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A human *ALDH1A2* gene variant is associated with increased newborn kidney size and serum retinoic acid

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Nephron number varies widely between 0.3 and 1.3 million per kidney in humans. During fetal life, the rate of nephrogenesis is influenced by local retinoic acid (RA) level such that even moderate maternal vitamin A deficiency limits the final nephron number in rodents. Inactivation of genes in the RA pathway causes renal agenesis in mice; however, the impact of retinoids on human kidney development is unknown. To resolve this, we tested for associations between variants of genes involved in RA metabolism (*ALDH1A2*, *CYP26A1*, and *CYP26B1*) and kidney size among normal newborns. Homozygosity for a common (1 in 5) variant, rs7169289(G), within an Sp1 transcription factor motif of the *ALDH1A2* gene, showed a significant 22% increase in newborn kidney volume when adjusted for body surface area. Infants bearing this allele had higher umbilical cord blood RA levels compared to those with homozygous wild-type *ALDH1A2* rs7169289(A) alleles. Furthermore, the effect of the rs7169289(G) variant was evident in subgroups with or without a previously reported hypomorphic *RET* 1476(A) proto-oncogene allele that is critical in determining final nephron number. As maternal vitamin A deficiency is widespread in developing countries and may compromise availability of retinol for fetal RA synthesis, our study suggests that the *ALDH1A2* rs7169289(G) variant might be protective for such individuals.

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In humans, nephrogenesis occurs exclusively during fetal life. As every nephron is linked to a branch of the collecting system, final nephron number is determined by the extent to which the ureteric bud arborizes between 4 and 36 weeks gestation.[1] Thus, genetic or environmental factors that alter the rate of branching nephrogenesis *in utero* may affect nephron number and relative kidney size by the time the baby is born.

Extensive studies of murine kidney development by Costantini and Shakya[2] have shown that ureteric bud branching is critically dependent on release of glial cell line-derived neurotrophic factor from the metanephric mesenchyme and expression of the tyrosine kinase receptor, RET, in cells of the ureteric bud tip, conferring responsiveness to the glial cell line-derived neurotrophic factor signal. In response to glial cell line-derived neurotrophic factor, RET(+) cells are recruited to the ureteric bud tip as it undergoes remodeling to initiate the next branching event.[3] Mice bearing homozygous mutations of the *Ret* gene are anephric.[4] We have recently identified a common variant, $RET^{1476(A)}$, within an exonic splicing enhancer of the human *RET* gene, which reduces fidelity of normal RET mRNA splicing; babies carrying this *RET* variant have 10% decrease in newborn kidney size.[5] Thus, RET expression in ureteric bud tip cells appears to be a crucial control point for regulation of renal branching morphogenesis and, thus, final nephron number.

Expression of RET in the ureteric bud tip is exquisitely sensitive to the paracrine retinoic acid (RA)-dependent signals from adjacent mesenchymal cells.[6] At least one such RA-responsive secreted branching morphogen, late-gestation lung protein 1, has been identified.[7] Fetal kidney RET expression is lost when mesenchymal retinoic acid receptors (RARα and RARβ) are knocked out.[8] Addition of RA (0.1–1 μM) to normal embryonic day E14 fetal rat kidney explants accelerates branching nephrogenesis.[9,10]

In fetal kidney, RA is synthesized from retinol derived from the maternal diet and transferred from the placenta through retinol binding protein in fetal blood. Retinol is

taken up by a specific receptor (STRA6) on fetal cells and converted to RA in a two-step enzymatic reaction. The first step, reversible oxidation of retinol into retinaldehyde, involves either alcohol dehydrogenases, or microsomal retinol dehydrogenases. The second step, irreversible oxidation of retinaldehyde into RA, can be catalyzed by at least four retinaldehyde dehydrogenases (RALDH1, 2, 3, and 4).[11,12] However, enzymatic studies[13] and *in situ* hybridization[14] indicate that RALDH2 is the dominant enzyme generating RA in embryonic mouse tissues. Knockout of the *Raldh2* gene results in early embryonic lethality caused by severe trunk, hindbrain, and heart defects.[15,16] The fate of intracellular RA in fetal kidney is regulated by cytochrome P450 hydroxylases, CYP26A1 and CYP26B1,[17,18] which convert RA into water-soluble hydroxylated end-products that are less active and readily excretable.[19,20]

