4 ± 6.8 mg/dl, $p < 0.05$). As previously mentioned, no difference was observed in the 3-4-week-old rats between obese and lean animals (see Table 1).

In the two rat age groups, the results of the DNA damage analysis, expressed as γ-H2AX levels, are shown in Figure 3 or Figure 4 according to the organs in which a genotype effect was or was not observed, respectively. For each sampled organ, we did not detect a significant difference in the basal DNA damage between fa+/fa+ and fa+/fa- 3-4-week-old rats. In the adult rats, the levels of γ-H2AX positive cells was quite variable among the analysed tissues, with values ranging from 0.0092 ± 0.0032 (pancreas) to 0.1210 ± 0.0323 (thyroid) in the lean group, and from 0.0495 ± 0.0246 (heart) to 0.1724 ± 0.0352 (gut) in the obese group. Statistically significant increases were observed in five organs of obese as compared to lean rats (Figure 3), with the highest and lowest difference observed in the pancreas (0.0624 ± 0.0195 vs. 0.0092 ± 0.0033, $p = 0.0447$) and in the kidney (0.1546 ± 0.0149 vs. 0.0686 ± 0.0318, $p = 0.0186$), respectively. The other values were (obese *vs*. lean): lung (0.1197±0.0217 *vs*. 0.0416±0.0185, $p=0.0352$), gut (0.1724 ± 0.0352 *vs*. 0.0703 ± 0.0239, $p = 0.0470$) and oesophagus 0.1230 ± 0.0351 *vs*. 0.0368 ± 0.0088, $p = 0.0249$). Also grouping together the γ-H2AX data from all the positive organs, we registered significantly higher values of DNA damage in the obese animals than in the lean rats (0.1264 ± 0.0287 *vs*. 0.0453 ± 0.0139, $p = 0.0234$). In the heart, liver and thyroid, the baseline frequencies of obese animals were, on average, comparable to those of the lean group (Figure 4).

With regard to the time-course of DNA damage, from animals aged 3-4 weeks to animals aged 9-13 weeks, the γ-H2AX levels of lean and obese rats showed a general increase in all the analysed organs, suggesting that the basal frequencies of DNA DSB increased with older age of the animal. However, this increase was more pronounced in the obese rats than in the lean ones, with the exception of heart and liver (thyroid was not sampled in 3-4-week-old rats): in these organs, in fact, the elevated increase in the basal DSB levels occurred at a comparable extent in both fa+/fa+ and fa+/fa- animals.

4. Discussion

The response of animal organs to genotoxic insults has been widely demonstrated using immunohistochemical detection of γ-H2AX levels in lung tissue of rats exposed to fine and ultrafine dusts [Rittinghausen et al., 2013]. Elevated immunostaining for this DSB marker has also been reported in renal tissue of obese mice as well as in the lungs of obese Zucker rats with altered expression of the endothelin axis [Del Ry et al., 2014; Mozaffari et al., 2012]. Despite the physiological presence of individual and organ variability, our results indicate that, irrespectively of their genotype, the levels of the DNA damage marker were significantly lower in all analysed organs of the 3-4-week-old rats as compared to the 9-13-week-old ones. Cell tissue alterations occurred in the 3-4-week-old obese rats were minor and less widespread among body organs than those observed in the corresponding 9-13-week-old age group. In other words, the tissue damage would seem to increase, at DNA level, as the age of the animals increased. This might be explained by the fact that in 3-4-week-old rats the level of inflammation/oxidative stress has not yet reached a sufficient intensity to affect DNA integrity. It is only in the 9-13-week old rats that the frequencies of cells containing DSB increase, in some organs, as a function of the presence of genetic predisposition to obesity. In fact, the levels of DNA damage we detected in the analysed tissues of the obese group delineated the following descending order: gut, kidney, oesophagus, lung, pancreas (organs that resulted significantly different from those of the lean animals), thyroid, liver and heart. As the parameter we used to quantify γ-H2AX (percent of positive nuclei adjusted for the degree of positivity to the γ-H2AX signal of the whole section) gives a rough estimate of the actual spontaneous levels of γ-H2AX in each tissue, any comparison among the various organs should be properly interpreted. Thyroid was found to express γ-H2AX signal to an extent much higher than other tissues such as, for example, the pancreas. However, it is not relevant that thyroid was, in general, the most reactive organ to γ-H2AX immunostaining, probably due to an overproduction of H_2O_2 by thyreocytes [Driessens et al., 2009], it is important that γ-H2AX levels in the

