



Mini-review

Clinical genetics of functionally mild non-coding GTP cyclohydrolase 1 (*GCH1*) polymorphisms modulating pain and cardiovascular riskAlexandra Doehring^a, Charalambos Antoniades^b, Keith M. Channon^b, Irmgard Tegeder^a, Jörn Lötsch^{a,*}^a pharmazentrum frankfurt/ZAFES, Institute of Clinical Pharmacology, Johann Wolfgang Goethe-University, Theodor Stern Kai 7, D-60590 Frankfurt am Main, Germany
^b Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, OX3 9DU Oxford, UK

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ABSTRACT

Guanosine triphosphate cyclohydrolase 1 (*GCH1*) is the first enzyme in the tetrahydrobiopterin (BH4) biosynthesis, an important co-factor for the formation of nitric oxide, biogenic amines and serotonin. Hereditary diseases such as DOPA-responsive dystonia and atypical phenylketonuria are known to be caused by coding or splice-site mutations in the *GCH1* gene, leading mostly to a dominant negative enzyme. However, recent evidence suggests a clinical genetics of *GCH1* beyond these hereditary loss-of-function diseases. That is, a non-coding *GCH1* haplotype has been associated with reduced pain hypersensitivity and with altered vascular endothelial function. Moreover, the presence of the non-coding c.*243C>T variant in the 3'-untranslated region (3'-UTR) of the *GCH1* gene has been associated with mildly increased heart rate and blood pressure. Here, we show that carriers of the pain-protective *GCH1* haplotype also carry the c.*243C>T variant and vice versa. We thus demonstrate that apart from the coding or splice-site variants causing DOPA-responsive dystonia and atypical phenylketonuria, there is a common clinically relevant *GCH1* genetics that is so far known to be related to unfavorable changes of endothelial function and a reduced risk for chronic pain.

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The enzyme guanosine triphosphate (GTP) cyclohydrolase 1 (*GCH1*) catalyzes the conversion of GTP to dihydroneopterin triphosphate. The latter is metabolized to tetrahydrobiopterin (BH4) by the enzymes 6-pyruvoyl tetrahydropterin synthase and sepiapterin reductase [1] (Fig. 1, upper part). In NO synthases, the

recycling of BH2 to BH4 is performed within the catalytic site of the NO synthase and is directly coupled to the two-step process of NO synthesis. BH4 oxidized to BH2 during hydroxylase catalysis is recycled to tetrahydrobiopterin by two independent enzymes, namely the pterin-carbinolamine dehydratase and quinoid dihydropteridine reductase. BH4 is an essential co-factor for the three isoforms of nitric oxide synthase catalyzing the synthesis of nitric oxide and citrulline from arginine, and for tyrosine-, tryptophan- and phenylalanine hydroxylases [2], which

* Corresponding author. Tel.: +49 69 6301 4589; fax: +49 69 6301 7636.
E-mail address: j.loetsch@em.uni-frankfurt.de (J. Lötsch).

