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Newborn Serum Retinoic Acid Level Is Associated With Variants of Genes in the Retinol Metabolism Pathway

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ABSTRACT: Retinoic acid (RA) is a critical regulator of gene expression during embryonic development. In rodents, moderate maternal vitamin A deficiency leads to subtle morphogenetic defects and inactivation of RA pathway genes causes major disturbances of embryogenesis. In this study, we quantified RA in umbilical cord blood of 145 healthy full-term Caucasian infants from Montreal. Sixty seven percent of values were <10 nmol/L (84 were <0.07 nmol/L) and 33% had moderate or high levels. Variation in RA could not be explained by parallel variation in its precursor, retinol (ROL). However, we found that the (A) allele of the rs12591551 single nucleotide polymorphism (SNP) in the *ALDH1A2* gene (*ALDH1A2*$^{rs12591551(A)}$), occurring in 19% of newborns, was associated with 2.5-fold higher serum RA levels. *ALDH1A2* encodes retinaldehyde dehydrogenase (RALDH) 2, which synthesizes RA in fetal tissues. We also found that homozygosity for the (A) allele of the rs12724719 SNP in the *CRABP2* gene (*CRABP2*$^{rs12724719(A/A)}$) was associated with 4.4-fold increase in umbilical cord serum RA. *CRABP2* facilitates RA binding to its cognate receptor complex and transfer to the nucleus. We hypothesize that individual variation in RA pathway genes may account for subtle variations in RA-dependent human embryogenesis. **(*Pediatr Res* 67: 598–602, 2010)**

In Western countries, pregnant women consume ~1–2 mg/d of the provitamin A, beta-carotene, from oranges, green leafy vegetables, and fortified beverages (1). Between 10 and 25% of this is converted to retinol (ROL) (1), which circulates in maternal blood in the form bound to a dimeric complex containing retinol binding protein (RBP) 4 and transthyretin (2,3).

During passage through the placenta, ROL is transferred to the fetal circulation, where it is picked up by fetal RBP4 or chylomicrons and delivered to fetal tissues (2,4,5). In the fetal tissues, ROL is converted to retinal and then to retinoic acid (RA) by the embryonic retinaldehyde dehydrogenase (RALDH) 2 (6). In mice, targeted disruption of *Raldh2* arrests development at midgestation (7–9), whereas administration of exogenous RA to the mother allows the development of *Raldh2*$^{-/-}$ embryos to proceed (7).

Within fetal tissues, RA may exit the cell or it may be targeted for degradation by binding to cellular retinoic acid binding protein (CRABP) 1 (10,11). Several studies (12–16) suggest an alternative fate in which RA is transferred to the nucleus *via* CRABP2 (12–16). In the nucleus, it associates with heterodimeric nuclear receptors (RARα,β,γ) that regulate transcription of target genes (17). Fetal RA is catabolized primarily by CYP26A1, a member of the cytochrome p450 system (18,19). Mice lacking either *Cyp26a1* or *Cyp26b1* genes die perinatally and exhibit abnormalities consistent with those seen in RA excess, including spina bifida, truncation of the tail, and abnormalities affecting the kidneys, urogenital tract, and hindgut (20–22).

Despite the vast literature attesting to the powerful effects of RA on embryogenesis and cell physiology, very little is known about the circulating levels of RA among human newborns. Also, there is no appreciable understanding of the factors that influence individual newborn RA level. Here, we report umbilical cord serum RA levels in 145 healthy full-term newborn Caucasian infants from Montreal. We show that RA level is unrelated to serum ROL concentration but that a subset of newborns bearing common variants of *ALDH1A2* (encoding RALDH2) and *CRABP2* genes have significantly elevated levels of umbilical cord RA.

METHODS

Reagents. The authentic standard retinoids (ROL and RA) were purchased from Sigma Chemical Co.-Aldrich (St-Louis, MO). The purity of the retinoids was verified by HPLC. Retinoids were handled under yellow light to prevent photoisomerization. HPLC-grade solvents were purchased from Fisher Scientific (Toronto, Ontario, Canada).

Study subjects. Healthy Caucasian infants (n = 145) who were born to mothers with uncomplicated pregnancies were recruited with informed parental consent at the prenatal clinic visit at the Royal Victoria Hospital

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D.C.M. and R.E.K. contributed equally to this study.

