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Probing the functional consequence and clinical relevance of *CD320* p.E88del, a variant in the transcobalamin receptor gene

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AUTHOR CONTRIBUTIONS

FP and DW drafted the manuscript. DW and DSR contributed the fibroblast studies. YC, ND and QC contributed the cellular localization studies MC, MM, MLB, and DMK coordinated New York state cohort selection and DNA extraction. DB, HOA and MK performed the genotyping. FP performed the statistical analyses. KS, CVR, OS, JS, and CPV provided clinical case follow-up. FP, DMK and LCB conceived and designed the study. All authors critically reviewed and approved the final submitted version of the manuscript, and take responsibility for the work.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Abstract

The biological and clinical significance of the p.E88del variant in the transcobalamin receptor, *CD320*, is unknown. This allele is annotated in ClinVar as likely benign, pathogenic, and of uncertain significance. To determine functional consequence and clinical relevance of this allele, we employed cell culture and genetic association studies. Fibroblasts from sixteen *CD320* p.E88del homozygotes exhibited reduced binding and uptake of cobalamin. Complete ascertainment of newborns with transiently elevated C3 (propionylcarnitine) in New York State demonstrated that homozygosity for *CD320* p.E88del was over-represented (7/348, $p<6x10^{-5}$). Using population data, we estimate that ~85% of the p.E88del homozygotes born in the same period did not have elevated C3, suggesting that cobalamin metabolism in the majority of these infants with this genotype is unaffected. Clinical follow-up of 4/9 homozygous individuals uncovered neuropsychological findings, mostly in speech and language development. None of these nine individuals exhibited perturbation of cobalamin metabolism beyond the newborn stage even during periods of acute illness. Newborns homozygous for this allele in the absence of other factors are at low risk of requiring clinical intervention, although more studies are required to clarify the natural history of various *CD320* variants across patient populations.

Keywords

transcobalamin receptor; *CD320* ; cobalamin; transcobalamin receptor deficiency; C3-acylcarnitine; propionylcarnitine; methylmalonic acid; newborn screening; rs150384171

1 INTRODUCTION

Severe vitamin B_{12} (cobalamin) deficiency results in hematologic and neurologic abnormalities, including megaloblastic anemia, sensory neuropathy, dementia and psychiatric disorders, and if untreated can result in death (Green et al, 2017). Moderate deficiency has been associated with a host of clinical symptoms, including cognitive impairment (Rosenberg & Miller, 1992; Smith et al., 2018), osteopenia (Feigerlova et al., 2016), stroke (Spence, 2006; Weikert et al., 2007), and maternal risk of having a child with a neural tube defect (NTD) (Candito et al., 2008; Kirke et al., 1993; Steen et al., 1998). Causes of vitamin B_{12} deficiency are inadequate intake, compromised absorption, and inborn errors of cobalamin transport and metabolism. Dietary deficiency can occur in those not consuming adequate animal products or supplements. In individuals consuming a diet containing adequate cobalamin, impaired absorption can be the result of pernicious anemia, an autoimmune disease that results in deficiency of the cobalamin-binding protein intrinsic factor, required for absorption of ingested cobalamin. Less severely decreased ability to absorb dietary vitamin B_{12} also occurs in 10-15% of those over the age of 60 (Baik & Russell, 1999), largely due to gastric dysfunction. Lastly, inborn errors that result in impaired intestinal uptake of cobalamin (intrinsic factor deficiency, Imerslund-Gräsbeck syndrome, *cblF* and *cblJ* disorders (Sloan et al., 1993) can result in decreased circulating

cobalamin, while a series of disorders affecting intracellular cobalamin metabolism can result in impaired cobalamin metabolism in the presence of normal circulating cobalamin levels (Watkins & Rosenblatt, 2011).

Pairs of cobalamin-binding proteins and specific receptors are required for cobalamin uptake at cell surfaces: intrinsic factor and the cubam receptor in the distal ileal epithelium, transcobalamin and megalin in the renal tubule, transcobalamin and the transcobalamin receptor, encoded by *CD320*, at the surface of all cells and tissues (reviewed in (Nielsen et al., 2012)). CD320 is expressed by all cells and is responsible for the uptake of circulating cobalamin in complex with transcobalamin. Targeting the *Cd320* gene for knockout in mouse models has recapitulated some of the human pathologies associated with severe cobalamin deficiency, such as anemia (Bernard et al., 2018) and nerve demyelination (Arora et al., 2019). The importance of the function of the *CD320* gene suggests that clinically relevant variants might exist.

An in-frame deletion variant in *CD320* (NM_016579.3:c.262_264delGAG (p.Glu88del); hereafter referred to as “p.E88del”) with potential clinical consequence was first reported in 2010 (Quadros et al., 2010). Newborn screening identified an infant with elevated C3 (propionylcarnitine, which can be indicative of a number of different inborn errors of metabolism based on defects that cause propionic acidemia and methylmalonic acidemia. Follow-up testing by Quadros et al., revealed moderately elevated methylmalonic acid (MMA), consistent with a cobalamin deficiency. At 14 days of life, a repeat screen of C3 was normal. At 21 days, a single dose of intramuscular cobalamin was administered, and within 24 hours circulating MMA was in the reference range. Circulating MMA remained normal at nine months. Circulating cobalamin was elevated, which would be consistent with reduced cellular cobalamin uptake by the CD320 receptor. The phenotype of such transiently elevated C3 is well established but unexplained (Chace et al., 2001; Chapman et al., 2008). In addition to this index case, four unrelated cases with unexplained, moderate elevation of C3 and MMA were identified by the authors. All five newborns were homozygous for *CD320* p.E88del. Concurrently, this allele was also nominally associated with risk of an NTD (Pangilinan et al., 2010). The identification of this allele in independent studies bolsters the case for it being a functional variant, or closely linked to such a variant, with potential clinical consequences.

This variant, *CD320* p.E88del (rs150384171), is a 3bp deletion that results in the loss of one of three consecutive glutamic acid residues near the end of the first of two conserved low-density lipoprotein receptor type A (LDLR-A) domains. Members of the LDLR family of proteins, including CD320, contain a variable number of these domains involved in ligand binding (Esser et al., 1988). Although mutational studies of CD320 demonstrated the importance of the LDLR-A domains in ligand binding (Jiang et al., 2013), structural studies of this *CD320* variant bound to holo-transcobalamin (holoTC) did not reveal significant changes in binding in an *in vitro* system (Alam et al., 2016). Alternately, some LDLR-A domains contain a DSSDE motif involved in protein localization, as observed in a model of polarized epithelial cells. When the DSSDE motif is intact, proteins localize specifically to the apical membrane. However, altering this motif in the eighth LDLR-A domain in corin or the second LDLR-A domain in CD320 results in a loss of this specificity (Zhang et al.,

2020). Although this DSSDE motif is not perfectly preserved in the first LDLR-A domain in CD320 (DGSDE), the final glutamic acid is the first of three consecutive glutamic acid residues, one of which is deleted in the p.E88del variant of *CD320*. The p.E88del variant could therefore potentially affect protein localization by disrupting this motif.

The functional consequence of this single amino acid deletion remains unclear, although there are multiple observations of its influence in human studies. As mentioned, fibroblasts from *CD320* p.E88del homozygotes show evidence of reduced cellular uptake of holoTC and increased levels of MMA and homocysteine (Quadros et al., 2010). Similarly, in large cohorts of healthy younger and older adults, this allele is significantly associated with increased circulating holoTC concentrations at the genome-wide level (Velkova et al., 2017). Two other genome-wide association studies have found other variants in or near this gene to be modestly associated with obsessive-compulsive disorder in males (Khramtsova et al., 2018) and small but increased levels of thyroid-stimulating hormone (Teumer et al., 2018).

In addition to the original report of five cases (Quadros et al., 2010), two additional *CD320* p.E88del homozygotes had relatively long-term follow-up (Hannah-Shmouni et al., 2018). These individuals were identified on newborn screening, exhibited transiently elevated MMA, and long term follow-up revealed normal neurodevelopment at ages 5 and 7, respectively. All of the reported cases have been detected on the basis of elevated C3 on newborn screening with the exception of one individual identified after bilateral central retinal artery occlusions at the age of seven weeks (Karth et al., 2012). The evidence annotated in ClinVar (VCV000203643.6, accessed June 29, 2021) is based on these and other studies, resulting in conflicting interpretations for this allele, including likely benign, pathogenic, other, and uncertain significance.

To clarify the effect of homozygosity of *CD320* p.E88del, we used cell culture systems to ask whether this variant affects cobalamin uptake, functional efficiency of cobalamin-dependent enzymes, and cellular localization of the protein. Additionally, we assessed genetic epidemiology and population genetics to examine this allele in the context of newborn screening. This allowed us to estimate the percentage of infants homozygous for *CD320* p.E88del that are detected during newborn screening for C3. We also asked the converse question: what percentage of *CD320* p.E88del homozygous newborns do not have elevated C3? Finally, we report the medical follow-up care of nine additional cases beyond the newborn period.

2 METHODS

2.1 Editorial Policies and Ethical Considerations

The specimens and data from the New York State Newborn Screening Program were deidentified before the initiation of genetic studies. Sample use was approved by the Institutional Review Board of the New York State Department of Health and reviewed by the Office for Human Research Protections at the National Institutes of Health.

