Complimentary and personal copy for

www.thieme.com

This electronic reprint is provided for non-commercial and personal use only: this reprint may be forwarded to individual colleagues or may be used on the author's homepage. This reprint is not provided for distribution in repositories, including social and scientific networks and platforms.

Publishing House and Copyright:

Georg Thieme Verlag KG
Postbox 30 11 20
70451 Stuttgart, Germany
ISSN

All rights are reserved by the publisher

Nicotinamide Nucleotide Transhydrogenase (*Nnt*) is Related to Obesity in Mice

Authors
Anne Kunath[1, 2], John T. Heiker[3], Matthias Kern[2], Joanna Kosacka[2], Gesine Flehmig[2], Michael Stumvoll[2], Peter Kovacs[4], Matthias Blüher[2, 3], Nora Klöting[1, 2, 3]

Affiliations
1 IFB Adiposity Diseases, University of Leipzig, Leipzig, Germany
2 Department of Medicine, Endocrinology and Diabetes, University of Leipzig, Leipzig, Germany
3 Helmholtz Institute for Metabolic, Obesity and Vascular Research (HI-MAG) of the Helmholtz Zentrum München at the University of Leipzig and University Hospital Leipzig, University of Leipzig, Leipzig, Germany
4 Interdisciplinary Center for Clinical Research, University of Leipzig, Leipzig, Germany

Key words
diet-induced obesity, genetic background, visceral fat mass, C57BL/6, Nnt, DIO

received 16.04.2020
accepted 09.06.2020

Bibliography
DOI https://doi.org/10.1055/a-1199-2257
Published online: 2020
Horm Metab Res
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 0018-5043

Correspondence
Nora Klöting
Helmholtz Institute for Metabolic, Obesity and Vascular Research (HI-MAG) of the Helmholtz Zentrum München at the University of Leipzig and University Hospital Leipzig
University of Leipzig
04103 Leipzig
Germany
Tel.: + 49 341 9713401, Fax: + 49 341 9713409
nora.kloeting@medizin.uni-leipzig.de
nora.kloeting@helmholtz-muenchen.de

ABSTRACT

The C57BL/6J (B6J) mouse strain has been widely used as a control strain for the study of metabolic diseases and diet induced obesity (DIO). B6J mice carry a spontaneous deletion mutation in the nicotinamide nucleotide transhydrogenase (*Nnt*) gene eliminating exons 7–11, resulting in expression of a truncated form of *Nnt*, an enzyme that pumps protons across the inner mitochondrial membrane. It has been proposed that this mutation in B6J mice is associated with epigonadal fat mass and altered sensitivity to diet induced obesity. To define the role of *Nnt* in the development of diet induced obesity, we generated first backcross (BC1) hybrids of wild type *Nnt* C57BL/6NTac and mutated *Nnt* C57BL/6JRj [(C57BL/6NTac × C57BL/6JRj) F1 × C57BL/6NTac]. Body weight gain and specific fat-pad depot mass were measured in BC1 hybrids under high fat diet conditions. Both sexes of BC1 hybrids indicate that mice with *Nnt* wild type allele are highly sensitive to DIO and exhibit higher relative fat mass. In summary, our data indicate that the *Nnt* mutation in mice is associated with sensitivity to DIO and fat mass.

Electronic reprint for personal use

Introduction

Experimental animal models offer a great opportunity to overcome heterogeneity and various environmental factors influencing obesity and associated metabolic disorders. C57BL/6J (B6J) is the single most widely used inbred strain and its genome has been extraordinarily well categorized with the most complete sequence data available produced by the Mouse Genome Sequencing Consortium [1]. The strain has been widely used as a control strain in the study of metabolic diseases or diet induced obesity (DIO) as well as background strain for transgenic and knockout mice [2, 3].