corresponding tissue of the obese rats resulted unchanged (thyroid) or significantly increased (pancreas). Moreover, although no information on the extent of DNA damage at single cell level could be drawn, as neither the number of γ-H2AX foci nor any other parameter related to the presence of DSB was assayed, the percent of γ-H2AX positive nuclei is considered a useful marker of DNA damage, and other authors used it to quantify DSB in some rodent tissues. Rittinghouse et al. [2013] found about 150 positive nuclei per mm^2 in the lung of female Wistar rats, while urinary bladder of 5 weeks old F344 rats showed approximately 2 γ-H2AX positive nuclei per 1000 cells counted [Toyoda et al., 2015]. Finally, no cell with the γ-H2AX signal or a very slight amount of nuclei was found in the brain of Wistar rats developing lymphosarcomas [Masutani et al., 2014] or in the duodenum of B6C3F1 mice, respectively [Thompson et al., 2015].

The fa^-/fa^- animals are also affected, on average, by the highest glucose levels that, indeed, is an intrinsic characteristic of these rats [Serpillon et al., 2009]. Hyperglycaemia is connected to oxidative stress as it causes, via ROS- and NOX1-mediated glycoxidation, a pro-inflammatory response and oxidative damage in the target tissue [Yoshida et al., 2000; Yang et al., 2011; Vlassara et al., 2011]. We have recently demonstrated that adolescents affected by type 1 diabetes but with BMI in the range of normality, have elevated levels of γ-H2AX foci in their peripheral lymphocytes [Giovannini et al.; 2014]. In this context, Nox 1-deficient hyperglycaemic mice exhibited reduced levels of γ-H2AX in their kidneys [Zhu et al., 2015]. We therefore suggest that the increased frequency of γ-H2AX positive cells observed among our fa+/fa- rats was probably due to the obesity status, in which a role might also be played by their constitutive hyperglycaemic condition, which was preferentially expressed in gut, kidney, oesophagus, lung and pancreas rather than in thyroid liver and heart.

With regard to a possible oncologic risk for humans from the animal data, we should take into account that DSB are still reparable DNA lesions, and the presence of γ-H2AX positive cells indicates the recruitment of the appropriate cellular response to remove the damage. However,

although only eight organs were studied, the obese rats, showing an increased occurrence of this type of DNA lesion, might be considered at increased risk of developing malignancies, especially in the above mentioned tissues. The excess of H2AX phosphorylation we have observed in the fa$^-$/fa$^-$ animals actually reflects the obesity condition that is capable of generating DSB and, in turn, organ damage. However, it cannot be ruled out that these γ-H2AX foci represent unrepaired DSB that persist and accumulate in the tissues, as also suggested by other authors [Siddiqui et al., 2013; Bhogal et al., 2010].

The presence of endocrine-metabolic alterations is compatible with an imbalance in plasma and intracellular antioxidant/pro-oxidant activities that produce DNA-reactive molecules through generation of chronic inflammation. In accordance with our data, some studies observed, in humans, a tight association between inflammatory-based lung diseases, colon and pancreatic cancers, and obesity, probably due to the excess of serum levels of leptin that can cause an imbalance of the immune response [Shore, 2010; Schatz et al., 2013; Youssef et al., 2013], or trigger a pro-inflammatory and anti-apoptotic response [Bardou et al., 2013; Huang et al., 2009; Yeo, 2015; Drew, 2012]. In addition, a prolonged obesity condition sustained by oxidative stress can trigger renal damage, as demonstrated in 3-month-old fa$^-$/fa$^-$ Zucker rats [Poirier et al., 2000]. Also oesophageal cancer and Barrett's oesophagus were clearly linked to adult obesity through overproduction of the insulin-like growth factor 1 (IGF-1), together with a reduced presence of IGF-binding proteins, due to hyperinsulinaemia [Calle et al., 2003; Kamat et al., 2009; Greer et al., 2012]. Indeed, the increase in the occurrence of γ-H2AX we observed in the cells of the respective tissues could reflect these conditions. It has been demonstrated that in mice, rats and hamsters the induction and persistence of γ-H2AX by ionizing radiation occurs in a tissue-specific manner, this in turn depends on the different ability of the organ's tissues to divide themselves [Koike et al., 2008; Firsanov et al., 2012]. The lack of an increase in the levels of DSB in the heart, thyroid and liver of obese rats, might, at least partially, rely on the proliferative status of the three organs. In