2.2 Cell Culture Experiments

2.2.1 Fibroblasts—Fibroblast cultures were established from individuals identified as having elevated levels of C3 levels on tandem mass spectrometry of newborn blood spots (eight from Minneapolis, MN; two each from Birmingham, AL, Iowa City, IA, Milwaukee, WI, Detroit, MI; and one each from Denver, CO, San Diego, CA, Cincinnati, OH, and Vancouver, BC) and submitted for analysis at the diagnostic laboratory at the Vitamin B12 Clinical Research Laboratory at the McGill University Health Centre. Metabolic studies were performed on all submitted fibroblast lines. Lines with evidence of decreased cobalamin uptake were genotyped for the *CD320* p.E88del variant using fragment-size analysis of a PCR product; in some cases additional Sanger sequencing was performed. Twenty individuals were genotyped: sixteen were homozygous for p.E88del in *CD320*; one was compound heterozygous for p.E88del and c.297delA (p.Gln99HisfsTer35); one was compound heterozygous for p.E88del and c.386G>T (p.Arg129Leu) (rs139064611); and one was heterozygous for p.E88del with no other *CD320* variant identified. Maternal cobalamin was available for only five of these cases. All five of these mothers had levels within the normal range. Control fibroblast cultures were derived from individuals with no known genetic disorder. Fibroblasts were cultured in modified Eagle's minimum essential medium with Earle salts plus non-essential amino acids, pyruvate and ferric nitrate, supplemented with 10% (v/v) fetal bovine serum, at 37°C and 5% CO_2.

2.2.2 Metabolic studies—Function of the cobalamin dependent enzymes methylmalonyl-CoA mutase and methionine synthase was determined in cultured fibroblasts by measurement of the incorporation of label from [1-^{14}C]propionate and 5-[^{14}C]methyltetrahydrofolate (Pharmaron Special Syntheses, Cardiff UK) respectively into cellular macromolecules in the presence or absence of 1.5μM hydroxocobalamin, as previously described (Watkins, 1998). Ability of cells to convert [^{57}Co]cyanocobalamin (MP Biomedicals, Solon, OH) to the active coenzyme derivatives adenosylcobalamin and methylcobalamin was assessed by incubation of fibroblast cultures in the presence of labeled cyanocobalamin bound to human transcobalamin for 96 h, followed by extraction of cobalamins in hot ethanol and separation of labeled cobalamin derivatives by high performance liquid chromatography (HPLC) on a Lichrosorb RP8 column (Phenomenex, Torrance, CA) as previously described (Watkins, 1998). Vitamer distribution is presented as a percent of total intracellular [^{57}Co]cobalamin.

2.2.3. HoloTC binding and uptake—Fibroblasts were plated into 35-mm tissue culture dishes at a density of 400,000 cells/dish. At time 0, medium was removed and replaced with medium supplemented with 10% human serum that had been preincubated for 30 minutes at 37°C with [^{57}Co]cyanocobalamin (MP Biomedicals) to allow formation of holoTC (final concentration: 25 pg/mL [^{57}Co]cyanocobalamin). Cultures were incubated for 1 hour at 5°C or for 1, 3 or 96 hours at 37°C. At each time point, medium was removed and cells were washed three times with phosphate-buffered saline and harvested by trypsinization. Radioactivity in pellets was determined by gamma counting. Values were normalized to protein concentration as determined by the Lowry method. At least three measurements were obtained for fibroblasts from each participant.

2.2.4 Membrane localization of exogenous human CD320 isoforms—

Madin-Darby Canine Kidney (MDCK) cells (China Center for Type Culture Collection, 3142C0001000000147, authenticated by STR profiling) were grown on glass coverslips in 12-well plates (Corning, 3513) containing Dulbecco's modified Eagle's medium (Corning, 10-0130CVRC) and 10% fetal bovine serum (Gibco, 16000-044) at 37°C with 5% CO_2. Plasmids expressing human *CD320* wild-type (WT) and variants p.E88del and p.S162A with an N-terminal Flag tag were transfected into the cells using PolyJet reagents (SignaGen Laboratories, SL100688), as described previously (Zhang et al., 2020). After 24 h, the cells were fixed with methanol at room temperature for 5 min and incubated with 5% (w/v) bovine serum albumin in phosphate-buffered saline at 37°C for 1 h. Immunostaining was conducted with an anti-Flag antibody (Sigma, F1804, 1:500) and an anti-ZO-1 antibody (Cell Signaling, 8193s, 1:300) and Alexa Fluro-488 or 594-labeled secondary antibodies (Thermo Fisher, A32766 and A32740, 1:500) (Zhang et al., 2020). The stained cells were examined with a confocal laser scanning microscope (Olympus, FV1000). X-Y plane photos (8 μs/picture) were taken at each 0.45 μm and used to reconstitute X-Z axis images. Fluorescent signals on apical (guided by ZO-1 staining) and basolateral (total staining minus apical staining) membranes were analyzed with Image J software to calculate the ratio of $F_{BL}/F_{Total,}$ where F_{BL} is the fluorescent intensity on basolateral membranes and F_{Total} is the total fluorescent intensity on apical and basolateral membranes. Values of black area without fluorescent staining were set as the background.

2.3 Genetic Association Studies of New York State Newborns

2.3.1 Congenital Heart Defect sample: a population-based sample—

The congenital heart defect (CHD) cohort is used as a population-based sample in this study, and has been previously described (Mamasoula et al., 2013). Briefly, this population-based sample was collected from the same catchment area as the transiently elevated C3 samples (Section 2.3.2) and included infants born in 1997 and 1998 in New York State. Cases were identified via the New York State Birth Defects Registry. Two controls were selected for each case matching for race/ethnicity and sex. Information from the registry was linked to records in the New York State Newborn Screening Program to obtain archived residual dried blood spots. DNA was extracted using the Gentra Puregene Blood Kit (Qiagen). Genotyping of *CD320* p.E88del was performed by allele-specific extension product mass, detected via matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrometry after undergoing iPlex assay chemistry (Sequenom, San Diego, CA, USA). Primer sequences and assay conditions are available upon request. The genotyping success rate was 97% (8811/9090). Replicate samples were 99.9% concordant (879/880).

2.3.2. Transiently elevated C3 sample—

This cohort of infants (n=397) was born in 2008 or 2009 and identified by the New York State Newborn Screening Program. During the study period, referrals and borderline results were based on concentration of C3 and the ratio of C3 to C2 (acetylcarnitine). Infants were considered positive if C3 was greater than or equal to 7.0 μmole/L, or if the C3 was greater than or equal to 5.0 μmole/L and the ratio of C3 to C2 was greater than or equal to 0.2, indicating a possible disorder in cobalamin or propionic acid metabolism. Of the total 397 infants, seventeen were subsequently excluded because elevated C3 was detected on a repeat specimen after

C3 concentration in an initial specimen was within acceptable limits. Cases with elevated C3 were then categorized according to outcomes based on: 1) subsequent testing, including repeat heel stick specimens received and evaluated by the Newborn Screening Program; or 2) follow-up information from treatment centers for infants who were referred to a genetics specialist for diagnostic work-up. Twelve infants were diagnosed with a confirmed genetic disorder (three with propionic acidemia, two with methylmalonic acidemia and seven with *cblC* deficiency) and were excluded. Seventeen additional cases without final outcomes (loss to follow-up, expired prior to diagnosis, etc.) were excluded. The final cohort consisted of 305 infants with transiently elevated C3 that were not referred (C3 was found to be normal on a repeat specimen from the same newborn) and 46 infants that were referred but found to be normal after diagnostic testing. Case-control matching was approximately 1:1 based on race/ethnicity and sex and selected from the same birth years. Race/ethnicity was obtained from the demographic data collected on the Guthrie card. Exclusion criteria for the control group included any specimens that screened positive for elevated C3 or methionine, those considered unsuitable for testing, and specimens from multiple birth gestations. Specimens were deidentified, and then DNA was extracted from archived residual dried blood spots (Saavedra-Matiz et al., 2013). Genotyping of *CD320* p.E88del was performed as for the CHD cohort. The genotyping success rate was 99% in cases (348/351) and 98% in controls (380/388). All 23 replicate samples were concordant.

Data on the incidence of transiently elevated C3 during comparable birth years were requested from other states. Screening protocols differ by state, and some state data were not comparable and were excluded. In Arkansas, the threshold for elevated C3 was 5.5 μmol/l. In Missouri, the threshold was 6.0 μmol/L (4.0 μmol/L for infants greater than seven days of age). Both states reported the number of confirmed cases of inborn errors of metabolism, and the number of infants with transiently elevated C3, which included outcomes determined by a normal value on repeat measure or by the absence of disease upon referral.

2.3.3 Neural Tube Defect sample: a replication study—An independent sample was selected to perform a replication study of the nominally significant association of *CD320* p.E88del with NTD risk (Pangilinan et al., 2010). This is a population-based, case-control sample based on 475 infants born in the state of New York between 1998 and 2005 with a diagnosis of spina bifida (n=375), anencephaly (n=18) or encephalocele (n=82), as recorded in the New York State Birth Defects Registry. Four controls born in the same period were selected for each case, matching for maternal race/ethnicity and infant sex, both obtained from birth certificates. Information from the registry was linked to records in the NYS Newborn Screening Program to obtain archived residual dried blood spots. After linkage, specimens were anonymized and DNA was extracted (Saavedra-Matiz et al., 2013). LGC Genomics (Herts, UK) performed genotyping of *CD320* p.E88del using KASP genotyping chemistry. All samples were genotyped at least two and up to four times. Only samples that were called on every run with consistent results were retained. The success rate was 93% in cases and 97% in controls. All 19 replicate samples were concordant.