Noteworthy, there are genetic and phenotypic differences among C57BL/6 substrains [2, 4, 5]. The most prominent genetic variation between C57BL/6J and C57BL/6NTac mice concerns the nicotinamide nucleotide transhydrogenase (*Nnt*) gene on chromosome 13 [6, 7]. Eukaryotic *Nnt* functions as a homodimeric redox-driven proton pump, catalysing the reversible reduction of $NADP^+$ by NADH and subsequent conversion of NADH into NAD^+ [8, 9]. Each subunit is composed of three principal domains: the first and third domains lie within the mitochondrial matrix and contain the NAD(H)- and NADP(H)-binding domains, respectively. The second consists of 14 transmembrane-spanning helices and harbours the proton-conducting pore [8] . In the B6J strain, a missense (methionine to threonine) mutation, in combination with the in-frame 5 exon deletion mutation (eliminating four putative transmembrane helices) results in a consequently truncated variant and markedly lower *Nnt* protein expression in liver and islets [6, 7]. It has been suggested that the reduced *Nnt* expression affects ATP production and thus impairs glucose stimulated insulin release [7, 10]. Concerning this matter, a recent study provided evidence that regardless of truncation, *Nnt* has little influence on insulin secretion under normal or low *Nnt* expression levels [11]. Also, they could demonstrate the loss of function character of the deletion mutant *Nnt* and that very likely other enzymes are able to compensate for reduced $NADP^+$ reduction activity considering normal *Nnt* expression levels [11]. Nonetheless, high level expression of native *Nnt* could significantly affect and enhance insulin secretion [11]. Furthermore, studies on high fat diet induced obesity have revealed differences between core substrains of C57BL/6, wild type *Nnt* expressing C57BL/6NTac and *Nnt*-mutant C57BL/6J regarding the ability to respond to HFD and develop DIO reflected by body weight gain and whole body fat mass [12]. Human NNT expression data from paired fat depot samples suggest a role of NNT in the development of obesity and fat distribution [13]. The C57BL/6JRj strain recently was found to be protected against DIO independent from physical activity and food intake [14].

To examine and assess the impact of *Nnt* mutation on the responsiveness to high fat diet induced obesity we genetically and phenotypically studied first backcross (BC1) hybrids of wild type *Nnt* expressing C57BL/6NTac and *Nnt*-mutant C57BL/6JRj mice [(C57BL/6NTac × C57BL/6JRj)F1 × C57BL/6NTac].

Materials and Methods

Animals and phenotyping

C57BL/6NTac mice were purchased from Taconic Farms, Inc. (Hudson, New York, USA) and were crossed with C57BL/6JRj from Janvier (Le Genest Saint Isle, France). The (C57BL/6NTacxC57BL/6JRj)F1 hybrids were backcrossed onto C57BL/6NTac to generate first backcross hybrids (BC1). There is abundant evidence that the maternal environment and phenotype can have an impact on offspring metabolism. To avoid the potential impact of maternally contributed mitochondria to the offspring, we used both F1 males and females to produce BC1 mice. F1 hybrids (n = 20 Female/Male 10/10) and all backcross hybrids (n = 190; Females: n = 93, Males: n = 97) were fed a sucrose chow containing 58 % fat in total calories (Ssniff, Soest, Germany) for 10 weeks beginning at 4 weeks of age. Animals were kept in groups of 5 in Macrolon cages (Size 2, Ehret GmbH, Emmerdingen Germany) and had free access to food and water. Body weight was recorded weekly and at the end of observation period liver weight, visceral fat mass, subcutaneous fat mass and HbA1c (%) were measured. The organs were weighed related to whole body mass to obtain relative organ weights.

All experiments were conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and were approved by the local authorities (Landesdirektion Leipzig, Approv. No. TVV16/08, T07/16) of the state of Saxony, Germany, as recommended by the responsible local animal ethics review board.

Nnt allele genotyping

Genomic DNA was extracted from tail tips using the DNeasy kit (Qiagen, Hilden, Germany). The *Nnt* allele was PCR genotyped using a three primer, two allele PCR assay that discriminates between *Nnt* wild type and truncated allele (lacking exon 7–11) as described [10, 12]. The primer sequences are *Nnt*-WT (5′-GGGCATAGGAAGCAA ATACCAAGTTG-3′) and *Nnt*-MUT (5′-GTGGAATTCCGCTGAGAGAACTCTT-3′) and *Nnt*-WT/Mut (5′-GTAGGGCCAACTG TTTCTGCATGA-3′), The "WT/Mut" primer participates in amplification of both alleles, while the "WT" and "MUT" primers are specific to the wild type and mutant alleles respectively. The amplification products are 579 bp for the wild type allele and 743 bp for the mutant allele. Primers with heterozygous mutant *Nnt* template produce an additional faint product at 1 kb that can assist in assignment of genotype [10]. Amplification conditions used were initial melt 95 °C, 5 minutes; then 35 cycles of 95 °C, 45 seconds, 58 °C, 30 seconds, 72 °C, 45 seconds; followed by a final extension of 5 minutes at 72 °C. Products were analyzed by electrophoresis through a 1 % agarose, 1 × TBE gel followed by staining in GelRed and visualized using a UV light box.