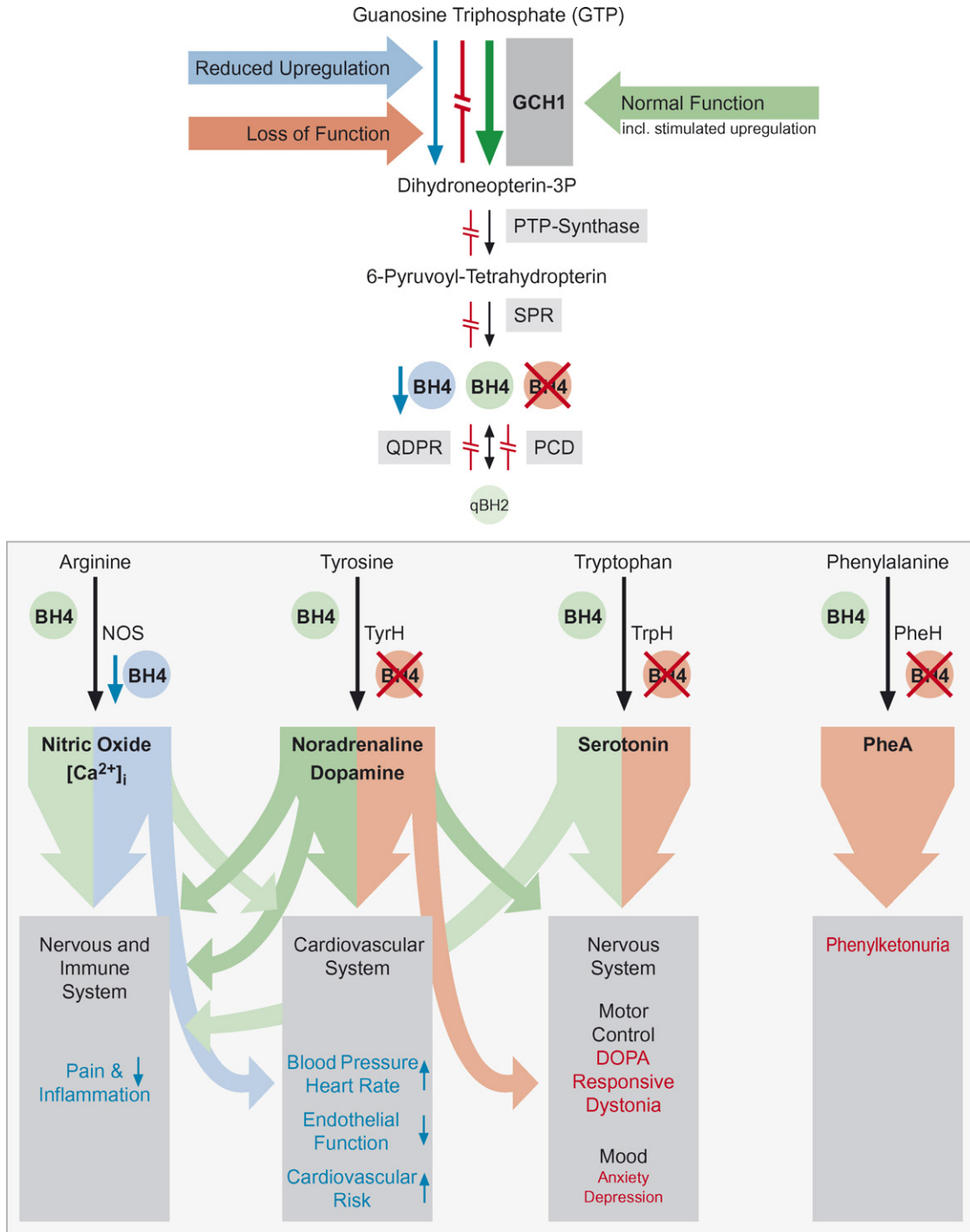


Fig. 1. Tetrahydrobiopterin pathway, with the rate-limiting enzyme GTP cyclohydrolase 1, and its functional clinical implications. GTP cyclohydrolase expression and/or activity are upregulated during inflammation, mast cell stimulation, following ischemic stroke or peripheral nerve injury leading to increased BH4 production. Excess BH4 in peripheral sensory neurons following axonal injury contributes to the manifestation of neuropathic pain. This is mediated in part by increasing calcium influx and nitric oxide production. Inhibition of GCH1 activity or reduced GCH1 upregulation reduces pain in various models. In blood vessels BH4 is required to produce nitric oxide by endothelial NOS (eNOS). Relative BH4 deficiency leads to an uncoupling of oxidation–reduction steps performed by eNOS resulting in increased production of reactive oxygen species, instead of nitric oxide, that contribute to endothelial dysfunction. Increasing endothelial BH4 improves vascular functions, particularly in diabetes models. In the brain BH4 is required for the production of dopamine and serotonin. BH4 deficiency due to loss-of-function mutations of GCH1 lead to DOPA-responsive dystonia, a Parkinson-like neurologic disease, or to atypical phenylketonuria. On the other hand, excess BH4 in the striatum contributes to the dying of dopaminergic neurons probably mediated by enhanced calcium influx and disturbance of the redox balance. Similarly, excess BH4 after stroke due to GCH1 upregulation contributes to neuronal death. *Abbreviations:* GTP, guanosine triphosphate; GCH1, GTP cyclohydrolase 1; PTPS, 6-pyruvoyl tetrahydropterin synthase; SPR, sepiapterin synthase; QDPR, quinoid dihydropteridine reductase; PCD, pterin-4 α -carbinolamine dehydratase; BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; nNOS, neuronal nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PheH, phenylalanine hydroxylase; PheA, phenylalanine; TyrH, tyrosine hydroxylase; DA, dopamine; NA, noradrenaline; 5-HT, serotonin; TrpH, tryptophan hydroxylase; ONOO⁻, peroxynitrite; BP, blood pressure; CAD, cardiovascular disease.