2.4 Case Studies

Seven cases homozygous for *CD320* p.E88del and one compound heterozygote (p.E88del/c.297delA) were identified via newborn screening. These cases came to attention via newborn screening in Minnesota and were detected via a two-tier strategy: 1) >5.25 μM C3, followed by; 2) >15 μmol/L tHcy or >5 μmol/L MMA. Initial newborn screening was performed by the Newborn Screening Program of the Minnesota Department of Health. Second tier testing was performed at the Mayo Clinic Laboratories. An additional older sibling was identified after genotyping based on family relationship. The University of Minnesota institutional review board approved review of medical records and waived informed consent.

A final case came to attention via newborn screening in Iowa (Table 5, Case 9). This case, in addition to three of the cases from Minnesota (Table 5, Cases 2, 3, 10) were evaluated at the NIH Clinical Center under a dedicated longitudinal natural history protocol "Clinical and Basic Investigations of Methylmalonic Acidemia" in compliance with the Helsinki Declaration (clinicaltrials.gov identifier: NCT00078078).

For cases, *CD320* genotyping was performed by University of Minnesota Molecular Genetics Laboratory and GeneDx (Gaithersburg, MD) by Sanger, NGS or fragment-size analysis of a PCR product.

2.5 Statistical methods

Weighted means and standard deviations were calculated using the Hmisc package in RStudio (Version 1.4.1717) for fibroblast culture assays when technical replicate measures were collected. Pairwise comparisons for holoTC binding assays were performed using Welch's t-test, and comparisons for the incorporation and distribution assays were performed using a nested t-test (Prism 9 for macOS). Exact tests for Hardy-Weinberg equilibrium (Wigginton et al., 2005) were performed using the HWExact package in RStudio.

3 RESULTS

3.1 Functional consequences

3.1.1 Fibroblast studies from newborn screening participants—To test the functional consequence of p.E88del, fibroblast cultures were used to examine activities dependent on or influenced by CD320. These include holoTC binding and uptake, as well as indirect assays of cobalamin-dependent enzymes.

CD320 genotype has been shown to influence holoTC binding to fibroblasts from several newborns with homozygosity for p.E88del (Quadros et al., 2010). To replicate this observation, we repeated these measures in fibroblasts from participants similarly identified by newborn screening that were *CD320* p.E88del homozygous (n=16), compound heterozygous (n=3), or controls homozygous (n=6) for the reference allele (Figure 1). Cell cultures were incubated for 1 hour at 5°C with radiolabeled holoTC and the amount of radioactivity on the cell surface was measured after 1 hour of incubation. Fibroblast cultures

homozygous for *CD320* p.E88del exhibited lower average binding to the CD320 receptor after 1 hour of incubation at 5°C with radiolabeled holoTC (0.56 pg holoTC/mg protein vs. 2.80 pg holoTC/mg protein, Figure 1). The compound heterozygotes showed similarly reduced average binding (0.32 pg holoTC/mg protein).

The p.E88del variant is thought to influence holoTC uptake, observed by comparing one case and two controls (Quadros et al., 2010). To test this further, we incubated fibroblasts representative of each p.E88del genotype with radiolabeled holoTC and measured its uptake over 96 hours at 37°C. At 1, 3 and 96 hours, fibroblasts from newborns homozygous (n=16) or compound heterozygous (n=3) for p.E88del exhibited reduced uptake of holoTC compared to fibroblasts from controls (Figure 2).

These results indicate a reduced ability of the p.E88del isoform to bind and import holoTC. To determine if there is sufficient change in receptor function to influence intracellular activities that depend on delivery of cobalamin into cells, incorporation assays were used to indirectly measure the function of the two cobalamin-dependent enzymes, the mitochondrial methylmalonyl-CoA mutase (encoded by *MMUT*) and the cytoplasmic methionine synthase (encoded by *MTR*). Incorporation of label from radiolabeled propionate or 5-methyltetrahydrofolate (5-methylTHF) into cellular macromolecules was measured to estimate flux through methylmalonyl-CoA mutase or methionine synthase, respectively (Figure 3). Propionate incorporation was decreased in affected (p.E88del homozygous and compound heterozygous) fibroblasts compared to controls, and uptake was increased by addition of hydroxocobalamin to the culture medium (unlike controls), indicating a deficiency in intact cellular methylmalonyl-CoA mutase function. There was also a decrease in 5-[^{14}C]methyl-THF incorporation, indicating decreased function of methionine synthase. Incorporation was stimulated in the presence of hydroxocobalamin to levels greater than that observed in controls (p=0.03). Although this level is high, such stimulation has been seen in fibroblasts from cases with inborn errors of metabolism, such as cblF (Armour et al., 2013).

The final question we asked in this fibroblast system was whether *CD320* p.E88del might alter intracellular processing of cobalamin. Fibroblasts from cases (p.E88del homozygotes and compound heterozygotes) and controls were incubated with radiolabeled holoTC. Average holoTC uptake was significantly different (13.2pg/10^6 cell in controls vs. 2.6pg/10^6 cells in cases, Welch's t-test, p=0.0004). The distribution of the intracellular forms of internalized radiolabelled cobalamin was also compared (Figure 4). The percentage of exogenous labeled cyanocobalamin converted to adenosylcobalamin, required for methylmalonyl-CoA mutase, was significantly decreased in affected fibroblasts (15.8% vs. 9.5%, p=0.04), although the decrease was smaller than seen in patients with inborn errors affecting intracellular methylmalonyl-CoA mutase function; conversion to methylcobalamin was unchanged from that in control fibroblasts (Figure 4).

3.1.2 Subcellular localization of the CD320 receptor—

To test whether the p.E88del variant influences CD320 localization, we expressed wild-type *CD320* or *CD320* p.E88del in MDCK cells (Figure 5). The *CD320* S162A construct replaces the serine with an alanine in the central residue of the DSSDE motif in the second LDLR-A domain of CD320. It exhibits loss of specific localization to the apical membrane (Zhang et al.,

2020), and was included as a control (Figure 5). There was no significant difference in the localization of wild-type *CD320* or *CD320* p.E88del, with both isoforms localizing on the apical surface.

3.2 Association Studies and Clinical investigations

3.2.1 Population allele frequencies—To address whether *CD320* p.E88del might have clinical manifestations, we first considered how common this deletion allele is in different populations as cataloged in the public resource gnomAD v.2.2.1 (Karczewski et al., 2020; Lek et al., 2016). This variant is observed in all populations, albeit at a low minor allele frequency (MAF) that ranges from 0.003 in the African group to 0.014 in the Hispanic group (Table 1). Of 138,234 individuals, twelve were homozygous for *CD320* p.E88del.

As the biochemical and clinical phenotypes of the gnomAD participants are not available, we genotyped a population sample of newborns in the state of New York. These consisted of a congenital heart defect (CHD) cohort, comprised of 2937 newborn cases and 5866 newborn controls, categorized into six race/ethnic groups. The frequency of *CD320* p.E88del did not differ between cases and controls. Therefore, these groups were combined (n=8803) to determine the frequency of *CD320* p.E88del in different race/ethnic populations from New York State (Table 2). We observed a single *CD320* p.E88del homozygous individual, consistent with Hardy-Weinberg equilibrium (HWE) (~1 homozygote expected in 10,000 individuals, p=0.42). Comparable to the gnomAD samples, the minor allele frequencies in the New York race/ethnic groups ranged from 0.002 in Black infants to 0.008 in Hispanic infants.

3.2.2 Association testing

3.2.2.1 Newborns with transiently elevated C3: Based on the initial observation of finding *CD320* p.E88del homozygosity in newborns with transiently elevated C3 (Quadros et al., 2010), we asked how often are newborns with otherwise unexplained transiently high C3 homozygous for this *CD320* variant ? We performed a population-based study by determining the *CD320* p.E88del genotype of all such newborns (n=351) detected in a two-year period in New York State with elevated C3 (Table 3). Seven homozygotes were detected, all among the group of infants with C3 that resolved upon repeat specimen testing. No homozygotes were identified among infants referred for diagnostic testing. The genotype distributions of all case and control groups for each race/ethnicity adhere to Hardy-Weinberg equilibrium (HWE) with the exception of the White and Hispanic case groups ($p \leq 6x10^{-5}$). This is likely due to the presence of two White and five Hispanic individuals that are *CD320* p.E88del homozygotes. At the observed minor allele frequency (MAF < 0.01) the expectation according to HWE would be to find approximately 1 in 10,000 homozygous individuals. We conclude that this variant contributes to the transiently high C3 in some newborn infants, but does not explain the majority of these cases. We then used a population genetics approach to determine the number of homozygotes in the newborn screening sample that did not present with an elevated C3. We estimate that ~50 *CD320* p.E88del homozygotes should have been born (1 in 10,000 among the 502,263 newborns screened in NYS in 2008-2009). However, only seven such homozygotes were

detected among newborns with initially elevated C3, indicating that most (~(50-7)/50 = ~86%) of these homozygous newborns have C3 levels below the state cutoff value.

