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CAPN2/CAPN8 locus on chromosome 1q associated with variation in serum α-carotene concentrations

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Abstract

Background/Aims—Alpha-carotene is a pro-vitamin A carotenoid present in fruits and vegetables. Higher serum concentrations of α-carotene have been associated with lower risk of cancer and all-cause mortality. Previous studies have suggested that genetic variants influence serum concentrations of pro-vitamin A carotenoids, but no variants have been robustly associated with serum α-carotene concentrations to date. The aim of this study was to identify genetic associations with serum α-carotene concentrations using the genome-wide association study (GWAS) approach.

Methods—A GWAS of serum α-carotene concentrations was conducted in 433 Old Order Amish adults who had consumed a 6-day controlled diet. Linear regression models adjusting for age, gender, and family structure were utilized to evaluate associations between genetic variants and serum α-carotene concentrations.

Results—Genome-wide significant associations with α-carotene concentrations were observed for loci on chromosome 1q41 between genes *CAPN2* and *CAPN8* (rs12137025, $p=3.55x10^{-8}$), chromosome 2p21 in *PRKCE* (rs2594495, $p=1.01x10^{-8}$), and chromosome 4q34 (rs17830069, $p=2.89x10^{-8}$).

Conclusions—We identified three novel loci associated with serum α-carotene concentrations among a population that consumed a controlled diet. While replication is necessary, the *CAPN2/CAPN8* locus provides compelling evidence for an association with serum α-carotene concentrations and may suggest a relationship with the development and progression of cancers.

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Keywords

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INTRODUCTION

The carotenoids are a class of pigments synthesized by plants and bacteria [1, 2]. Carotenoids are not produced endogenously in humans and other animals and are obtained from the diet [1]. Only 40 of the 600 identified carotenoids are contained in foods, the most abundant carotenoid-containing foods being fruits and vegetables [3]. Approximately half of the dietary carotenoids can be found in human serum and tissues, with α-carotene, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin representing 90% of all the carotenoids in the human body [4]. Pro-vitamin A carotenoids can be converted to retinol in the body and include β-carotene, α-carotene, and β-cryptoxanthin. Alpha-carotene is obtained in the diet mainly from yellow-orange fruits and vegetables, especially carrots, pumpkin, and squash [2].

Although observational studies have consistently associated eating carotenoid-containing fruits and vegetables with lower risk of a variety of chronic diseases, interventions with carotenoid-rich diets and supplementation have not shown consistent health benefits [5–7]. In fact, β-carotene supplementation has been associated with an increase in mortality among smokers, although it should be noted that a high daily dose of synthetic β-carotene was utilized in this study [8]. The discrepancy between observational and clinical studies may be due in part to genetic differences in how circulating carotenoid concentrations and their physiological effects respond to carotenoid intake. Alpha-carotene has not been studied as extensively as β-carotene, but it is believed to have numerous beneficial health effects. Alpha-carotene is a potent antioxidant that prevents lipid oxidation, scavenges free radicals, and enhances gap junction communication [9]. In-vivo studies suggest the ability of α-carotene to inhibit proliferation of cancer cells is approximately 10 times more potent than β-carotene [10]. In addition, α-carotene has been associated in observational and animal studies with a decreased risk for gastric cancers, liver and lung cancers, and other chronic diseases, including diabetes, cardiovascular disease, and chronic lower respiratory diseases [2, 11, 12]. Due in part to its antioxidant properties, low levels of α-carotene have also been associated with diseases that are worsened by excessive oxidative stress, such as glaucoma and atrophic gastritis [13, 14]. Furthermore, data from the National Health and Nutrition Examination Surveys revealed an inverse association between serum α-carotene concentrations and the risk of all-cause mortality as well as mortality from cardiovascular disease, cancer, and all other causes not related to cardiovascular disease or cancer [2]. Therefore, a greater understanding of genetic associations with circulating α-carotene concentrations in humans may have important public health implications.