fact, heart is a non-dividing tissue and thyroid and liver are considered quiescent tissues with a slow proliferation ability. These tissues, therefore, would be less responsive to the low-grade oxidative stimuli present in the obesity condition than more proliferating tissues such as gut and lung. Variation among the analysed organs may also depend on a different expression and/or functioning of their antioxidant systems. At the same time, the protective action of some natriuretic peptides produced by the cardiac tissue and of adipokines such as visfatin cannot be ruled out [Rittinghausen et al., 2013; Habbu et al., 2006; Xiao et al., 2013]. Moreover, additional researches to investigate these topics should be performed.

The present study conducted in Zucker fatty rats indicates there may be an association between obesity and the occurrence of potentially genotoxic/carcinogenic lesions such as DSB. In addition, although we could not detect these signs of DNA damage earlier in animal life (*i.e.* in the rats of age comparable to that of children/adolescents) as we did in a previous study [Scarpato et al., 2011], the Zucker model has shown the potential to identify organs which could be considered at risk of developing obesity-mediated cancers later in human life.

Author Contributions

RS designed the study and wrote the paper. AA performed all the experimental parts of the study and wrote the paper. AC and EF performed all the experimental parts of the study. PI participated to the design of the study, she and was the responsible for the Zucker rat model. MG performed the collection and processing of rat organs. SM prepared paraffin-embedded tissue sections. FB, BP and SP participated in writing the manuscript. All authors approved the final manuscript.

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References

Azzarà A, Pirillo C, Giovannini C, Federico G, Scarpato R. 2016. Different repair kinetic of DSBs induced by mitomycin C in peripheral lymphocytes of obese and normal weight adolescents. Mutat Res. 789:9-14.

Bardou M, Barkun AN, Martel M. 2013. Obesity and colorectal cancer. Gut 62: 933-947.

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Ziegerm K, Guldberg P, Sehested M, Nesland JM, Lukas C, et al. 2005. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 434: 864–870.

Bhogal N, Kaspler P, Jalali F, Hyrien O, Chen R, Hill RP, Bristow RG. 2010. Late residual gamma-H2AX foci in murine skin are dose responsive and predict radiosensitivity in vivo. Radiat Res 173:1-9.

Brenner DR. 2014. Cancer incidence due to excess body weight and leisure-time physical inactivity in Canada: implications for prevention. Prev Med 66:131-139.

Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ. 2003. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. N Engl J Med: 1625-1638.

Cozzi J, Anegon I, Braun V, Gross AC, Merrouche C, Cherifi Y. 2009. Pronuclear DNA injection for the production of transgenic rats. Methods Mol Biol 561:73–88.

Del Ry S, Cabiati M, Salvadori C, Guiducci L, Caselli C, Prescimone T, Facioni MS, Azzarà A, Chiaramonte A, Mazzoni S, et al. 2014. Transcriptional alterations of ET-1 axis and DNA damage in lung tissue of a rat obesity model. DNA Cell Biol 34:170-177.

DeVallance E, Fournier SB, Donley DA, Bonner DE, Lee K, Frisbee JC, Chantler PD. 2015. Is obesity predictive of cardiovascular dysfunction independent of cardiovascular risk factors? Int J Obes 39: 244-253.