catalyze the production of biogenic amines (noradrenaline, dopamine, adrenaline), the production of serotonin, and the degradation of phenylalanine to tyrosine, respectively.

Given by this variety of transmitters that require BH4 to be synthesized, BH4 deficiency affects various biological systems (Fig. 1, lower part). Since GCH1 plays a rate-limiting role for BH4 production, changes in its net function due to alterations of gene transcription, mRNA stability, enzyme phosphorylation or conformation are of major clinical relevance affecting several biological systems [3]. Since the GCH1 gene is highly polymorphic [4], with 231 human single nucleotide polymorphisms (SNPs) currently listed in the NCBI SNP database (www.ncbi.nlm.nih.gov/SNP/; accessed at 16 October 2007), part of the interindividual variability in GCH1 function is likely to be under genetic control. Here, we systematize the genotype–phenotype association of GCH1 polymorphisms focusing on the recently described SNPs that do not cause a loss-of-function of the enzyme but modify the adaptation to increased requirements.

1. Functional genetic polymorphisms of GCH1

1.1. Loss-of-function mutations

GCH1 mutations of clinical importance have been first recognized in coding regions or splicing sites (Fig. 2) [5–7]. These

mostly autosomal-dominant loss-of-function mutations that are typically private in a particular pedigree, lead to rare Mendelian congenital neurological diseases. For example, DOPA-responsive dystonia [5,6] characterized by Parkinson-like symptoms, results from deficiency of dopamine and other neurotransmitters; atypical phenylketonuria [7], with progressive neuronal degeneration, results from the accumulation of neurotoxic phenylalanine metabolites, due to the dependence of phenylalanine hydroxylase on BH4 availability.

1.2. Non-loss-of-function variants

1.2.1. A “pain-protective” GCH1 haplotype

In late 2006, variants in non-coding and non-splicing regions of the GCH1 gene, with allelic frequencies of approximately 15–20% in Caucasians, were recognized to play a clinical role by decreasing the risk or intensity of chronic pain [8]. These variants are associated with only moderate changes of BH4 availability, due to changes in GCH1 upregulation after stimulation and are therefore not associated with hereditary neurological disorders. Specifically, a particular GCH1 haplotype comprising 15 GCH1 SNPs (Fig. 2) has been found to be associated with improved outcome in patients with chronic neuropathic pain, and with reduced sensitivity to experimental pain [8]. Although one retrospective analysis was unable to find a genotype–phenotype association with pain