There is one previously reported genome-wide association study (GWAS) of α-carotene concentrations. In a meta-analysis that combined results across three diverse study populations (n = 3,881 subjects total), a single locus was found to be associated with pro-

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vitamin A carotenoids at a genome-wide threshold for statistical significance. In this study, single nucleotide polymorphisms (SNPs) in the gene *BCO1* were associated with significantly higher circulating concentrations of β-carotene and the same locus was associated to a lesser extent with α-carotene concentrations [15]. *BCO1* catalyzes the first step of pro-vitamin A carotenoids to vitamin A (retinol) in the small intestine. *BCO1* is a 15–15′ dioxygenase that performs a 15–15′ central cleavage that generates one molecule of retinol from each molecule of α-carotene. The association between SNPs in *BCO1* and α-carotene was weak (p=0.001), relative to its association with β-carotene ($p=1.6x10^{-24}$), and did not have convincing evidence of consistency in the original report (p=0.057 in the replication cohort). Little else is known about genetic variants that may influence serum α-carotene concentrations and no robustly associated genetic associations with this important micronutrient have been identified to date.

The goal of this study was to identify novel genetic associations with serum α-carotene concentrations. We studied a relatively genetically-homogenous population of Old Order Amish men and women living in Lancaster County, Pennsylvania. Study participants were administered a 6-day controlled diet, which helped reduce variability in serum α-carotene concentrations associated with varying intake of α-carotene, fat, and other dietary factors that influence its absorption. We hypothesized that the controlled diet would enable us to more closely isolate the genetic influences of serum α-carotene concentrations. While relatively brief, the controlled diet helped reduce potential confounding of the relationship between genetic variants and circulating α-carotene concentrations introduced by varying dietary intake..

METHODS

Study Population

The study sample was composed of the 433 participants from the Heredity and Phenotype Intervention (HAPI) Heart Study who completed a controlled diet and had frozen blood samples obtained during a clinic visit on the final day of the controlled diet. The design of the HAPI Heart Study has been described previously [16]. Briefly, the initial aim of the HAPI Heart Study was to identify genetic and environmental determinants of the responses to intervention affecting cardiovascular risk factors. Of the 868 Old Order Amish adults recruited into the HAPI Heart study, 469 were administered the controlled dietary intervention and subsequently provided a blood sample at the conclusion of the 6-day diet. There were 27 samples of insufficient quality to measure serum α-carotene concentrations and 8 participants with incomplete genotype data who were excluded from analysis. The study was approved by the Institutional Review Board of the University of Maryland School of Medicine and all participants provided written informed consent.

Controlled Diet

Research staff prepared the controlled diet for study participants. A registered dietitian visited several Old Order Amish households to obtain diet histories and observe meals and foods that were in their homes. All meals in the controlled diet were designed to be representative of the typical diets of Old Order Amish adults and were delivered to the

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homes of study participants during the 6-day controlled diet period. Study participants did not consume any prescribed or over the counter medications or dietary supplements during this 6-day period. The complete menus for the 6-day controlled diet that the study participants consumed are provided in the **Supplementary Material**. The controlled diet contained an average of 3,277 kilocalories per day, with 49% from carbohydrate, 36% from fat, and 15% from protein. There was an average of 525 mg of cholesterol per day in the diet. The diet contained approximately 1,724 mcg of α-carotene per day, coming primarily from carrots, green beans, and lettuce.

Compliance with the controlled diet was assessed by comparing sodium, potassium, and creatinine levels from first morning urine samples obtained: 1) prior to consuming the 6-day controlled diet that participants consumed in this study; 2) on the final day of the 6-day controlled diet that participants consumed in this study; and 3) on the final day of a second 6-day controlled diet that was low in salt and consumed after the blood draw that was used to conduct the GWAS in this study. The compliance data have been reported in detail previously [17]. In brief, while not a direct measure of carotenoid intake, the excreted sodium, potassium, and creatinine levels reflecting the varying salt content in the different diets suggest that compliance with the controlled diet in this study was excellent.

Serum micronutrient measurement

Frozen blood samples taken from the final day of the 6-day controlled diet were assayed for serum concentrations of α-carotene, other key carotenoids (β-carotene, lycopene, lutein, zeaxanthin, cryptoxanthin), vitamin E (γ-tocopherol, α-tocopherol), and retinol (pre-formed vitamin A) in the Johns Hopkins University Nutritional Biochemistry Laboratory. Reverse-phase high-pressure liquid chromatography (HPLC) was utilized to assess serum α-carotene concentrations from each 200 μL frozen blood sample [18]. The intra-assay and inter-assay coefficients of variability for α-carotene were 8.2% and 19.4%, respectively.