Drew JE. 2012. Molecular mechanisms linking adipokines to obesity-related colon cancer: focus on leptin. Proc Nutr Soc 71: 175-180.

Driessens N, Versteyhe S, Ghaddhab C, Burniat A, De Deken X, Van Sande J, Dumont JE, Miot F, Corvilain B. 2009. Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. Endocr Relat Cancer 16: 845-856.

Firsanov D, Vasilishina A, Kropotov A, Mikhailov V. 2012. Dynamics of γH2AX formation and elimination in mammalian cells after X-irradiation. Biochimie 94: 2416-2422.

Geurts AM, Cost GJ, Freyvert Y, Zeitler B, Miller JC, Choi VM, Jenkins SS, Wood A, Cui X, Meng X, et al. 2009. Knockout rats via embryo microinjection of zinc-finger nucleases. Science 325:433.

Giovannini C, Piaggi S, Federico G, Scarpato R. 2014. High levels of γ-H2AX foci and cell membrane oxidation in adolescents with type 1 diabetes. Mutat Res 770: 128-135.

Greer KB, Thompson CL, Brenner L, Bednarchik B, Dawson D, Willis J, Grady WM, Falk GW, Cooper GS, Li L, et al. 2012. Association of insulin and insulin-like growth factors with Barrett's oesophagus. Gut 61: 665-672.

Habbu A, Lakkis NM, Dokainish H. 2006. The obesity paradox: fact or fiction?. Am J Cardiol 98: 944-948.

Huang XF, Chen JZ. 2009. Obesity, the PI3K/Akt signal pathway and colon cancer. Obes Rev 10: 610-616.

Imai T, Cho YM, Takahashi M, Kitahashi T, Takami S, Nishikawa A, Ogawa K. 2013. High susceptibility of heterozygous (+/fa) lean Zucker rats to 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis. Oncol Rep 29: 1914-22.

Ishii N, Wei M, Kakehashi A, Doi K, Yamano S, Inaba M, Wanibuchi H. 2011. Enhanced Urinary Bladder, Liver and Colon Carcinogenesis in Zucker Diabetic Fatty Rats in a Multiorgan Carcinogenesis Bioassay: Evidence for Mechanisms Involving Activation of PI3K Signaling and Impairment of p53 on Urinary Bladder Carcinogenesis. J Toxicol Pathol 24: 25-36.

Ishizaki S, Nishiyama M, Hagiwara A. 2013. Long-term Branched Chain Amino Acid Supplementation Ameliorates Diethylnitrosamine-induced Liver Glutathione S-transferase-p Positivity in Zucker Fatty Rats. J Clin Exp Hepatol 3: 192-7.

Kamat P, Wen S, Morris J, Anandasabapathy S. 2009. Exploring the association between elevated body mass index and Barrett's esophagus: a systematic review and meta-analysis. Ann Thorac Surg 87: 655-662.

Karagozian R, Derdák Z, Baffy G. 2014. Obesity-associated mechanisms of hepatocarcinogenesis. Metabolism 63:607-17.

Koch TC, Briviba K, Watzl B, Bub A, Barth SW. 2008. Obesity-related promotion of aberrant crypt foci in DMH-treated obese Zucker rats correlates with dyslipidemia rather than hyperinsulinemia. Eur J Nutr 47:161-70.

Koike M, Sugasawa J, Yasuda M, Koike A. 2008. Tissue-specific DNA-PK-dependent H2AX phosphorylation and gamma-H2AX elimination after X-irradiation in vivo. Biochem Biophys Res Commun 376: 52-55.

Marcello MA, Cunha LL, Batista FA, Ward LS. 2014. Obesity and thyroid cancer. Endocr Relat Cancer 21: T255-T271.

Masutani M, Baiseitov D, Itoh T, Hirai T, Berikkhanova K, Murakami Y, Zhumadilov Z, Imahori Y, Hoshi M, Itami J. 2014. Histological and biochemical analysis of DNA damage after BNCT in rat model. Appl Radiat Isot 88: 104-108.