Abbreviations: ***ALDH1A2***, aldehyde dehydrogenase 1 family, member A2; **CEPH,** Centre d'Étude du Polymorphisme Humain; ***CRABP1***, cellular retinoic acid binding protein 1; ***CRABP2,*** cellular retinoic acid binding protein 2; ***CYP26A1***, cytochrome P450, family 26, subfamily A, polypeptide 1; ***CYP26B1***, cytochrome P450, family 26, subfamily B, polypeptide 1; **RA,** retinoic acid; **RALDH2,** retinaldehyde dehydrogenase 2, **ROL,** retinol; **SNP,** single nucleotide polymorphism

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(Montreal, Quebec, Canada). The study was approved by the Montreal Children's Hospital Research Ethics Board. Mothers with twins, diabetes, intrauterine growth restriction, genetic abnormalities, or delivery <36 wk were excluded. Babies with Apgar scores <7 at 1 min were excluded.

Cord blood analyses. At birth, cord blood was obtained for isolation of leukocyte DNA and retinoid (ROL and RA) assay. Genomic DNA was isolated with the FlexiGene DNA kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's protocol. DNA was quantified using the Quant-iT PicoGreen dsDNA Assay Kit *2000 (Invitrogen, Carlsbad, CA). 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 10,000 rpm at 4°C. Serum samples were stored in 1.5-mL brown cryotubes, coded, and stored at −80°C until analysis.

Extraction of retinoids from cord blood for HPLC analysis. Retinoids from the blood samples were extracted by butanol/acetonitrile (equal volumes) essentially as described by McClean *et al.* (23), except that the method was adapted to a smaller sample volume. Serum samples stored at −80°C were defrosted on ice and centrifuged for 10 min at 4°C at 3000 rpm to obtain a clear supernatant. Two hundred microliters 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 after that, we added to it a salt solution of 72 mg $K_2HPO_4 \cdot 3H_2O$, previously diluted in 20 μL of water (15.8 molar and pH 9.0). Then, the mixture was vortexed again for 30 s. The extraction mixture was centrifuged at 3000 rpm for 15 min in a Sorvall RC3C Plus centrifuge precooled 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 LC-10ADVP equipped with a SIL-HTC autosampler and cooling system (Man-Tech, Guelph, Ontario, Canada). Retinoids were separated on a Partisil 10-ODS analytical column (250 × 4.5 mm; Grace Discovery Sciences, IL) 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), which collected spectra between 200 and 500 nm. Calibration curves for RA and ROL were obtained using standard pure solutions of retinoids. Characteristic UV spectra and retention times identified each retinoid, and peak areas were measured at $\lambda_{max-330}$ in a Shimadzu SZ-228 data system. The detection limit for retinoids was 2 pg (0.14 nmol/L).

Choice of single nucleotide polymorphisms. The NCBI dbSNP database was screened for coding and noncoding single nucleotide polymorphism (SNPs) in *ALDH1A2*, *CYP26A1*, *CYP26B1*, *CRABP1*, and *CRABP2* genes with minor allele frequency of >5% in Caucasian populations. For each gene, *ALDH1A2*, *CYP26A1*, *CYP26B1*, *CRABP1*, and *CRABP2*, the haplotype tagging SNPs were chosen from a region that spanned the entire gene plus 10 kb at both the 5′ and 3′ flanking segments, using the HapMap human genome database (http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B36/). All known SNPs from the Centre d'Étude du Polymorphisme Humain (CEPH; http://www.cephb.fr) population (white individuals from Western Europe who settled in Utah) for the *ALDH1A2*, *CYP26A1*, *CYP26B1*, *CRABP1*, and *CRABP2* genes regions were downloaded into Haploview (Haploview version 4.1; http://www.broadinstitute.org/mpg/haploview) (24), and a plot of linkage disequilibrium (LD) between SNPs was obtained. By using Haploview's pairwise "Tagger" program (25), we chose htSNP (r^2 >0.8) that occurred in at least 5% of the population.

SNP genotyping. A total of 135 patients were genotyped. For each subject, 15 ng of genomic DNA was used for multiplex genotyping, using Sequenom iPLEX PCR technology (Sequenom, San Diego, CA). 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 Assay Design software (Sequenom).

Statistical methods. We analyzed data with R software (http://cran.r-project.org; version 2.7.1). Genotype frequencies for each SNP were examined for divergence from Hardy-Weinberg equilibrium. The χ^2 test was conducted for genotype analysis. The dependency between RA and ROL was tested by Kendall's tau. Because exact values for RA cannot be known below a certain limit of detection, we adapted the methodology of Oakes (26) to left-censored data to estimate Kendall's tau.