Interestingly, adjustments in intracellular RA metabolism cannot fully compensate for reduced availability of retinol substrate when maternal dietary vitamin A intake drops below some threshold. In the early 1950s, Wilson *et al.*[21] observed that maternal vitamin A deficiency in rats caused renal hypoplasia among the pups and this could be prevented by vitamin A administration during pregnancy.[9] Even mild-to-moderate maternal vitamin A deficiency in rats (50% reduction in circulating retinol level) causes renal hypoplasia among the offspring.[9] At 14 days of age, the kidneys of pups exposed to gestational retinol deficiency weighed 24% less and contained 20% fewer nephrons than controls.[22,23]

As rodent nephrogenesis is limited by modest maternal vitamin A deficiency, we reasoned that optimal kidney development must require full efficiency of the metabolic pathways regulating intracellular RA levels. We hypothesized, therefore, that metabolic bottlenecks due to hypofunctional genes of the RA pathway might slow ureteric bud branching and reduce kidney size *in utero*; conversely, genes with regulatory elements that confer high expression might increase branching nephrogenesis. We screened a cohort of normal Caucasian newborns from Montreal for an association between polymorphic variants of *ALDH1A2* (coding for RALDH2 enzyme), *CYP26A1* or *CYP26B1* genes and both: (A) a shift in umbilical cord RA concentration; (B) a parallel shift in newborn kidney size.

RESULTS

Characteristics of study subjects

Characteristics of the newborn cohort ($n = 113$) are shown in Table 1. Mean ($\pm$ s.d.) newborn weight, height, and body surface area (BSA) were 3.59 ± 0.46 kg, 51.31 ± 2.18 cm, and 0.223 ± 0.018 m^2, respectively. Unadjusted left, right, and total kidney volumes were 14.39 ($\pm$ 3.91), 15.93 ($\pm$ 3.68), and 30.33 ($\pm$ 6.58) ml, respectively. These values are similar to those of a large cohort of Danish newborns who were studied within the first 5 days of life.[24] Total (left + right) kidney volume corrected for BSA was 135.85 ($\pm$ 27.77) ml/m^2.

A polymorphic variant of the human *ALDH1A2* gene is associated with increased newborn renal volume

To define the common variants of *ALDH1A2* among normal Caucasian newborns in Montreal, we genotyped 113 healthy term newborns for 19 known single-nucleotide polymorphisms (SNPs) spanning the *ALDH1A2* gene (Supplementary Table S1). SNP frequencies were similar to those reported for the Centre d'Étude du Polymorphisme Humain (CEPH) population, indicating no appreciable genetic drift in our cohort. Genotype distributions for each SNP locus conformed to the expected Hardy–Weinberg equilibrium.

Using an analysis of variance, we detected an association between total kidney volume normalized to BSA and the *ALDH1A2*^{rs7169289} SNP ($p = 0.039$). A multiple comparisons testing procedure showed that newborns with homozygosity for the rs7169289(G), a fairly common variant (minor allele frequency = 20%), had a 22% increase in newborn kidney volume adjusted for BSA ($p = 0.023$). Total renal volume/BSA (161 ml/m^2) among homozygous rs7169289G/G infants (4.4% of the cohort) was 22% higher than that (132 ml/m^2) of subjects who were homozygous for the more common rs7169289A/A genotype (63.7% of the cohort; (Figure 1)). Total renal volume/BSA (140 ml/m^2) in heterozygous rs7169289A/G infants (31.9% of the cohort) was intermediate between the two homozygous subgroups (Figure 1).