Genotyping

Genotypes were obtained using either the Affymetrix 500k or Affymetrix 1M SNP chip v6.0 by the Genomics Core Laboratory at the University of Maryland. Genotyping calls were made separately for the two chips using BRLMM (500K array) and Birdseed version 2 (1M array), which is part of the Birdsuite tools [19, 20]. Called genotypes were then synthesized and filtered by excluding markers with > 5% missing and extreme Hardy-Weinberg equilibrium (HWE $p < 5x10^{-6}$). A total of 397,704 SNPs passed quality control standards, were called per each calling algorithm, were in common on both arrays and had a minor allele frequency (MAF) ≥ 1%. These SNPs were then passed to the imputation phase. Imputation was conducted with MACH using the HapMap CEU reference sample [21]. An imputation quality score of ≥ 30% was used as a final quality control filter. Variants with an imputed MAF ≥ 2% were used in association analyses, for a final analyzed SNP count of 2,193,082.

HaploReg Version 4.1 [22] was utilized to pull data from ENCODE and the Roadmap Epigenomic projects to annotate the functions of any variants associated with α-carotene concentrations in our analyses. The ENCODE and the Roadmap Epigenomic projects

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provide data across a variety of tissue and cell types. For each associated variant, we looked for its predicted chromatin state across multiple cell types, its effect on regulatory motifs, and potential enrichment of cell type-specific enhancers.

Statistical Methods

Descriptive statistics were computed to characterize the study sample at baseline and determine the mean serum α-carotene concentrations. We estimated the effect of genotype on α-carotene levels at each SNP, adjusting for the effects of age and sex utilizing a general linear model. Genotype was coded as the number of copies of the reference allele (0, 1, or 2), thereby corresponding to an additive genetic model. GWAS analyses were performed using the MMAP software, which accounts for family structure [23]. Statistical analysis was performed using a variance component approach to account for relatedness among study participants. This approach has been shown to provide valid estimates of regression parameters [24]. We estimated that our sample provided 80% power to detect SNPs accounting for 9–10% of trait variation at genome-wide thresholds for statistical significance ($p < 5\times10^{-8}$).

Our secondary aim of the statistical analysis was to perform replicative association testing of previously reported associations. Ferrucci et al previously reported associations with serum α-carotene, with one locus achieving genome wide significance [15]. We report here replicative association tests for rs6564851 at the *BCO1* locus.

RESULTS

Baseline characteristics of the study sample are provided in Table 1. There were 252 men and 181 women in the study. The mean age of participants was 43.1 years and they had a mean BMI of 26.4 kg/m^2. Mean α-carotene concentrations were 0.29 μmol/L. The residual heritability of α-carotene concentrations after accounting for age and sex was estimated to be 0.23 ± 0.11.

A Manhattan plot summarizing results of the GWAS is provided in Figure 1. There was little evidence for genomic inflation (lambda = 1.00) as illustrated in Figure 2. Details of the genome-wide significant ($p < 5x10^{-8}$) associations are given in Table 2.

Three novel loci with genome-wide significant associations with α-carotene concentrations were detected on chromosomes 1, 2 and 4. The strongest genetic evidence of an association exists on chromosome 1q41. Figure 3 provides a regional association plot of the genome-wide significant association on chromosome 1. Several SNPs in linkage disequilibrium tag this association. The minor allele at the lead SNP (rs12137025, $p=3.55 \times 10^{-8}$) was associated with a 0.19 μmol/L increase in serum α-carotene and this locus accounted for 7.1% of the variation in α-carotene levels. The minor allele at the locus (C) is present in the Old Order Amish (MAF = 0.069) at similar frequencies to other populations (Hapmap CEU = 0.062).

Two other genome-wide significant associations were identified, though there was little corroborating evidence from near-by SNPs at those loci. The strongest of the associations

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with α-carotene concentrations was a locus on chromosome 2p21 (SNP = rs2594495; $p=1.01x10^{-8}$). Each copy of the A allele was associated with a 0.37 μmol/L increase in serum α-carotene, and this locus accounted for 7.2% of the variation in α-carotene levels. Figure 4 provides a regional association plot of the genome-wide significant association on chromosome 2. The MAF at the locus (A) is present in the Old Order Amish (MAF = 0.039) though it is more frequent in other populations (Hapmap CEU = 0.11). The rs2594495 variant is in an intronic region of the *PRKCE* gene.

