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Diet-induced variability of the resistin gene (*Retn*) transcript level and methylation profile in rats

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Abstract

Background: Adipose tissue is recognized as a highly active metabolic and endocrine organ. The hormones secreted by this tissue play an important role in many biochemical processes. It is known that dysfunction of adipocytes can cause insulin resistance, type 2 diabetes or hyperlipidemia. One of the important factors produced in fat tissue is resistin (Retn). It has been postulated that this hormone is involved in glucose homeostasis and insulin resistance. In the present study, the impact of five diet types (*ad libitum* normal, restricted, high-carbohydrate, high-fat and high-protein) on the *Retn* gene transcription and methylation profile was evaluated in rats of different ages.

Results: Transcript levels and methylation status of the *Retn* gene were studied in three tissues (muscle, subcutaneous and abdominal fat) in rats at 30, 60 and 120 days of age. We found an effect of tissue type on the *Retn* transcription in all diet types, as well as an effect of feeding type and age on the mRNA levels for high-fat and high-protein diets. The DNA methylation levels depended only on tissue type.

Conclusions: The obtained results demonstrate a tissue-specific expression pattern and a characteristic DNA methylation profile of the *Retn* gene in rats. *Retn* expression seems to be sensitive to nutritional changes, but only in the case of high-fat and high-protein diets. Moreover, an effect of age on *Retn* mRNA content was observed in these diets. Because no correlation between the transcript level and methylation status was found, we assumed that the transcription control of this gene by DNA methylation of the promoter seems to be unlikely.

Keywords: Resistin, Rat, Obesity, Diets, Gene expression, DNA methylation

Background

Obesity is a growing problem worldwide, which results in an increasing number of diagnosed type 2 diabetes characterized by insulin resistance. Adipose tissue is not only an energy reservoir, but also plays an important role as an endocrine organ. It secretes many adipokines, such as leptin, resistin (Retn), adiponectin, visfatin, apelin, and omentin, as well as sex steroids and various growth factors. Retn is cysteine-rich protein belonging to the RELMs (resistin-like molecules) family. The genetic structure of the *Retn* gene varies between mammals and the similarity in the coding sequence ranges from approximately 60 % for rodents to 80 % for livestock [1]. The expression of this gene in rodents occurs mostly in mature adipocytes, but it was also found in other tissues. Retn is considered a factor linking obesity and insulin resistance. In obesity, its expression increases, leading to enhanced resistance of tissues to insulin [2].

An interesting issue is how different factors, such as nutrition or age, can modulate expression of the *Retn* gene. It is known that diet type has an influence on *Retn* mRNA as well as serum levels in rodents. In mice during fasting the *Retn* mRNA in the adipose tissue is reduced, while upon refeeding transcription increases. Fasting also reduces circulating Retn concentrations. Conversely, in diet-induced obese mice serum Retn levels are enhanced, while transcription of the *Retn* gene is decreased (for a review see: [3]). It is also well documented that aging is associated with changes in gene expression levels in many tissues, including adipose tissue [4]. Moreover, it has been shown that aging leads to both global and local alterations in the DNA methylation profile [5].

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Bearing in mind the role of Retn in the development of obesity, many studies have been performed on animals fed with a high-fat diet (HFD) to serve as a model for human obesity. Such studies, conducted by Kim and Park [6], found that insulin resistance, dyslipidemia and fatty liver developed in rats with obesity induced by HFD. The authors analyzed the expression levels of different genes in adipose tissue and found that among many genes, *Retn*, *TNF*-α and *leptin* expression levels were upregulated 1.5-, 3.4- and 7.0-fold, respectively, in rats on a HFD when compared with the control group. Interesting studies concerning changes in *Retn* expression depending on the age of animals were performed by Oliver [7]. They analyzed site-specific *Retn* expression patterns from 4 different depots (epididymal, inguinal, mesenteric and retroperitoneal) of white adipose tissue (WAT) and brown adipose tissue (BAT), as well as circulating Retn levels. The analysis covered the period from suckling to 1 year of age. In general, it was noticed that in BAT and retroperitoneal WAT, *Retn* transcription levels were higher than in epididymal, inguinal, and mesenteric WAT depots. Moreover, at 2 months old the mRNA levels decreased in inguinal WAT and increased in epididymal and retroperitoneal WAT. These time-specific differences could be explained by the change of feeding from suckling to a chow diet. However, the *Retn* transcript levels in BAT were quite stable during the study period, while the circulating levels increased during development as a result of the increasing body fat content [7]. The results mentioned above suggest sensitivity of the *Retn* expression to nutritional changes.