RNA isolation and quantitative real-time PCR analysis

RNA isolation and quantitative real-time PCR was performed as previously described [15]. In brief mRNA expression was measured in a fluorescence temperature cycler using the Taq Man assay; fluorescence was detected on an ABI PRISM 7000 sequence detector (Applied Biosystems, Darmstadt, Germany). Total RNA was isolated using Trizol (Life Technologies, Grand Island, NY, USA), and 1 µg RNA was reverse transcribed with standard reagents (Life Technologies). From each RT-PCR, 1 µl was amplified in a 20 µl PCR reaction using Power Sybr Green Master Mix (ABI, Foster City, CA USA) according to the manufacturer's protocol. Samples were incubated in the sequence detector for an initial denaturation at 95 °C for 10 minutes, followed by 40 PCR cycles, each cycle consisting of 95 °C for 15 seconds, 60 °C for 30 seconds and 72 °C for 32 seconds. *Nnt* primer sequences are forward CAATAGCTCAGGGCTATGATGCT and backward GCTGTGATCTGCCCAGTGAAA. Specific mRNA expression was calculated relative to *36B4*, which was used as an internal control due to its resistance to glucose-dependent regulation [16]. Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR. Expression levels are determined as follows: expression level = (copy number geneX per µl cDNA)/(copy number 36B4 per µl cDNA) and relativized to con-

Electronic reprint for personal use

trol cell results. All values are expressed as mean ± SD if not indicated otherwise.

Data analysis and statistics

Data are given means ± SD. Datasets were analyzed for statistical significance using a two-tailed unpaired *t*-test, or differences were assessed by one-way ANOVA using the Statistical Package for Social Science, version 20.0 (SPSS, Chicago, IL, USA). p-Values < 0.05 were considered significant.

Results

Backcross and F1 hybrids - frequency and phenotype

As shown in ▶ **Fig. 1a**, F1 hybrids offspring showed intermediate responses to diet. As expected, first backcross animals carrying the *Nnt* mutation were obtained with the expected Mendelian frequency of 50 percent.

Phenotyping in all BC1 hybrids indicated a significant association between *Nnt* mutation and body weight gain, relative epigonadal and subcutaneous (SC) fat-pad weight. Under a high fat diet *Nnt*-mutant female and male mice exhibited a less pronounced body weight gain (▶ **Fig. 2a** and **b**), as well as significantly lower relative epigonadal (▶ **Fig. 2c** and **d**) and SC fat mass (▶ **Fig. 2e** and **f**) than mice carrying the wild type allele. There were no differences in serum concentrations of adiponectin, insulin and leptin between both *Nnt* variants (▶ **Table 1**). Taking this into account, the presence or absence of the *Nnt* mutation did not have any effect on serum parameters of glucose homeostasis and insulin sensitivity.

