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The *TCN2* 776C>G polymorphism correlates with vitamin B_{12} cellular delivery in healthy adult populations

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ABSTRACT

Objectives: Vitamin B_{12}, or B_{12}, is an essential nutrient for humans, and its deficiency is a public health problem, especially in elderly population. Around 30% of circulating total B_{12} levels are attached to transcobalamin II (TCN2), being referred as holotranscobalamin (holo-TC), and representing the biologically active fraction. After cellular uptake, B_{12} participates in the homocysteine (Hcy) metabolism. The potential influence of the described *TCN2* 776C>G polymorphism upon B_{12} intracellular delivery is a current target of research and we aimed to investigate its biochemical significance upon a healthy adult population.

Design and methods: The *TCN2* 776C>G polymorphism was screened by PCR-RFLP in 122 individuals. Concentrations of plasma total B_{12}, holo-TC, total Hcy and folate, as well as red blood cell folate, were determined.

Results and conclusions: The studied polymorphism is common in the Portuguese population and significantly affects holo-TC but neither total B_{12} nor total Hcy plasma concentrations, confirming that the *TCN2* 776C>G genotype exerts a significant influence upon B_{12} cellular delivery.

Introduction

Vitamin B_{12} or cobalamin is an essential nutrient for humans, because two of its derivatives, methylcobalamin and adenosylcobalamin, act as coenzymes in important metabolic reactions [1,2]. Methylcobalamin is a cytosolic methyl carrier in the folate dependent remethylation of homocysteine (Hcy) to methionine, reaction mediated by methionine synthase; in mithocondria, adenosylcobalamin is required for the isomerization of methylmalonyl-CoA into succinyl-CoA, reaction catalyzed by methylmalonyl-CoA mutase. Therefore, Hcy and methylmalonic acid accumulation are often used as biomarkers for vitamin B_{12} status. Additionally, increased circulating levels of total Hcy are generally accepted as risk factor, not only for vascular disease, but also for Alzheimer's disease, osteoporosis and congenital disorders like neural tube defects [2–4].

However, and unlike micro-organisms, mammals are not capable of synthesizing vitamin B_{12} and consequently developed complex biochemical and physiological processes for its conversion from dietary into active coenzyme forms. Cobalamin, only present in animal products, enters the stomach bound to animal proteins and is released from the proteins by pepsin and gastric acid. The free vitamin B_{12} is then bound to R-binder (haptocorrins) produced by salivary glands. In the ileum, haptocorrin is degraded by pancreatic enzymes, and vitamin B_{12} is transferred to the intrinsic factor (IF), a protein synthesized in the gastric parietal cells, by means of a pH dependent process. In the terminal ileum, the complex IF-cobalamin binds to IF receptors on the membrane surface of enterocytes and is then transferred through the ileal membrane. Vitamin B_{12} is subsequently released and enters circulation where it is coupled to transcobalamin II (TC) or to haptocorrin [5–8]. A maximum of 30% of circulating vitamin B_{12} is attached to transcobalamin II and is referred as holotranscobalamin (holo-TC), representing the biologically active fraction that is delivered to all tissues in the body. The remaining circulating vitamin B_{12} is bound to haptocorrin which is thought to transport the surplus of vitamin B_{12} to the liver [9].

Vitamin B_{12} deficiency is nowadays a major public health problem, and since a deficiency in this vitamin can lead to hematologic abnormalities and irreversible neurological damage, early diagnosis is essential [10]. In recent years, holo-TC has been suggested to be a more sensitive indicator of vitamin B_{12} status, because the most common cause of vitamin B_{12} deficiency, especially among the elderly population, is failure at one of the steps to internalize cobalamin rather than its dietary lack. Accordingly, variations in the TC protein can affect the binding characteristics of vitamin B_{12} to TC or the recognition of the holo-TC complex by the receptors responsible for its endocytosis.

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Transcobalamin II (TC) protein is encoded by the *TCN2* gene which has been mapped to chromosome 22q12-13. The gene spans around 20 kb of genomic DNA, is structured in nine exons separated by introns and displays considerable heterogeneity, being described more than 10 single nucleotide polymorphisms (SNP) [11]. One of them, the c.776C>G (dbSNP ID: rs1801198) substitution originating the P259R missense mutation, has been reported as common and potentially interfering with vitamin B_{12} intracellular availability [12,13].

