Vitamin B₁₂ and transcobalamin in bovine milk: Genetic variation and genome-wide association with loci along the genome

Grum Gebreyesus,^{1*} Nina Aagaard Poulsen,² Mette Krogh Larsen,^{2,3} Lotte Bach Larsen,² Esben Skipper Sørensen,⁴ Christian Würtz Heegaard,⁴ and Bart Buitenhuis¹

Graphical Abstract

Summary

This study reports significant associations between the log-transformed transcobalamin content in the Danish Holstein milk and 28 single nucleotide polymorphisms (SNPs) across *Bos taurus* autosomes (BTA). Of these 28 significantly associated SNPs, 24 were on BTA17, where strong associations were detected, with $-\log_{10}(P\text{-value})$ up to 62.93. The QTL region on BTA17 spanned between 71.71 and 71.79 Mbp.

Highlights

- High heritability (0.61 ± 0.13) was found for milk transcobalamin content in Danish Holstein.
- Moderate heritability (0.37 ± 0.18) was found for vitamin B₁₂ content in Danish Holstein.
- Twenty-eight SNPs were detected with strong association to the milk content of transcobalamin.
- A strong QTL region was detected for transcobalamin on BTA17 (71.71–71.79 Mbp).

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Abstract: In human nutrition, bovine milk is an essential source of bioavailable vitamin B₁₂ and B₁₂-binding proteins, including transcobalamin. In this study, we estimated genetic parameters for milk content of vitamin B₁₂ and transcobalamin using milk samples from 341 and 663 Danish Holstein cows, respectively. Additionally, we conducted whole-genome association analysis to identify SNP and genes associated with vitamin B₁₂ and transcobalamin. Our results indicated moderate to high heritability for vitamin B₁₂ (0.37 ± 0.18) and transcobalamin (0.61 ± 0.13) content in the Danish Holstein. With a significance threshold of $-\log_{10} P\text{-value} > 5.87$, significant associations were detected between SNP in *Bos taurus* autosome (BTA)17 and the log-transformed transcobalamin content of milk; no significant association was detected for vitamin B₁₂. The significant region in BTA17 was imputed to full sequence for further fine mapping, and the SNP with the most significant associations to transcobalamin were assigned to the transcobalamin 2 (*TCN2*) gene.

In human nutrition, milk and dairy products are important sources for several vitamins, including cobalamin (vitamin B₁₂; Rooke et al., 2010). The primary origin of water-soluble vitamins, including B₁₂, is through microorganism biosynthesis in the rumen (Schwab et al., 2006) and products from ruminants, such as milk and meat, which are generally rich in B₁₂ vitamins. Studies have established links between vitamin B₁₂ deficiency and some serious disorders in humans, including neurodegeneration and anemia (Hahn et al., 1988; Green and Lindsay, 2017). Humans are ultimately dependent on animal sources of vitamin B₁₂, and deficiency is caused by inadequate intake, inadequate bioavailability, or malabsorption. Deficiency can affect individuals at all ages but particularly elderly individuals and vegetarians (Song et al., 2010; Green and Lindsay, 2017).

Studies indicate that vitamin B₁₂ in milk from cows has a high bioavailability (Matte et al., 2014). Bovine bulk milk contains approximately 2 to 6 µg/L of vitamin B₁₂ (Duplessis et al., 2016); therefore, 1 L of milk contains more than the current recommended dietary allowance for adults (2.4 µg/d; Institute of Medicine, 1998). In milk from cows, B₁₂ is evenly distributed between the whey and the casein micelle fraction, bound to transcobalamin (TC) in the whey, and via coordination to histidine residues of the caseins, respectively (Fedosov et al., 2018, 2019). In humans, TC functions primarily as a circulatory protein, which binds B₁₂ following its absorption and delivers it to peripheral tissues via the receptor CD320. Recently, we found that cow milk-derived TC–B₁₂ complex was more effective at stimulating receptor-mediated passage of B₁₂ across polarized monolayers of human intestinal epithelial (Caco-2) cells than human TC–B₁₂ complex (Juul et al., 2019). Clinical studies have shown that 8 wk of daily intake of

whey protein isolate improved biomarkers of B₁₂ status in elderly Australians with subclinical B₁₂ deficiency and, more recently, long-term daily whey powder intake was shown to reinforce B₁₂ status in healthy elderly Danes (Dhillon et al., 2017; Greibe et al., 2020). Furthermore, it has been reported that whey powder supplement provided over 4 wk was as efficient as synthetic B₁₂ vitamin pills in improving biomarkers of B₁₂ deficiency in lactovegetarians (Naik et al., 2019).