Finally, we identified a genome-wide significant association with a locus on chromosome 4q34 (lead SNP = rs17830069; $p=2.89x10^{-8}$). Each copy of the G allele was associated with a 0.38 μmol/L increase in serum α-carotene and this locus accounted for 7.5% of the variation in α-carotene levels. The minor allele at the locus (G) is present in the Old Order Amish (MAF = 0.023) as well as other populations (Hapmap CEU = 0.050). Figure 5 provides a regional association plot of the genome-wide significant association on chromosome 4. Rs17830069 is in an intergenic region near the non-protein coding RNA *LINC00290*. There are no obvious candidate genes in the region.

We also performed look-ups for SNPs at the *BCO1* locus previously reported to be associated with serum β-carotene levels in the GWAS meta-analysis reported by Ferrucci et. al [15]. There was no evidence of association between this locus and α-carotene concentrations in our study population (lead SNP: rs6564851, p=0.28).

GWAS was also performed for the other carotenoids (β-carotene, lycopene, lutein, zeaxanthin, cryptoxanthin), vitamin E (α-tocopherol, γ-tocopherol), and retinol that were assayed. In brief, there were no meaningful associations noted between retinol, vitamin E, or any of the other carotenoids and any other genetic loci ($p > 5\times10^{-8}$, data not shown) with the exception of lycopene. Serum lycopene concentrations among our study population were associated ($p=3.41\times10^{-9}$) with the variant rs7680948 on chromosome 4, located in the intron region of the *SETD7* gene [25]. In addition, our results offered nominal support ($p=3.79\times10^{-4}$) for the association previously noted between *SCARB1* and serum lycopene levels, albeit with a different variant (rs11057841) in the region.

DISCUSSION

We report the results of a genome-wide association study of serum α-carotene concentrations among a study sample that consumed a controlled diet. Three genome-wide significant associations are identified, though all three require further replication from independent studies and fine mapping to help determine the potential functional effects of these associations. The *CAPN2/CAPN8* locus on chromosome 1 has the strongest evidence for association due to the association being identified with relatively common genetic makers (MAF ~ 7%) and multiple SNPs. The other two loci, the *PRKCE* locus on chromosome 2 and the chromosome 4q34 locus, lack strong collaborating evidence from nearby SNPs. While having associated SNPs in strong linkage disequilibrium is not a requirement of a causal genetic determinant, it lends supporting statistical evidence for a meaningful association and lowers the probability of an association being identified due to genotype error. We were unable to replicate the association previously noted between a

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variant on *BCO1* and α-carotene concentrations. However, this is not surprising because this SNP was more strongly associated with β-carotene than α-carotene concentrations and did not have strong replicative evidence in the initial publication [15]. GWAS performed on retinol, vitamin E (α-tocopherol, γ-tocopherol), and the other carotenoids (β-carotene, lycopene, lutein, zeaxanthin, cryptoxanthin), assayed in this study revealed no other meaningful genetic associations with the exception of lycopene [25].

Our results introduce a potentially interesting relationship between *CAPN2/CAPN8*, α-carotene, and gastric cancer. Calpains are a group of calcium-sensitive cysteine proteases that are ubiquitously expressed in mammals. The calpain system has been shown to be dysregulated in cancer [26]. *CAPN8* is a stomach-specific calpain whose expression is localized to gastric pit cells. Functioning *CAPN8* genes produce nCL-2 which is a cysteine protease essential for mucosal defense [27]. Dysfunction of this protease and the pit cells in which they are expressed can lead to atrophic gastritis and metastatic gastric cancers [28]. We determined that the genetic variant rs12137025, found near *CAPN8*, was associated with higher serum α-carotene concentrations. Higher levels of α-carotene have been shown to reduce the risk of atrophic gastritis, a precancerous condition associated with gastric cancer [29]. Alpha-carotene protects the gastric mucosa by scavenging free radicals and preventing the initiation or propagation of lipid peroxidation reactions [30]. While our findings suggest that there could potentially be a relationship between dysfunction of *CAPN8*, lower circulating α-carotene concentrations, and gastric cancer mediated by reduced protection of the gastric mucosa from lipid peroxidation, this potential relationship would need to be evaluated in future studies.