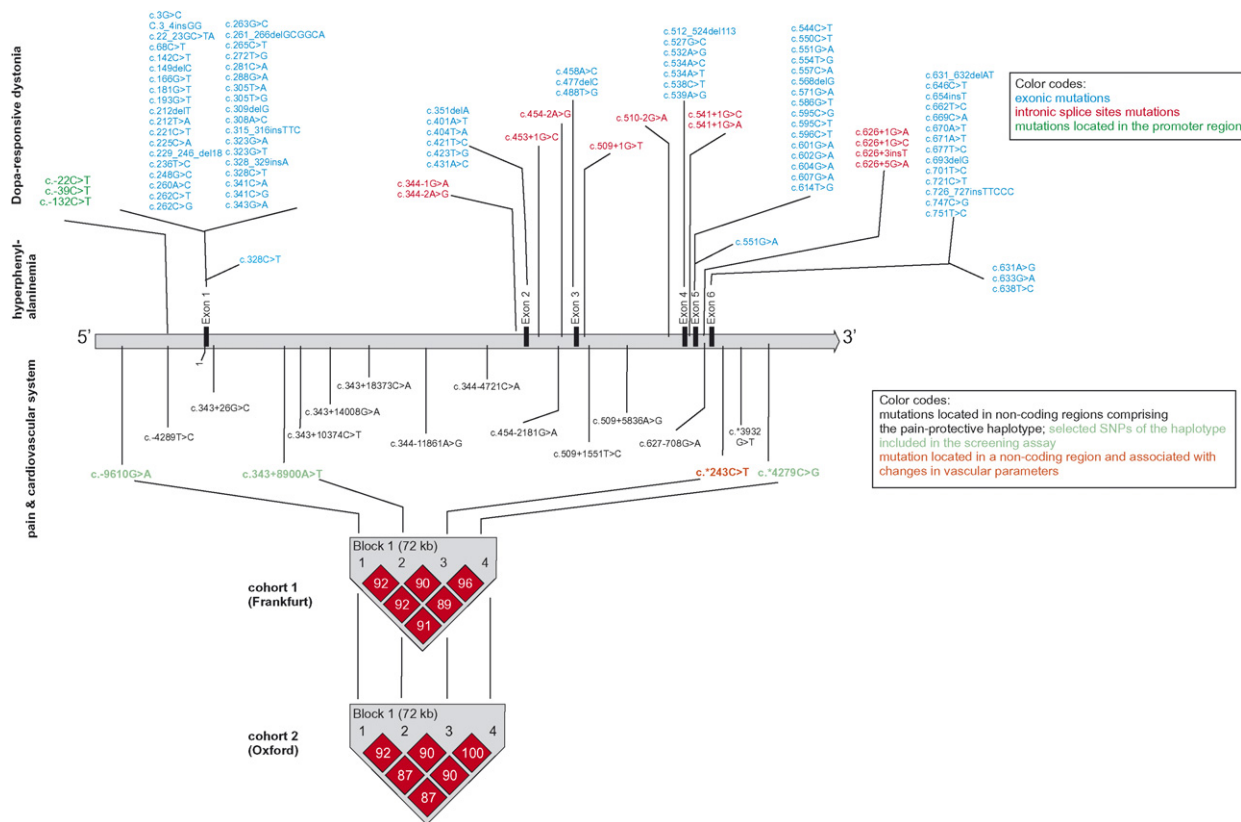


Fig. 2. Top: Distribution of functional single nucleotide polymorphisms (SNPs) along the GCH1 gene (blue: exons; red: splicing sites; green: promoter; black: non-coding regions; turquoise and orange: selected SNPs included in the screening assays) which are associated with DOPA-responsive dystonia, hyperphenylalaninemia (data taken from the BIoDEF and BIoMDB databases available at www.bh4.org) or with pain and adaptive processes in the vascular system [8,11]. The SNPs are given in the notation suggested in <http://www.hgvs.org/mutnomen> [28]: nucleotide 1 is the A of the ATG-translation initiation codon, nucleotides toward 5' of the ATG-translation initiation have negative numbers, the nucleotide 3' of the translation stop codon is *1, the next *2, etc. Intronic nucleotides for a coding DNA reference sequence are named as follows: beginning of the intron; the number of the last nucleotide of the preceding exon, a plus sign and the position in the intron, like c.453+1G>C, and end of the intron; the number of the first nucleotide of the following exon, a minus sign and the position upstream in the intron, like c.454–2A>G. Bottom: Linkage between the three SNPs used to detect the 15-SNP pain-protective haplotype and the c.*243C>T SNP is shown as D' values according to a Haploview analysis, separately for the two here analyzed cohorts. All four SNPs are located in a single haploblock.

following dental surgery [9], a phenotype consequence of the “pain-protective” haplotype has been prospectively reproduced and found to especially apply to pain associated with sensitization involving GCH1 and BH4 upregulation [10].

At the molecular level, the GCH1 “pain-protective” haplotype has been found to be associated with reduced GCH1 mRNA and protein upregulation after forskolin or lipopolysaccharide stimulation [8,10]. As a consequence, BH4 levels in white blood cells or plasma increase only weakly after stimulation in carriers of the GCH1 haplotype [10], whereas baseline BH4 production is not altered [8,10]. The reduced upregulation of BH4 is also associated with decreased iNOS upregulation and lower nitric oxide production following stimulation. However, at the molecular genetics level it had not been identified which, if any, of the 15 SNPs composing the haplotype confers these molecular consequences.