Despite the acknowledged value of bovine milk as a B₁₂ source (Rooke et al., 2010), very few studies have documented the drivers for B₁₂ and TC content variation in milk within and across bovine breeds. Rutten et al. (2013) quantified B₁₂ in 544 first-lactation Dutch Holstein-Friesian cows and found an average content of 4.40 mg/L (range 1.0 to 12.9 mg/L). The study estimated a moderate heritability of 0.37, suggesting that vitamin B₁₂ content in milk could be altered through genetic selection. A genome-wide association study identified 68 significant SNP associated with B₁₂, but none of these was associated with genes involved in B₁₂ transport (Rutten et al., 2013).

The aim of this study was to determine the contents of B₁₂ and TC in milk from Danish Holstein cows and estimate genetic influence on these traits. An additional aim was to identify QTL associated with the variability in milk B₁₂ and TC contents. To our knowledge, this is the first study to screen B₁₂ and TC at the same time in milk from a large number of cows.

Morning milk samples were collected from 663 Danish Holstein cows in 21 herds in Denmark, as described by Gebreyesus et al. (2017). Of the collected milk samples, only 341 were used to quantify B₁₂, whereas TC was quantified in all samples.

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Table 1. Descriptive statistics and genetic parameters for milk vitamin B₁₂ and milk transcobalamin contents in milk from Danish Holstein cows¹

Variable	Mean	CV (%)	σ_a^2 (SE)	σ_e^2 (SE)	h^2 (SE)
B ₁₂ (µg/L)	3.93	41.0	0.008 (0.090)	0.014 (0.118)	0.37 (0.18)
Transcobalamin (pmol/L)	557.00	81.0	0.036 (0.190)	0.023 (0.151)	0.61 (0.13)

¹ σ_a^2 = genetic variation, σ_e^2 = residual variation, and h^2 = heritability; genetic and residual variation and heritability were estimated based on the log-transformed data.

All procedures to collect the samples followed the protocols approved by the National Guidelines for Animal Experimentation and the Danish Animal Experimental Ethics Committee. Milk sampling was restricted to routine on-farm procedures that did not cause any inconvenience or stress to the animals; hence, no specific permission was required.

Total B₁₂ was measured by the standard procedure for the Advia Centaur CP System (Siemens Healthcare Diagnostics), using hydroxo-B₁₂ as standard. The endogenous B₁₂ was extracted from the milk samples as previously described (Fedosov et al., 2019).

The in-house bovine TC ELISA protocol was performed as previously described (Fedosov et al., 2019). A custom-made polyclonal rabbit antibody and a monoclonal antibody, both raised against recombinant bovine TC, were used as capture and detect

antibodies, respectively. Recombinant bovine TC was used as calibrator.

Genotyping was performed as described in Buitenhuis et al. (2013). In short, genomic DNA was extracted from ear tissues of 663 Danish Holstein cows. In total, 341 animals were genotyped with the BovineHD BeadChip (Van Tassell et al., 2008), whereas the remaining 322 animals were genotyped with the Bovine50K BeadChip (Illumina Inc.). The genotyping was accomplished using an Illumina Infinium II Multisample assay device. iScan and Beadstudio version 3.1 software (Illumina Inc.) were used for scanning and analysis of the SNP chips. Quality parameters for the selection of SNP were as outlined by Buitenhuis et al. (2013), and individuals with average GenCall scores <0.65 were excluded, following Teo et al. (2007). Based on the overlap between the 2 SNP chips,

Figure 1. Manhattan plot for association analysis of log₁₀ B₁₂ (top) and log₁₀ transcobalamin (bottom) using 50K genotype data.

37,458 SNP were used to calculate the genomic relationship matrix and the initial association analysis. Genomic relationship matrix was calculated following method 1 of VanRaden (2008).

Genotype on BTA17 was imputed to full sequence in a 2-step procedure, as described in detail in Gebreyesus et al. (2016). First, the group of cows genotyped using the Bovine50K chip were imputed to the BovineHD (777K) level using a reference of 3,383 animals, including the 341 cows from the current study. In this step, only the 50K SNP that passed quality control (i.e., minor allele frequency >0.05 and GenCall scores ≥0.65) were used in the target population. Subsequently, the true and imputed high-density data for both groups of cows were merged, and imputation was undertaken to the whole-genome sequence level for BTA17 using a reference of 1,228 animals from run4 of the 1,000 Bull Genomes project (Daetwyler et al., 2014). In both steps, data sets of different densities were pre-phased with Beagle 4 r1398 (Browning and Browning, 2013) and imputed using IMPUTE2 v2.3.1 (Howie et al., 2011). After imputation, a total of 391,026 variants were available on BTA17 for the fine-mapping study. The SNP positions were based on the *Bos taurus* genome assembly UMD 3.1 (Zimin et al., 2009).