RESULTS

Characteristics of study subjects. Body measurements were available for 121 babies (53 girls and 68 boys) from the cohort (Table 1). Mean (±SD) newborn weight, height, and body surface area (BSA) were 3.57 ± 0.46 kg, 51.2 ± 2.25 cm, and 0.22 ± 0.01 m², respectively.

Table 1. *Characteristics of study subjects*

Characteristics	Mean value (SD)	Minimum value	Maximum value
Weight (kg)	3.57 (0.46)	2.62	5.05
Height (cm)	51.20 (2.25)	46	58
Body surface area (m²)	0.22 (0.01)	0.18	0.27

The mean and standard deviation (SD) measurements of weight, height, and body surface area are presented for 121 newborns from the cohort for whom complete morphometric data were available.

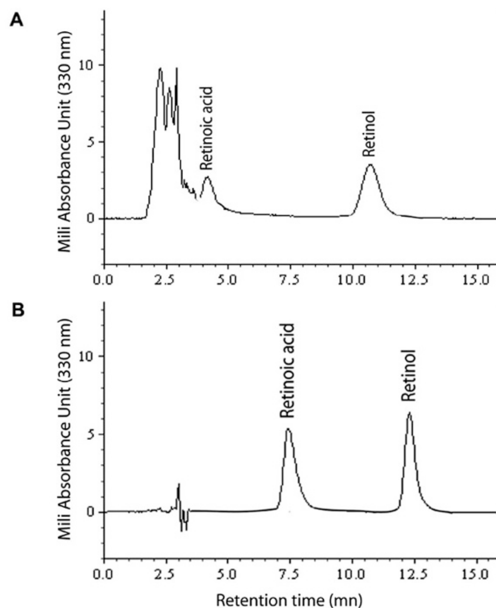

Figure 1. HPLC chromatogram of umbilical cord serum sample (*A*) and standard retinoids (*B*). RA and ROL are present and well separated.

Retinoid levels in cord blood. Numerous retinoids, in addition to ROL and retinyl esters, are present in plasma at nanomolar concentrations (~5–10 nM). These include all-*trans* RA, 13-*cis* RA, 13-*cis*-4-oxo RA, and others (27,28). We optimized an extraction procedure adapted to HPLC separation (23) for measuring RA in cord blood of 145 infants in Montreal. Figure 1*A* shows an HPLC chromatogram of an umbilical cord serum sample. RA and ROL were present and well separated, and their presence was confirmed by characteristic UV absorption maxima (data not shown). In this HPLC system, the isomers of RA such as 13-*cis*/9-*cis* are not separated, and therefore the retinoids eluting as peak 1 constitute a mixture of RAs. Note that in Figure 1*A*, there is a shift in the elution times of RA and ROL compared with the elution times of standard retinoids (Fig. 1*B*). This could be due to the presence of lipids in the cord blood, which interfere with interactions between retinoids and column materials. Addition of dipotassium phosphate to the extraction mixture enhanced the recovery of retinoids. Recovery studies, with known amounts of retinoids added to the blood samples, resulted in yields of RA and ROL of >99%. However, repeated freezing and thawing of the blood samples resulted in significant loss of retinoids. Storage of blood samples

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at −20°C for 1 year resulted in 63% loss of retinoids. Samples for this study were stored at −80°C; at this temperature, degradation of retinoids is minimal.

Marked variation in RA levels (0.07–550nmol/L) was observed in the 145 cord samples analyzed. The distribution was skewed, with the appearance of three distinct subgroups: 1) two thirds of the samples (97 infants) fell below a RA concentration of 10 nmol/L and, in most (84 infants), RA was below the limit of detection (0.07 nmol/L); 2) RA concentrations in 28% of samples were spread between 10 and 100 nmol/L; 3) in 5% of samples (eight infants), RA concentrations were substantially higher (100–550 nmol/L; Fig. 2). The skewed distribution of umbilical cord RA could not be explained by parallel variation in its precursor, ROL. ROL was normally distributed, with an average of 1150 nmol/L and values ranging between 51.4 and 3892 nmol/L. Mean cord ROL levels in the three subgroups were 1294 nmol/L (group 1) 793 nmol/L (group 2), and 1194 nmol/L (group 3), respectively (Table 2). In fact, there was weak inverse correlation between ROL and RA levels (Kendall tau = −0.2733, $p < 0.01$).

