Mildly compromised tetrahydrobiopterin cofactor biosynthesis due to *Pts* variants leads to unusual body fat distribution and abdominal obesity in mice

Running title: Abdominal obesity in mice with mildly compromised BH_4 biosynthesis

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Compliance with Ethics Guidelines

Conflict of interest:

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Informed Consent: no studies with human subjects are included in this manuscript.

Animal Rights: All institutional and national guidelines for the care and use of laboratory animals were followed. Animal experiments were carried out in accordance with the guidelines and policies of the State Veterinary Office of Zurich and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Animal studies presented here received approval from by the Cantonal Veterinary Office, Zurich, and the Cantonal Committee for Animal Experiments, Zurich, Switzerland.

Details of the contributions of individual authors:

Author contributions: GK, TS, DA, AR, MC, AR, RS, DH, BL, DK, CW, MH, BR, MK, and JB have conducted the experiments, and HI, EW, VG-D, HF, MHA, NB, JR, and BT were involved in planning and reporting of the work described.

Abstract Tetrahydrobiopterin (BH_4) is an essential cofactor for the aromatic amino acid hydroxylases, alkylglycerol monooxygenase and nitric oxide synthases (NOS). Inborn errors of BH_4 metabolism lead to severe insufficiency of brain monoamine neurotransmitters while augmentation of BH_4 by supplementation or stimulation of its biosynthesis is thought to ameliorate endothelial NOS (eNOS) dysfunction, to protect from (cardio-) vascular disease and/or prevent obesity and development of the metabolic syndrome. We have previously reported that homozygous knock-out mice for the 6-pyruvolytetrahydropterin synthase (PTPS; *Pts*-ko/ko) mice with no BH_4 biosynthesis die after birth. Here we generated a *Pts*-knock-in (*Pts*-ki) allele expressing the murine PTPS-p.Arg15Cys with low residual activity (15% of wild-type *in vitro*) and investigated homozygous (*Pts*-ki/ki) and compound heterozygous (*Pts*-ki/ko) mutants. All mice showed normal viability and depending on the severity of the *Pts* alleles exhibited up to 90% reduction of PTPS activity concomitant with neopterin elevation and mild reduction of total biopterin while blood L-phenylalanine and brain monoamine neurotransmitters were unaffected. Yet, adult mutant mice with compromised PTPS activity (i.e. *Pts*-ki/ko, *Pts*-ki/ki or *Pts*-ko/wt) had increased body weight and elevated intra-abdominal fat. Comprehensive phenotyping of *Pts*-ki/ki mice revealed alterations in energy metabolism with proportionally higher fat content but lower lean mass, and increased blood glucose and cholesterol. Transcriptome analysis indicated changes in glucose and lipid metabolism. Furthermore, differentially expressed genes associated with obesity, weight loss, hepatic steatosis and insulin sensitivity were consistent with the observed phenotypic alterations. We conclude that reduced PTPS activity concomitant with mildly compromised BH_4-biosynthesis leads to abnormal body fat distribution and abdominal obesity at least in mice. This study associates a novel single gene mutation with monogenic forms of obesity.

275 words

Key words: tetrahydrobiopterin, endothelial dysfunction, eNOS/NOS3, neopterin, metabolic syndrome, monogenic obesity

Introduction

Tetrahydrobiopterin (BH_4) is synthesized *de novo* from guanosine triphosphate (GTP) by the three enzymes GTP cyclohydrolase I (GTPCH), 6-pyruvoyltetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (Thöny et al 2000; Werner et al 2011). BH_4 is an essential cofactor for the aromatic amino acid mono-oxygenases, i.e. the phenylalanine hydroxylase (PAH), the tyrosine hydroxylase (TH) and the two tryptophan hydroxylases (TPH1, TPH2). Besides providing L-tyrosine (L-Tyr) for protein and catecholamine biosynthesis, the major role of the hepatic PAH is the prevention from systemic L-phenylalanine (L-Phe) accumulation, which is toxic in the brain. TH and TPH1/2 are the key enzymes in the biosynthesis of L-3,4-dihydroxyphenylalanine (L-Dopa) and 5-hydroxy-L-tryptophan (5-HTP), respectively. BH_4 is also a cofactor for the three nitric-oxide synthases (NOS) isoenzymes neuronal NOS (nNOS/NOS1), cytokine-inducible NOS (iNOS/NOS2) and endothelial NOS (eNOS/NOS3) for nitric oxide production as well as for the alkylglycerol mono-oxygenase (AGMO) which catalyzes the hydroxylation of alkylglycerols or ether lipids (Werner et al 2011).