Records for both B₁₂ and TC were log-transformed for the genetic analyses following tests for normality. The REML approach in DMU (Madsen and Jensen, 2007) was used to estimate the genetic parameters and variance components using the following model in the analysis

$$Y_{ijkl} = \mu + herd_i + parity_j + b_1 DIM_k + b_2 e^{-0.05 \times DIM_k} + animal_l + e_{ijkl}, \quad [1]$$

where Y_{ijkl} represents the phenotype of individual l in herd i and parity j , μ is the overall mean, $herd_i$ ($i = 1, 2, \dots, 21$ for TC, and $i = 1, \dots, 3$ for B₁₂) and $parity_j$ ($j = 1, 2, \dots, 5$) are fixed effects; b_1 and b_2 are regression coefficients for DIM_k and $e^{-0.05 \times DIM_k}$, respectively, where DIM_k is a covariate of days in milk (d 4 to 877), and $e^{-0.05 \times DIM_k}$ is the Wilink adjustment of DIM (Wilink, 1987); $animal_l$ is a random additive genetic effect of animal l based on the genomic relationship matrix G ; and e_{ijkl} is the random residual effect.

Milk samples were collected once on each farm and during the same season across farms; therefore, a season effect was not fitted into the model.

Heritability (h^2) estimate was defined as

$$h^2 = \frac{\sigma_a^2}{(\sigma_a^2 + \sigma_e^2)}, \quad [2]$$

where σ_a^2 is the genetic variation, and σ_e^2 is the residual variation based on univariate analyses.

The association analysis was performed based on an extension of the linear model [1] with an allele substitution effect (b_3) and a covariate SNP_m , indicating whether a SNP was heterozygous (1) or homozygous (0, 2). The effect of the SNP was tested using a Wald test with a null hypothesis of $H_0: b = 0$. For both traits, SNP effects were declared significant if the corresponding $-\log_{10} P$ -value was >5.87 (based on genome-wide Bonferroni correction). Additional association analysis was implemented to fine-map QTL regions

Figure 2. Manhattan plot for the association analysis of $\log_{10}$ transcobalamin using imputed full sequence data on BTA17. The SNP within the *TCN2* gene region are highlighted in green.

detected in BTA17 using imputed sequence variants and using a similar model.

Table 1 presents descriptive statistics and genetic parameters for milk B₁₂ and TC content in the Danish Holstein. The mean B₁₂ content was 3.93 µg/L, with a coefficient of variation of 41%. Accordingly, B₁₂ content in the Danish Holstein varied from 1.06 to 10.28 µg/L. The content and ranges found in this study for B₁₂ are in line with the findings by Rutten et al. (2013), and with levels generally reported from bovine milk (Akins et al., 2013; Duplessis et al., 2016).

For TC, the mean content was 577 pmol/L, and the value varied from 96 to 4,672 pmol/L, with a relatively higher coefficient of variation (81%). To our knowledge, TC has never been evaluated in such a large number of milk samples from individual cows, although the mean value found here is in line with those reported for a pooled sample of milk from Danish Holstein cows (Fedosov et al., 2019).

Genetic variance explained a substantial part of the variation, and estimated heritabilities were moderate to high for B₁₂ (0.37 ± 0.18) and TC (0.61 ± 0.13), respectively. The heritability estimate found for B₁₂ in this study was similar to the estimate for the Dutch Holstein-Friesian ($h^2 = 0.37$; Rutten et al., 2013) but higher than estimates reported for the Scottish Holstein-Friesian ($h^2 = 0.10$; Denholm et al., 2019).

Performing a GWAS on the log-transformed data revealed no significant SNP for B₁₂ (Figure 1). This is in contrast to the findings of Rutten et al. (2013), which reported 68 SNP that showed significant association with milk B₁₂ content in the Dutch Holstein-Friesian. This could be partly explained by the stringent significance threshold used in this study ($-\log_{10} P\text{-value} = 5.87$) compared with the less stringent significance threshold used in the study of Rutten et al. (2013) ($-\log_{10} P\text{-value} = 3.0$).

In contrast, 28 significant SNP were associated with TC (Figure 1). For the significant SNP, minor allele frequency ranged between 0.07 and 0.49. A very strong QTL was detected for TC on BTA17, and significant associations were detected with 24 SNP. Further fine-mapping using imputed full sequence data showed a QTL region spanning between 71.71 and 71.79 Mbp on BTA17, with the most significant SNP (rs209672492) having a $-\log_{10} P\text{-value}$ of 62.93 (Figure 2). The SNP within this region were assigned to the *TCN2* gene, which encodes transcobalamin, suggesting that the main driver of TC variation relates to variation within the *TCN2* gene or to regulatory elements in close proximity. It should be noted that this study used relatively small sample sizes for both the heritability and GWAS analysis and that results should be interpreted with caution.

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Notes

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The authors declare that they have no competing interests.