The transcobalamin (TCN2) 776C>G polymorphism affects homocysteine concentrations among subjects with low vitamin B12 status

ORIGINAL ARTICLE

The transcobalamin (*TCN2*) 776C > G polymorphism affects homocysteine concentrations among subjects with low vitamin B$_{12}$ status

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Background/Objectives: Methionine synthase catalyzes the conversion of 5-methyltetrahydrofolate to tetrahydrofolate and homocysteine (Hcy) to methionine using vitamin B$_{12}$ as a cofactor. Transcobalamin is the main transporter of vitamin B$_{12}$ from blood into cells. This study was undertaken to assess the relationship between the transcobalamin P259R (*TCN2* 776C > G) polymorphism and both serum vitamin B$_{12}$ and total Hcy (tHcy) levels.
Subjects/Methods: The population comprised 613 men from Northern Ireland, aged 30–49 years, for whom tHcy, serum vitamin B$_{12}$ and serum folate concentrations were available. *TCN2* 776C > G genotypes were determined using a TaqMan 5′ nuclease Real-Time PCR assay. Standard statistical tests of association were applied to assess the relationships between the polymorphism and phenotypic variables.
Results: The *TCN2* 776CC homozygous genotype was associated with lower serum vitamin B$_{12}$ concentrations compared with the 776CG ($P_{\text{unadjusted}} = 0.01$; $P_{\text{adjusted}} = 0.03$) and 776GG genotypes ($P_{\text{unadjusted}} = 0.015$; $P_{\text{adjusted}} = 0.045$). Among individuals with vitamin B$_{12}$ concentrations in the lower half of the distribution, tHcy concentrations were higher in *TCN2* 776GG homozygotes than in individuals with the other genotypes ($P_{\text{unadjusted}} = 0.015$; $P_{\text{adjusted}} = 0.06$).
Conclusions: These data suggest that, relative to transcobalamin with arginine at position 259 (776G), transcobalamin with proline at this position (776C) is either more efficient at vitamin B$_{12}$ transport from blood to tissues or has higher affinity for vitamin B$_{12}$. Furthermore, vitamin B$_{12}$ status influences the relationship between *TCN2* 776C > G genotype and tHcy concentrations. Thus, the *TCN2* 776C > G polymorphism may contribute to the risk of pathologies associated with a low B$_{12}$, and high tHcy phenotype.
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Introduction

Vitamin B$_{12}$ (cobalamin) is an enzyme cofactor that is synthesized only in microorganisms and, in humans, must be obtained from food or supplements (Scott and Weir,

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1994). Serum vitamin B$_{12}$ concentrations decrease with age (Baik and Russell, 1999), and among elderly patients with clinically apparent vitamin B$_{12}$ deficiency, 75–90% develop neurological complications such as sensory and motor disturbances and cognitive impairment (Baik and Russell, 1999). Low B$_{12}$ status is associated with increased levels of total homocysteine (tHcy) and methylmalonic acid (Pennypacker *et al.*, 1992), and low maternal B$_{12}$ status in early pregnancy has been associated with an increased risk of spina bifida in the offspring (Kirke *et al.*, 1993; Molloy *et al.*, 2009).

Vitamin B$_{12}$ is transported from the intestine to the bloodstream in a complex with intrinsic factor (Seetharam, 1999), whereas its transport from blood to cells depends

mainly on transcobalamin (Seetharam *et al*., 1999). Inadequate *cellular* levels of vitamin B$_{12}$ may be due to impairment in either of these processes. Intracellular vitamin B$_{12}$ is a cofactor of methionine synthase, which catalyzes the conversion of Hcy to methionine, concomitant with the conversion of 5-methyl-tetrahydrofolate (5-methyl-THF) to THF (Figure 1). Hence, altered levels of B$_{12}$ may cause changes in folate/Hcy metabolism (Selhub *et al*., 2007). It has been suggested that the inhibition of methionine synthase caused by B$_{12}$ deficiency creates a 'methyl-trap', which results in the accumulation of 5-methyl-THF and hence the elevation of tHcy concentrations (Scott and Weir, 1981).