BH_4 deficiency is a heterogeneous group of rare disorders associated with a spectrum of phenotypes ranging from mild, peripheral symptoms including hyperphenylalaninemia (HPA) due to lowered hepatic PAH activity to severe morbidity due to compromised monoamine neurotransmitter synthesis by dysfunction of TH and TPH in the brain (Blau et al 2010). Oral supplementation with BH_4 - in combination with neurotransmitter precursors - has been successfully employed to treat patients. Besides cofactor for the aromatic amino acid hydroxylases, BH_4 is an intracellular antioxidant and a key regulator of cellular redox-signaling, and conditions of low BH_4 for NOS lead to NOS uncoupling and production of superoxide rather than NO (Werner et al 2011; McNeill and Channon 2012). Since NO is required to maintain vascular function, limited bioavailability of the NOS cofactor BH_4 is associated not only to cell toxicity but also to vascular dysfunction (McNeill and Channon 2012). Thus, perturbed homeostasis of BH_4 does not only lead to oxidative stress but is thought to be associated with pathogenesis of cardiovascular and neurodegenerative diseases.

Over the last years, numerous experiments with rodents or patients were performed under conditions of increased BH_4 by augmentation of cofactor through pharmacological supplementation, stimulation of biosynthesis or protection from oxidation, and they basically all confirmed correction of eNOS

dysfunction to protect from (cardio-) vascular disease (Shi et al 2004; Forstermann and Munzel 2006). Furthermore, the bioavailability of endothelial BH_4 for eNOS was found also to be important, besides probably many other dietary factors (Wu and Meininger 2002), for the control of glucose and lipid homeostasis (Duplain et al 2001; Wyss et al 2005), and various experiments in animal models and patients suggest a role in, or progression to, type 2 diabetes mellitus (T2DM)(Meininger et al 2000; Alp et al 2003; Ihlemann et al 2003; Pannirselvam et al 2003; Meininger et al 2004; Nystrom et al 2004). Oral supplementation of BH_4 over several weeks in rats prevented endothelial dysfunction and restored adiponectin levels, a hormone secreted from adipose tissue and regulating glucose and fatty acid catabolism (Wang et al 2007). It was speculated based on such experiments with animals and in patients with T2DM that BH_4 might be a candidate for the treatment of the metabolic syndrome. Increase of abdominal obesity is known to contribute to insulin resistance and metabolic abnormality which is linked to development of T2DM and cardiovascular disease (Despres and Lemieux 2006; Fox et al 2007; Rader 2007). However, the underlying mechanisms for the relation between arterial hypertension, insulin resistance, and the metabolic syndrome are unclear (Despres and Lemieux 2006).

Various transgenic animal models are available to study pathophysiology and disease mechanism of BH_4 cofactor deficiency (Werner et al 2011). We and others have reported on the perinatal lethal phenotype of a homozygous *Pts*-knock-out mouse (*Pts*-ko/ko)(Sumi-Ichinose et al 2001; Elzaouk et al 2003). This mouse mutant exhibited complete absence of PTPS biosynthesis activity accompanied by systemic HPA, severe brain monoamine neurotransmitter deficiency, IGF-1 depletion and dwarfism, while whole brain NOS activity was normal. Due to its severe morbidity and perinatal mortality, this mouse model turned out to be difficult for further and detailed studies on the natural history and development of pathophysiology for classical BH_4 deficiency. We thus aimed at generating a mouse model with a milder form of BH_4 deficiency. Here we report on the generation and characterization of a *Pts*-knock-in (*Pts*-ki) allele with low but residual PTPS activity. Surprisingly, homozygous *Pts*-ki/ki or heterozygous *Pts*-ki/ko mutant animals exhibited normal L-Phe levels and brain monoamine neurotransmitters but abnormal body fat distribution and abdominal obesity.

Materials, methods and animal husbandry

Pts gene targeting

A genomic clone containing the murine *Pts* gene was as described previously, isolated from a 129/Sv-λ phage library (Turri et al 1998). For targeting vector (pMSY211) construction, a 1.3 kb fragment of the *Pts* gene spanning exon 1 was used as short arm of homology (see Supplementary Figure S1B). A phosphoglycerate kinase promoter (*Pgk*)-diphtheria toxin (DT) gene cassette, essential for the negative selection of the embryonic stem (ES) cells, was added 5' to the short arm of homology. The long arm of homology was a 5.1 kb fragment containing exons 2, 3 and 4 of the *Pts* gene, and as a positive selective marker, the "floxed" *Pgk*-neomycin resistance gene (*neo*) cassette that was introduced between the short and the long arm of homology. After successful construction, the pMSY211 targeting vector was linearized and electroporated into ES cells derived from 129S6/SvEvTac strain. ES cell clones with correct homologous recombination were confirmed by nested PCR under standard amplification conditions with 40 cycles with primers MSY220: 5'-GCACCCCAAGGTAGCCAAGAATTTG-3' and MSY221: 5'-TTCTTCGCCCACCCCGAAATTGATG-3', followed by 25 cycles with primers MSY226: 5'-ACCGGGCTGGAGAACATCTGATAAG-3' and MSY228: 5'-TCAGCAGCCTCTGTTCCACATACAC-3'. For further confirmation of correctly targeted ES cell clones, Southern blot analysis was performed (not shown). One correctly targeted ES cell clone was chosen for blastocyst injection. Blastocyst injection (FVB/N host embryos) led to generation of one 50% chimeric male that, when sexually mature, was mated with FVB females. The chimera revealed germline transmission resulting in the generation of heterozygous *Pts*-R15C knock-in (*Pts*-ki) targeted mice. Correct genotype was confirmed on genomic DNA from tail or ear biopsies by *Pts*-ki or *Pts*-ko genotyping PCR (for genotyping, see Supplementary Fig. S1C and S1D plus supplementary information).