The *ALDH1A2*^{rs7169289} SNP is not tightly linked ($r^2 < 0.3$) to any of the HapMap SNPs in the 132 kbp *ALDH1A2* gene region (chr15:56022918.56155197). We analyzed the rs7169289 sequence region for transcription factor binding motifs using two different software programs, TFBIND (http://tfbind.ims.u-tokyo.ac.jp/) and AliBaba2 (http://www.generegulation.com/pub/programs/alibaba2/index.html). Both

Table 1 | Characteristics of study subjects

Characteristic	Mean value (s.d.)	Minimum value	Maximum value
Weight (kg)	3.59 (0.46)	2.62	5.05
Height (cm)	51.31 (2.18)	46	58
BSA (m^2)	0.223 (0.018)	0.181	0.275
Left kidney volume (ml)	14.39 (3.91)	3.31	23.75
Right kidney volume (ml)	15.93 (3.68)	8.43	28.54
Total kidney volume (ml)	30.33 (6.58)	16.06	47.43
Total kidney volume/BSA (ml/m^2)	135.85 (27.77)	80.31	209.61

The mean and s.d. measurements of weight, height, body surface area (BSA), and the left and right kidney volume for the cohort of white newborns (n=113) are presented. Total kidney volume (left+right) and total kidney volume normalized for BSA are also shown.

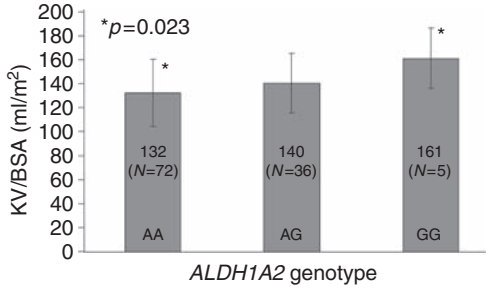

Figure 1 | The *ALDH1A2* variant (rs7169289G) is associated with increased newborn renal volume. Kidney volume (KV)/body surface area (BSA) (161 ml/m^2) among rs7169289G/G homozygous infants (4.4% of the cohort) was 22% higher than that (132 ml/m^2) of subjects who were homozygous for the more common rs7169289A/A genotype (63.7% of the cohort; $p = 0.023$). KV/BSA (140 ml/m^2) in heterozygous rs7169289A/G infants (31.9% of the cohort) was intermediate between the two homozygous subgroups.

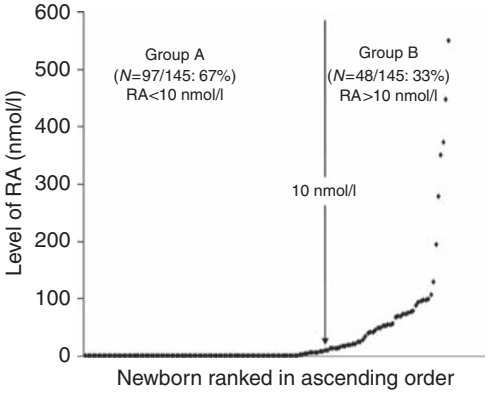

Figure 2 | Levels of retinoic acid (RA) in newborns. Our cohort demonstrates a skewed distribution with the appearance of two distinct subgroups: (Group A) two-thirds of the samples (97 out of 145) fell below an RA concentration of 10 nmol/l; (Group B) the remaining third (48 out of 145) of RA values were spread evenly across a wide range of higher concentrations (10–550 nmol/l).

programs predicted that the *ALDH1A2*^{rs7169289} SNP is located within an Sp1 (Cys2His2 zinc-finger domain) consensus sequence.

We also genotyped each infant for 13 SNPs covering the *CYP26A1* gene (Supplementary Table S2) and 15 SNPs in *CYP26B1* gene (Supplementary Table S3). However, none of these polymorphic variants was significantly associated with newborn kidney volume.

The *ALDH1A2*$^{rs7169289(G)}$ variant is associated with higher cord RA levels

If the *ALDH1A2*$^{rs7169289(G)}$ variant affects newborn kidney size by increasing local production of RA, we reasoned that this should be reflected in an increase in RA circulating in fetal blood.

To quantify levels of RA at the lower end of the spectrum, we modified a standard high-pressure liquid chromatography (HPLC) method to ensure clean separation of RA from retinol and other retinoids. Inspection of cord RA levels in our cohort demonstrates a skewed distribution with the appearance of two distinct subgroups: (Group A) two-thirds of the samples fell below a RA concentration of 10 nmol/l; (Group B) the remaining third of RA values were spread evenly across a wide range of higher concentrations (10–550 nmol/l) (Figure 2). Interestingly, retinol levels (1348 nmol/l) in babies with RA <10 nmol/l were less than those (862 nmol/l) in babies with RA >10 nmol/l. Umbilical cord retinol levels were normally distributed in our cohort (data not shown).