Gene expression analysis

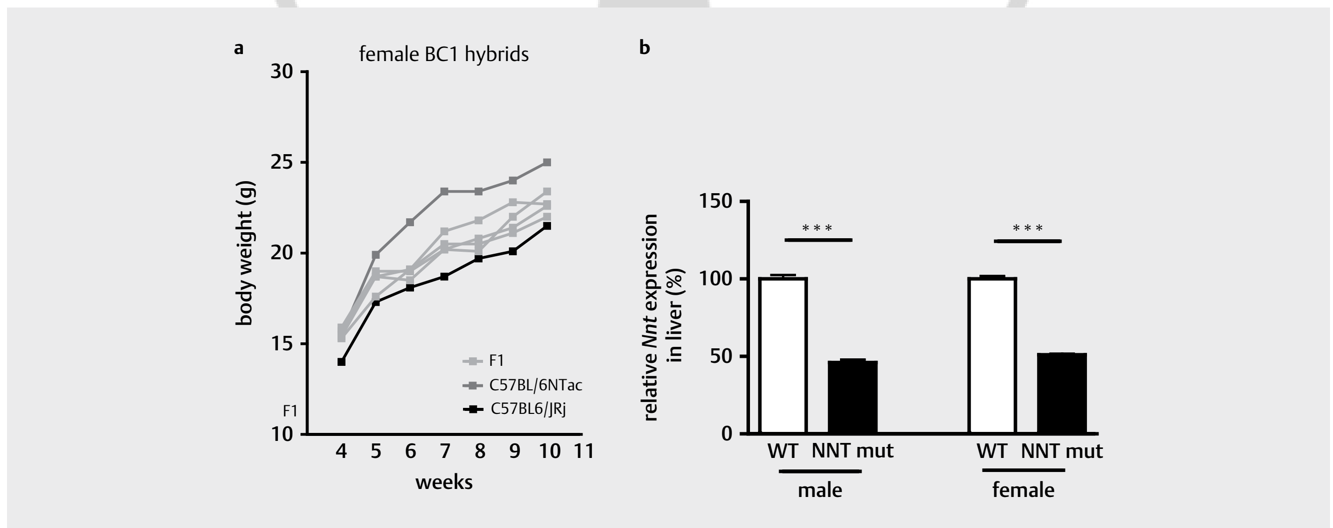
To elucidate if the genotype affects *Nnt* mRNA levels we detected an association between genotype and expression levels for *Nnt* gene (▶ **Fig. 1b**). In BC1 animals carrying the *Nnt* mutation *Nnt* expression was reduced by about 50 % in liver samples (▶ **Fig. 1b**).

Discussion and Conclusion

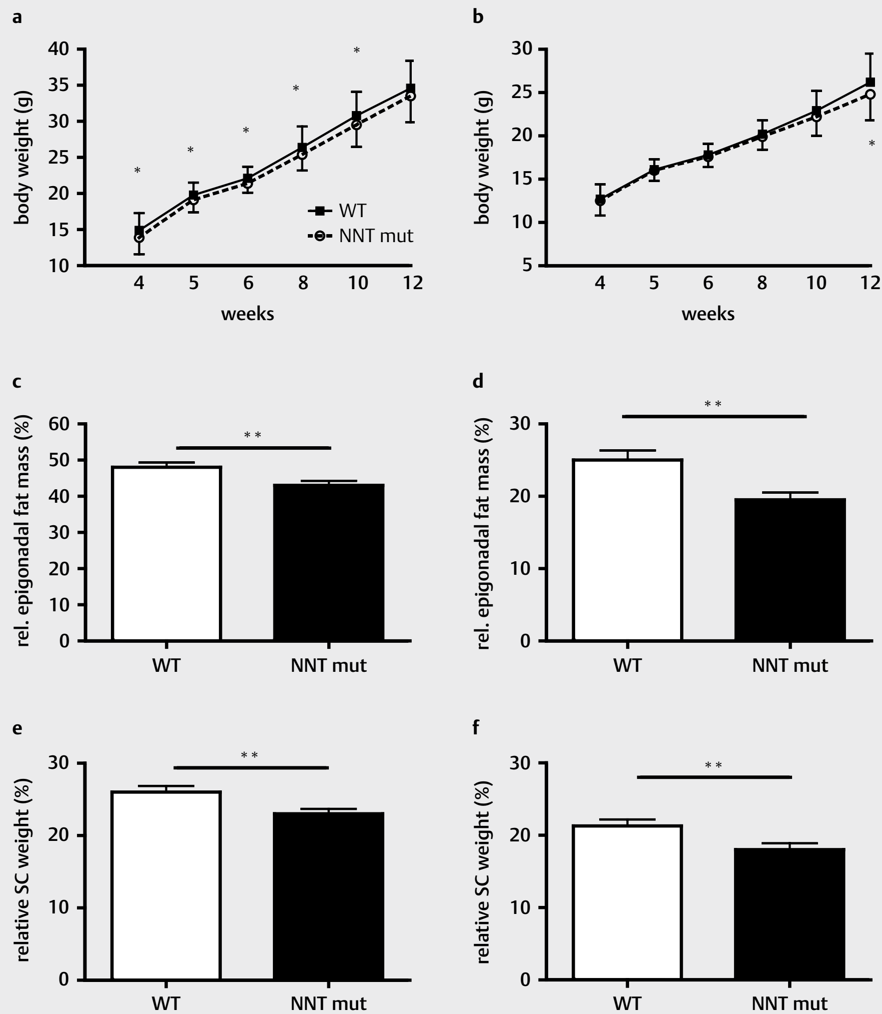
The present backcross study demonstrates that *Nnt* genotype variants are related to responsiveness to DIO independently from sex in mice. The *Nnt* wild type allele contributes to DIO sensitivity with markedly greater body weight gain. This is in accordance with the study of Nicholson et al. [12]. While they found both strains responsive to DIO, *Nnt* wild type carrying male B6NJ mice (Jackson Laboratory) developed greater adiposity than *Nnt*-mutant male B6J mice under a 10 % fat diet. Since Nicholson et al. applied dual emission X-ray absorptiometry (DEXA), to determine whole body fat mass, the individual contribution of fat depots was not differentiable [12]. Here, we show that greater adiposity in BC1 hybrids carrying the intact *Nnt* wild type allele is based on increased SC as well as epigonadal fat-pad depot masses independently from sex.