Although the number of observed *CD320* p.E88del homozygotes was too small to test for significance, the distribution by race/ethnicity was striking. This is unlikely to be due to *CD320* p.E88del genotype as the allele frequency does not differ drastically by race/ethnicity as measured in gnomAD (Table 1) or New York State-based controls (Tables 2 and 3). To determine if race/ethnicity could contribute to this transient elevation of C3 irrespective of *CD320* p.E88del genotype, we calculated the incidence of transiently elevated C3 in the three largest race/ethnic groups over these birth years in New York State (Figure 6). The 95% confidence intervals indicate a significantly higher incidence of transiently elevated C3 in Hispanic newborns. To determine whether this over-representation might be observed in other regions, we obtained data from two other states, Arkansas and Missouri (Figure 6). Although the number of observations is too small (<20) in some groups to reliably estimate the confidence intervals (Ventura et al., 2000), the Hispanic group had the greatest incidence of newborns with transiently elevated C3 in all three states.

3.2.2.3 Infants with neural tube defects: As *CD320* p.E88del was previously found to be nominally associated with NTDs in the Irish population (Pangilinan et al., 2010), we performed a replication study by genotyping this variant in an NTD cohort from New York State (Table 4). We did not observe any significant differences between cases and controls in any race/ethnicity, although power is low considering the low MAF of this variant and lower sample size of the replication cohort (less than half). The minor allele frequencies for each race/ethnicity were not significantly different from those observed in the CHD cohort.

3.2.3 Case studies—Nine *CD320* p.E88del homozygous individuals have been reported in the literature, with follow-up provided for only two (summarized in (Hannah-Shmouni et al., 2018)). We report the status of nine new cases with long-term follow-up, up to 15 years of age (Table 5). Follow-up is additionally reported for a case that is a *CD320* compound heterozyote (p.E88del/c.297delA). No consanguinity was reported. Maternal cobalamin measures were only available for two homozygous cases; total circulating cobalamin and homocysteine were in the normal range in these two mothers.

Nine of ten cases were identified via newborn screening for elevated C3 and corresponding elevations in biomarkers of cobalamin deficiency (Table 5). When measured, all cases identified via newborn screening also exhibited circulating cobalamin levels above the reference range (Table 5), consistent with a reduction in binding and uptake by the p.E88del isoform of the transcobalamin receptor. All ten children had biochemical measures of cobalamin metabolism (e.g., MMA, homocysteine) within the normal range after resolution of the transient elevations observed in the newborn period, whether receiving cobalamin supplementation or not. Nine cases have received some form of cobalamin treatment, although six (Cases 1, 4, 6, 7, 8, 10) discontinued this treatment before one year of age. Neuropsychological findings in five cases included language acquisition deficit, working memory deficits, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD), and anxiety. The five remaining cases appear to be developing normally.

4 DISCUSSION

While endocytosis of holoTC after binding to its cell surface receptor, encoded by the *CD320* gene, represents a critical first step in cellular cobalamin metabolism, no clinical entity associated with a block in this step has been described. A variant in *CD320*, p.E88del, has been independently identified by several groups, based on relatively uncommon phenotypes diagnosed in newborns: transiently elevated C3 (Quadros et al., 2010) and NTDs (Pangilinan et al., 2010). While significantly over-represented in individuals with these phenotypes, homozygosity for *CD320* p.E88del is present in only a small fraction of these individuals, who – as reported in the literature - are typically not followed clinically. Attempting to identify adults with this genotype to investigate health outcomes is also impractical due to the low population frequency (~1 in 10,000).

Cell culture assays support a role for the p.E88del variant in receptor function but not membrane localization. The p.E88del variant did not alter the apical membrane localization of CD320 in polarized MDCK cells. Although the p.E88del variant could have affected the DSSDE motif in the first LDLR-A domain in CD320, we note that this motif is not intact in the reference genome (DGSDE), and it may be the second LDLR-A domain in CD320 that is essential for the specificity of localization. Studies of fibroblasts derived from cases with elevated newborn C3 levels and homozygous for the *CD320* p.E88del variant demonstrated reduced binding of radiolabeled holoTC to cells after incubation for 1 hour at 5°C, as well as decreased uptake of labeled cobalamin at all timepoints tested. This was accompanied by decreased synthesis of the cobalamin coenzyme derivatives adenosylcobalamin and methylcobalamin and decreased function of the cobalamin dependent enzymes methylmalonyl-CoA mutase and methionine synthase. While average cobalamin uptake was reduced to 19.7% of that in controls, *CD320* variant fibroblasts were able to convert exogenous cyanocobalamin to adenosylcobalamin and methylcobalamin in normal or nearly normal proportions. The percentage of intracellular labeled cobalamin present as adenosylcobalamin was 60% of the level in control fibroblasts, while the percentage conversion to methylcobalamin was unchanged from control cells. Based on indirect measures of cobalamin-dependent function, *CD320* variant fibroblasts were able to largely compensate for the decreased levels of cobalamin uptake. Although function of the cobalamin-dependent enzymes methylmalonyl-CoA mutase and methionine synthase were reduced compared to control fibroblasts, the degree of reduction was much less than seen in fibroblasts from cases with inborn errors of cobalamin metabolism (Armour et al., 2013). Overall, the individuals identified by newborn screening with long-term follow-up data had some degree of elevation of serum or urine methylmalonic acid, and several had elevated serum total homocysteine levels. For the most part, there were no obvious clinical phenotypes related to altered cobalamin metabolism in the fibroblast donors, although one had bilateral central retinal artery occlusion which the authors suggest may be related to elevated total homocysteine (Karth et al, 2012), although the observed elevations of 17μM and 23μM were modest compared to the levels reached (>100μM) in other inborn errors of cobalamin metabolism and classical homocystinuria (Lawrence de Koning et al., 2003).

While cellular perturbations are detectable in *in vitro* systems, cobalamin metabolism in some homozygous newborns is impacted resulting in C3 elevations during newborn

screening. Based on the allele frequency (MAF ~0.01), approximately 1 in ~10,000 individuals in the general population are expected to be homozygous for *CD320* p.E88del. However, in the New York State cohort we found seven of 348 newborns with transiently elevated C3 homozygous for *CD320* p.E88del. Observing seven homozygotes among this small number is a significant over-representation ($p<6x10^{-5}$), but its absence in the majority of these cases indicates it is not a common explanation for the transient elevation of C3 detected at newborn screening. In this New York State cohort, we estimate that ~14% (~7/50) of all *CD320* p.E88del homozygous newborns are detected by transiently elevated C3 in newborn screening. In parallel with this, another study based in Minnesota (Sarafoglou et al., 2011) covering birth years 2005-2010 reported detection of five *CD320* p.E88del homozygous newborns among 46 infants presenting with elevated C3. A total of 363,649 newborns were screened, allowing the estimation that ~36 of all newborns must have been *CD320* p.E88del homozygotes, with ~14% (~5/36) of them detected by elevated C3 in newborn screening. These independent estimates of *CD320* p.E88del homozygous newborns with elevated C3 are in alignment, suggesting that >85% of newborns with this genotype do not come to attention via newborn screening.

The newborn screen for C3 was designed to detect inborn errors of metabolism caused by single gene defects. Transiently elevated C3 remains largely unexplained, though some factors have been implicated such as very low birth weight (Slaughter et al., 2010), early collection of the newborn specimen (Peng, Tang, Cowan, et al., 2020), and maternal cobalamin deficiency (Chapman et al., 2008; Garg & Smith, 2017; Rossi et al., 2020). We note that in this study conducted over two birth years, there were 12 diagnoses of organic acidemias as a result of this screen. The majority of newborns exhibiting elevated C3 (n=351) in this primary screen were ultimately found to be unaffected. We observed a small but significant fraction of newborns presenting with C3 were homozygous for *CD320* p.E88del. This provides evidence that this genotype can contribute to the transient elevation of C3, likely in the presence of other unknown factors.

We speculate that the increased detection of transient elevation of C3, independent of genotype, in Hispanic newborns may be due to these unknown factors. The apparent relative increase of these cases in Hispanic newborns observed in three states (Figure 6) is consistent with recent studies in California that observed an increased number of Hispanics among 605 screen positive newborns (elevated C3 and/or C3/C2 ratio (Peng et al., 2019)), and increased levels of C3 levels in Hispanic newborns generally (Peng, Tang, Gandotra, et al., 2020). More studies are required to confirm this overrepresentation Hispanic newborns with elevated C3. The *CD320* variant is unlikely to be a contributing factor this overrepresentation. The p.E88del variant is of low frequency and doesn't differ greatly between populations; furthermore, the corresponding homozygous genotype is present in <15% of transiently elevated C3 cases. This does not rule out other genetic modifiers that may be more common in this population. Alternately, the shared cultural, sociological and environmental factors, especially those related to maternal diet during pregnancy, may contribute to the C3 levels in this population. These factors remain unexplored.