1.2.2. The cardiovascular important GCH1 c.*243C>T SNP

In 2007, another GCH1 variant, the c.*243C>T SNP (dbSNP rs841), located in the 3'-UTR, was reported to be associated with decreased nitric oxide excretion in urine, mildly increased blood pressure and heart rate and dysfunction of the baroreceptor reflex in homozygous carriers of the variant allele [11]. In twins with European ancestry, of five frequent SNPs (minor allele frequency > 5%) spanning the whole GCH1 gene, only the c.*243C>T SNP was associated with biochemical parameters dependent upon GCH1 activity, including NO and dopamine biosynthesis [11]. In addition, evidence for functional associations of the c.*243C>T variant was found by means of luciferase reporter assays in transfected PC12 chromaffin cells showing reduced luciferase activity with the variant c.*243T allele compared with the common c.*243C allele [11].

1.2.3. Carriers of the GCH1 pain-protective haplotype and carriers of the cardiovascular important GCH1 c.*243C>T SNP are the same persons

The GCH1 c.*243C>T SNP appears to be associated with similar changes of BH4 availability as the “pain-protective” haplotype [8]. However, the GCH1 c.*243C>T SNP was not among the 15 SNPs originally used to define the “pain-protective” haplotype [8]. Nevertheless, the “pain-protective” GCH1 haplotype has been found to be associated with reduced BH4 levels in both plasma and vascular tissue, and with reduced GCH1 mRNA expression in patients with coronary artery disease [12]. These biochemical differences were in turn associated with reduced nitric oxide-mediated endothelial function and with increased vascular superoxide production in carriers of the “pain-protective” GCH1 haplotype.

Thus, both the “pain-protective” haplotype [8,10,12] and the c.*243C>T SNP [11], share functional genetic properties suggesting that they might be indicators of the same genetic modulation of GCH1 consisting of a moderately decreased GCH1 expression and reduced production of BH4 or of downstream substances. That is, both the haplotype and the c.*243C>T SNP are found at a comparable allelic frequency of 15–20%. In addition, both are located in non-coding and non-splice-site regions of the GCH1 gene. Moreover, the GCH1 gene locus spanned by the “pain-protective” haplotype [8] encompasses the c.*243C>T SNP (dbSNP rs841), which is located 4036 nucleotides upstream of the most 3' end located c.*4279 SNP (dbSNP rs10483639C>G) of the “pain-protective” haplotype (Fig. 2).

To systematize the genetics of non-functional loss GCH1 variants, we analyzed whether the GCH1 “pain-protective” haplotype and the c.*243C>T SNP, both associated with distinct but also partly overlapping clinical symptoms, are carried by the same subjects, i.e., represent the same genetic modulator of GCH1. For this, from 891 healthy unrelated Caucasians (cohort 1, Frankfurt) screened

previously for the GCH1 haplotype [13] (informed consent and Ethics approval obtained), all 23 homozygous carriers, and a random sample of 60 heterozygous and 79 non-carriers were analyzed for the c.*243C>T SNP (for assay details, see Appendix A). In addition, from 347 patients with coronary artery disease (cohort 2, Oxford) of the cohort analyzed in a GCH1 cardiovascular association context [12], all 7 homozygous carriers of the pain-protective haplotype and a randomly drawn sample of 48 heterozygous and of 120 non-carriers were analyzed for the c.*243C>T SNP.

The three GCH1 SNPs analyzed to reliably diagnose the pain-protective haplotype [13] and the GCH1 c.*243C>T SNP were found to be in strong linkage disequilibrium (values of D' \geq 89 and r^2 \geq 75 for the four GCH1 SNPs in cohort 1, and values of D' \geq 87 and r^2 \geq 58 for the four GCH1 SNPs in cohort 2; for details about the genetics statistics, see Appendix A). This agreed with the localization of all four SNPs within one haploblock according to a solid spine of linkage disequilibrium analysis implemented in the Haploview software [14]. On a subjects' level, non-carriers, heterozygous or homozygous carriers of the variant GCH1 c.*243T allele were in almost all cases also non-carriers, heterozygous or homozygous carriers of the GCH1 pain-protective haplotype, respectively (χ^2 test: p < 0.001 for both cohorts). The high agreement between test allocations as non-carriers, heterozygous or homozygous carriers of either the haplotype or the c.*243T allele was also reflected in a value of Cohen's κ [15] of 0.82 in cohort 1, and 0.74 in cohort 2, which indicates very good or good agreement, respectively [16]. Moreover, the test sensitivity [17] to detect the pain-protective GCH1 haplotype by assessing the GCH1 c.*243C>T SNP was 91.7 and 100% for heterozygous and homozygous carriers, respectively, with respective test specificities [17] of 88.2 and 97.1% in cohort 1. In cohort 2, the respective values of test sensitivity were 85.4 and 100% for heterozygous and homozygous carriers, respectively, with test specificities of 88.2 and 95.2%, respectively.