So far, many studies concerning Retn in the rat focused only on protein levels and incomplete information about transcript levels of the gene is available. Also, most studies tested HFDs; knowledge about other feeding types is quite limited. Thus, the aim of this study was the comprehensive analysis of the impact of different diets and age on *Retn* transcript levels. Moreover, given the lack of data concerning the epigenetic mechanisms regulating its expression, we also analyzed the DNA methylation profile. The study was performed on two types of adipose tissue, as well as muscle tissues, in animals of different ages.

Methods

Ethics statement

Tissue sampling was carried out according to standard Polish veterinary protocols. All animal experiments were approved by the local Bioethical Commission for Animal Care and Use in Poznan, Poland (approval No: 7/2009).Treatment of animals was in accordance with the Arrive Guidelines for Reporting Animal Research (Additional File 1: ARRIVE Checklist).

Animals and diets

Male Wistar rats (180 ± 10 g in weight) from Lab Animal Breeding (Brwinow, Poland) were housed under standard conditions (22 ± 2 °C; 12-h light/dark cycles) with unlimited access to water. After 10 days of adaptation, the animals were assigned to 6 different groups (A–F) in terms of diet, divided in two separate experiments. In experiment 1, the animals received one of the following diets: group A = *ad libitum* normal diet Labofed B; group B = restricted diet (i.e., 75 % dose compared with group A); and group C = high-carbohydrate diet (i.e., normal diet + 40 % starch). In experiment 2, the animals received one of the follow diets: group D = *ad libitum* normal diet Labofed B; group E = high-fat diet (i.e., normal diet + 20 % triglycerides); and group F = high-protein diet (i.e., normal diet + 20 % soy protein). All animals were euthanized by decapitation. Tissue samples (muscle = MT, subcutaneous fat = ST, and abdominal fat = AFT) from each of the diet groups were collected at three time points (30, 60 and 120 days of age). Each diet*age group consisted of 8 animals.

Expression analysis

RNA was extracted in two independent technical replicates with the use of TriPure Isolation Reagent (Roche) according to a standard procedure. An aliquot of 2 ug of RNA was reversely transcribed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche). The semi-quantitative transcript level analysis was performed in duplicate using the Fast Start DNA Master^{Plus} SYBR Green I kit (Roche) on a capillary real-time PCR LightCycler 2.0 (Roche). The relative transcript level was calculated after correction via transcript levels of two reference genes (Hypoxanthine-guanine phosphoribosyltransferase [*Hprt*] and TATA box binding protein [*Tbp*]) using standard curves designed for all analyzed genes [8]. The primer sequences and amplicon lengths are shown in Table 1. Representative qPCR amplification and melting curves are presented in Additional file 2: Figure S1.

Methylation analysis and detection of CpG islands

The region 2 Kb upstream of the *Retn* gene was selected for CpG island searching with the use of the Cpgplot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/). Using standard criteria (Obs/Exp ratio > 0.6; min. C + G content 50 %; length min. 200 bp), no CpG islands were identified. Thus, we decided to reduce the minimum length of the island to 100 bp. A similar approach was applied in studies concerning the human *RETN* gene [9], where a small CpG island (162 bp) located upstream of the *RETN* gene was analyzed. Applying reduced size criteria, we identified a region of 140 bp as a potential CpG island located from −945 to −1193 bp upstream of the first exon of the rat *Retn* gene. The primers were designed

Table 1 PCR primer sequences, amplicon length and annealing temperature used in *Retn* gene analysis