Mouse husbandry

Animal experiments were carried out in accordance with the guidelines and policies of the State Veterinary Office of Zurich and Swiss law on animal protection, the Swiss Federal Act on Animal Protection (1978), and the Swiss Animal Protection Ordinance (1981). Animal studies presented here received approval from by the Cantonal Veterinary Office, Zurich, and the Cantonal Committee for Animal Experiments, Zurich, Switzerland. All mice, including the wild-type controls, are based on C57BL/6-background. The high fat diet was from Research Diets D12331 (with 58% kcal% fat

w/sucrose Surwit Diet) for up to 10 weeks of feeding mice *ad libitum*. At the GMC mice were maintained in IVC cages with water and standard mouse chow (Altromin 1314, Altromin, Lage, Germany) according to the GMC housing conditions and German laws. All tests performed at the GMC were approved by the responsible authority of the district government of Upper Bavaria, Germany.

More materials and methods are described in the Supporting Materials and Methods.

RESULTS

Generation of a *Pts* knock-in mouse (*Pts*-ki)

To generate a viable mouse model for BH_4 deficiency, we chose to knock-in a single point mutation in the murine *Pts* gene, c.43C>T leading to mPTPS-p.Arg15Cys. This mutation corresponds to the human mutation *PTS*-c.46C>T/hPTPS-p.Arg16Cys which was found in a patient with a mild phenotype with lowered BH_4 biosynthesis in the periphery but normal BH_4 and neurotransmitter levels in the CNS (Supplementary Fig. S1A)(Thöny et al 1994; Oppliger et al 1995). Expression studies of recombinant hPTPS-p.Arg16Cys and mPTPS-p.Arg15Cys in COS-1 cells revealed enzyme activity of 12% and 15%, respectively, compared to wild-type PTPS (not shown). Details for the targeting vector construct and strategy for knocking-in the mPTPS-p.Arg15Cys allele (*Pts*-ki), including mouse genotyping, are described in Materials and Methods and are illustrated in Supplementary Figures S1B - S1D.

Homozygous *Pts*-ki/ki or compound heterozygous *Pts*-ki/ko mice exhibit lowered PTPS activity, elevated neopterin and lowered BH_4 in liver and brain, but normal plasma L-Phe levels

Upon breeding *Pts*-ki mice to homozygosity, we found the expected Mendelian ratio for a recessive allele with ~25% *Pts*-ki/ki mice, and no behavioral or visible abnormalities compared to wild-type littermates. In the following, we bred all possible viable *Pts* genotypes, excluding homozygous knock-outs which are perinatal lethal, and analyzed in 10-12 weeks old adults for PTPS expression, pterin content in liver and brain, L-Phe in blood, and monoamine neurotransmitter metabolites in the brain.

First we quantified *Pts* gene expression in liver and brain by RT-PCR and western analyses (Table 1). For the *Pts*-mRNA, we found no difference in *Pts*-ki/wt and *Pts*-ki/ki compared to homozygous wild-type controls, and an expected ~50% reduction in mice with one *Pts*-ko null allele. For the PTPS protein, we found a roughly 50% reduction in the liver of mice with one *Pts*–ko null allele compared to wild-type and *Pts*-ki/wt mice. An exception was the somewhat unprecedented elevation of PTPS expression in *Pts*-ki/ki mice, which might be due to a compensatory action due to low PTPS activity. The PTPS protein in (whole) brain extracts could not be quantified, as expression levels were below detection limit for our anti PTPS-antibody.

Next, we investigated PTPS enzyme activity in different tissues from mice carrying various *Pts* alleles. As depicted in Fig. 1A, PTPS activity was only slightly but not significantly reduced in liver and brain of *Pts*-ki/wt, and in brain of *Pts*-ko/wt compared to wild-type mice, while *Pts*-ki/ki, *Pts*-ko/wt and *Pts*-ki/ko

mice showed a strong reduction of activity in brain and liver. Taken together, progressive reduction of PTPS activity in mice with different *Pts* alleles was as follows: ko/wt > ki/ki > ki/ko > ko/ko (for *Pts*-ko/ko see (Elzaouk et al 2003)).