In 2005, two mutations were identified in the coding region of the mouse *Nnt* gene [7]. The first being a missense (methionine to threonine) mutation in the mitochondrial leader sequence of the precursor protein (exon 1) and the second an in-frame 5-exon deletion removing 4 predicted transmembrane helices and connecting linkers [7]. Both mutations result in decreased *Nnt* protein expression in liver and islets which is thought to affect ATP production and thus, impair glucose stimulated insulin release [7, 10]. In accordance to that, we detected reduced *Nnt* mRNA level in livers of BC1 hybrids carrying *Nnt* mutation. But our data does not support this proposed mechanism, as backcross hybrids reveal no association between metabolic parameters of glucose homeostasis, insulin sensitivity and *Nnt* genotype. There was no correlation between serum concentrations of insulin, adiponectin nor percent of HbA1c and *Nnt* variant in mice. Although in vitro, Freeman et al. demonstrated that *Nnt* plays an important role in the regulation of insulin secretion from pancreatic beta cells [10]. In accordance with our findings, a recent study compared wild type *Nnt* B6N and *Nnt*-mutant B6J mice concerning mutant *Nnt* associated reduced insulin secretion and glucose intolerance, but found that truncated *Nnt* did not affect insulin secretion and glucose tolerance on the

Electronic reprint for personal use



▶ **Fig. 1** **a** The intermediate body weight gain showing individual female first backcross hybrid (BC1) curves and the relative *Nnt* expression; **b** in the liver of the F1 hybrids and wild-type (WT) animals. Bars represent mean ± SD. ***p < 0.001.



► **Fig. 2** Backcross hybrid phenotype. Body weight gain of males **a**; Body weight gain of females **b**; Relative epigonadal fat mass of males **c**; Relative epigonadal fat mass of females **d**; Relative subcutaneous fat mass of males **e**; and Relative subcutaneous fat mass of females **f**. Bars represent mean ± SD. *p < 0.05; **p < 0.01.

► **Table 1** Metabolic parameters of wild-type and BC1 hybrids.

	Female		**Male**	
	Wild-type n = 46	***BC1*** **n = 47**	**Wild-type n = 50**	***BC1*** **n = 47**
HbA1c (mmol/mol)	19.1 ± 0.4	19.1 ± 0.4	23.5 ± 0.5	23.5 ± 0.5
Leptin (ng/ml)	8.3 ± 5.4	6.8 ± 4.5	15.5 ± 6.9	14.9 ± 6.9
Insulin (ng/ml)	0.69 ± 0.37	0.66 ± 0.46	1.1 ± 0.6	1.1 ± 0.7
Adiponectin (µg/ml)	151 ± 77	124 ± 77	264 ± 15	270 ± 16

Data are means ± SD.

B6J background [11]. In addition, Hull and co-workers performed a comparison of insulin secretory responses, in vitro and in vivo, among different C57BL/6J substrains and four commonly used C57BL/6N substrains. In vitro, they found that islets from C57BL/6N substrains secrete significantly more insulin in response to glucose than islets from C57BL/6J substrains. In contrast, in vivo insulin re-

Electronic reprint for personal use

sponses to glucose showed no differences among any of the six substrains [17].