Several studies have been performed in order to elucidate possible associations between this SNP in the *TCN2* gene and plasma levels of vitamin B_{12}, folates, methylmalonic acid or Hcy [12–17]. Though controversy exists, the most consistent finding is that holo-TC levels are lower in plasma from individuals homozygous for the 776G allele, than in those bearing the wild-type genotype (776CC) [13–16,18,19]. On the other hand, the clinical significance of the 776C>G SNP may be reflected by birth outcomes and, accordingly, some studies have found its association with spontaneous abortion and cleft lip or palate [20–22].

During the last years, our group has been devoted to elucidate the mechanisms underlying the correlation between an impaired Hcy metabolism and vascular disease [2,23–26]. Moreover, besides the known influence of folate status upon total Hcy levels, recently it has been suggested that vitamin B_{12} status can also modulate the same Hcy levels. Accordingly, the aim of the present study was to investigate the biochemical significance of the *TCN2* 776C>G SNP upon the cellular delivery of vitamin B_{12} in a healthy adult population and its potential reflex on Hcy concentrations. Therefore, we correlated the different *TCN2* genotypes with various plasmatic biomarkers of vitamin B_{12} status, namely holo-TC, total vitamin B_{12} and total Hcy concentrations.

Methods

Subjects

A group of 122 healthy Portuguese individuals (42M and 80F), volunteers from the Faculty of Pharmacy staff and students with a mean age of 45.9 ± 12.6 years, has been enrolled in this study. Details of lifestyle (i.e. smoking, alcohol consumption, medication, physical exercise, as well as personal and family history) and routine biochemical measurements were established using standardized questionnaires and protocols. The following criteria for inclusion were used: routine biochemical values within the normal range, normal hemogram, no history of metabolic, renal or vascular pathology and no supplementary intake of vitamins within the 2 months prior the study. The protocol was approved by the local ethics committee, and written informed consent was obtained from all participants.

Homocysteine, folate and vitamin B12 analysis

Overnight fasting (12 h) blood samples were drawn by venipuncture from all participants. Blood was collected either into EDTA-containing tubes kept on ice or into sodium citrate light-protected tubes. EDTA-blood samples were used for measurement of plasmatic levels of total Hcy (protein-bound plus free oxidized and reduced species), total vitamin B_{12} and holo-TC, as well as for preparation of genomic DNA. Sodium citrate-blood samples were used for determination of folate levels, either in plasma or red blood cells (RBC). Plasma was promptly separated by centrifugation at 4 °C, divided in aliquots and stored at −20 °C until analysis. All metabolite analysis was performed by specific immunoassays (ASSYM, Abbott Laboratories, Abbott Park, IL, USA).

Genetic analysis

Genomic DNA was isolated from peripheral blood leukocytes according to standard methods [27,28]. The 776C>G polymorphism was screened by enzymatic hydrolysis with *Scr*F1 after *in vitro* amplification of the target region in exon 6 of the *TCN2* gene, as previously described [16].

Statistical analysis

Descriptive analysis was used in frequency tables for categorical (absolute) variables, and mean and standard deviation for continuous variables. The Wilcoxon rank sum test was used to compare continuous variables between two groups. A multivariate analysis was performed using a linear regression model considering total Hcy a dependent variable and total B_{12}, holo-TCN2, RBC folate, plasma folate, age, gender, and *TCN2* C776G polymorphism as independent variables. All analyses were adjusted for subjects' age and sex. Odds ratios were then calculated and were useful to further clarify interactions between blood concentrations of holotranscobalamin, total B_{12}, total Hcy, plasma and RBC folate and *TCN2* 776 C>G genetic polymorphism; the odds and 95% confidence intervals were determined. The software used was SPSS for Windows 12.0 (SPSS Inc., Chicago, USA 2004). All *P* values are two-sided; for all statistics, significance was accepted at the 5% probability level.

Results

The characteristics of the studied population divided by transcobalmin II genotype are presented in Table 1. The analysis of the 776C>G SNP in the cohort of 122 individuals under study revealed that both alleles display similar relative frequencies: 51.6% for C allele and 48.4% for G allele. Concerning the three different possible genotypes, we could observe that 38 individuals (31.2%) carry the wild-type genotype (776CC), while 34 (27.9%) harbor the homozygous mutant genotype (776GG); the remaining 50 individuals, representing 41.0% of the studied population, proved to be heterozygous for this SNP.