Systemic accumulation of neopterin, the oxidized and dephosphorylated substrate of the PTPS enzyme, is one of the diagnostic hallmarks of PTPS deficiency (Werner et al 2011). In accordance with the observation of lowered PTPS activity, we found slightly elevated neopterin in *Pts*-ki/ki mice at least in liver (but not in brain), but significantly elevated neopterin in liver and brain of the more severely affected *Pts*-ki/ko mice (Fig. 1B). Furthermore, mice mutants with severely reduced PTPS activity had a two to maximally three-fold reduction of total biopterin while the ratio of BH_4 versus 7,8-BH_2 remained without any significant changes (Fig. 1C-D). We also analyzed the biopterin content in mammary glands of females because it was reported that this tissue had probably the highest biosynthesis activity and thus concentration of biopterin, and a potential reduction of biopterin in milk might have an effect on the development of offsprings (Leeming et al 1976; Matsubara and Gaull 1985). Yet, there was no difference in mouse mother milk between *Pts*-wt/wt, *Pts*-ki/wt, *Pts*-ko/wt and *Pts*-ki/ki (Fig. 1E). In all mice, blood L-phenylalanine was unaffected as we found no indication for (systemic) elevation of L-Phe or L-Tyr analyzed in peripheral blood (Fig. 1F). Plasma L-Phe remained also unchanged when *Pts*-ki/ki mice were exposed to high levels of L-Phe (300 mg/l) for 5 days in the drinking water (not shown).

Brain monoamine neurotransmitter levels and TH expression are not altered in *Pts* mice with lowered PTPS activity

Since compound heterozygous *Pts*-ki/ko and homozygous *Pts*-ki/ki mice were compromised in their brain PTPS activity with elevated neopterin and reduction of total biopterin (Figs. 1A-D), we analyzed the brain monoamine neurotransmitter metabolites dopamine, norepinephrine, epinephrine and serotonin. As depicted in Fig. 1G, these compounds did not differ in the different *Pts* backgrounds. Next, tyrosine hydroxylase (TH) was analyzed in the brain of these mice because TH expression and/or stability were reported to be reduced under conditions of BH_4 and/or PAH deficiency (Sumi-Ichinose et al 2001; Joseph and Dyer 2003; Embury et al 2007). However, we found no difference in TH expression in adult brains between *Pts*-ki/ko mice compared to their *Pts*-wt/wt, *Pts*-ki/wt and *Pts*-ko/wt controls (Supplementary Fig. S2).

In summary, we did not observe any abnormality in homozygous *Pts*-ki/ki or compound heterozygous *Pts*-ki/ko mice regarding brain TH expression and monoamine neurotransmitter biosynthesis, despite the reduction of BH_4 biosynthesis.

Heterozygous *Pts* mutant mice exhibit abnormal body weight and intra-abdominal fat content

Since heterozygous *Pts*-ko/wt mutant synthesized potentially less BH_4 as we found a ~50% reduction at least after birth but normal levels at later ages (Elzaouk et al 2003; Thöny et al 2004), we initially hypothesized that these animals might be prone to cofactor limitation under for instance acute hyperglycemia. Yet, in standard oral glucose tolerance tests we could not see any difference in glucose clearance between groups of wild-type and *Pts*-ko/wt mice (not shown). At the same time, we found that *Pts*-ko/wt mice tend to have a slightly higher relative increase in body weight and in intra-abdominal fat than their wild-type litter mates. This phenomenon seemed to be more pronounced in male mice than in females, and we therefore limited the following analyses to male mutants. By serendipity, we further observed in the same male mice during autopsies an increase in intra-abdominal fat content. A representative quantification of such an early observation is summarized in Table 2: upon feeding limited number of male mice (n = 4) over a period of several weeks with high fat diet (58 kcal% fat with sucrose compared to normal diet with 11 kcal% fat with corn starch), we saw a two-fold increase in intra-abdominal adipose tissue compartments in *Pts*-ko/wt mice compared to an only 1.6-fold increase in wild-type control mice. For these first observations, we decided to dissect and weigh the sum of epididymal (or perigonadal) fat tissues, termed ‘intra-abdominal fat’, as a marker for fat increase. An *in vivo* determination of whole-body fat in mice using time-domain magnetic resonance analysis (TD-NMR) was only performed later to confirm these observations in *Pts*-ki/ki mouse mutants (see below). Next we extended our investigations with male *Pts*-ko/wt male mice by analyzing various metabolic parameters in a larger cohort of mice fed with high fat diet (see Supplementary Table S1). This study corroborated the previously observed increase in intra-abdominal fat in *Pts*-ko/wt mice ($p < 0.05$), while metabolic, inflammatory and oxidative stress parameters were either unchanged or only slightly and statistically not significantly increased in *Pts*-ko/wt mice compared to wild-type controls. As shown in Supplementary Table S1, these parameters included triglycerides in liver and plasma, plasma cholesterol and HDL, blood glucose, and adiponectin and *Il6* gene expression in fat tissue.