Transcobalamin is synthesized by hepatocytes, endothelial cells and enterocytes (Ermens *et al*., 2003). Although most circulating B$_{12}$ is bound by haptocorrin, transcobalamin binds 22–37% of plasma B$_{12}$ (Refsum *et al*., 2006a), and is responsible for up to 99% of B$_{12}$ transport into tissues (Baik and Russell, 1999). In humans the gene encoding transcobalamin (*TCN2*) is polymorphic (Daiger *et al*., 1978). To date, six nonsynonymous single nucleotide polymorphisms have been identified (Li *et al*., 1994; Afman *et al*., 2002), the most extensively studied of which is *TCN2* 776C>G (rs1801198) (P259R) in exon 6. Higher holo-transcobalamin (transcobalamin–vitamin B$_{12}$ complex) levels are observed among *TCN2* 776CC homozygotes than in those with the other *TCN2* 776C>G genotypes (Miller *et al*., 2002).

Furthermore, in the highest quartile of the vitamin B$_{12}$ distribution, *TCN2* 776CC homozygotes have lower tHcy concentrations compared with those with the other genotypes (Lievers *et al*., 2002). The latter observation suggests that plasma vitamin B$_{12}$ levels are a determinant of the relationship between *TCN2* 776C>G genotype and tHcy concentrations. In addition, the *TCN2* 776GG genotype has been associated with a lower proportion of vitamin B$_{12}$ bound to transcobalamin (calculated as a holo-transcobalamin/total transcobalamin ratio) (Afman *et al*., 2002), lower holo-transcobalamin levels *per se* (Afman *et al*., 2002; Wans *et al*., 2003; von Castel-Dunwoody *et al*., 2005), lower apo-transcobalamin (that is, transcobalamin not bound to vitamin B$_{12}$) levels (Namour *et al*., 1998, 2001), lower total transcobalamin levels (Namour *et al*., 2001; Afman *et al*., 2002), higher methylmalonic acid concentrations (Miller *et al*., 2002; Fredriksen *et al*., 2007) and higher tHcy concentrations (Alessio *et al*., 2007; Gueant *et al*., 2007). The association between the *TCN2* 776C>G polymorphism and tHcy levels has also been reported to be underpinned, in part, by folate concentrations (Gueant *et al*., 2006). In epidemiological studies, the *TCN2* 776C>G polymorphism has been associated with the risk of several conditions including Alzheimer's disease (McCaddon *et al*., 2004), spontaneous abortion (Zetterberg *et al*., 2002) and spina bifida (Pietrzyk and Bik-Multanowski, 2003). However, the association with
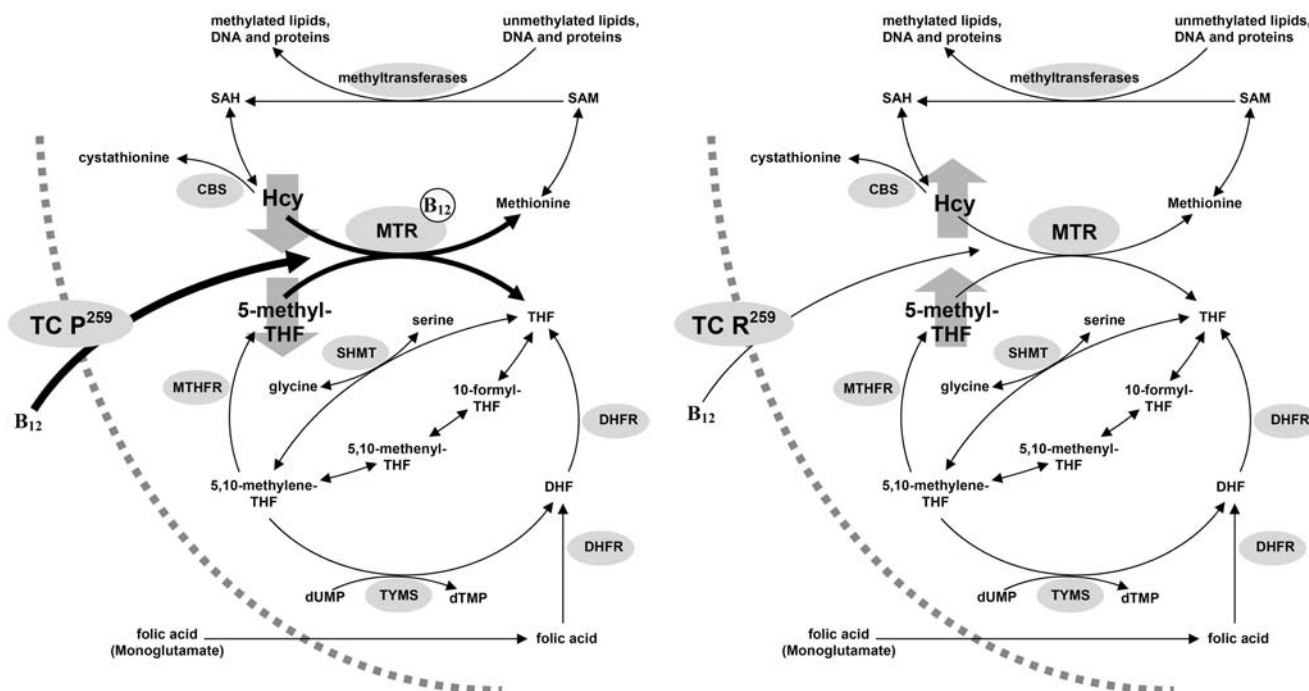
Figure 1 Schematic representation of folate/Hcy metabolism, incorporating a model for potential phenotypic consequences of having TC P^{259} (left) or TC R^{259} (right). CBS, cystathionine β-synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, deoxythymidine monophosphate; dUMP deoxyuridine monophosphate; Hcy homocysteine; MTHFR, 5,10-methylenetetrahydrofolate reductase; MTR, methionine synthase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SHMT, serine hydroxymethyltransferase; TC, transcobalamin; THF, tetrahydrofolate; TYMS, thymidylate synthase.