Although the long-term clinical implications of homozygosity for *CD320* p.E88del remain unknown, the case studies summarized here and concurrent reports of extended follow-up

of individuals up to 15 years of age indicate that ongoing management with oral or injected forms of cobalamin supplementation may be unnecessary in the absence of other clinical concerns. Among the total of 20 cases reported to date (Table 5 (n=10), Hannah-Shmouni et al., 2018 (n=2), Karth et al., 2012 (n=1), Pappas, Younan & Conway, 2022 (n=7)) and followed beyond the neonatal period, six have exhibited clinical outcomes with an unknown relationship to their *CD320* p.E88del genotype. Perhaps the most severe is the case that presented at 7 weeks of age with bilateral central retinal arterial occlusions (Karth et al., 2012), which are exceedingly rare in infants. Only four other cases in children or infants have been reported, each attributed to varying conditions: elevated homocysteine (Wilson & Ruiz, 1969), disseminated intravascular coagulation (Axer-Siegel et al., 1997), Henoch-Schonlein purpura (Wu et al., 2002); and severe hypernatremic dehydration (Ozer et al., 2016).

The potential role of *CD320* p.E88del homozygosity in this previously reported case is additionally confounded by the history of consanguinity (parents and paternal grandparents). In contrast to this rare condition, the observed neuropsychological findings in Table 5 are more common. Estimated prevalences include 1.74% for ASD (Zablotsky et al., 2019), 11% for ADHD (Visser et al., 2014), 5.9% for speech disorders, and 3.3% for language disorders (Black et al., 2015; Rosenbaum et al., 2016, pp. 63-64). Additionally, because of the clinical follow-up of this population, biased ascertainment of neuropsychological symptoms may have occurred. It remains unknown whether modulation of cobalamin metabolism could have been a contributing factor in the development of these conditions, especially during prenatal development, and further surveillance of other cases is warranted.

Ultimately, treatment for these vision and neuropsychological conditions is primarily guided by the diagnoses. In the remaining 14 case reports of individuals with this genotype, it is only transiently elevated C3 as a newborn that has brought them to clinical attention via newborn screening. It could be that metabolic stress in the perinatal/newborn period may induce transient cobalamin deficiency in these individuals that is ultimately not of consequence. It is possible that physiologic stress creates conditions where the *CD320* p.E88del genotype increases susceptibility for altered cobalamin metabolism. Evidence against this hypothesis is the observation that three cases (Cases 2, 3 and 4) had normal values for cobalamin biomarkers during emergency department visits and inpatient admissions for acute illnesses. It is unknown if other forms of metabolic stress later in life (e.g., puberty, impaired cobalamin absorption in the aged) could alter the clinical consequence of homozygosity for this variant. While our data suggest that *CD320* p.E88del is contributing to the clinical phenotype in these cases, it is neither necessary nor sufficient; other factors contributing to the phenotypes in these cases remain unidentified. One model would be that p.E88del homozygote embryos are more sensitive to maternal nutritional deficiencies or other metabolic disturbances during development.

We expect that newborns homozygous for *CD320* p.E88del will continue to be identified in areas where clinicians use DNA sequencing as a follow-up to newborn screening. Due to the uncertainty surrounding the impact of this genotype on clinical phenotype, periodic follow-up is warranted in these identified homozygotes. However, it would be premature to recommend *CD320* p.E88del genotyping solely on the basis of an elevated C3 during

newborn screening. Although there is strong evidence that this variant affects the function of the transcobalamin receptor, it appears that >85% of homozygous newborns are not detected biochemically during newborn screening for C3, and most of those that have been followed appear to have no clinical symptoms related to cobalamin metabolism. We conclude that individuals homozygous for *CD320* p.E88del detected via transiently elevated C3 rarely require clinical intervention in the absence of any other factors.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are included in the article. Further details may be available from the corresponding author upon reasonable request.

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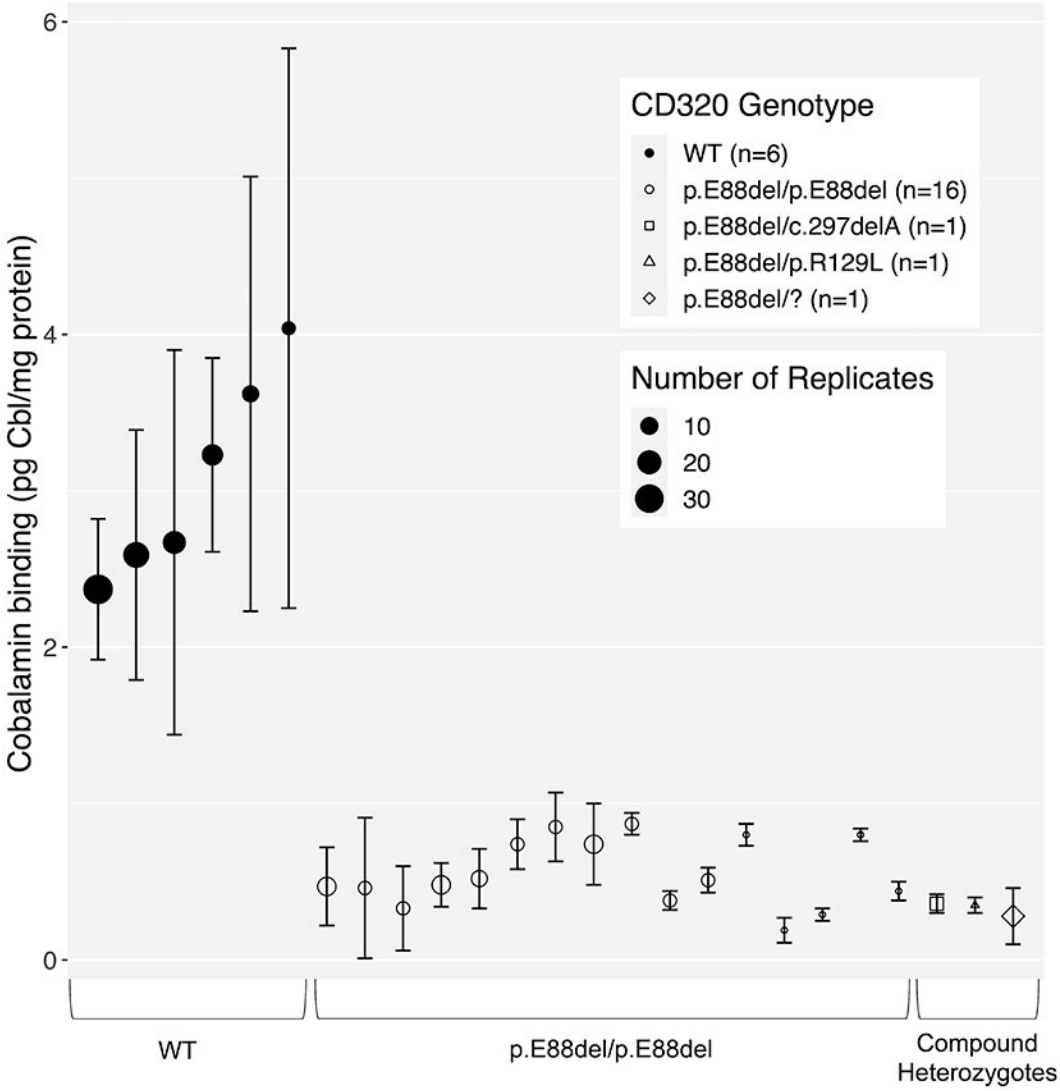

Figure 1.

Binding of holoTC to CD320 in fibroblasts from cases and unaffected controls. HoloTC binding is shown as an average ± standard deviation (pg [^{57}Co]cobalamin /mg protein). *CD320* genotype is indicated by shape (WT, solid circles; p.E88del/p.E88del, open circles; p.E88del/c.297delA, open square; p.E88del/p.R129L, open triangle, p.E88del/?, open diamond). Number of replicates is indicated by size.