The observation of body weight increase in mildly compromised *Pts* (male) mutants, i.e. *Pts*-ko/wt compared to wild-type, was also seen in a parallel study including *Pts*-ki/ki males (see Table 3). Here we found differences in body weight (but not in plasma glucose) when mice were kept over several weeks under standard chow *or* under high fat diet. From these results we concluded that a somewhat lowered PTPS activity that is connected to detectable reductions of BH_4 is a potential risk factor for weight increase with a tendency for abdominal obesity at least in male mice. For further analysis, we undertook in a next step a comprehensive and standardized analysis towards a potential metabolic phenotype with our homozygous *Pts*-ki/ki mutant mice.

Comprehensive phenotyping of *Pts*-ki/ki mice revealed higher fat content and lower lean mass, and an increase in fasting plasma glucose, plasma cholesterol and triglycerides

Pts-ki/ki mice were systematically characterized in the standardized "primary screen" of the German Mouse Clinic (Gailus-Durner et al 2005; Gailus-Durner et al 2009). 78 mice (40 mutants and 38 wild-type littermates, age of 12-13 weeks) were analyzed in the screens dysmorphology, behavior, neurology, eye, nociception, energy metabolism, clinical chemistry, immunology, allergy steroid metabolism, cardiovascular function, lung function, and pathology. In addition, liver and brain tissue samples were used for microarray based analysis of differential gene expression. *Pts*-ki/ki mice showed phenotypic alterations indicating a mild metabolic phenotype. Despite no difference in body mass in 13 weeks old mutants compared to wild-type controls, fat mass was increased especially in male mutants whereas lean mass was reduced (Figs. 2A-C and Table 4). The monitoring of daily energy expenditure and substrate utilization by indirect calorimetry in male control and mutant mice revealed no differences between genotypes (see Supplementary Table S2). Clinical chemistry analyses of plasma samples revealed for both sexes a mild increase of fasting glucose levels in *Pts*-ki/ki mice and significantly higher cholesterol and triglyceride concentrations in plasma of *ad libitum* fed mutant mice as compared to corresponding controls. Additionally, alkaline phosphatase activity in plasma of mutant mice was slightly increased compared to controls, pointing towards a potential liver dysfunction (Supplementary Table S3). The remaining parameters analyzed did not show significant genotype-related differences.

Liver and brain transcriptome profiles of *Pts*-ki/ki mice

To potentially identify differential gene regulation in *Pts*-ki/ki mice with reduced PTPS enzyme activity and elevated neopterin in brain and liver, transcriptome profiles of these organs were performed (see Supplementary Tables S4 for liver and S5 for brain). Slightly increased expression levels of *Pts* were detected in both organs comparing *Pts*-ki/ki with *Pts*-wild-type mice (fold change: brain 1.45 ± 0.48; liver 1.66 ± 0.33) which is similar to what we found by RT-qPCR (see Table 1) and which might be a compensatory effect due to reduced PTPS enzyme activity. Statistical analysis revealed 36 significantly regulated genes in brain and 347 in liver of *Pts*-ki/ki mice (see Suppl. Table S4 and S5). An overlap of 22 differentially down-regulated genes was found between the analyzed organs: *Alg*, *Atm*, *Cd14*, *Cd207*, *Ch25h*, *Hp*, *Hspb1*, *Lcn2*, *Lrg1*, *Mkks*, *Ms4a6d*, *Miacr1*, *Osmr*, *Retnlg*, *S100a8*, *S100a9*, *Serpina3f*, *Serpina3g*, *Socs3*, *Srpr*, *Tmem25*, and *Zfp235*. Several of these common genes were associated with cytokine activity (*Mkks, Osmr, Serpina3f, Serpina3g*, and *Socs3*), immune processes (*Atm, Cd14, Cd207, Lrg2, S100a8* and *Tmem25*) and metabolism (*Ch25h, Lcn2, Osmr, S100a8* and *Socs3*).