2. Common functional consequences of non-coding, non-splice GCH1 polymorphisms

Pointed at by available reports about functional consequences of non-loss-of-function GCH1 variants [8,10–12] and verified by the genetics statistics performed in the context of this overview, it appears that carriers of the GCH1 pain-protective haplotype are also carriers of the GCH1 c.*243C>T SNP and vice versa. Thus, genetic variants of the GCH1 gene that have been independently associated with either protection against pain [8,10] or mildly increased blood pressure and heart rate [11] essentially represent the same clinically relevant genetic variation of GCH1. It affects non-coding and non-splice-site GCH1 regions (5'-UTR, non-splicing sites of introns, 3'-UTR). Its phenotypic consequences are decreased GCH1 upregulation upon stimulation or tissue inflammation but not a complete loss of GCH1 function. Decreased BH4 production mainly manifests in pathophysiological situations when its production would be normally increased due to upregulation of GCH1, e.g. at inflammatory sites or in injured neurons [8,10] or in blood vessels exposed to high blood pressure, high cholesterol or other cellular stress factors [12,18,19]. The moderate consequences of this variant contrast to the serious neurological pathologies associated with GCH1 variants in coding regions or splicing sites that cause the production of a dominant negative GCH1 and thus strongly reduced BH4 levels.

Thus, the clinical result of genetically altered GCH1 net function depends on the extent of GCH1 dysfunction and BH4 deficiency. So far, the main clinical evidence is available for genetic variants associated with decreased GCH1 function resulting in BH4 deficiency. However, since BH4 has been shown to induce

dopaminergic cell death involving apoptosis [20] and GCH1 upregulation contributed to neuronal cell death in stroke models [21], it is conceivable that relative BH4 overproduction in specific brain regions causes further clinically relevant pathophysiological changes in humans that might possibly contribute to, for example, the risk for Parkinson's disease or other neurologic and mental disorders.

While *GCH1* genetic variants with moderate functional consequences for BH4 expression appear to affect various pathophysiological processes in line with the respective importance of BH4, their quantitative modulation of pain, of the cardiovascular risk or of other symptoms is nevertheless modest. This is to be expected from the polygenic nature of the affected symptoms [22–25] that are additionally shaped by environmental pressures. With modest phenotypic consequences such as for pain, variants in other genes are likely to contribute to the phenotype to a similar extent, sometimes with opposite or cancelling out effects as proposed previously [26]. This might also be a reason for non-reproductions of genetic associations in polygenically controlled symptoms, as presently occurred for the pain-protection by *GCH1* variants [8,9].

In conclusion, the clinically relevant common genetic variation in *GCH1* appears to be reducible to a single gene composition that is associated with decreased but not abolished GCH1 expression and thus reduced but not absent BH4 production. Several linked *GCH1* SNPs have been reported to be indicators for this reduced GCH1 expression in the particular person; we now show that they describe in fact the same persons. Which of the variants is chosen for genetic screening, the c.*243C>T SNP or one of the SNPs able to identify the “pain-protective haplotype” [27], appears to be of minor diagnostic importance. Due to their frequency of 15–20% and due to the critical biochemical role of BH4, these variants may be genetic markers with clinical importance for various pathologies such as cardiovascular disease, chronic pain or other neurological disease states.

Conflict of interest statement

None.