Interestingly, though the relationship noted between the *PRKCE* locus and α-carotene concentrations relies on a single SNP, *PRKCE* may also have implications in cancer as uncontrolled chronic activation of *PRKCE* has been shown to lead to malignant tumor development [31]. Additional studies are clearly needed to confirm whether α-carotene concentrations have a role in mediating the oncogenic activity of these genes. The significant association on chromosome 4q34 is in a region with no obvious candidate genes. More work is required, and replication evidence needed, to verify this and all other associations reported here.

There are several notable strengths of the study which may have resulted in successful identification of associated loci. This was the first GWAS aimed at identifying genetic associations with serum α-carotene concentrations that was conducted among participants who had consumed a controlled diet. While we did not assess serum micronutrient concentrations prior to the controlled diet as determine the specific effects of this diet on α-carotene concentrations was beyond the scope of the current study, the controlled diet minimized the potential for confounding of the relationship between serum α-carotene and genetic variants due to differences in dietary intake among people consuming variable diets. The controlled diet enabled us to more closely isolate the genetic contributions to the variance in serum α-carotene. The diet was designed to be culturally-appropriate, representative of the typical diet of our study population, and was delivered to study participants at their homes to support compliance. It should be noted that the controlled diet contained more α-carotene (1,724 mcg), as is typical of the diet of our Old Order Amish

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study population, than the average 451 mcg adult population of the United States as expressed in the most recent publicly available National Health and Nutrition Examination Surveys data [32]. Urinary excretion tests suggested that adherence to the controlled diet was excellent. The Old Order Amish study population also provided unique advantages in a study of this nature. The relationship structure of the Old Order Amish enabled us to perform the first estimate of the heritability of serum α-carotene concentrations in humans. The Old Order Amish are also a relatively homogenous population with respect to both genetics and lifestyle. The genetic homogeneity provided increased power to detect genetic variants associated with α-carotene and the similar lifestyles further minimized potential sources of confounding of the study findings.

There were also a number of key limitations of this study. The relatively small sample size (n=433) provided power to detect associations of genetic variants with relatively large effect sizes only. Despite our relatively small sample size, our study was able to identify three novel loci associated with serum α-carotene concentrations. We believe that these successes may be attributable in part to the controlled diet administered to study participants prior to the blood draw and the relatively similar lifestyle habits of the Old Order Amish study population which enabled us to more closely isolate the genetic contributions to serum α-carotene concentrations. While the novel association between a variant near *CAPN8* and serum α-carotene concentrations, both of which have been associated with gastritis and gastric cancer, may provide the rationale for further study into the specific mechanisms of this relationship, this study did not collect data on family history of gastric cancer or other markers of the disease and thus no direct inference can be made. Bioinformatics analysis utilizing HaploReg demonstrated that some of the variants associated with α-carotene concentrations, or other variants in high linkage disequilibrium with them, have potential regulatory function by virtue of being associated with enhancer or promoter activity across multiple tissues. However, without more direct experimental evidence it is not possible to ascribe a regulatory function of these SNPs to nearby genes (e.g., *CAPN2* or *CAPN8*), nor the nearby genes to regulation of α-carotene concentrations.

In summary, this was the first study to identify genome-wide significant associations with serum α-carotene concentrations. Three novel genome-wide associations were noted with serum α-carotene levels: the *CAPN2*/*CAPN8* locus, *PRKCE*, and the chromosome 1q41 locus. These findings suggest that genetics may influence serum concentrations of α-carotene. Further studies are needed to replicate these findings and to identify potential mechanisms underlying the relationships between the identified genetic loci, α-carotene concentrations, and clinical endpoints such as gastric cancer.