Further overlap was detected among the over-represented functional annotations of the regulated genes: proliferation and differentiation of cells, cell death, leukocyte migration and vascular disease (Supplementary Table S6) which might be an indication for inflammatory processes. Exclusively, genes annotated with glucose (e.g. *Cxcl14, Dusp1, Fabp5, Myd88, Nnmt, Nos3, Pilrb, Ptpn1, Retnlb, Serpina3, Stat3, Timp1, Tlr2, Vcam1, Xbp1*) and lipid metabolism (e.g. *Abcb1b, Adora1, Adrb2, Apoa4, Atf3, Cebpb, Fabp5, Fas, Lbp, Lcn2, Lgals3, Ptpn1, Saa1, Stat3, Xbp1*), protein synthesis (e.g. *Arntl, Bag3, Casp4, Gdf9, Hdc, Hmox1, Lgmn, Mkks, Mt1e, Mt1h, Myd88, Rcan1, S100a9, Sgms1, Slc39a14, Thbd, Tlr2*), obesity (e.g. *Adora1, Adrb2, Atf3, Cebpb, Fabp5, Gas6, Hhex, Icam1, Lbp, Mfsd2a, Mkks, Mt1e, Mt1h, Ppargc1b, Socs3, Stat3*), weight loss (e.g. *Adh7, Apcs, Arntl, Atf3, Bag3, Cdkn1a, Ikbke, Mt1e, Mt1h, Nfkb2, Tlr2, Tpmt*), hepatic steatosis (e.g. *Adora1, Atf4, Cyp4a11,, Fabp5, Igfbp1, Il18, Il1b, Lbp, Mfsd2a, Retnlb, Ripk2, Stat3, Steap4, Tlr2*) and insulin sensitivity (e.g. *Arntl, Cebpb, Ptpn1, Socs3, Spp1, Stat3, Tgm2, Tlr2, Xbp1*) were over-represented in liver. These gene ontology (GO) terms might be of particular interest with regard to changes in fat content and elevated blood glucose and cholesterol levels in *Pts*-ki/ki mice. It has to be emphasized that we found reduced gene expression levels for *Nos3* only in hepatic transcriptome profiling analysis which would give evidence towards a mildly compromised eNOS/NOS3 function, whereas a “validation” by RT-qPCR did not necessarily confirm this in the various *Pts*-mice tested (see Supplementary Table S7).

Furthermore, we did not find any measurable difference in NOS activity in liver of fat tissues between mice with the various genotypes (not shown). Nevertheless, a potential association between the reduced BH_4-biosynthetic activity, abnormal body fat distribution and abdominal obesity, and the reduced gene expression levels of e*Nos*/*Nos3* found in liver will be discussed below.

Discussion

The here presented *Pts*-ki mouse was initially thought to represent a hypomorphic model that mimics human BH_4 deficiency due to severely reduced PTPS activity which, if untreated, may be lethal in patients but *not* at birth as it is observed in *Pts*-ko/ko mice. We found that a reduction of up to 90% of PTPS activity and lowered biopterin biosynthesis (in *Pts*-ki/ko mice) does *not* lead to systemic hyperphenylalaninemia concomitant with brain monoamine neurotransmitter abnormality. Unexpectedly, such mice turned out to exhibit compromised or limited cofactor availability without classical signs of BH_4 deficiency but rather with abnormal body fat distribution and abdominal obesity. An indirect measure of BH_4 limitation due to low PTPS activity is the elevated neopterin that is clearly detectable in liver and less striking in brain in at least *Pts*-ki/ko mice. As described in the introduction, it was found that conditions of increased BH_4 may protect from cardiovascular diseases, endothelial dysfunction and potentially also from progression to T2DM through endothelial BH_4 for eNOS (for references see Introduction). Yet, whereas the role of increased BH_4 in abdominal obesity or the metabolic syndrome has been investigated, the opposite condition i.e. *decreased* BH_4 – but not classical BH_4 deficiency – in these processes has not been studied to our knowledge under *in vivo* conditions. By serendipity, we found in our first mouse model with potentially limited BH_4, i.e. in the heterozygous *Pts*-ko/wt mice, abnormal fat distribution which was later confirmed also in homozygous *Pts*-ki/ki mice.

A follow-up study by a comprehensive and standard systemic and phenotype analysis of *Pts*-ki/ki mice revealed slight alterations in energy metabolism with proportionally higher fat content and lower lean mass, and mildly increased fasting blood glucose as well as cholesterol and triglyceride levels in these mutant animals. Transcriptome analysis of liver indicated changes in glucose and lipid metabolism, including genes such as *Adora1, Adrb2, Apoa, Atf3, Atf4, Cebpb, Cxcl14, Dusp1, F13a1, Fabp5, Map3k14, Nos3, Ppargc1a, Rgs16, Socs3, Stat3, Steap4* and *Zc3h12a.* Furthermore, several of the differentially regulated genes in liver are associated with obesity, weight loss, hepatic steatosis and insulin sensitivity, which are consistent with the phenotypic alterations found in *Pts*-ki/ki mice. Genes such as *Adrb2*, *Apoa4*, *Adora*, *Atm* and *Ripk2* play roles in lipid accumulation in liver and hepatosteatosis. Deficiency of *Ripk2*, also down-regulated in our study, exacerbates hepatosteatosis (Wang et al 2013). However, *Adrb2* and *Atm*, recently linked with activation of fatty liver-induced