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Appendix A

A.1. DNA sample origin

From 891 healthy unrelated Caucasians (cohort 1, Frankfurt) screened previously for the *GCH1* haplotype (informed consent and Ethics approval obtained), all 23 homozygous carriers, and a random sample of 60 heterozygous and 79 non-carriers were analyzed for the c.*243C>T SNP. The second cohort (cohort 2, Oxford) was pooled from two cohorts in whom we found a functional association of the *GCH1* “pain-protective” haplotype with increased vascular superoxide production and reduced endothelial function in a cardiovascular context has been recently shown [12]. It consisted of 347 patients, from whom again all 7 homozygous carriers of the pain-protective haplotype and a randomly drawn sample of 48 heterozygous and of 120 non-carriers were analyzed for the c.*243C>T SNP. In both initial

cohorts from which the samples for the present analysis were drawn, the distribution of the pain-protective haplotype corresponded to the Hardy–Weinberg equilibrium [29]. However, due to the selection of all homozygous carriers of the haplotype but random samples of heterozygous and non-carriers, the presently analyzed cohorts were not random samples and contained an increased fraction of homozygous carriers to enhance assessment of the detection of the rare homozygous subjects.

A.2. Genetic screening of the *GCH1* “pain-protective haplotype”

For the *GCH1* “pain-protective” haplotype [8], a screening assay [27] provided the genetic diagnosis with 100% sensitivity and specificity by screening for just three *GCH1* genetic variants that span the entire DNA range of the 15-SNP haplotype: c.-9610G>A (dbSNP rs8007267G>A) in the 5'-untranslated region, c.343+8900A>T (dbSNP rs3783641A>T) in intron 1, and c.*4279 (dbSNP rs10483639C>G) in the 3'-untranslated region [27] (nomenclature according to <http://www.hgvs.org/mutnomen> [28]).

A.3. Genetic screening of *GCH1* c.*243C>T

For the *GCH1* c.*243C>T SNP (dbSNP rs841) located 4036 nucleotides upstream of the c.*4279 SNP (dbSNP rs10483639C>G) included in the haplotype screening assay, we developed and validated a new screening assay. Genomic DNA was extracted from 200 µl blood samples on a BioRobot EZ1 workstation applying the blood and body fluid spin protocol provided in the EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany). The *GCH1* segment of interest was amplified by means of polymerase chain reaction (PCR), performed in a 50 µl assay volume on a Mastercycler ep gradient S instrument (Eppendorf, Hamburg, Germany), using 5 µl genomic DNA (20–30 µg/ml), mixed with 0.25 µl HotStarTaq plus DNA Polymerase (5 U/µl) (Qiagen, Hilden, Germany), 5 µl 10× PCR buffer, 10 µl 5× Q-Solution, 1 µl of dNTP mix (10 mmol/l each) (Qiagen, Hilden, Germany), 0.1 µl of each PCR primer (forward: 5'-CGTTGGTTGCCGATCGTA-3'; reverse: 5'-biotin-CAGGCCCTCTGGT-TATCT-3'; each 100 µmol/l) and 28.55 µl HPLC-purified water. Thermocycling conditions were as follows: initial denaturation step (5 min 95 °C), 45 cycles of 30 s 95 °C, 30 s 60 °C, 30 s 72 °C, and terminal extension (5 min 72 °C). PCR primers and the sequencing primer were designed using the Pyrosequencing™ assay design software (Version 1.0.6; Biotage AB, Uppsala, Sweden). Specificity of primers was verified by alignment (<http://www.ncbi.nlm.nih.gov/Blast/>).

The sequence of interest around dbSNP rs841 was analyzed by means of Pyrosequencing™ [30], technically performed according to the manufacturer's instructions (Biotage AB, Uppsala, Sweden). In brief, during Pyrosequencing™, a sequence-specific oligonucleotide (sequencing primer: 5'-TGAAGTCTAATAGTGTAAG-3') binds to purified single-stranded DNA close to the mutation site and is elongated by specific dispensing of deoxynucleoside triphosphates (dNTPs). If the dispensed deoxynucleoside triphosphate matches the next nucleotide of the DNA, it is incorporated into the oligonucleotide and pyrophosphate is released. The pyrophosphate together with adenosine-5-phosphosulfate (APS) is converted to ATP, which triggers a luciferase catalyzed luciferin-to-oxyluciferin conversion. The resulting light is shown as a peak with a height proportional to the number of incorporated nucleotides in the so-called pyrograms. For assay validation, three samples of each genotype (i.e., *GCH1* c.*243CC, CT, and TT) were conventionally sequenced (AGOWA GmbH, Berlin, Germany) and implemented as positive controls during Pyrosequencing™ (Fig. A1).