The presented study here shows that decreased *Nnt* level, as a result of *Nnt* mutation, causes a different sensitivity to diets. From siRNA silencing studies it is known that *Nnt* suppression by siRNA knockdown experiments results in decline of NADPH production and an oxidized cellular redox status, and a less negative redox potential [16]. Furthermore, a decrease in NADPH supply in *Nnt* -knockdown cells results in an augmented H_2O_2 release, which induces mitochondrion dependent intrinsic apoptosis and results in decreased cell viability [18]. Therefore a possible mechanism in adipose tissue could be an activation of redox-sensitive signaling by H_2O_2 which induces mitochondrion dependent intrinsic apoptosis resulting in reduced adipocyte viability. In conclusion, the *Nnt* mutation is related to the extent of obesity in mice.

Funding Information

Deutsche Forschungsgemeinschaft: SFB1052 B01 (MB) B04 (NK).

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Mural RJ, Adams MD, Myers EW et al. A comparison of whole-genome shotgun-derived mouse chromosome 16 and the human genome. Science 2002; 296: 1661–1671

[2] Mekada K, Abe K, Murakami A et al. Genetic differences among C57BL/6 substrains. Exp Anim 2009; 58: 141–149

[3] Collins S, Martin TL, Surwit RS et al. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiol Behav 2004; 81: 243–248

[4] Petkov PM, Ding Y, Cassell MA et al. An efficient SNP system for mouse genome scanning and elucidating strain relationships. Genome Res 2004; 14: 1806–1811

[5] Zurita E, Chagoyen M, Cantero M et al. Genetic polymorphisms among C57BL/6 mouse inbred strains. Transgenic Res 2011; 20: 481–489

[6] Aston-Mourney K, Wong N, Kebede M et al. Increased nicotinamide nucleotide transhydrogenase levels predispose to insulin hypersecretion in a mouse strain susceptible to diabetes. Diabetologia 2007; 50: 2476–2485

[7] Toye AA, Lippiat JD, Proks P et al. A genetic and physiological study of impaired glucose homeostasis control in C57BL/6J mice. Diabetologia 2005; 48: 675–686

[8] Hoek JB, Rydström J. Physiological roles of nicotinamide nucleotide transhydrogenase. Biochem J 1988; 254: 1–10

[9] Rydström J. Mitochondrial transhydrogenase–a key enzyme in insulin secretion and, potentially, diabetes. Trends Biochem Sci 2006; 31: 355–358

[10] Freeman HC, Hugill A, Dear NT et al. Deletion of nicotinamide nucleotide transhydrogenase: A new quantitive trait locus accounting for glucose intolerance in C57BL/6J mice. Diabetes 2006; 55: 2153–2156

[11] Wong N, Blair AR, Morahan G et al. The deletion variant of nicotinamide nucleotide transhydrogenase (Nnt) does not affect insulin secretion or glucose tolerance. Endocrinology 2010; 151: 96–102

[12] Nicholson A, Reifsnyder PC, Malcolm RD et al. Diet-induced obesity in two C57BL/6 substrains with intact or mutant nicotinamide nucleotide transhydrogenase (Nnt) gene. Obesity (Silver Spring) 2010; 18: 1902–1905

[13] Heiker JT, Kern M, Kosacka J et al. Nicotinamide nucleotide transhydrogenase mRNA expression is related to human obesity. Obesity (Silver Spring) 2013; 21: 529–534

[14] Kern M, Knigge A, Heiker JT et al. C57BL/6JRj mice are protected against diet induced obesity (DIO). Biochem Biophys Res Commun 2012; 417: 717–720

[15] Klöting N, Graham TE, Berndt J et al. Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass. Cell Metab 2007; 6: 79–87

[16] Yin F, Sancheti H, Cadenas E. Silencing of nicotinamide nucleotide transhydrogenase impairs cellular redox homeostasis and energy metabolism in PC12 cells. Biochim Biophys Acta 2012; 1817: 401–409

[17] Hull RL, Willard JR, Struck MD et al. High fat feeding unmasks variable insulin responses in male C57BL/6 mouse substrains. J Endocrinol 2017; 233: 53–64

[18] Lopert P, Patel M. Nicotinamide nucleotide transhydrogenase (Nnt) links the substrate requirement in brain mitochondria for hydrogen peroxide removal to the thioredoxin/peroxiredoxin (Trx/Prx) system. J Biol Chem 2014; 289: 15611–15620

Electronic reprint for personal use