399 steatoapoptosis and fibrosis (Daugherity et al 2012; Ghosh et al 2012), were down-regulated in liver of
400 *Pts*-ki/ki mice. Additionally, over-expression of *Apoa4* and *Adora*, both genes associated with
401 reduction of lipid accumulation (VerHague et al 2013; Yang et al 2013), give evidence for protection of
402 liver dysfunction. Several genes associated with insulin sensitivity showed decreased expression in
403 *Pts*-ki/ki mutants. While *Atf3* has antidiabetic effects (Park et al 2010), *Tgm2* null mice were glucose
404 intolerant (Burke et al 2012) and *Fabp5* was described to modulate systemic glucose metabolism and
405 insulin sensitivity (Babaev et al 2011). Changes in fat content correlated also to the down-regulation of
406 genes annotated with obesity, e.g. the adipocyte specific transcription factor *Cebpb* (Wang et al 2013),
407 *Dusp1*, expressed in visceral adipose tissue of several obese man (Guenard et al 2013) and *Nik*, a
408 gene that protect against hyperglycemia and glucose intolerance in obese mice (Sheng et al 2012).
409
410 A potential direct link between reduced BH_4 biosynthetic activity to abnormal body fat distribution and
411 abdominal obesity can potentially be through a mildly compromised eNOS/NOS3 function as
412 suggested at least by the hepatic transcriptome profiling analysis with reduced expression of
413 e*Nos*/*Nos3* in liver. It was reported that increased NO signaling inhibits insulin-induced glycogen
414 synthesis in hepatocytes (Tsuchiya and Accili 2013), therefore reduced NO signaling might increase
415 hepatic gluconeogenesis and fasting glucose levels. Furthermore, expression and stability of e*NOS*-
416 mRNA are influenced by many epigenetic and external factors that could also account for differences
417 seen in the degree of reduction in gene expression (Tai et al 2004). Since we have not found yet a
418 molecular mechanism, including no changes in NOS activity at least in liver and fat tissues, we can
419 only speculate about an influence of potential BH_4-cofactor limitation in e.g. endothelial tissues that is
420 propagated in the organism through a (mildly) compromised eNOS/NOS3. For instance, we observed
421 that BH_4/BH_2 ratios were generally higher in brain (between 2.35. to 7.02 in Fig. 1D) compared to liver
422 (between 0.08 to 2.55 in Fig. 1C). This might explain a peripheral or “metabolic” rather than a central
423 brain phenotype (with normal neurotransmitter homeostasis) due to the relative higher content of BH_2
424 which might act as a competitive antagonist for NO production in the liver. An alternative link between
425 the mildly reduced biopterin biosynthesis and the observed obesity could be accumulation of the by-
426 product neopterin which was detectable at least in liver tissue from *Pts*-ki/ko and *Pts*-ki/ki mice while
427 *Pts*-ko/wt mice had only an insignificant neopterin increase after birth (Elzaouk et al 2003). Neopterin
428 was proposed to reflect oxidative stress induced by immune system activation in general, and was
429 found to be elevated in patients with inflammation and atherosclerosis (De Rosa et al 2011). Recently,

neopterin was also shown to negative affect expression of various transporters involved in cellular cholesterol efflux and foam cell formation and thus to have an aggravating effect on atherosclerosis (Yan et al 2013). Clearly, more studies are required to confirm a connection to eNOS/NOS3 and/or neopterin. Nevertheless, our study associates a single gene mutation with monogenic forms of obesity, a well known phenomenon related to the so-called leptin-melanocortin pathway, that regulates energy balance and food intake, and, if compromised, may lead to obesity (for a review see (Farooqi and O'Rahilly 2005)). Association of recessive mutations in the pterin-carbinolamine dehydratase (PCD), required for biopterin recycling (Werner et al 2011), leading to a monogenetic MODY-form of diabetes was recently found for the PCD-encoding gene *PCBD1*, as the PCD protein has a second "moonlight" function as DCoH1, i.e. dimerization-cofactor of the liver-specific transcription factor HNF-1a (Simaite et al 2014).

In conclusion, a reduction in BH_4-biosynthetic activity caused by a single heterozygous gene mutation leads in mice to abnormal body fat distribution and abdominal obesity. Whether such an effect is also visible in humans that are carriers of a mutation in the *PTS* gene (or in other BH_4-cofactor metabolizing genes) needs to be verified by future studies.