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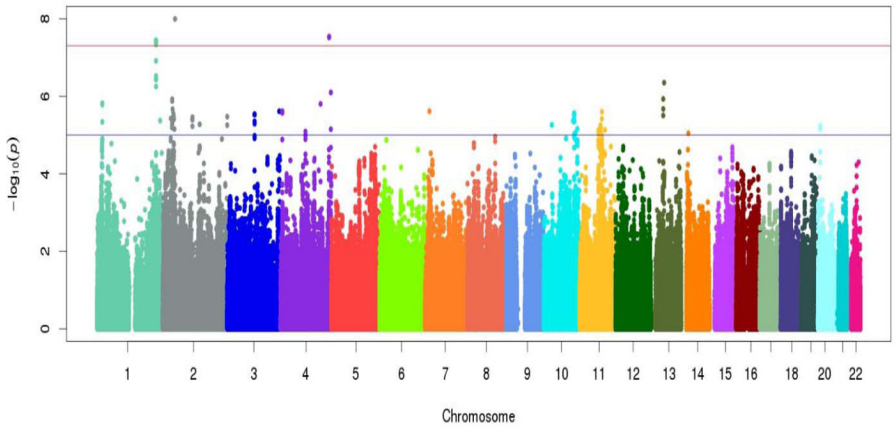

Figure 1.

Manhattan plot for the GWAS of serum α-carotene concentrations in the study population following a 6-day controlled diet. The x-axis represents chromosomal position along the genome. The y axis shows the p-value for association test at each locus on the log scale.

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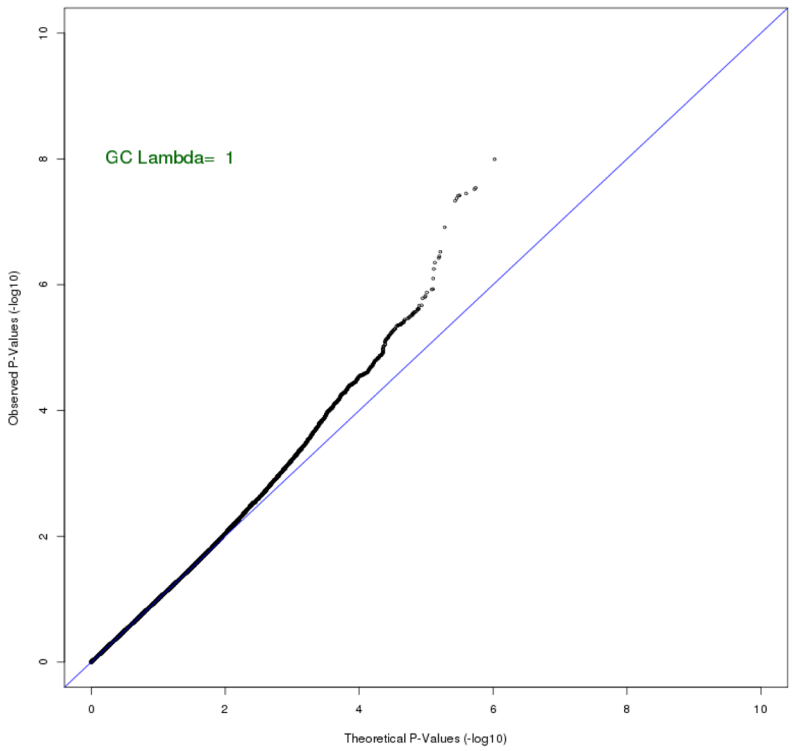

Figure 2.

Quantile-quantile (QQ) plot of GWAS of serum α-carotene concentrations. The axes plot the observed (y-axis) vs theoretical (x-axis) association p-values on the log scale for all SNPs with minor allele frequency greater than 1%. The Old Order Amish are a closed founder population with little admixture expected. The genomic control lambda is estimated to be 1.00, indicating little bias due to population stratification.