spina bifida has not been observed in all studies of this condition (Afman *et al.*, 2002; Swanson *et al.*, 2005).

The aim of this study was to investigate the relationships between the *TCN2* 776C>G genotype, vitamin B$_{12}$, folate and tHcy concentrations in a population of Northern Irish men aged 30–49 years and to identify particular genotype/phenotype combinations that might confer risk of pathologies that are known to be associated with elevated tHcy concentrations (Mattson *et al.*, 2002; Wald *et al.*, 2002).

Materials and methods

Study population
The study population has been previously described (Harmon *et al.*, 1996; Woodside *et al.*, 1998). Briefly, between January and March 1995, 765 male volunteers aged 30–49 years, from a single Belfast-based industrial workforce, were invited to a screening clinic. Men from all grades of staff (that is, manual, clerical, administrative and executive) were eligible to participate in the study. After giving informed consent, a fasting venous blood sample was obtained from each study subject and a brief medical history was taken; 140 individuals who were either diabetic, had been administered a general anesthetic within the previous 3 months, or were using vitamin supplements, were excluded. The study was approved by the Research Ethics Committee of the Faculty of Medicine, the Queen's University of Belfast, in compliance with the guidelines of the Royal College of Physicians of London.

Genetic analysis
TCN2 776C>G (rs1801198) genotypes were determined using a TaqMan 5′ nuclease Real-Time PCR assay on a PTC-200 DNA Engine (Bio-Rad, Hercules, CA, USA) with fluorescence detection by a Chromo4 Real-Time PCR detector (Bio-Rad). Individual PCR amplification reactions (final volume of 20 μl each) contained 4–25 ng of sample DNA, 1 × TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA, USA), 0.5 μM forward primer (5′-TCTATCACCAGTTCCTCATGA-3′) and 0.5 μM reverse primer (5′-GCCTTGAGACATGCTGTTC-3′), 50 nM C-specific probe (VIC-CCCCAGGCATGGG) and 50 nM G-specific probe (FAM-CCCCACGCATGGG). Probe sequences were derived from the SNP500Cancer website (http://snp500cancer.nci.nih.gov). The probes were synthesized by Applied Biosystems. For the PCR amplification, initial incubation was at 95 °C for 10 min followed by 60 cycles of denaturation at 95 °C for 30 s and extension/5′ nuclease steps at 62 °C for 40 s. Dual fluorescence was detected after each completed 70 s cycle. Genotypes were assigned using Opticon Monitor 3 analysis software (Bio-Rad).