Using a standard two independent proportions test, we analyzed the relationship between *ALDH1A2*^{rs7169289} genotype and cord RA level in the newborn cohort. Overall, 50% of subjects with RA >10 nmol/l carried the *ALDH1A2*$^{rs7169289(G)}$ allele, whereas only 30% of subjects with RA <10 nmol/l carried this allele ($p<0.001$). Also, when we used the tobit model, we found that mean RA for infants with one or more *ALDH1A2*$^{rs7169289(G)}$ alleles (43 nmol/l), was

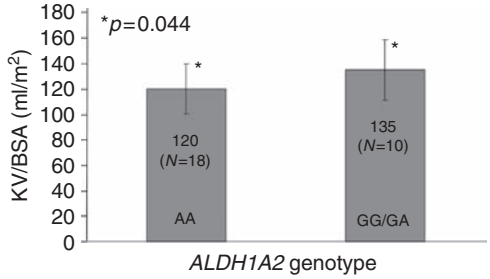

Figure 3 | The *ALDH1A2*$^{rs7169289(G)}$ variant increases kidney volume among infants with a dysfunctional *RET*$^{1476(A)}$ allele. In *RET*$^{1476(A)}$ group, the presence of the *ALDH1A2*$^{rs7169289(G)}$ allele increases newborn kidney volume from 120 to 135 ml/m^2.

significantly ($p = 0.006$) higher than cord blood RA in homozygous *ALDH1A2*$^{rs7169289(A)}$ subjects (17 nmol/l).

Cord RA level was unrelated to any of the other 19 *ALDH1A2* SNPs, which had no association with newborn kidney volume. Furthermore, cord RA level was uncorrelated with SNPs in the *CYP26A1* or *CYP26B1* genes.

The *ALDH1A2*$^{rs7169289(G)}$ variant is associated with increased newborn kidney size in subgroups defined by *RET*^{1476} genotype

In a previous study, we identified a partially dysfunctional allele of the RET tyrosine kinase receptor that is associated with a 10% decrease in newborn kidney volume.[5] In this study we examined the effect of the *ALDH1A2*$^{rs7169289(G)}$ variant among babies in whom the hypomorphic *RET*$^{1476(A)}$ isoform had been previously identified. Interestingly, in *RET*$^{1476(A)}$ babies the presence of a single *ALDH1A2*$^{rs7169289(G)}$ allele increases newborn kidney volume from 120 to 135 ml/m^2 ($p = 0.044$) (Figure 3), bringing it close to that of babies with wild-type *RET* alleles (138 ml/m^2).

We also examined the effect of the *ALDH1A2*$^{rs7169289(G)}$ allele among those babies with proven homozygous wild-type *RET* alleles. Newborn kidney size of babies with the

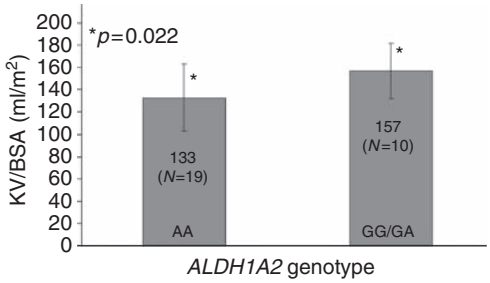

Figure 4 | The *ALDH1A2*$^{rs7169289(G)}$ variant increases kidney volume among infants with two *RET*$^{1476(wildtype)}$ alleles. In *RET*$^{(wildtype)}$ group, the *ALDH1A2*$^{rs7169289(G)}$ babies have significantly greater kidney volume (KV)/body surface area (BSA) (157 ml/m^2) than that of babies with the common *ALDH1A2*$^{rs7169289(A)}$ allele (133 ml/m^2) ($p = 0.02$).

ALDH1A2$^{rs7169289(G/G\ or\ G/A)}$/*RET*$^{(wildtype)}$ (157 ml/m^2) was significantly greater than that of babies with the common *ALDH1A2*$^{rs7169289(A/A)}$/*RET*$^{(wildtype)}$ genes (133 ml/m^2) ($p = 0.022$) (Figure 4).