The potential effect of the *TCN2* 776C>G polymorphism upon the concentrations of the major biomarkers for vitamin B_{12} status was evaluated and is shown in Table 1, where subjects are divided into

Table 1
Characteristics of studied population according to *TCN2* genotype.

	TCN2 genotype		
	CC	CG	GG
N	38	50	34
Age (year)	47 (40–49)	49 (46–53)	48 (42–50)
Total B12 (pmol/L)	349.0 (310.0–396.42)	316.5 (301.6–386.0)	309.9 (290.1–384.7)
Holo-TC (pmol/L)	66.3 (55.8–73.6)	60.7 (52.0–60.4)	54.2 (44.0–60.3)*
Plasma Hcy (μmol/L)	7.8 (7.5–9.2)	8.5 (8.0–9.4)	8.2 (7.6–9.7)
Plasma folate (nmol/L)	14.5 (13.8–20.5)	12.9 (12.3–16.4)	14.0 (12.8–17.2)
RBC folate (nmol/L)	554.9 (463.9–645.7)	563.3 (476.4–675.8)	556.7 (448.4–665.8)

Values represent medians (95% CI).
* $P<0.05$.

three groups according to their genotype. Holo-TC plasma concentrations were significantly different between the individuals carrying the wild-type and the homozygous mutant genotype. Accordingly, individuals homozygous for the 776G allele displayed significantly ($P<0.05$) lower holo-TC values (median 54.2 pmol/L, 95% CI 44.0–60.3) when compared to the ones harboring the wild-type 776CC genotype (median 66.3 pmol/L, 95% CI 55.7–73.6). On the other hand, this SNP displayed no detectable effect on either total vitamin B_{12} or total Hcy plasma levels, once no significant statistical differences were observed in their concentrations among the different genotypes; however, we could observe that individuals carrying the heterozygous genotype displayed a tendency to higher Hcy values than those harboring the homozygous, either wild-type or mutant, genotype (Table 1 and Fig. 1). Furthermore, as folate status is a major determinant of total Hcy levels, we analyzed plasma and RBC folate concentrations in order to discard biased total Hcy values; the results showed no significant differences (Table 1) among individuals.

Trying to find a correlation between the 776C>G SNP and total Hcy values, we further stratified our population into quartiles according to their holo-TC concentrations and compared total Hcy concentrations among the three different genotypes in each quartile (Fig. 2). The results showed that, in the lowest quartile, total Hcy concentrations in 776GG individuals (median 10.4 μmol/L, 95% CI 7.2–11.7) were higher than in those harboring the wild-type genotype (median 8.0 μmol/L, 95% CI 7.0–9.5), but the observed difference was not statistically significant ($P=0.16$) . However, in the same lowest quartile, when we compared total Hcy concentrations of all individuals bearing a 776G allele (homozygous mutant plus heterozygous) *versus* those harboring only the wild-type genotype, we could observe a decrease on the *P* value (median 9.8 μmol/L, 95% CI 8.6–12.0 *versus* median 8.0 μmol/L, 95% CI 7.0–9.5, respectively; $P=0.07$), although not reaching statistical significance.