Determination of tHcy, serum vitamin B$_{12}$ and serum folate concentrations
The methods for determining tHcy (that is, free plus protein-bound Hcy), serum vitamin B$_{12}$ and serum folate have previously been described (Harmon *et al.*, 1996). In brief, tHcy was quantified using the modified high-performance liquid chromatography method (Ubbink *et al.*, 1991). Serum vitamin B$_{12}$ and serum folate concentrations were ascertained using a kit from ICN Pharmaceuticals (Costa Mesa, CA, USA).

Statistical methods
Descriptive analyses of the study variables were conducted using data from all study subjects, and included means, medians and percentiles for continuous variables (that is, tHcy, serum vitamin B$_{12}$ and serum folate) and proportions for categorical variables (that is, genotypes). In addition, departure of the *TCN2* 776C>G genotype distribution from Hardy–Weinberg equilibrium was assessed using χ^2 analysis.

The distributions of tHcy, serum vitamin B$_{12}$ and serum folate were skewed, even after log transformation. Therefore, non-parametric and ranked analyses were performed. Differences in tHcy, vitamin B$_{12}$ and serum folate concentrations between the *TCN2* 776C>G genotypes in the population as a whole and within strata defined by vitamin B$_{12}$ and folate levels were tested using the Kruskal–Wallis test. Tests with associated *P*-values $\leqslant 0.05$ were followed by pairwise comparisons using Wilcoxon rank-sum tests, corrected for multiple comparisons using Bonferroni method. Adjusted *P*-values of $\leqslant 0.05$ were considered statistically significant. All statistical analyses were carried out using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA).

Results

A total of 625 men participated in this study. However, biochemical and genetic data were unavailable for 10 subjects. In addition, two subjects had extreme tHcy concentrations of 58.5 and 76.9 μmol/l and were excluded, leaving data from 613 subjects for analysis. The characteristics of the study subjects are summarized in Table 1. The distribution of *TCN2* 776C>G genotypes was not significantly different than expected under the assumption of Hardy–Weinberg equilibrium (*P* = 0.2).

In the whole population, serum vitamin B$_{12}$ concentrations were significantly associated with the *TCN2* 776C>G genotype (*P* = 0.01), with significantly lower B$_{12}$ concentrations among *TCN2* 776CC homozygotes, as compared to those with the *TCN2* 776CG ($P_{\text{unadjusted}} = 0.01$; $P_{\text{adjusted}} = 0.03$) or GG genotypes ($P_{\text{unadjusted}} = 0.015$; $P_{\text{adjusted}} = 0.045$) (Table 2). In contrast, tHcy levels were not significantly related to *TCN2* 776C>G genotype in the total study population (*P* = 0.4, Table 2). However, following subdivision of the study population into those with B$_{12}$ concentrations at or below the median ($\leqslant 261.96$ pmol/l) and above the median (>261.96 pmol/l),

an association between the *TCN2* 776C>G genotype and tHcy concentration was observed in the former (Table 2). In this subset, *TCN2* 776GG homozygotes had higher median tHcy concentration compared with 776C allele carriers (8.5 vs 7.5 μmol/l; $P_{unadjusted} = 0.015$). However, after Bonferroni adjustment for the four comparisons, this observation was no longer significant ($P_{adjusted} = 0.06$). No such association was observed among subjects with B$_{12}$ levels above the median. Furthermore, the *TCN2* 776C>G polymorphism was not associated with altered serum folate concentrations in any of the subgroups defined by vitamin B$_{12}$ levels (data not shown).

When the study population was analyzed after sub-division according to folate concentrations, that is, at or below the median (≤10.81 nmol/l) and above the median (>10.81 nmol/l), there was no significant association between the *TCN2* 776C>G genotype and tHcy concentration in either subgroup (data not shown). Further, when the study population was analyzed after subdivision according to a combination of folate and vitamin B$_{12}$ concentrations

(that is, folate ≤10.81 nmol/l and B$_{12}$ ≤261.96 pmol/l; folate >10.81 nmol/l and B$_{12}$ >261.96 pmol/l; folate ≤10.81 nmol/l and B$_{12}$ >261.96 pmol/l; folate >10.81 nmol/l and B$_{12}$ ≤261.96 pmol/l;), there was no significant association between the *TCN2* 776C>G genotype and tHcy concentration in any of the subgroups (data not shown).