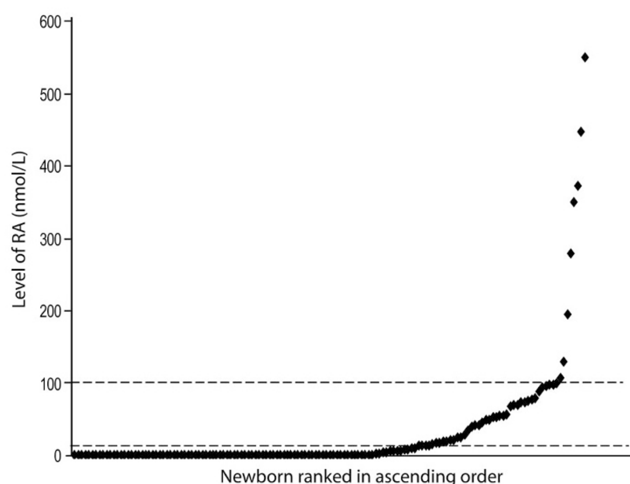

Figure 2. Distribution of umbilical cord RA levels in full-term newborns. The distribution of umbilical cord RA levels in our cohort was skewed, with the appearance of three distinct subgroups: 67% of the samples fell below 10 nmol/L (group 1); 28% of samples were between 10 and 100 nmol/L (group 2); 5% were >100 nmol/L (group 3).

Table 2. *Umbilical cord serum ROL levels in the three RA groups*

	Group 1 (RA <10 nmol/L)	Group 2 (10 nmol/L < RA <100 nmol/L)	Group 3 (RA >100 nmol)
No. newborns	97	40	8
Percentage	67	28	5
Mean ROL levels (SEM)	1294 (75)	793 (96)	1194 (126)

Mean (±SEM) ROL levels (nmol/L) in the three RA groups are presented.

Association between cord RA and SNPs in genes encoding enzymes of RA synthesis and degradation. Because the skewed distribution of RA levels could not be explained by variation in ROL, we explored the possibility that circulating levels of RA were influenced by variation in genes involved in RA synthesis (*ALDH1A2*) or degradation (*CYP26A1*; *CYP26B1*). Each subject was genotyped for SNPs spanning each candidate gene. SNP frequencies were similar to those of the HapMap Caucasian (CEPH) population, indicating no appreciable genetic drift in our cohort. Genotype distributions for each SNP locus conformed to Hardy-Weinberg predictions. By using a χ^2 test, we found that heterozygous $ALDH1A2^{rs12591551(A/C)}$ babies (19% of the cohort) are associated with high values of umbilical cord serum RA (p = 0.035; Table 3). In fact, heterozygous (rs12591551C/A) babies showed a 2.5-fold increase in cord RA (62.2 nmol/L) compared with subjects who were homozygous for the wildtype allele (rs12591551C/C; 25 nmol/L). None of the SNPs within the *CYP26A1* and *CYP26B1* genes showed significant association with RA levels.

Association between RA and SNPs in genes encoding intracellular RA binding proteins. We also considered the possibility that circulating levels of RA in the fetus were influenced by variation in genes encoding intracellular RA binding proteins (*CRABP1* and *CRABP2*). We chose htSNPs and cSNPs in the CEPH population, and all subjects were genotyped at these loci; SNP frequencies in our cohort were similar to those of the CEPH population, indicating no appreciable genetic drift. Genotype distributions for each SNP locus conformed to the expected Hardy-Weinberg equilibrium.

By using the χ^2 test, we identified an association between the $CRABP2^{rs12724719A/A}$ genotype and higher levels of RA ($p = 0.013$; Table 3). Homozygous $CRABP2^{rs12724719A/A}$ newborns (3% of the population) showed a 4.4-fold increase (141.3 *versus* 32.3 nmol/L) of umbilical cord serum RA compared with homozygous $CRABP2^{rs12724719G/G}$ babies (75% of the population).

DISCUSSION

Fetal RA is derived from fetal metabolism of maternally supplied ROL in developing tissues (29). We used a refined

Table 3. *Genotype distribution in the three RA groups*

Gene	SNP	Genotype	RA <10 nmol/L, *n* (%)	10 nmol/L < RA <100 nmol/L, *n* (%)	RA >100 nmol/L, *n* (%)	χ^2	*p*
ALDH1A2	rs12591559	CC	77 (71)	27 (25)	5 (4)	6.72	0.035
		CA	11 (44)	11 (44)	3 (12)		
CRABP2	rs12724719	GG	71 (72)	21 (21)	7 (7)	18.61	0.013 (GG vs AA)
		AG	16 (53)	14 (47)	0 (0)		
		AA	1 (25)	2 (50)	1 (25)		

Heterozygous $ALDH1A2^{rs12591551C/A}$ babies showed a 2.5-fold increase in cord RA compared with subjects who were homozygous for the wildtype allele $ALDH1A2^{rs12591551C/C}$. Homozygous $CRABP2^{rs12724719A/A}$ newborns showed a 4.4-fold increase of umbilical cord serum RA compared with homozygous $CRABP2^{rs12724719G/G}$ babies.