Type of analysis	Gene	Primer sequence	Amplicon length	Anneal.temp.
Transcript level analysis	*Retn*	F: 5′ CCACGTACTTAACAGGATG 3′	195 bp	62 °C
		R: 5′ GAGGAGACTGACCAGCAAT 3′		
	Hprt	F: 5′ CAGTCAACGGGGGACATAAAAG 3′	146 bp	62 °C
		R: 5′ ATTTTGGGGCTGTACTGCTTGA 3′		
	Tbp	F: 5′ ATCCTTCACCAATGACTCCTATG 3′	190 bp	62 °C
		R: 5′ ATGATGACTGCAGCAAACC 3′		
Methylation analysis (converted sequence)	*Retn*	F: 5′ GTGGAAAGGAGGAATGTATTATTTG3′	249 bp	56 °C
		R: 5′ACAAATAAAAAAACTTTAATATTTATTCA3′		

Reference sequences for the *Retn* - GenBank: NC_005111.3; the *Hprt* - GenBank: S79292 and the *Tbp* – GenBank:NM_001004198

using MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The amplified fragment 249 bp in length overlapped 13 CG dinucleotides in the CpG island (Fig. 1). The primer sequences are shown in Table 1. DNA was extracted using a standard protocol with the phenol:chloroform:isoamyl alcohol acid mixture (25:24:1, Sigma). The quality and quantity of isolates were controlled using the Nanodrop. Using the EZ DNA Methylation kit (ZymoResearch), 1 ug of DNA was bisulfite converted. After conversion, the touch-down amplification was performed (annealing temperature reduced from 60° to 56 °C every first 8 cycles, the following 32 cycles at 56 °C). PCR products were cloned into the pGEM T-Easy vector (Promega) following the transformation of DH5α competent cells (Invitrogen) according to the supplier's protocol. Transformed cells were harvested on agar plates (37 °C, overnight) with X-Gal, IPTG and ampicillin. For each sample, 12–16 white colonies were selected and amplified overnight with the Illustra Templi-Phi Amplification Kit (GE Healthcare). Afterwards, the clones were sequenced using the Big Dye Terminator v1.1 Sequencing kit (Life Technologies) on the Genetic Analyzer 3130 (Applied Biosystems). The methylation level was calculated as a mean for at least 8 clones with the use of QUMA software (http://quma.cdb.riken.jp/).

Statistical analysis

Associations between diets and transcript levels, as well as diets and methylation levels, were evaluated using linear fixed models. The models for both experiments were identical. We started from long models the included the tissue, age (as a factor), diet and three two-way interactions between these variables as fixed factors. Because in experiment 1 we obtained only one significant effect of interaction (diet-by-age; $P = 0.028$) and for experiment 2 we did not calculate any significant effects of

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                           GTGGAAAGGAGGAATGCACCACCTGAATCCACT
                           ||||||||||||||||:|::|::|||||::|:|
                           GTGGAAAGGAGGAATGTATTATTTGAATTTATT

TAACGCTGAAGAGGAAAGGCAAATCTCGGGCAGGGGCTGCGGGTCAGCAAGCTCTAGCGC
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TAACGTTGAAGAGGAAAGGTAAATTTCGGGTAGGGGTTGCGGGTTAGTAAGTTTTAGCGT

ATGCGCGGAGCAGTGCATCCCTGCCATCCCGGCGTGGAGAACGCAGGAGGGTCAGGCGCT
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ATGCGCGGAGTAGTGTATTTTTGTTATTTCGGCGTGGAGAACGTAGGAGGGTTAGGCGTT

TAAGGTCATTCTTGGCTACACAGTTCAAACTTGCGTAGGCTGCGTAAGAGCTCGGATTTA
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TAAGGTTATTTTTGGTTATATAGTTTAAATTTGCGTAGGTTGCGTAAGAGTTCGGATTTA

AATAAATGAATAAATATTAAAGTCTTCTCACTTGTC
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AATAAATGAATAAATATTAAAGTTTTTTTATTTGTT
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Fig. 1 The fragment of 249 bp in the 5′ flanking region of the *Retn* gene analyzed in DNA methylation studies (upper line: sequence before BS conversion; lower line: sequence after BS conversion; underlined: primer sequence; in the box: CG dinucleotides)

interactions, we excluded all interactions effects from the models. To obtain a normal distribution of residuals in an ANOVA, the data concerning the transcript level were logarithmically transformed. Normality of the distribution was tested by the Kolmogorov–Smirnov test.