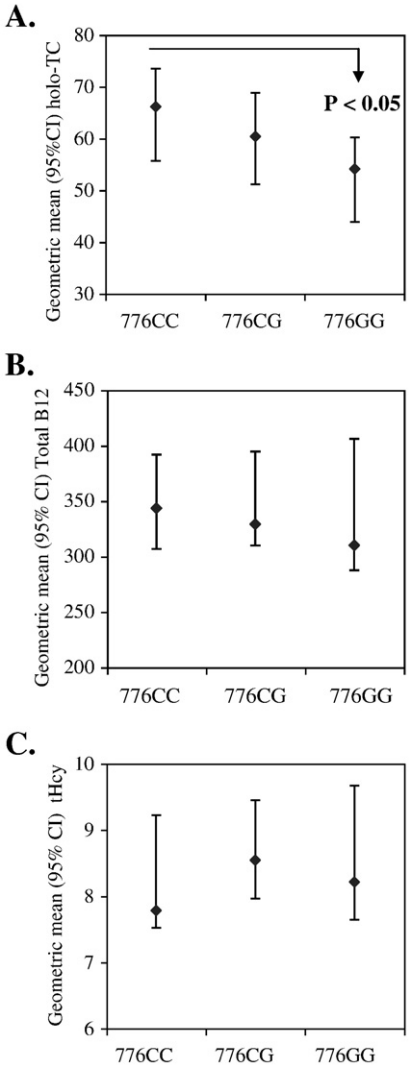

Fig. 1. *TCN2* 776C>G genotype and holo-TC (pmol/L), total B_{12} (pmol/L) and total Hcy (μmol/L) plasma concentrations.

Discussion

The potential influence of the *TCN2* 776C>G polymorphism (P259R) upon indices of vitamin B_{12} status is a current target of research, though the results are very confounding. Then, the present work aims to contribute for the elucidation of this question.

Genotyping of a cohort of 122 healthy adult Portuguese individuals showed that the frequency of the 776G allele is similar to those reported for other European Caucasian populations (45% and 47% in France and Netherlands, respectively), proving that this polymorphism is also extremely common in the Portuguese population [29]. In fact, this SNP reveals different worldwide incidence, ranging from a low prevalence in African populations (36%) to a high prevalence in Asian populations, namely in central China (56%); in between, we can find the Afro-Americans, the Caucasians and the Hispanics [15,18,19,29–31]. Though the studied population could be considered in Hardy–Weinberg equilibrium, its relative small size advises the estimate of the *TCN2* 776C>G allele frequencies to be confirmed in a larger cohort.

The biochemical significance of the 776C>G SNP upon several indicators of vitamin B_{12} status has been evaluated in previous studies, and the most consistent finding is that holo-TC values are lower in plasma from individuals homozygous for the 776G allele than from those homozygous for the wild-type allele [13–16,18,19]. In accordance, the major finding of our study is that *TCN2* 776C>G genotype significantly affects holo-TC but not total B_{12} plasma concentrations (Fig. 1).

The lack of correlation between plasma total vitamin B_{12} concentrations and *TCN2* genotypes is not surprising, since the major part of plasma cobalamin is transported bound to haptocorrin, and only a maximum of 30% circulates attached to transcobalamin II, the assumed metabolically active fraction. Therefore, total vitamin B_{12} levels within the normal range can not reliably rule out a functional cobalamin deficiency [19].

The relationship between plasma total Hcy levels and *TCN2* genotypes was also evaluated. However, we were unable to detect any significant differences among the studied genotypes; our results match those reported by Namour et al. [19] who observed that heterozygous individuals display higher total Hcy levels than individuals homozygous for the wild-type or mutant allele. Later on, Afman et al. [16] reported that the homozygous mutant genotype was associated with higher total Hcy concentrations and Alessio et al. [32] observed a statistically significant increase in total Hcy levels in children bearing the GG genotype *versus* the CC genotype. Unfortunately, these findings could not be confirmed by several other reports [12,13,15,17] and these results seem to confirm that Hcy metabolic pathway is very complex and under the control of many factors, both genetic and non-genetic.

Recently, it has been suggested that the *TCN2* 776C>G genotype effect upon total Hcy concentrations could be modulated by vitamin B_{12} status [13]. Accordingly, to elucidate the simultaneous effect of both holo-TC levels and 776C>G genotype on total Hcy levels, the studied population was stratified into quartiles according to their holo-TC concentrations (Fig. 2) and further divided in conformity to the displayed genotype. Our results showed that, only in the lowest quartile of holo-TC concentrations, the individuals homozygous for the mutant 776G allele have higher total Hcy concentrations than the