DISCUSSION

Kidney organogenesis is the result of interactions between the undifferentiated metanephric blastema and the ureteric bud. In humans, final nephron number has been determined by late gestation and displays wide individual variation (0.3–1.3 million nephrons per kidney).[25,26] Although once dismissed as a benign reflection of human diversity, it has been proposed that individuals born at the low end of the nephron endowment spectrum may have increased risk for developing 'essential' hypertension and renal insufficiency later in life.[27] In fact, patients with essential hypertension have 46% fewer nephrons than normotensive age-matched control subjects.[28] We have hypothesized that congenital nephron number is a multifactorial trait controlled by the interaction of environmental factors (for example, maternal vitamin A intake) and genetic variants that influence the extent of branching nephrogenesis during fetal life.

In humans, it is as yet unknown whether maternal retinol levels influence fetal nephrogenesis as they do in rodents. However, a pilot study showed that newborn kidney volume was significantly smaller in Bangalore, India (where maternal vitamin A deficiency is widespread) than in Montreal (where most pregnant women are vitamin A replete).[29] When measured in the newborn period, kidney size correlates with congenital nephron number before the association is masked by compensatory renal hypertrophy.[5] We recently identified a common variant of the human *RET* gene that is associated with relatively small newborn kidney size and function;[5] interestingly, RET expression in fetal kidney is exquisitely sensitive to local RA concentrations.[6] This observation prompted us to consider the possibility that variation in genes of RA metabolism might also influence nephrogenesis in fetal life. Specifically, we focussed on the principal genes involved in the synthesis (*ALDH1A2*) and degradation (*CYP26A1*/*CYP26B1*) of RA in fetal kidney.

We identified a relatively common (20% of the Montreal Caucasian population) *ALDH1A2* variant, rs7169289(G), that is associated with a 22% increase in mean newborn total kidney volume/BSA. It could be that the rs7169289G is linked to an unidentified variant that regulates RALDH2 expression. However, rs7169289 SNP is located 1.9 kb 3′ of the *ALDH1A2* coding elements, within an Sp1 site. Cawley *et al.*[30] have shown that about one-third of the transcription factor binding sites that regulate gene expression lie within introns or 3′ to the coding sequence. Thus, we hypothesized that the rs7169289G variant might increase RALDH2 expression, thereby increasing local RA concentrations in fetal kidney. Lelièvre-Pégorier *et al.*[9] have shown that a single injection of RA given to pregnant rats at mid-gestation induces supernumerary nephrons.

If the *ALDH1A2*$^{rs7169289(G)}$ variant increases RALDH2 expression in fetal kidney, we reasoned that it might be reflected by increased levels of RA in umbilical cord blood. To measure RA in fetal blood, we developed a sensitive HPLC assay, which allowed quantification in the low range and overcame the tendancy for retinoids to isomerize and undergo oxidative degradation during manipulation. In our cohort of newborns, nearly two-thirds of the umbilical cord blood samples had RA levels below 10 nmol/l. However, the distribution was skewed. About one-third of newborns had fairly high levels of RA, which could not be explained by high levels of cord blood substrate (retinol). We found that the *ALDH1A2*$^{rs7169289(G)}$ allele was significantly associated with the high cord RA subgroup. This observation offers a plausible mechanism by which the *ALDH1A2*$^{rs7169289(G)}$ allele might affect fetal kidney RALDH2 activity, increased RA synthesis and, thus, kidney size. Furthermore, as RA levels presumably reflect fetal RALDH2 activity, the fact that both newborn RA level and newborn kidney size were associated with the *ALDH1A2*$^{rs7169289(G)}$ allele argues strongly that the two associations were not each identified by chance.

Two cytochrome P450 hydroxylases (CYP26A1 and CYP26B1) regulate degradation of RA in fetal kidney. This is important to protect fetal kidney cells from excessive RA.[19,31] Mice lacking either *Cyp26a1* or *Cyp26b1* die *in utero*, or shortly after birth, and exhibit abnormalities consistent with those seen in RA teratogenesis. These anomalies include spina bifida, truncation of the tail, and abnormalities affecting the kidneys, urogenital tract, and hindgut.[32–34] However, no *CYP26A1* or *CYP26B1* SNP was associated with either newborn kidney size or cord RA levels. Our study had 80% power to detect a difference in mean kidney volume of 12% or more, assuming a minor allele frequency of $>20\%$ for each SNP.