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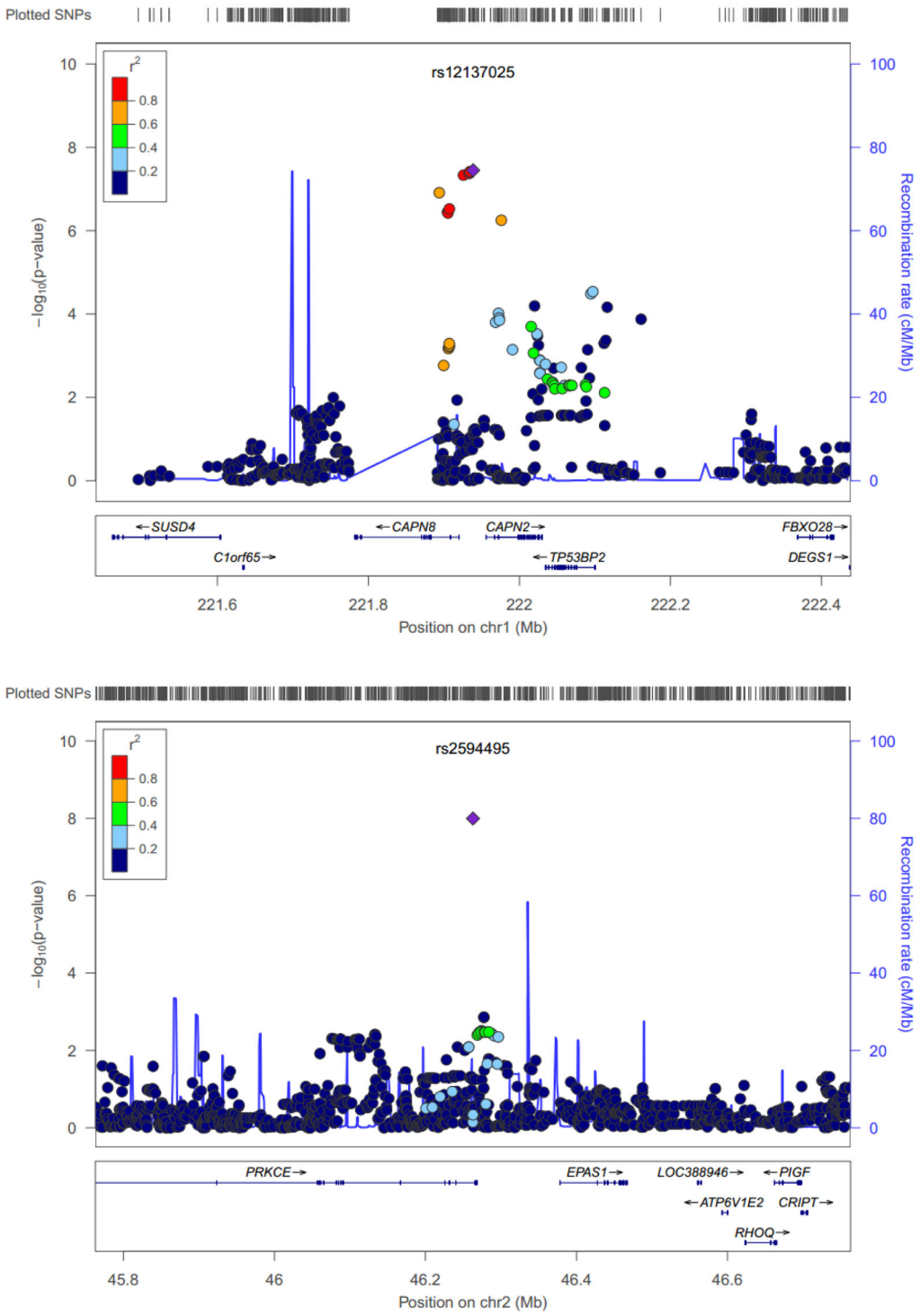