The linear relationship between transcript (in log scale) and methylation levels was expressed as Pearson's correlation after adjusting for fixed effects. To adjust for fixed effects, both traits were first analyzed in the linear models described above and residuals were extracted for correlation calculation. All statistical calculations and plots were performed using R software v3.1.1 (R Development Core Team).

Results and discussion

Retn gene transcript levels in restricted and high-carbohydrate diets versus control

The relative transcript level of the *Retn* was analyzed in three tissues (MT, ST and AFT) and the highest concentration was found in both fat tissues, while in MT it was almost a hundred times lower. In experiment 1, in which three feeding types were compared [normal (A), restricted (B) and high-carbohydrate (C) diets], the statistical analysis showed significant differences in transcript levels depending on tissue type ($P = 1.7 \times 10^{-13}$). There was no effect of the diet type ($P = 0.129$) or age of the animals ($P = 0.142$). However, in the restricted diet group an increase in transcription was noticed in AFT and MT at 60 days of age. This is in agreement with previous findings described by Oliver [6], in which an increase of *Retn* mRNA levels (in 2-month-old rats) was found in epididymal and retroperitoneal WAT. Those authors observed similar results also for other genes such as *Leptin* or *UPC2* for different depots of WAT. They explained that this type of reaction could have been caused by the feeding system change (from milk to chow diet). The impact of food restriction versus an *ad libitum* diet on the mRNA levels of the *Retn* gene in three age groups of rats (3, 8 and 24 months old) was analyzed by Fernandez [10]. In *ad libitum* fed animals, they found a significant reduction of mRNA levels in epididymal visceral adipose tissue (VAT) in 8- and 24-month-old rats when compared with 3-month-old animals, while there was no effect of age in retroperitoneal VAT. Conversely, food restriction resulted in a greater decrease of *Retn* transcript contents in 8- than 3-month-old animals in both analyzed VAT depots, while in 24-month-old rats the mRNA levels were much higher than in 8-month-old ones. Food restriction in terms of the *Retn* and *adiponectin* expression in VAT and ST adipose tissue was also studied by Milan [11]. They compared lean and obese (Zucker) rats. The authors observed a significant reduction of adiponectin and Retn expression levels only in VAT of obese rats when compared with lean ones. After food restriction, the adiponectin expression levels were restored to the normal, in contrast with Retn, for which levels decreased continuously.

In the high-carbohydrate diet, we noticed a decrease of the transcript levels in ST and MT depending on the age of the animal, in contrast with AFT fat where it increased; however, the observed trends were not significant (data not shown). This type of diet was also analyzed by Stroubini [12], who investigated serum Retn concentrations. These authors found that a high-carbohydrate diet reduced circulating levels of Retn when compared with high-fat and high-protein feeding. Unfortunately, they did not test the tissue *Retn* transcript levels; thus, it is hard to compare their results with our observations. A high-sucrose diet was studied by Polson and Thompson [13]; however, the authors did not notice any significant effects of this type of diet on mRNA levels of *Retn* in WAT, which is in agreement with our results.

Retn gene transcript levels in high-fat and high-protein diets versus control

In experiment 2, we tested normal (D), high-fat (E) and high-protein (F) diets and the statistical analysis showed significant differences in transcript levels in terms of tissue type ($P = 5.8 \times 10^{-12}$), diet ($P = 0.030$) and age of the animals ($P = 0.048$). In detail, in group E (high-fat), *Retn* transcript levels decreased with the age of the animals in all analyzed tissues. In the high-protein diet (F) group, mRNA levels increased in ST and decreased in AFT depending on the age of the animals (Fig. 2). When comparing diets from experiment 2, we noticed that in all the tissues the highest mRNA levels of *Retn* were observed in the high-protein diet group (E) in contrast with the high-fat diet, for which transcription was lower than in the other feeding regimens, especially over the long-term (Fig. 3). Similar diets were applied by Stroubini [12], who tested three diets for 13 weeks in rats: high fat (HFD), high carbohydrate (HCD) and high protein