In a previous study, we identified a *RET*$^{1476(A)}$ variant associated with reduced newborn kidney size and increased cord cystatin C level (reduced renal function).[5] Interestingly, RET expression in fetal kidney is highly sensitive to local RA.[6] We considered the possibility that the *ALDH1A2*$^{rs7169289(G)}$ variant might influence kidney size in babies bearing the

slightly dysfunctional $RET^{1476(A)}$ allele. Our prediction was confirmed by analyzing the distribution of the rs7169289(G) variant among babies with the dysfunctional $RET^{1476(A)}$ variant. The presence of a single $ALDH1A2^{rs7169289(G)}$ allele influences newborn kidney volume (renal volume/BSA = 135.4 ml/m^2), making it likely to be close to that of babies with wild-type *RET* alleles (renal volume/BSA = 138.6 ml/m^2). The effect of the $ALDH1A2^{rs7169289(G)}$ variant was also evident among the sub-population of babies who were homozygous for the wild-type $RET^{1476(G)}$ allele. These observations suggest a robust association between $ALDH1A2^{rs7169289(G)}$ and newborn kidney size, which is demonstrable even within independent subgroups of the cohort.

In conclusion, we show that the $ALDH1A2^{rs7169289(G)}$ variant, present in 20% of healthy Caucasians from Montreal, is associated with a 22% increase in newborn kidney volume (adjusted for BSA). The effect of this variant is evident in subgroups of the study cohort bearing the previously reported hypomorphic $RET^{1476(A)}$ allele or the most common (wild-type) $RET^{1476(G)}$ allele. The $ALDH1A2^{rs7169289(G)}$ variant is also associated with high cord blood RA levels, suggesting that it influences RALDH2 activity within the fetal kidney. This represents the first report of a common genetic variant linked to increased newborn kidney size and could exert a protective effect in developing countries where maternal vitamin A deficiency is common.

MATERIALS AND METHODS

Study subjects

Healthy white infants who were born to women with uncomplicated pregnancies ($n = 113$) were recruited with informed parental consent at the final prenatal clinical visit to the Royal Victoria Hospital (Montreal, QC, Canada). The study (PED-04-016) was approved by the Montreal Children's Hospital Research Ethics Board. Mothers with twins, diabetes, intrauterine growth restriction, genetic abnormalities, renal malformations or hydronephrosis, or delivery <36 weeks, and who had newborns with low birth weight (<2500 g) or low serum albumin were excluded. RET^{1476} genotype was previously determined on half of the subjects.[5]

Renal volume

The left and right kidney volumes were measured by ultrasonography in newborns during the first 48 h of life using the following formula: (kidney volume = 4/3π (length/2) (height/2) (width/2)). BSA was calculated as the square root of (length (cm) and weight (kg)/3600) according to Mosteller.[35]

Cord blood analyses

At birth, cord blood was obtained for isolation of leukocyte DNA and determination of retinoid levels (retinol and RA). Genomic DNA was isolated with the FlexiGene DNA kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's protocol. DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit *2000 (Invitrogen, Carlsbad, CA, USA). To measure retinoids, the cord blood was collected in a 7 ml vacutainer tube containing a silica gel-based clotting activator, previously wrapped with aluminum foil to minimize the exposure to light. The blood was immediately processed in a dark room and serum was collected by centrifugation at 12,500 *g* at 4°C (AccuSpin Micro R, Fisher Scientific, Toronto, ON, Canada). Serum samples were stored in 1.5 ml brown Eppendorf cryotubes, coded and stored at −80°C until analysis. We observed that multiple freeze/thaw cycles of the blood sample resulted in 100% loss of retinoids and that storage at −20°C for 1 year resulted in 63% loss of retinoids. Thus, all samples were stored in the dark at −80°C and thawed only once for analysis.