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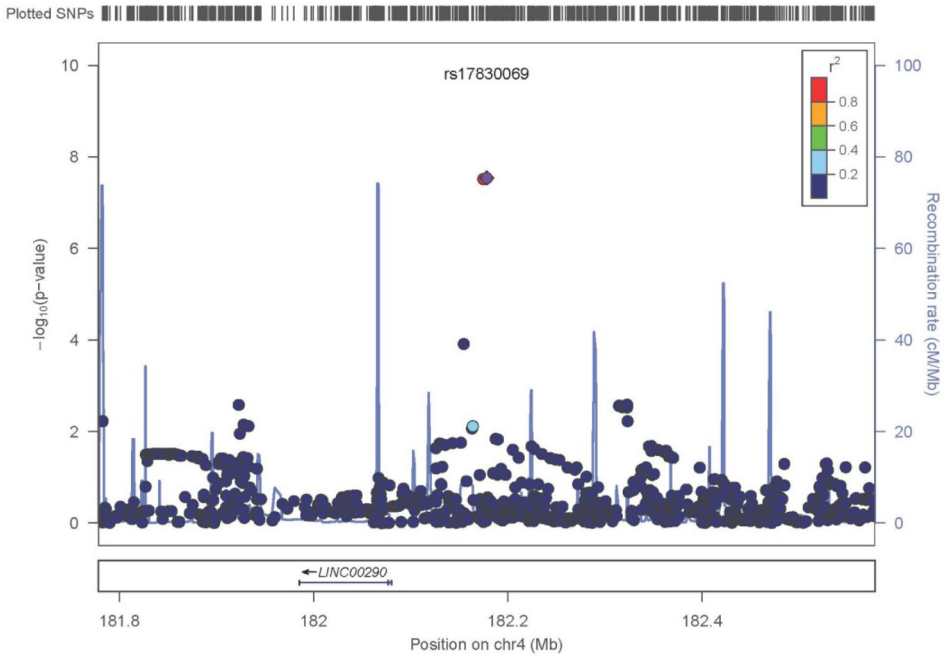

Figures 3–5.

Regional association plots of chromosome 1q41 (Figure 3), chromosome 2p2 (Figure 4), and chromosome 4q34 (Figure 5). The x-axis represents chromosomal position with the location of genes at the locus annotated. The left y-axis shows the p-value for association tests at each locus (dot) on the log scale. The right y-axis provides recombination rates in centimorgans per megabase in the chromosomal region identifying recombination hotspots in the region (blue line). The most significantly associated variant in the region is indicated by a purple diamond. All other variants in the region are indicated with colored solid dots. The color reflects the linkage disequilibrium (measured in r^2) between the variant and the top hit. Variants in high linkage disequilibrium ($r^2 > 0.8$) are in red, variants in low linkage disequilibrium are in dark blue ($r^2 < 0.2$), with all other colors representing linkage disequilibrium in between ($0.2 \leq r^2 \leq 0.8$) as described in the color legend in the upper left hand corner of the figures.

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Table 1

Characteristics of Old Order Amish study population from Lancaster County, PA after consuming a 6-day controlled diet

Characteristic	All (n=433)	Female (n=181)	Male (n=252)	p-value*
Age (years)	43.1 (12.3)	45.6 (13.1)	41.4 (12.5)	< 0.0001
BMI (kg/m^2)	26.4 (4.22)	27.8 (5.06)	25.4 (3.13)	< 0.0001
Lycopene (μmol/L)	0.73 (0.37)	0.71 (0.33)	0.74 (0.40)	0.2
Lutein (μmol/L)	0.25 (0.10)	0.24 (0.09)	0.27 (0.10)	0.004
Zeaxanthin (μmol/L)	0.12 (0.06)	0.11 (0.05)	0.13 (0.06)	0.01
Cryptoxanthin (μmol/L)	0.16 (0.07)	0.16 (0.07)	0.16 (0.06)	0.9
Alpha-carotene (μmol/L)	0.29 (0.23)	0.32 (0.25)	0.27 (0.21)	0.04
Beta-carotene (μmol/L)	0.70 (0.50)	0.78 (0.58)	0.64 (0.42)	0.006
Retinol (μmol/L)	1.53 (0.37)	1.51 (0.37)	1.55 (0.36)	0.3
Gamma-tocopherol (μmol/L)	4.68 (1.68)	4.71 (1.81)	4.66 (1.58)	0.6
Alpha-tocopherol (μmol/L)	30.5 (7.79)	31.4 (8.02)	29.8 (7.57)	0.07

*Student's t-test utilized to calculate p-values for differences in all variables between females and males

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Table 2

Genome-wide ($p< 5x10^{-8}$) associations with serum α-carotene concentrations

SNP	Chromosome	Position	Gene	MAF	Coded Allele	Beta (SE)	P value
rs2594495	2	46262970	*PRKCE*	0.04	A	0.37 (0.06)	$1.01x10^{-8}$
rs17830069	4	182415300		0.02	A	−0.38 (0.07)	$2.89x10^{-8}$
rs12137025	1	221938674	*CAPN8, CAPN2*	0.07	C	0.19 (0.03)	$3.55x10^{-8}$