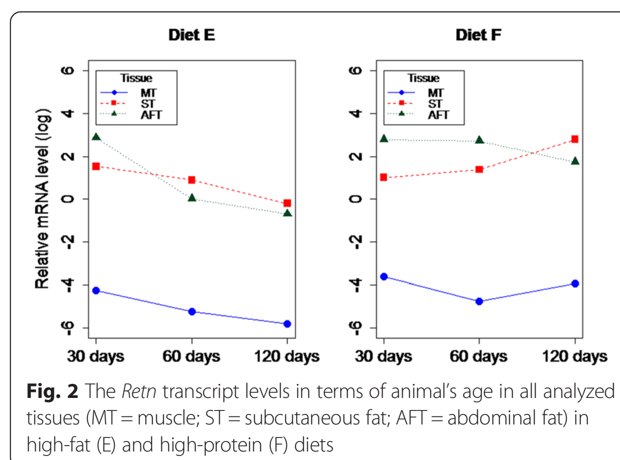

Fig. 2 The *Retn* transcript levels in terms of animal's age in all analyzed tissues (MT = muscle; ST = subcutaneous fat; AFT = abdominal fat) in high-fat (E) and high-protein (F) diets

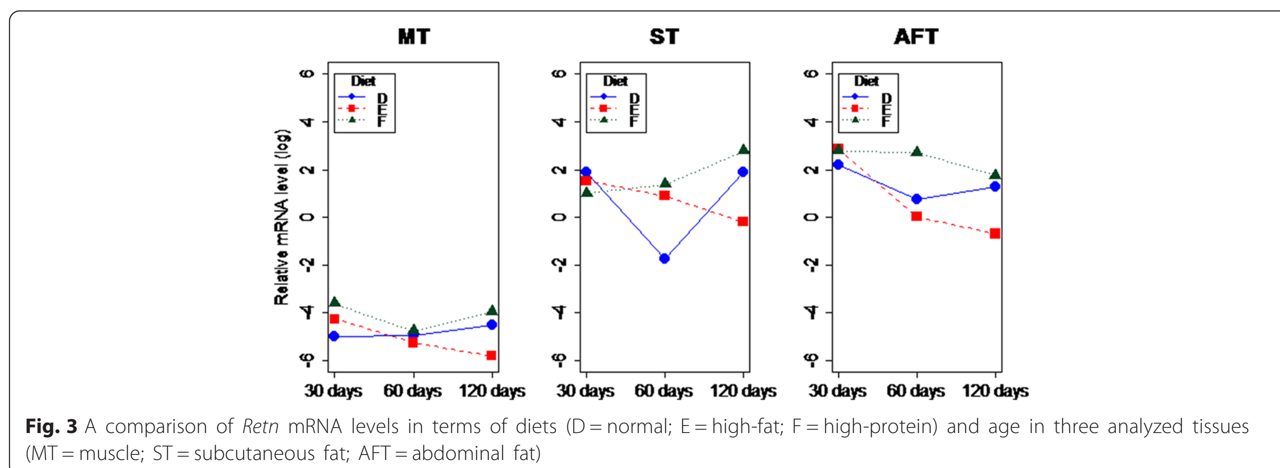

Fig. 3 A comparison of *Retn* mRNA levels in terms of diets (D = normal; E = high-fat; F = high-protein) and age in three analyzed tissues (MT = muscle; ST = subcutaneous fat; AFT = abdominal fat)

(HPD) diets. Moreover, after 10 weeks of dietary manipulation, the animals received sibutramine, a drug usually administered in humans with exogenous obesity, at two different doses. The authors measured body weight and the fat/lean ratio, as well as serum adiponectin and Retn concentrations. They found that a HFD elevated body weight and the fat/lean ratio; however, after drug treatment at the higher dose the fat/lean ratio was reduced in the HCD and HPD groups only, probably as an effect of appetite suppression. Serum adiponectin levels were higher after sibutramine administration in the HFD group compared with the HCD and HPD groups, while Retn concentrations were not altered by drug treatment in any diet type. No tissue *Retn* transcript level analysis was undertaken in that study [12], so we are not able to compare the results with our data.