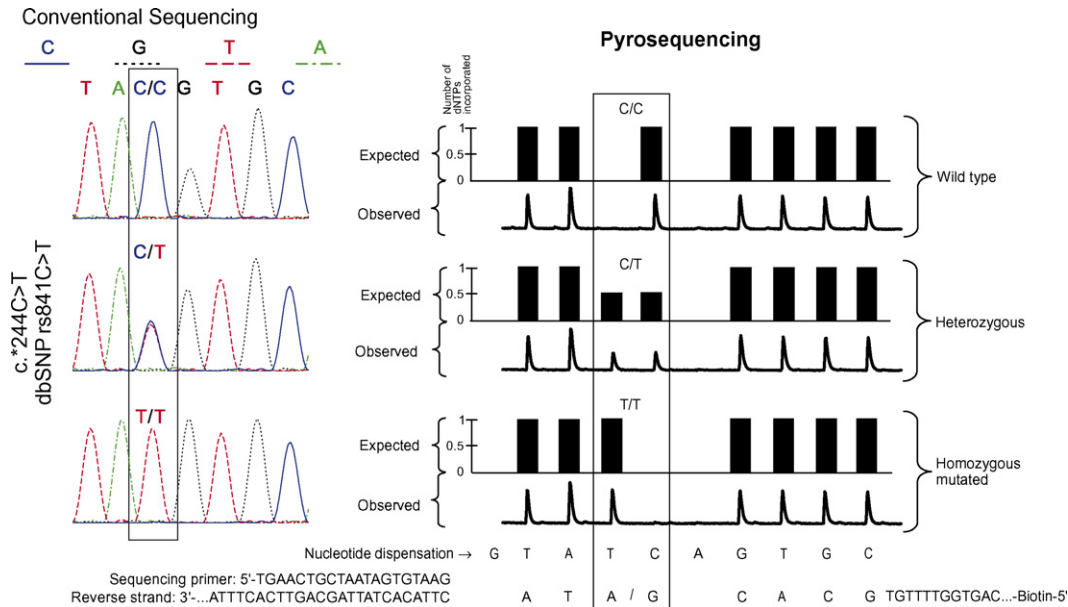


Fig. A1. Overview of the here designed forward simplex Pyrosequencing™ genetic screening assay with expected and observed pyrograms for detecting the *GCH1* polymorphism c.*243C>T (rs841). The relevant positions for genotype identification are framed. The DNA sequence on the reverse DNA strand following the sequencing primer accords to the sequence to be analyzed and determines the nucleotide dispensation order. The pyrograms denote the three possible genotypes for each DNA position. Note that the initial part in the pyrograms corresponding to the dispensation of enzyme and substrate mixes has been omitted. From conventional *GCH1* sequencing only the fragments relevant for identifying the SNPs and corresponding to the nucleotides analyzed in the Pyrosequencing™ assay (marked with frames) are displayed (different style lines and color codes for the nucleotides). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

A.4. Statistics

GCH1 haploblocks and linkage disequilibrium between the three *GCH1* SNPs of the “pain-protective” haplotype screening assay and the *GCH1* c.*243C>T SNP, with parameters D' and r^2 , were analyzed employing the solid spine of linkage disequilibrium algorithm implemented in the Haploview computer program [14]. The agreement between subjects not carrying, heterozygously or homozygously carrying the “pain-protective” *GCH1* haplotype and the *GCH1* variant rs841C>T was analyzed by means of χ^2 statistics. In addition, the sensitivity and specificity (sensitivity [%] = $100 \times \text{correctly positive} / (\text{correctly positive} + \text{false negative})$; specificity [%] = $100 \times \text{correctly negative} / (\text{correctly negative} + \text{false positive})$) [17] by which the pain-protective *GCH1* haplotype could be detected by the newly developed assay for the c.*243C>T SNP was calculated, and agreement between results of screening for the *GCH1* pain-protective haplotype and those for screening for the c.*243C>T SNP was assessed by calculating Cohen's κ [15].

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