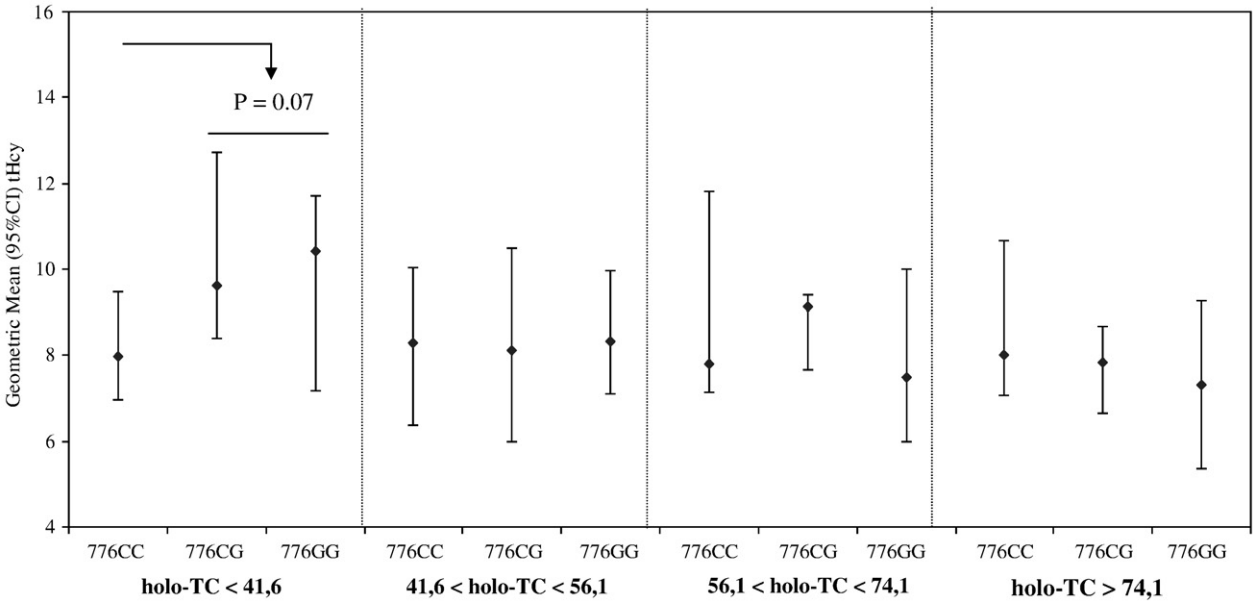

Fig. 2. Association between the *TCN2* 776C>G genotype and total Hcy concentrations (μmol/L) after stratification in quartiles for holo-TC concentrations (pmol/L).

subjects bearing the wild-type genotype, though this difference had no statistical significance ($P=0.16$). Furthermore, in order to increase statistical power, we further compared total Hcy concentrations between individuals harboring a G allele (heterozygous plus homozygous mutant) and those only bearing the C allele (wild-type); the observed statistical value was lower ($P=0.07$), but the small sample size precludes any extrapolation for a biological significance.

As we referred earlier, the major finding of this study is the clear correlation ($P<0.05$) between the *TCN2* 776C>G genotype and plasma holo-TC levels. However, the molecular basis of this correlation remains to be elucidated, although several hypotheses have been advanced.

This 776C>G biallelic polymorphism originates two different proteins carrying either a proline or an arginine at amino acid in position 259 (P259R), respectively. *In silico* modeling predicts that this substitution can potentially affect the secondary structure of the protein, thus probably influencing the binding ability of cobalamin to transcobalamin II [16]. Recently, however, Wuerges et al. [33] solved the structural basis for mammalian vitamin B_{12} transport by TC and stated that proline 259 is part of the solvent-exposed flexible loop between helices α10 and α11, spatially near the N terminus, thus suggesting that this P259R polymorphism will influence neither the binding ability of cobalamin to TC nor the stability of the α-domain. Additionally, an involvement of this substitution in receptor recognition of holo-TC seems to be ruled out due to high sequence variability among seven mammalian TC proteins in the vicinity of proline 259.

The other hypothesis to explain the diminished intracellular availability of vitamin B_{12} resides at the transcriptional level. In fact, Namour et al. [19] observed lower levels of 776G-containing *versus* 776C-containing transcripts in heterozygous cultured cells; RNA secondary structure prediction showed that the 776C-containing transcript had an additional stem loop, potentially inducing a greater stability of this transcript *versus* de 776G-containing one.

In summary, our results allow to reconfirm that the TCN2 776C>G genotype exerts a significant influence upon holo-TC cellular pool; however, the cellular enzymatic reactions dependent on vitamin B_{12}, namely the Hcy remethylation here evaluated, are modulated by other factors, genetic and non-genetic, and further studies are necessary to evaluate the effect of the aforementioned correlation upon the biomarkers linked to the dysfunction of vitamin B_{12}-dependent reactions.

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