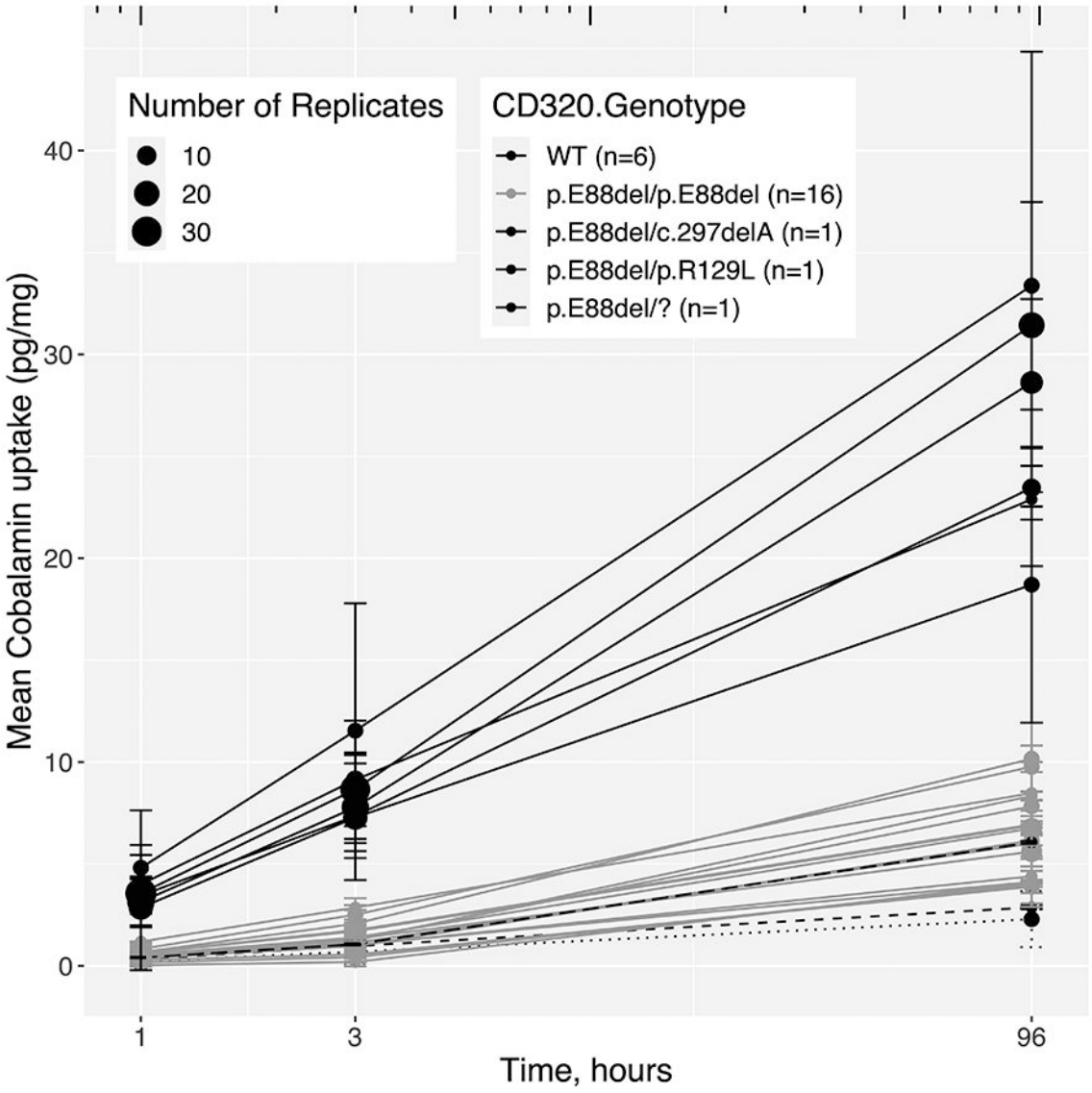

Figure 2.

Time course of holoTC uptake in fibroblasts from cases and unaffected controls. HoloTC uptake was measured at 1, 3 and 96 hours. *CD320* genotype is indicated by line type (WT, solid black; p.E88del/p.E88del, solid gray; p.E88del/c.297delA, dashed black; p.E88del/p.R129L, long dashed black; p.E88del/?, dotted black). Number of replicates is indicated by size. X-axis, time in hours (log-scale); Y-axis, mean cobalamin uptake (pg [^{57}Co]cobalamin /mg protein), ± standard deviation.

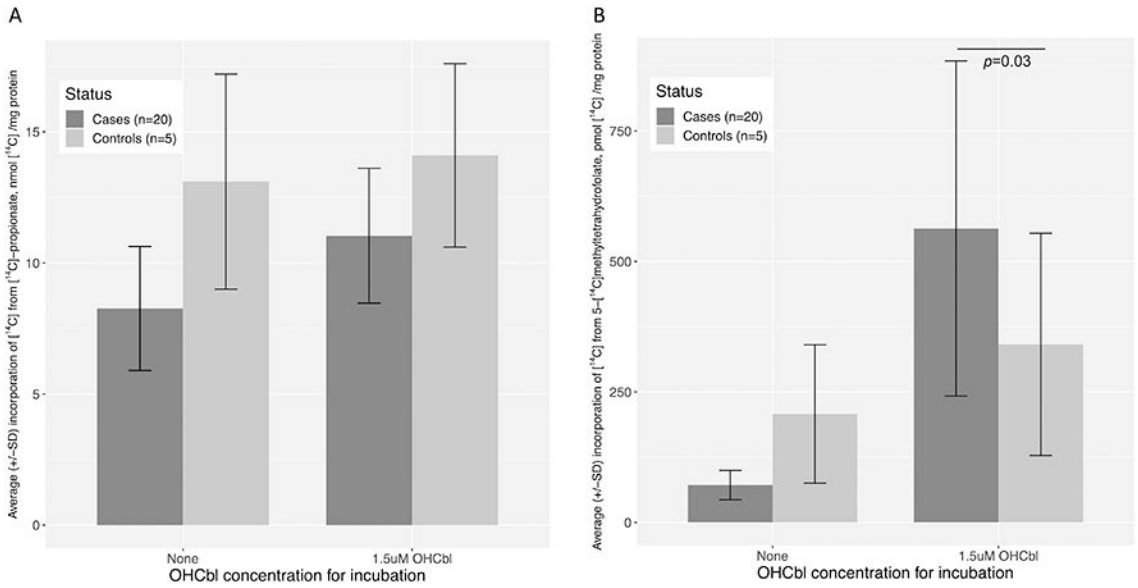

Figure 3.

Function of mitochondrial methylmalonyl-CoA mutase and cytoplasmic methionine synthase, as measured by incorporation of radiolabel from [^{14}C]propionate or 5-[^{14}C]methyltetrahydrofolate, respectively. Case or control fibroblasts were incubated with (A) [^{14}C]propionate; (B) 5-[^{14}C]methylTHF. Cells were harvested after 18 hours and measured for incorporation of radioactivity into cellular macromolecules. Incorporation is shown as average nmol [^{14}C] / mg protein (± SD) for cases (n=19) and controls. Control data involves up to 127 repeated measures of 5 participants and is displayed as the weighted mean (± SD). Case data involves one measure per participant and is displayed as an arithmetic mean (±SD). A nested t-test was performed to compare cases and controls. Each assay was performed in the absence or presence of 1.5uM hydroxocobalamin, as shown.

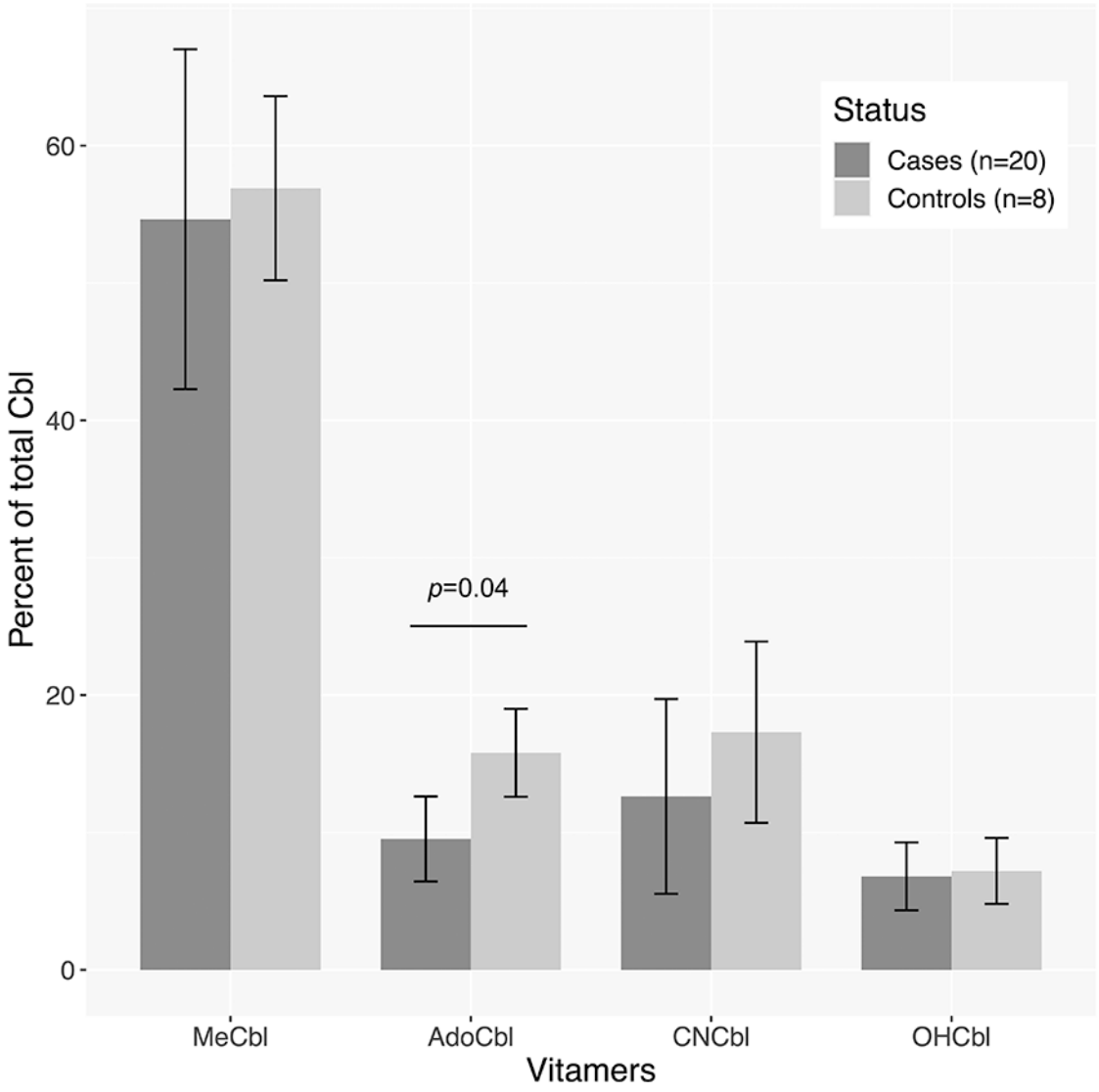

Figure 4.