Mitchell EA, Beasley R, Björkstén B, Crane J, García-Marcos L, Keil U. 2013. The association between BMI, vigorous physical activity and television viewing and the risk of symptoms of asthma, rhinoconjunctivitis and eczema in children and adolescents: ISAAC Phase Three. Clin Exp Allergy 43: 73-84.

Mozaffari MS, Baban B, Abdelsayed R, Liu JY, Wimborne H, Rodriguez N, Abebe W. 2012. Renal and glycemic effects of high-dose chromium picolinate in db/db mice: assessment of DNA damage. J Nutr Biochem 23: 977–985.

Nuciforo PG, Luise C, Capra M, Pelosi G, d'Adda di Fagagna F. 2007. Complex engagement of DNA damage response pathways in human cancer and in lung tumor progression. Carcinogenesis 28: 2082–2088.

Poirier B, Lannaud-Bournoville M, Conti M, Bazin R, Michel O, Bariéty J, et al. 2000. Oxidative stress occurs in absence of hyperglycaemia and inflammation in the onset of kidney lesions in normotensive obese rats. Nephrol Dial Transplant 15: 467-476.

Redon CE, Nakamura AJ, Martin OA, Parekh PR, Weyemi US, BonneR WM. 2011. Recent developments in the use of γ-H2AX as a quantitative DNA double-strand break biomarker. Aging 3: 168–174.

Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. 2008. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet 371: 569–578.

Rittinghausen S, Bellmann B, Creutzenberg O, Ernst H, Kolling A, Mangelsdorf I, Kellner R, Beneke S, Ziemann C. 2013. Evaluation of immunohistochemical markers to detect the genotoxic mode of action of fine and ultrafine dusts in rat lungs. Toxicology 303:177-186.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 273: 5858–5868.

Rosenkranz SK, Townsend DK, Steffens SE, Harms CA, Ford ES. 2005. The epidemiology of obesity and asthma. J Allergy Clin Immunol 115: 897–909.

Scarpato R, Verola C, Fabiani B, Bianchi V, Saggese G, Federico G. 2011. Nuclear damage in peripheral lymphocytes of obese and overweight Italian children as evaluated by the gamma-H2AX focus assay and micronucleus test. FASEB J 25: 685–693.

Schatz M, Zeiger RS, Zhang F, Chen W, Yang SJ, Camargo CA Jr. 2013. Overweight/obesity and risk of seasonal asthma exacerbations. J Allergy Clin Immunol Pract 1: 618-622.

Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. 2000. Central nervous system control of food intake. Nature 404:661-671.

Serpillon S, Floyd BC, Gupte RS, George S, Kozicky M, Neito V Recchia F, Stanley W, Wolin MS, Gupte SA. 2009. Superoxide production by NAD(P)H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac

dysfunction, The role of glucose-6-phosphate dehydrogenase-derived NADPH. Am J Physiol Heart Circ Physiol 297: H153-162.

Shore SA. 2010. Obesity, airway hyperresponsiveness, and inflammation. J Appl Physiol 108: 735-743.

Siddiqui MS, Filomeni E, François M, Collins SR, Cooper T, Glatz RV,Taylor PW, Fenech M, Leifert WR. 2013. Exposure of insect cells to ionising radiation in vivo induces persistent phosphorylation of a H2AX homologue (H2AvB). Mutagenesis 28: 531-41.

Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Mori K, Tamura N, Hosoda K, Nakao K. 1996. Molecular cloning of rat leptin receptor isoform complementary DNAs--identification of a missense mutation in Zucker fatty (fa/fa) rats. Biochem Biophys Res Commun 225: 75-83.

Thompson CM, Seiter J, Chappell MA, Tappero RV, Proctor DM, Suh M, Wolf JC, Haws LC, Vitale R, Mittal L, Kirman CR, Hays SM, Harris MA. 2015. Synchrotron-based imaging of chromium and γ-H2AX immunostaining in the duodenum following repeated exposure to Cr(VI) in drinking water. Toxicol Sci 143: 16-25.