Discussion

In our Northern Irish male study population, the *TCN2* 776C>G (P259R) genotype is associated with serum vitamin B$_{12}$ concentrations, the 776CC genotype being associated with lower concentrations compared with those with other genotypes. Other investigators have reported that the *TCN2* 776C>G polymorphism shows no association with altered vitamin B$_{12}$ concentrations in several heterogeneous populations (Miller *et al.*, 2002; Wans *et al.*, 2003; Zetterberg *et al.*, 2003; von Castel-Dunwoody *et al.*, 2005; Alessio *et al.*, 2007; Fredriksen *et al.*, 2007). However, in two of these populations (Miller *et al.*, 2002; Fredriksen *et al.*, 2007), the *TCN2* 776C>G polymorphism was associated with altered concentrations of methylmalonic acid, a good indicator of cellular vitamin B$_{12}$ status. Our observations therefore may indicate that the different transcobalamin variants differentially affect the distribution of vitamin B$_{12}$ between the circulation and tissues such that there is less circulating vitamin B$_{12}$ in *TCN2* 776CC homozygotes than in 776CG heterozygotes or 776GG homozygotes.

Recently, the two-domain structure of transcobalamin, comprising an N-terminal domain of 12 α-helices plus a 3/10 helix and a C-terminal domain with mostly β-strands, was solved by X-ray crystallography (Wuerges *et al.*, 2006). The polymorphic site at amino-acid residue 259 is located in the N-terminal domain in the turn preceding helix α11. Any change in the properties of this turn could alter the tertiary

Table 1 Characteristics of male industrial workers in Belfast, Northern Ireland

Biochemical variables	N (%)	Mean (s.d.)	Median (25th–75th percentile)
Homocysteine (μmol/l)	613	7.5 (±2.8)	7.1 (5.9–8.6)
B$_{12}$ (pmol/l)[a]	597	279.8 (±112.5)	262.7 (202.8–344.1)
Folate (nmol/l)[b]	598	12.4 (±6.1)	11.0 (8.5–14.0)
TCN2 776C>G genotype			
CC	195 (31.8)		
CG	286 (46.7)		
GG	132 (21.5)		

Abbreviation: *TCN2*, transcobalamin gene.
[a]Vitamin B$_{12}$ levels were not known for 16 subjects.
[b]Serum folate levels were not known for 15 subjects.

Table 2 Associations between *TCN2* 776 C>G genotype and biochemical parameters in the population as a whole and in subsets after stratification by vitamin B$_{12}$ concentration[a]

	Biochemical parameter	TCN2 776CC	TCN2 776CG	TCN2 776GG	P-value by Kruskal–Wallis
All subjects					
	Homocysteine (μmol/l)	7.2 (5.8–8.9) (195)	6.9 (5.9–8.4) (286)	7.2 (5.9–8.6) (132)	0.4
	Vitamin B$_{12}$ (pmol/l)	243.5 (182.8–333.0) (190)	269.7 (212.8–343.7) (276)	279.7 (213.9–351.5) (131)	0.01[b]
Vitamin B$_{12}$ rank					
≤261.96 pmol/l lowest 50%	Homocysteine (μmol/l)	7.5 (6.1–9.4) (108)	7.4 (6.2–8.9) (132)	8.5 (6.5–10.9) (58)	0.04[c]
>261.96 pmol/l highest 50%		6.6 (5.5–8.4) (82)	6.5 (5.4–7.6) (144)	6.6 (5.6–7.5) (73)	0.6