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HPLC method to measure RA levels in umbilical cord blood of healthy full-term newborn Montreal babies. Interestingly, the majority of infants had low circulating RA level (<10 nmol/L). However, the distribution of RA levels in our cohort was skewed by two apparent subpopulations of newborns with much higher RA levels ranging from 10–100 nmol/L and >100 nmol/L, respectively. Berggren Soderlund *et al.* (30) measured RA levels in umbilical cord blood from 10 Swedish newborns (mean = 3.4 nmol/L). However, the reported range of RA values was much narrower than in our study. Conceivably, our identification of a subgroup with significantly higher RA levels was because of our improved extraction method. None of the mothers in our cohort were taking supplementary retinoids.

Although fetal RA is generated in fetal tissues from maternally supplied ROL, we found no relationship between umbilical cord RA and the corresponding level of umbilical cord ROL. This result is concordant with the findings of Berggren Soderlund *et al.* (30). Therefore, we reasoned that individual variation in newborn blood RA likely reflects variation in fetal genes involved in ROL metabolism, RA degradation, or RA delivery to the nucleus where it exerts its effects on transcription of target genes.

After its uptake by fetal tissues, ROL is reversibly converted to retinal by alcohol dehydrogenase and then irreversibly to all-*trans* RA by RALDH2. Because the latter is the rate-limiting step, we investigated whether fetal RA levels might be influenced by common variants of the human *ALDH1A2* gene. We screened a panel of SNPs spanning the *ALDH1A2* locus and found that newborns heterozygous (A/C) for the *ALDH1A2*^{rs12591551} SNP (intron 7) had a 2.5-fold increase in umbilical cord RA level compared with those who were homozygous for the wildtype allele, *ALDH1A2*$^{rs12591551(C)}$. Approximately 19% of our healthy full-term newborns were heterozygous for this variant allele, demonstrating that is a fairly common normal variant and might confer a biologic advantage in settings where maternal ROL availability is marginal.

The *ALDH1A2*^{rs12591551} SNP is located in intron 7. Interestingly, Cawley *et al.* (31) have shown that ~one third of the transcription factor binding sites that regulate gene expression lie within introns or 3′ to the coding sequence. Thus, the *ALDH1A2*^{rs12591551} may mark a variant regulatory sequence, which enhances RALDH2 expression in fetal tissues, increases intracellular RA pools, and, thereby, raises circulating RA levels in cord blood. It seems unlikely that umbilical cord RA level is determined by the maternal *ALDH1A2* genotype, because this gene is expressed primarily in fetal tissues (7).

Intracellular RA bound to CRABP1 is targeted for degradation in fetal tissues by CYP26A1 and CYP26B1 members of the cytochrome P450 system (32). This pathway is clearly a powerful regulator of the intracellular RA pool in fetal tissues. Mice with inactivation of *Cyp26A1* or *Cyp26B1* genes die *in utero* (33). However, these genes are highly conserved, and no common variants of these two human CYPs were associated with umbilical cord RA in our cohort.

Intracellular RA may also bind to CRABP2, which facilitates delivery to the nucleus (12,34), where it activates RAR transcriptional regulation of target genes (35). In our cohort, the four babies who were homozygous for the *CRABP2*^{rs12724719A} variant had substantially increased levels of cord blood RA compared with newborns who were homozygous for the wildtype G allele (141.3 *versus* 32.3 nmol/L, respectively). However, newborns who were heterozygous (*CRABP2*$^{rs12724719A/G}$) for the SNP had levels of umbilical cord RA similar to wildtypes. We speculate that the *CRABP2*^{rs12724719A} allele is slightly dysfunctional in the homozygous state and reduces the level of CRABP2 expression, thereby compromising delivery of RA to the nucleus. Because reduced RA signaling induces feedback up-regulation of RALDH2 expression (36), this might increase RA level in fetal tissues and, consequently, in umbilical cord RA.

Our studies show for the first time that the distribution of RA level in human neonates is skewed by the presence of two subgroups with relatively high RA. We found that this is unrelated to the availability of circulating ROL substrate but is affected by fetal genotype for variant alleles of the *ALDH1A2* and *CRABP2* genes.

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