Toyoda T, Cho YM, Akagi JI, Mizuta Y, Hirata T, Nishikawa A, Ogawa K. 2015. Early detection of genotoxic urinary bladder carcinogens by immunohistochemistry for γ-H2AX.Toxicol Sci 148: 400-408.

Trésallet C, Seman M, Tissier F, Buffet C, Lupinacci RM, Vuarnesson H, Leenhardt L, Menegaux F. 2014. The incidence of papillary thyroid carcinoma and outcomes in operative patients according to their body mass indices. Surgery 156: 1145-1152.

Vlassara H, Striker GE. 2011. AGE restriction in diabetes mellitus: a paradigm shift. Nat Rev Endocrinol 7: 526-539.

Watters GP, Smart DJ, Harvey JS, Austin CA. 2009. H2AX phosphorylation as a genotoxicity endpoint. Mutat Res 679:50–58.

Weiss R, Dziura J, Burgert TS, Tamborlane TS, Taksali WV, Yeckel SE, Allen K, Lopes M, Savoye M, Morrison J, et al. 2004. Obesity and the metabolic syndrome in children and adolescents. N. Engl. J. Med 350: 2362–2374.

Wright SM, LJ Aronne. 2012. Causes of obesity. Abdom Imaging 37: 730-732.

Xiao J, Sun B, Li M, Wu Y, Sun XB. 2013. A novel adipocytokine visfatin protects against H_2O_2-induced myocardial apoptosis: a missing link between obesity and cardiovascular disease. J Cell Physiol 228: 495-501.

Yang H, Jin X, Wai Kei LC, Yan SK. 2011. Review: oxidative stress and diabetes mellitus. Clin Chem Lab Med 49: 1773-1782.

Yeo TP. 2015. Demographics, epidemiology, and inheritance of pancreatic ductal adenocarcinoma. Semin Oncol 42: 8-18.

Yoshida K, Kikutani H. 2000. Genetic and immunological basis of autoimmune diabetes in the NOD mouse. Rev Immunogenet 2: 140-146.

Youssef DM, Elbehidy RM, Shokry DM, Elbehidy EM. 2013. The influence of leptin on Th1/Th2 balance in obese children with asthma. J Bras Pneumol 39: 562-568.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. 1994. Positional cloning of the mouse obese gene and its human homologue. Nature 372: 425–432.

Zhu K, Kakehi T, Matsumoto M, Iwata K, Ibi M, Ohshima Y, Zhang J, Liu J, Wen X, Taye A, et al. 2015. NADPH oxidase NOX1 is involved in activation of protein kinase C and premature senescence in early stage diabetic kidney. Free Radic Biol Med 83: 21-30.

Table 1. Genetic, phenotypic characteristics and biochemistry measurements of male Zucker rats grouped according to age. All values are expressed as mean±S.E.

Animal groups	Genotype[a]	Body weight (g)	Hyperphagia	Blood glucose (mg/dl)	Insulin (pg/ml)	Total cholesterol (mg/dl)
9-13 weeks						
Lean	fa+/fa-	289.3±21.4	no	209.4±35.6	750±199	110.4±6.8
Obese	fa-/fa-	353.4±19.3	yes	305.2±28.1[b]	2944±341[c]	162.5±12.8[b]
3-4 weeks						
Lean	fa+/fa-	67.6±4.6	no	159.8±9.4		
Obese	fa-/fa-	64.1±5.3	no	175.0±14.5		

[a]fa- indicates the presence of the missense mutation (C→A) at nucleotide 806 of the leptin receptor gene.

[b]Significantly different *vs*. lean rats ($p < 0.05$, Student's t-test).

[c]Significantly different *vs*. lean rats ($p < 0.001$, Student's t-test).