Abbreviation: *TCN2*, transcobalamin gene.
[a]Median (25th–75th percentile) (*n*, number of individuals).
[b]$P_{unadjusted} = 0.015$ for comparison of *TCN2* 776CC with GG; $P_{unadjusted} = 0.01$ for comparison of *TCN2* 776CC with CG; and $P_{unadjusted} = 0.5$ for comparison of *TCN2* 776CG with GG genotype.
[c]$P_{unadjusted} = 0.05$ for comparison of *TCN2* 776CC with GG; $P_{unadjusted} = 0.5$ for comparison of *TCN2* 776CC with CG; $P_{unadjusted} = 0.01$ for comparison of *TCN2* 776CG with GG; and $P_{unadjusted} = 0.015$ for comparison of *TCN2* 776C allele with GG genotype.

positions of the flanking α-helices and ultimately affect the overall structure of the protein and its vitamin B$_{12}$-binding capability. That the substitution at residue 259 is a proline, known as a helix breaker, increases the likelihood that the two forms of the transporter specified by *TCN2* 776C>G are functionally distinct. Indeed, it has been suggested by others that transcobalamin P259R variants may exhibit different vitamin B$_{12}$-binding affinities (Afman *et al.*, 2002; Miller *et al.*, 2002). Another mechanism by which the *TCN2* 776C>G polymorphism might affect vitamin B$_{12}$ distribution is suggested by the reported differences in 776C and 776G transcript concentrations (Namour *et al.*, 2001).

Our data indicate that the *TCN2* 776C>G genotype is also associated with tHcy concentrations in subjects in the lower half of the vitamin B$_{12}$ distribution; *TCN2* 776GG homozygotes in this subset have higher tHcy concentrations than 776C allele carriers ($P_{unadjusted} = 0.015$; $P_{adjusted} = 0.06$). The apparently paradoxical observation of high circulating vitamin B$_{12}$ (in the population as a whole) and high tHcy concentrations (in the lower half of the B$_{12}$ distribution) associated with the *TCN2* 776G allele may be the result of less efficient vitamin B$_{12}$ transport from the circulation to tissue and a consequent biased distribution of the available pool of B$_{12}$ favoring retention in the circulation. With respect to remethylation of Hcy to methionine, the reduced levels of tissue B$_{12}$ in *TCN2* 776GG homozygotes may become biologically relevant when overall B$_{12}$ status is suboptimal. A possible mechanism for the observed association between the *TCN2* 776C>G polymorphism and tHcy is suggested by the 'methyl-trap' hypothesis (Scott and Weir 1981). According to this hypothesis, when inadequate amounts of cellular vitamin B$_{12}$ are available, Hcy levels increase due to impairment of the activity of methionine synthase, which requires vitamin B$_{12}$ as a cofactor (Figure 1). Less efficient vitamin B$_{12}$ transport into the tissues of *TCN2* 776GG homozygotes may therefore result in increased tHcy concentrations among individuals who have low serum vitamin B$_{12}$ levels. Under this scenario, a genetically mandated reciprocal relationship with tHcy would not be apparent among subjects with high serum vitamin B$_{12}$ status and consequent optimally active methionine synthase. However, we were unable to test this possible explanation in our study population as cellular 5-methyl-THF concentrations were not available.

In conclusion, we report that in a study population of 613 Northern Irish men, the *TCN2* 776C>G genotype is associated with circulating vitamin B$_{12}$ concentrations. This is most likely attributable to higher vitamin B$_{12}$ transport from blood to tissues associated with the transporter encoded by the *TCN2* 776C allele (which results in a proline at residue 259 in the amino-acid sequence) than that associated with the transporter encoded by the *TCN2* 776G allele (which results in an arginine at residue 259). However, biochemical and biological studies of the relative transfer efficiencies and B$_{12}$ affinities of the transcobalamin variants will be needed in order to define the precise mechanism

underlying the above observation. After subdivision of the study population according to serum vitamin B$_{12}$ status, the 776GG genotype (carried by 21.5% of subjects in our study population) was associated with elevated tHcy concentrations among subjects in the lower half of the B$_{12}$ distribution, although this finding will need to be confirmed in a larger study. The above observations suggest that the *TCN2* 776GG genotype may be a modest risk factor for lower *cellular* vitamin B$_{12}$ levels and concomitantly elevated tHcy concentrations. This genotype may therefore contribute to the risk of pathologies such as cardiovascular disease that have been associated with a low folate, low B$_{12}$, and high tHcy phenotype (Selhub 2006; Refsum *et al.*, 2006b).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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