Cobalamin vitamer distribution in case and control fibroblasts. Abbreviations: MeCbl, methylcobalamin; AdoCbl, adenosylcobalamin; CNCbl, cyanocobalamin; OHCbl, hydroxocobalamin. Cells were incubated with radiolabeled holoTC for 96 hours, and cobalamin vitamers were measured by the relative amount of radioactivity in the relevant HPLC fractions. Average (±SD) vitamers as a percent of total counts are shown for cases (n=20) and controls. Control data involves up to 106 repeated measures of 8 participants and is displayed as the weighted mean (± SD). Case data involves one measure per participant and is displayed as an arithmetic mean (±SD). A nested t-test was performed to compare cases and controls.

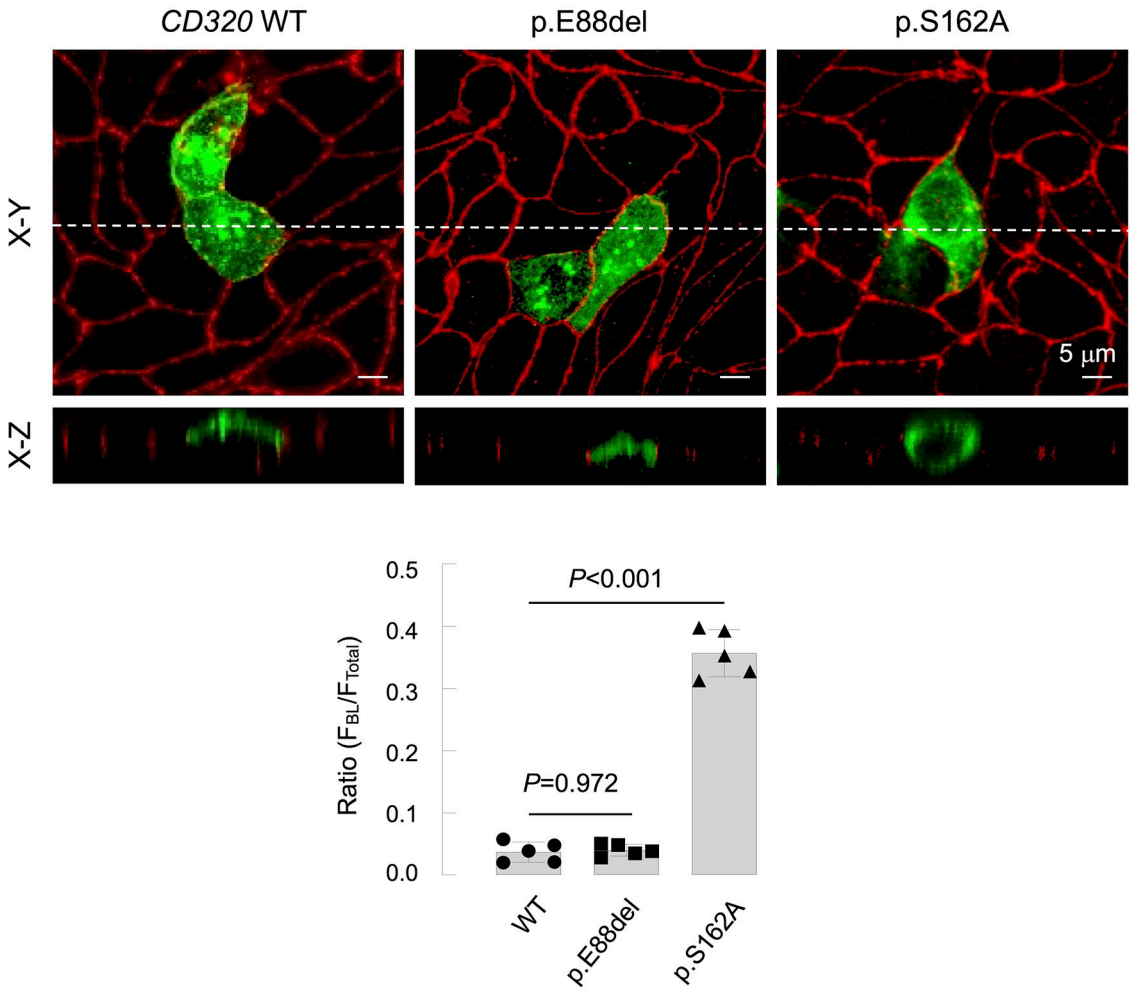

Figure 5.

Membrane distribution of human CD320 in transfected MDCK cells. Human CD320 (WT) and two variant isoforms, p.E88del and p.S162A, were expressed in polarized MDCK cells. Immunostaining of CD320 (green) and ZO-1 (red) (an indicator of apicolateral tight junctions) was analyzed by confocal microscopy. X-Y and X-Z views are shown in top and bottom panels, respectively. Scale bars: 5 μm. Each image represents data from five experiments. Fluorescent signals along the X-Z axis were analyzed with Image J software to calculate the ratio of F_{BL}/F_{Total}, where F_{BL} is the fluorescent intensity on basolateral membranes and F_{Total} is the total fluorescent intensity on apical and basolateral membranes. Quantitative data (means ± S.D.) from five experiments are presented in the bar graph. Statistical analysis was done with one-way ANOVA.

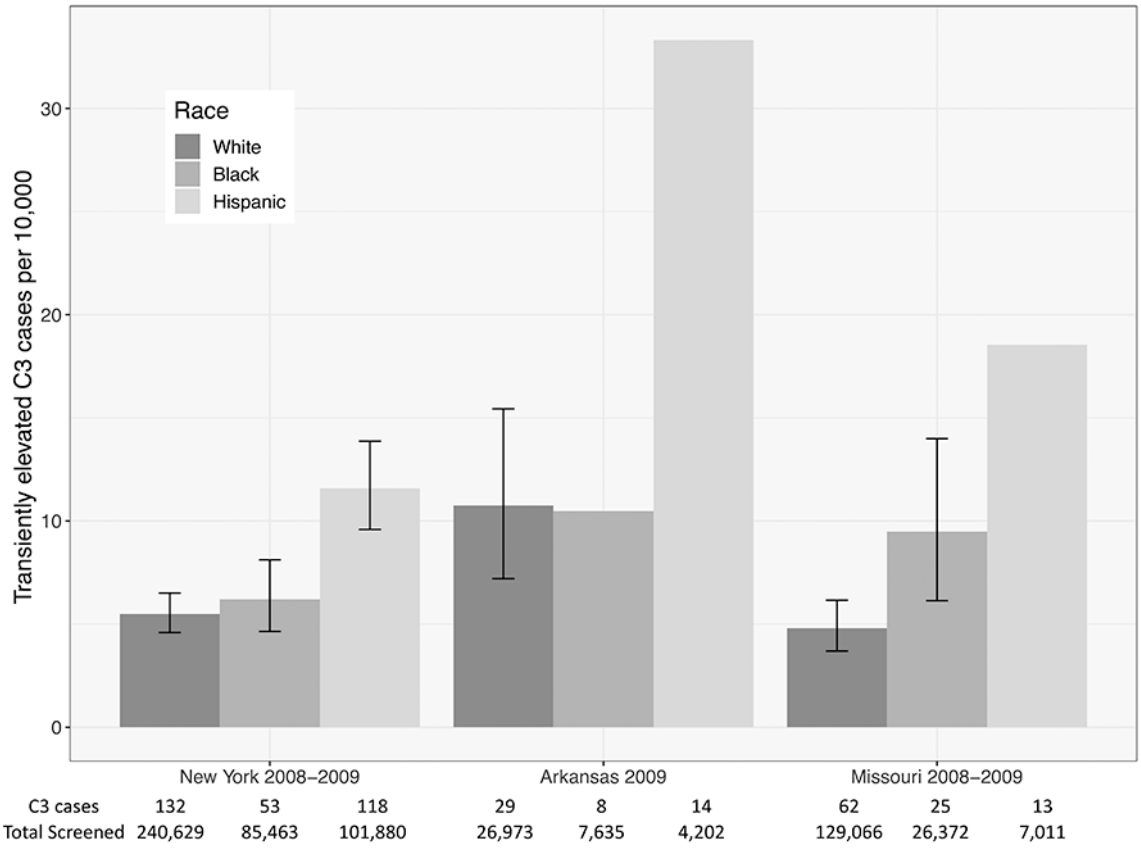

Figure 6.

Newborns with transiently elevated C3 per 10,000 screened newborns by race/ethnicity in three states. Incidence was calculated based on counts of screened newborns with transiently elevated C3 divided by the total number of infants screened by race/ethnicity in each state for the indicated birth years. Vertical bars indicate the 95% confidence interval; this is omitted (NA) when <20 cases were observed.

Table 1.