Extraction of retinoids from cord blood

Retinoids from the blood samples were extracted by butanol/acetonitrile (equal volumes), essentially as described elsewhere[36] except that the method was applied for smaller sample volume. Serum samples stored at −80°C was defrosted on ice and centrifuged for 10 min at 4°C at 2000 *g* (Multifuge 3SR Plus, Thermo Scientific, Ottawa, Ontario, Canada) to obtain a clear supernatant. A volume of 200 µl of serum was transferred to a borosilicate tube wrapped in aluminum foil and 200 µl of butanol/acetonitrile (1:1) was added. The mixture was vortexed for 1 min and then added to 72 mg of hydrated K_2PO_4 in 20 µl of water and vortexed again for 30 s. The extraction mixture was centrifuged at 2000 *g* for 15 min in a Multifuge 3SR Plus centrifuge pre-cooled to 4°C. One hundred microliters of clear supernatant was injected onto HPLC. Recovery studies were performed with the addition of retinoids (5–50 ng/100 µl range) to three separate cord blood samples.

HPLC analysis of retinoids

For retinoid analysis we used a Shimadzu (Toronto, Ontario, Canada) LC-10ADVP equipped with a SIL-HTC autosampler and cooling system (Man-Tech, Guelph, ON, Canada). Retinoids were separated on a Partisil 10-ODS analytical column (250 × 4.5 mm, Grace Discovery Sciences, Bannockburn, IL, USA), and eluted with a mobile phase of acetonitrile/water 65:35, containing 10 mM ammonium acetate, at a flow rate of 1.2 ml/min. Retinoids were detected in a photodiode array detector (Shimadzu Model SPD-M10 AVP) that collected spectra between 200 and 500 nm. Calibration curves for RA and retinol were obtained using standard pure solutions of retinoids purchased from Sigma-Aldrich (St Louis, MO, USA). The purity of retinoid standards were verified by HPLC. Retinoids were handled under yellow light to prevent photoisomerization. HPLC grade solvents were purchased from Fisher Scientific.

Characteristic ultraviolet spectra and retention times identified each retinoid, and peak areas were measured at $\lambda_{max\text{-}330}$ in a Shimadzu SZ-228 data system. The detection limit for retinoids was 2 pg. In this HPLC system, the isomers of RA such as 13-cis/9-cis are not separated and therefore, the retinoid eluate constitutes a mixture of RAs.

Choice of SNPs

The NCBI dbSNP database was screened for common coding and 5′UTR/3′UTR SNPs in *ALDH1A2*, *CYP26A1,* and *CYP26B1* genes. Also, haplotype tagging SNPs, with minor allele frequency of >5% in white populations, were chosen from a region that respectively spanned *ALDH1A2*, *CYP26A1,* and *CYP26B1* genes plus 10 kb at both the 5′- and 3′-flanking segments, using the HapMap human genome database (http://hapmap.org/cgi-perl/gbrowse/hapmap24_B36/). All known SNPs from the CEPH population (white individuals from Western Europe who settled in Utah) for the *ALDH1A2*, *CYP26A1,* and *CYP26B1* gene regions were downloaded into Haploview (Haploview version 4.1),[37] and a plot of linkage

disequilibrium between SNP was obtained. Using Haploview's pairwise 'Tagger' program,[38] we chose haplotype tagging SNP ($r^2 > 0.8$) that occurred in at least 5% of the population.

SNP genotyping

For each subject, 15 ng of genomic DNA was used for multiplex genotyping, using Sequenom iPLEX PCR technology (Sequenom, San Diego, CA, USA). This system involves extension of the PCR amplicon with modified nucleotides to distinguish SNP alleles by matrix-assisted laser desorption ionization-time of flight technology. Primers for SNP detection were designed using MassARRAY AssayDesign software (Sequenom, San Diego, CA, USA).

Statistical methods

We analyzed data with R software (http://www.cran.r-project.org, version 2.7.1). Genotype frequencies for each SNP were examined for divergence from Hardy–Weinberg equilibrium.[39] To investigate associations between each outcome variable and each SNP, we performed an analysis of variance by treating genotype as the dependent variable. When this test yielded a significant difference, we performed a multiple comparison testing procedure.[40]

To analyze the relationship between *ALDH1A2*^{rs7169289} genotype and cord RA level in the newborn cohort, we used a standard two independent proportions test. We also conducted a parametric test to compare the two independent left-censored samples by assuming a tobit model as suggested by Zhang *et al.*[41] Such a model requires the specification of the detection limit; we set this limit at 0.05 nmol/l.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. *ALDH1A2* SNPs characteristics.
Table S2. *CYP26A1* SNPs characteristics.
Table S3. *CYP26B1* SNPs characteristics.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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