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461 **Supplementary information is available at Journals website.**

462

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578

579 **Figure Legends**

580

581 **Figure 1. Biochemical analysis of blood, milk, liver and brain tissues from (male and female)**
582 **mice carrying the various *Pts* alleles *Pts*-wt/wt, *Pts*-ki/wt, *Pts*-ki/ki, *Pts*-ko/wt and *Pts*-ki/ko.** (**A**)
583 PTPS enzyme activity (μU/mg protein) in liver and brain, (**B**) neopterin (pmol/mg protein) in liver and
584 brain, (**C-D**) BH_4, 7,8-BH_2 and total biopterin (pmol/mg protein) in liver and brain, (**E**) biopterin and
585 neopterin in mother milk (nmol/l), (**F**) blood amino acids L-Phe and L-Tyr concentrations (μmol/l), and
586 (**G**) brain monoamine neurotransmitter metabolites dopamine, norepinephrine, epinephrine and
587 serotonin (pmol/mg protein). Five mice, 10-12 weeks old, were used for all measurements, with the
588 exception of 3 mice per group in (**E**). Genotypes are indicated by bar color (except for (**E**)): *Pts*-wt/wt
589 (white), *Pts*-ki/wt (left striped), *Pts*-ko/wt (right striped) *Pts*-ki/ki (gray), and *Pts*-ki/ko (black). Significant
590 difference from the corresponding wild-type value is indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***,
591 0.001 (Student's two tailed *t*-test). The age of the animals was between 3-6 months (young adults).

592

593

594 **Figure 2. Body composition analysis by non-invasive NMR. (A)** Body mass (in g). (**B**) Non-
595 invasive NMR scans to determine the lean mass (in g), and (**C**) fat mass (in g). Open circles, *Pts*-wt/wt
596 females (n = 9); grey circles, *Pts*-ki/ki females (n = 10); open squares, *Pts*-wt/wt males (n = 10); grey
597 squares, *Pts*-ki/ki males (n = 10). The age of the animals was between 12-13 weeks (young adults),
598 and all mice were analyzed at the same day. Significant difference from the corresponding wild-type
599 value is indicated by asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, 0.001 (Student's two tailed *t*-test).

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603 **Supplementary Figure Legends**

604

605 **Supplementary Figure S1.** Generation of the murine *Pts*-ki allele. **(A)** Primary amino acid sequence
606 alignment of human and mouse PTPS, which share 82.1% sequence identity. The human mutation
607 *PTS*-p.Arg16Cys (hR16C) and the corresponding mouse mutation *Pts*-p.Arg15Cys (mR15C), both
608 located in exon 1, are marked with arrows. **(B)** Schematic representation of genomic structure of the
609 murine *Pts* wild-type allele (top), the targeting vector pMSY211 including the mR15C mutation (E1'),
610 the p.L16L mutation to destroy the *Bss*SI restriction site, a *Pgk*-DT-gene-cassette (DT) for negative
611 selection, and a "floxed" *Pgk*-neo-gene-cassette (PGK neo) for positive selection (middle), and the
612 resulting targeted mutant allele (bottom). **(C)** Schematic representation of the genotyping concepts for
613 the *Pts*-wt, *Pts*-ki and *Pts*-ko alleles with genomic DNA and the primer pairs a/b (*Pts*-ki PCR) and c/d/e
614 (*Pts*-ko PCR). *Pts*-ki PCR: primers a and b are located upstream and downstream from exon 1 (E1),
615 respectively. They generate a 730 bp for the wild-type/knock-out alleles and a 751 bp PCR fragment
616 for the knock-in allele (due to additional targeting vector sequence; see **C**). Digestion with restriction
617 enzyme *Bss*SI, 3 bp downstream of the mR15C-c.43C>T mutation, leads to a 444 bp and a 286 bp
618 fragment for the wild-type/knock-out PCR products. The PCR fragment derived from the *Pts*-ki allele
619 can not be digested with *Bss*SI because the silent p.L16L/c.48C>G mutation destroys the *Bss*S1-
620 recognition site. The *Pts*-wt and the *Pts*-ko alleles can not be distinguished by the *Pts*-ki genotyping
621 using primer pair a/b. *Pts*-ko PCR: genotyping according to our previously published method (Elzaouk
622 et al 2003). Primer c is upstream of exon 2 (E2), primer d is specific for exon 2 and primer e is specific
623 for the *lacZ* gene. The primer pair c/d results in a wild-type fragment of 287 bp and a knock-in
624 fragment of 316 bp whereas primer pair c/e generates mutant fragment of 355 bp (due to the
625 difference in the *Pts*-intron 1 sequence between the 129/Ola and C57BL/6J mice strains; see **C**). **(D)**
626 Conventional 2% agarose gel representative PCR-genotyping for the *Pts*-ki allele (top; after *Bss*SI
627 digestion) and *Pts*-ko allele (bottom).

628

629

630 **Supplementary Figure S2. TH protein expression in brain of *Pts*-ki/ko mice.** Western blot analysis
631 and densitometric quantification of TH in brains from (**A**) newborn mice (n = 3 *Pts*-wt/wt, 5 *Pts*-ko/wt,
632 24 *Pts*-ki/wt, and 19 *Pts*-ki/ko) and (**B**) young adult animals (n = 3 *Pts*-wt/wt, 7 *Pts*-ko/wt, 12 *Pts*-ki/wt,
633 and 13 *Pts*-ki/ko); always males and females. For details see also Materials and Methods.