CD320 p.E88del frequency in gnomAD v.2.2.1 cohort

***CD320* p.E88del**	**gnomAD European (non-Finnish) Population**	**gnomAD European (Finnish) Population**	**gnomAD African Population**	**gnomAD Latino Population**	**gnomAD East Asian Population**	**gnomAD South Asian Population**	**gnomAD Ashkenazi Jewish Population**
WT/WT	62809 (0.98)	9969 (0.991)	12305 (0.995)	17199 (0.971)	17199 (0.971)	15201 (0.993)	5063 (0.98)
WT/Del	1293 (0.02)	86 (0.009)	64 (0.005)	504 (0.028)	504 (0.028)	100 (0.007)	104 (0.02)
Del/Del	9 (0.0001)	0 (0)	0 (0)	2 (0.0001)	2 (0.0001)	0 (0)	1 (0.0002)
Total	64111	10055	12369	17705	17705	15301	5168
WT Freq.	0.99	0.996	0.997	0.986	0.986	0.997	0.99
Del Freq.	0.01	0.004	0.003	0.014	0.014	0.003	0.01

Table 2.

CD320 p.E88del in NYS CHD samples (1997-1998)

***CD320* p.E88del**	**White**	**Black**	**Hispanic**	**Asian**	**Other**	**Native American**
WT/WT	4704 (0.982)	1971 (0.996)	1356 (0.984)	250 (0.965)	390 (0.992)	6 (1)
WT/Del	83 (0.017)	8 (0.004)	22 (0.016)	9 (0.035)	3 (0.008)	0 (0)
Del/Del	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total	4788	1979	1378	259	393	6
WT Freq.	0.991	0.998	0.992	0.983	0.996	1.000
Del Freq.	0.009	0.002	0.008	0.017	0.004	0.000

Table 3.

CD320 p.E88del in NYS newborns with transiently elevated C3 and controls (2008-2009)

	***CD320* genotype**	**White***	**Black**	**Hispanic****	**Asian**	**Other**
High C3 Cases	WT/WT	128 (0.985)	49 (0.98)	104 (0.889)	14 (1)	36 (0.973)
	WT/Del	0 (0)	1 (0.02)	8 (0.068)	0 (0)	1 (0.027)
	Del/Del	2 (0.015)	0 (0)	5 (0.043)	0 (0)	0 (0)
	Total (n=348)	130	50	117	14	37
	WT Freq.	0.985	0.990	0.923	1.000	0.986
	Del Freq.	0.015	0.010	0.077	0.000	0.014
Controls	WT/WT	133 (0.993)	57 (1)	129 (0.985)	17 (1)	41 (1)
	WT/Del	1 (0.007)	0 (0)	2 (0.015)	0 (0)	0 (0)
	Del/Del	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total (n=380)	134	57	131	17	41
	WT Freq.	0.996	1.000	0.992	1.000	1.000
	Del Freq.	0.004	0.000	0.008	0.000	0.000

*Exact test of HWE p=4.5x10^{-5} in transiently elevated C3 cases

**Exact test of HWE p=5.9x10^{-5} in transiently elevated C3 cases

Table 4.

CD320 p.E88del in NYS NTD samples (1998-2005)

	***CD320* p.E88del**	**White**	**Black**	**Hispanic**	**Asian**	**Other**	**Native American**
NTD cases	WT/WT	211 (0.995)	93 (1)	88 (0.957)	18 (1)	17 (1)	3 (1)
	WT/Del	1 (0.005)	0 (0)	3 (0.033)	0 (0)	0 (0)	0 (0)
	Del/Del	0 (0)	0 (0)	1 (0.011)	0 (0)	0 (0)	0 (0)
	Total	212	93	92	18	17	3
	WT Freq.	0.998	1.000	0.973	1.000	1.000	1.000
	Del Freq.	0.002	0.000	0.027	0.000	0.000	0.000
NTD controls	WT/WT	909 (0.984)	404 (1)	376 (0.977)	80 (0.976)	80 (0.988)	12 (1)
	WT/Del	15 (0.016)	0 (0)	9 (0.023)	2 (0.024)	1 (0.012)	0 (0)
	Del/Del	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Total	924	404	385	82	81	12
	WT Freq.	0.992	1.000	0.988	0.988	0.994	1.000
	Del Freq.	0.008	0.000	0.012	0.012	0.006	0.000

Table 5.

Summary of biochemical and clinical findings for *CD320* p.E88del homozygous cases

Case	Sex	NBS* positive?	NBS C3 (abnormal >5.25uM)	NBS C3:C2 ratio (abnormal >0.22)	NBS tHcy*, umol/L (abnormal > 15 uM)	NBS MMA*, units (abnormal >5uM)	Newborn total B_{12}, pg/mL (RR*: 247-911)	Treatment	Age at last visit	Clinical Findings	Evidence of abnormal cobalamin metabolism?
1	Male	Yes	Elevated	NR	Elevated	NR	>2000	Daily 0.2 mg B_{12} injections and Propimex started at 3 weeks. Stopped at 3 months.	10 years	Language acquisition deficit; below average working memory	Normal MMA, tHcy since 3 weeks of age
2 [1]	Male	Yes	Normal (3.44 uM)	0.24	16.4	13.7	1908	Daily 1mg B_{12} injections started within a month of birth; stopped by 4 months of age. 2mg B_{12} injections twice a week for three months at age 4. Remains on 100ug oral B_{12}, started at last visit (age 6).	6 years	ADHD, autism spectrum, anxiety, residual deficits with aspects of expressive language and language processing, working memory deficits	At age 4 years, plasma MMA, plasma C3, total plasma homocysteine, urine organic acids, MCV of red blood cells were normal and normal at subsequent ED* visits
3 [1]	Male	No	Normal	NR	NR	NR	>1966	Remains on 100 mcg B12, started at age 8.	9 years	Mild muscle weakness	At age 5 years and 9 months, plasma MMA, plasma C3, total plasma homocysteine, urine organic acids, MCV of red blood cells were normal even during ED visits
4	Male	Yes	Elevated	NR	13.3	11.5	NR	Treated with Propimex and B_{12} injections 3 times a week for 3 months until diagnosis of B_{12} receptor was made.	8 years	None	Normal MMA beginning at 1 month and during ED visits and inpatient admissions
5	Female	Yes	Elevated (6.04 uM)	0.27	21.9	22.8	1000	NA	1 year	None	Normal MMA & tHcy at 1 year
6	Male	Yes	Elevated (6.63 uM)	NR	12	6.41	>2000	Started on 1 mg B_{12} injections daily, B6 at 50mg, betaine 150 mg twice a day, and folate at 5 mg/day. Treatment lasted for 6 weeks.	15 years	Admitted for sepsis/pneumonia at 11 weeks of life; Speech and language delay at 19 months of age, ongoing mental health issues and	Normal MMA and tHcy at 11 weeks of life

Case	Sex	NBS* positive?	NBS C3 (abnormal >5.25uM)	NBS C3:C2 ratio (abnormal >0.22)	NBS tHcy*, umol/L (abnormal > 15 uM)	NBS MMA*, units (abnormal >5uM)	Newborn total B_{12}, pg/mL (RR*: 247-911)	Treatment	Age at last visit	Clinical Findings	Evidence of abnormal cobalamin metabolism?
										learning disabilities	
7	Female	Yes	Elevated (6.94 uM)	NR	20	11.5	NR	Started on 1 mg B_{12} injections 3 times a week until 6 weeks of age.	12 weeks	None	Normal MMA and tHcy at 6 weeks of life and again at 6 weeks off therapy
8	Male	Yes	Elevated (6.63 uM)	NR	13.3	10.2	>2000	Started at 1 month of age on 50 mcg oral B_{12} daily until 10 months of age	10 months	None	Normal urine MMA and plasma tHcy at 2 months of age
9 [2]	Male	Yes	Elevated[2] (7.8 uM)	0.27	9	NR	NR	Hydroxocobalamin three times a week at 2 weeks of age, changed to oral B_{12} 1 mg/day at 7 months of age - biochemical parameters remained normal after switching to oral B_{12}; oral levocarnitine. Remains on 1mg oral cyanocoblamin daily.	7 years	Seizures, ADHD	At age 7 years and 9 months, plasma MMA, plasma C3, total plasma homocysteine, urine organic acids, MCV of red blood cells were normal
10	Female	Yes	2.11	NA	19	17.0	normal	1mg hydroxocoblamin injections daily with carnitine and folate then decreased frequency until 9 months discontinued medications. Started monthly 1mg hydroxocobalamin injections at most recent visit.	14 years	Premature 36 weeks, admitted 2 days hypoglycemia resolved. Normal development, ADHD, chronic pain syndrome likely familial.	Elevated B12 2260pg/ml, low carnitine and acylcarnitine, normal MMA and homocysteine at age 13. Elevated homocysteine at age 14 normalized after first hydroxocobalamin injection.

*Abbreviations: NBS – newborn screening; tHcy – total homocysteine; MMA – methylmalonic acid; RR – reference range; NR – Not recorded/reported; ED – emergency department.

[1]Siblings.

[2]Case ascertainment occurred in Iowa. NBS screening thresholds: C3 > 7uM, C3/C2 ratio > 0.22.

Note: All patients were homozygous for p.E88del except for case 10 who is compound heterozygous for p.E88del and c.297delA