The effects of a HFD (cafeteria diet, CAF) was analyzed by Ribot [14], who measured serum as well as tissue adiponectin and Retn levels in rats of both sexes. mRNA levels were analyzed in different WAT depots: gonadal, retroperitoneal (visceral) and inguinal (subcutaneous). It was found that the CAF diet increased energy intake and weight of adipose tissue (more significantly in females). Moreover, the effective adiponectin and Retn production (the parameter calculated as a serum level of both adipokines divided by the total weight of WAT depots) was decreased in the CAF group. In three WAT depots, there was no difference in adiponectin mRNA levels in response to the CAF diet. In agreement with our findings, the *Retn* transcript levels were reduced in CAF-fed male and female rats in gonadal WAT depots. A similar trend (although non-significant) was also observed for females in retroperitoneal and inguinal WAT, suggesting sex specific differences [14].

Methylation analysis of the Retn *gene*

We also performed methylation analysis for diet*age samples in three studied tissues and found that methylation status was dependent on tissue type ($P = 0.0057$). The lowest level of methylation was observed in MT, while it was highest in AFT (Figs. 4 and 5). We did not notice any effect of diet ($P = 0.363$) or age of the animals ($P = 0.829$) on methylation levels. Moreover, no correlation was found between the transcript level and methylation ($r_g = 0.027$). There is an increasing amount of data that environmental factors may alter gene expression profiles through epigenetic mechanisms [15]. However, it seems that the impact of such factors on the epigenome depends on developmental stages. It is widely accepted that the gestational period is especially susceptible to epigenetic modifications. Many studies have focused on the role of early life nutrition on the modulation of DNA methylation [16]. It has been

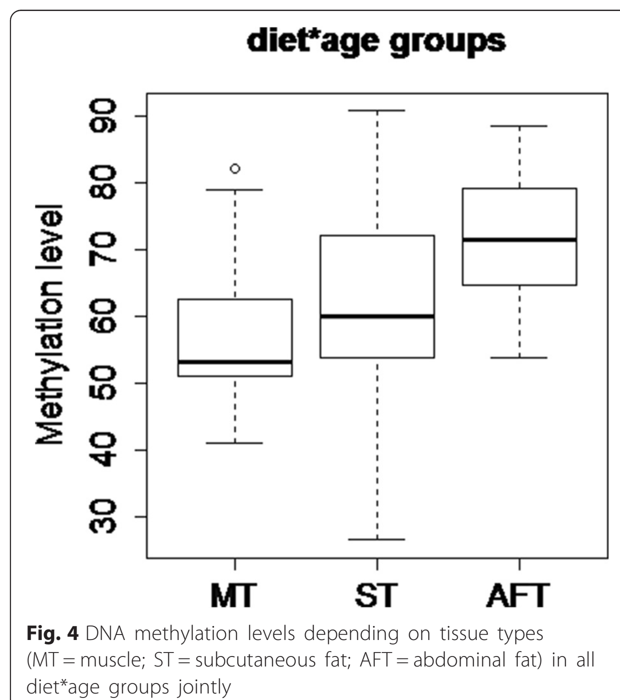

Fig. 4 DNA methylation levels depending on tissue types (MT = muscle; ST = subcutaneous fat; AFT = abdominal fat) in all diet*age groups jointly

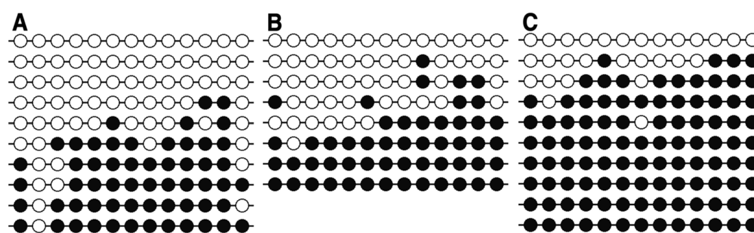

Fig. 5 DNA methylation analysis in the F diet (high-protein) at 60 days of age (% of methylation: **a** 44.6 % in muscle; **b** 51 % in subcutaneous fat; **c** 78.5 % in abdominal fat); black dot = cytosine methylated, white dot = cytosine unmethylated