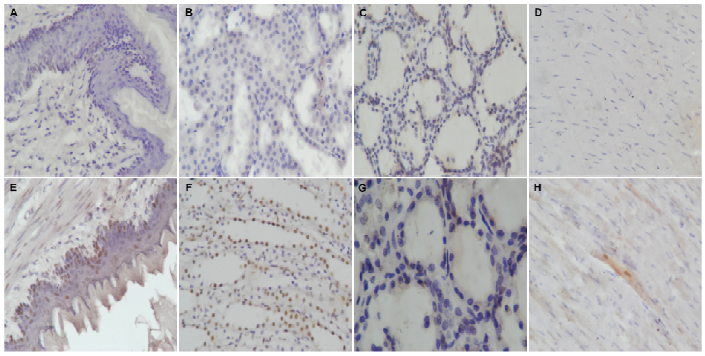


Figure 1

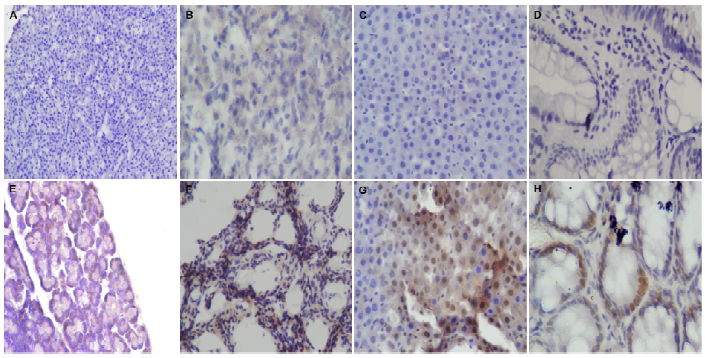
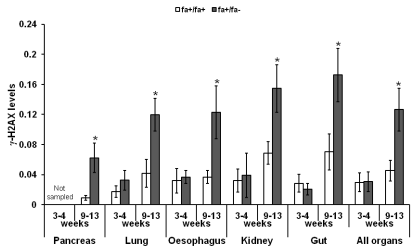


Figure 2



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2 **Figure 3**

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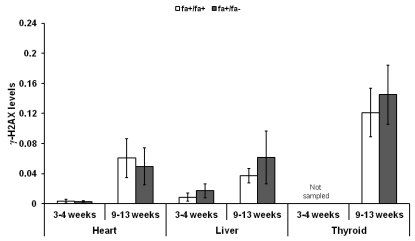

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5 **Figure 4**

Figure legends

Figure 1. Images of organ sections from Zucker rat after immunohistochemistry with γ-H2AX primary antibody detected with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (hematoxylin counterstaining). A-D: areas of oesophagus, kidney, thyroid and heart showing only blue stained γ-H2AX-negative nuclei; E-H, areas of the same organs showing several brown coloured γ-H2AX-positive nuclei. A-F, 200X magnification; G and H, 400X magnification.

Figure 2. Images of organ sections from Zucker rat after immunohistochemistry with γ-H2AX primary antibody detected with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (hematoxylin counterstaining). A-D: areas of pancreas, lung, liver and gut showing only blue stained γ-H2AX-negative nuclei; E-H, areas of the same organs showing several brown coloured γ-H2AX-positive nuclei. A, 100X magnification; C, D, E, F and G, 200X magnification; B and H, 400X magnification.

Figure 3. Levels of the DNA damage marker (γ-H2AX) in 3-4 week-old rats and 9-12 week-old rats. Data refer to those organs in which a genotype effect was observed (fa+/fa+ = lean rats, fa+/fa- = obese rats). γ-H2AX levels are expressed as percentage of γ-H2AX positive nuclei adjusted for the degree of positivity to the γ-H2AX signal of the whole section. Bars represents the mean ± S.E. of each animal group (two replicate sections per animal). Asterisks denote a significant difference (* $p < 0.05$, Student's t-test) *vs*. the respective fa+/fa- rats.

Figure 4. Levels of the DNA damage marker (γ-H2AX) in 3-4 week-old rats and 9-12 week-old rats. Data refer to those organs in which a genotype effect was not observed (fa+/fa+ = lean

rats, fa+/fa- = obese rats). γ-H2AX levels are expressed as percentage of γ-H2AX positive nuclei adjusted for the degree of positivity to the γ-H2AX signal of the whole section. Bars represents the mean ± S.E. of each animal group (two replicate sections per animal).