found that dietary components (e.g., folate, vitamin B6 and B12, methionine), which affect the amount of the universal methyl donor S-adenosylmethionine (SAM), can change the methylation profile of particular genes and have long-term effects on the epigenome of the offspring [17]. Conversely, environmental factors acting postnatally are restricted only to adult cells or, in case of adult stem cells, to a specific tissue. Although the epigenome of fully differentiated cells is more stable and not very susceptible to environmental signals, there are reports showing that postnatal diets also influence DNA methylation. For example, a methyl donor–deficient diet post-weaning affected the methylation status of the *Igf2* gene in mice [18] or mice fed a HFD from weaning has shown a significant increase in DNA methylation in the promoter of the μ-opioid receptor [19]. In addition, application of genome-wide DNA methylation methods allowed the detection of changes in the methylation profile of adipose tissue after intervention, such as exercise [20] or weight loss following gastric bypass [21].

Interesting studies including epigenetic factors were undertaken by Satoor [22]. Firstly, they analyzed the transcript levels of different adipokines in 8 adipose fat depots and observed that mRNA concentrations of *adiponectin*, *leptin*, *Retn* and *visfatine* genes were 10,000 to a million-fold lower in VAT than in ST. Thus, the authors performed an autologous transplantation of VAT into peripheral (ST) sites to determine the metabolic effects. After transplantation into thigh or chest regions, the transcription levels of four studied genes increased in the VAT grafts. To verify if the observed changes were an effect of higher expression or lower mRNA degradation, the authors performed ChIP analysis to check the histone-methylation antibodies to active (H3K4) or inactive (H3K9) marks of lysine methylation. They found that promoter regions of the studied genes acquired methylation at H3K4me2 after transplantation. This region recruits less LSD1 and/or KMT1a enzymes, known as regulators of chromatin compaction and transcription repressors, causing an increase of transcription in grafts when compared with the original depots. Unfortunately, the authors did not determine the DNA methylation profiles [22]. In our studies, we did not analyze histone modifications; however, the regulation of *Retn* transcription through this mechanism could be possible. Because the *Retn* locus is poor in CG rich regions, we have analyzed only a small fragment upstream of the gene. However, it cannot be excluded that other CpG islands located near or within the *Retn* gene may also influence expression via other than classic promoter regulation mechanisms. Based on the Genome Browser Database (https://genome.ucsc.edu/index.html) for humans, it has been shown that a CpG island of the *RETN* is located within the gene body. Our analysis of the corresponding region in the rat genome did not indicate such a sequence. It can be expected that the availability of more accurate databases for the rat will allow the identification of other potential CpG islands in the *Retn* locus.

Conclusions

Based on the obtained results, we can conclude that tissue type has the highest significance for *Retn* transcript levels. Diet- and age-induced alternations in transcript levels were observed in HFD and HPD. However, no influence of diet on DNA methylation and no correlation of methylation with transcript levels were observed. Thus, we assume that transcription regulation of the rat *Retn* gene by DNA methylation of its promoter is doubtful. However, it cannot be excluded that other CpG islands are involved in *Retn* regulation; however, the lack of information concerning their location makes such analysis difficult. Also, other epigenetic processes, including histone modifications, should be taken into consideration.

Additional files

Additional file 1: ARRIVE checklist. (PDF 369 kb)

Additional file 2: Figure S1. Representative qPCR amplification curves of serial dilutions and melting curves of the *Retn* gene (A and B) as well as two reference genes: *Hprt* (C and D) and *Tbp* (E and F). (BMP 5071 kb)

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JNW carried out the molecular analysis (transcription experiments and DNA methylation analysis) and drafted the manuscript; EPO coordinated the

animal feeding experiments and collected the tissues; MS and SS performed the statistical analysis; IS carried out the molecular analysis, transcriptional experiments and drafted the manuscript. All authors read and approved the final manuscript.

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