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Urinary Excretion of Purine and Pyrimidine Metabolites in the Neonate

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ABSTRACT. Random urine samples from 614 neonates were screened for metabolites of purine and pyrimidine metabolism using an adapted column chromatographic method. A restricted number of metabolites and a number of unidentified peaks appeared on the chromatograms. No inborn errors of this metabolism were found. The chromatograms were identical in term and in premature or dysmature neonates, except for the presence of more unidentified peaks in the latter group. The pattern was not influenced by the type of feeding or i.v. nutrition. Metabolites of different medications were identified. One female neonate with an increased excretion of uracil was shown to be heterozygous for ornithine carbamyl transferase deficiency. (*Pediatr Res* 34: 762–766, 1993)

Abbreviations

OCT deficiency, ornithine carbamyltransferase deficiency

Inherited disorders of purine and pyrimidine metabolism may be more prevalent than is currently believed (1). Some are associated with a severe clinical picture and a fatal outcome, whereas others have a discrete clinical expression and a relatively benign evolution. For some of them treatment is available, for others recognition is important for genetic counseling and prenatal diagnosis in subsequent pregnancies. The majority of these inherited disorders can now be identified from specific metabolites in the urine (Tables 1 and 2). Systematic screening of newborns has, however, not yet been performed. This paper reports the results of a screening of more than 600 newborns, on the 4th d of life, using an adapted column chromatographic method.

MATERIALS AND METHODS

A random urine sample was obtained from 614 neonates on the 4th d of life and stored at −20°C immediately after collection. Data concerning the perinatal period were collected; the diagnosis of hypoxia was made on the basis of data provided by monitoring during delivery, Apgar score, and blood gas analysis shortly after birth. Information was obtained on the feeding and the medication given to the mother shortly before delivery or during lactation or to the child. Two categories of neonates were investigated. Group A was formed by 510 neonates, born successively in the same maternity ward, who had a birth weight above 2800 g and were born after an uneventful pregnancy and delivery. There were 234 boys and 276 girls; 296 (58%) were breast fed, 199 (39%) were formula fed, and 15 (3%) received mixed feeding. An adapted start formula containing 1.4–1.9 g/100 mL protein, 5.2–7.1 g/100 mL lactose, and 3.2–3.6 g/100 mL fat was used. In this group, no medication was given to mother or child. Group B comprised 104 neonates, 62 boys and 42 girls, who were admitted to the neonatal intensive care unit of the same hospital. Ninety-four were born prematurely with a mean gestational age of 32 ± 2.5 wk. Ten were considered to be dysmature on the basis of a low birth weight for gestational age: mean birth weight was 1822 ± 585 g. Of the 104 neonates, 26 (25%) were breast fed, 39 (37%) were formula-fed, and seven (7%) had mixed feeding. The formula contained 2.0–2.2 g/100 mL protein, 4.0–6.0 g/100 mL lactose, and 3.4–4.0 g/100 mL fat. Twelve of these breast-fed or formula-fed neonates had additional parenteral nutrition. Thirty-two infants (31%) received only parenteral nutrition. The parenteral nutrition consisted of the usual mixtures of glucose, amino acids (Primene 10%, Clintec Benelux, Brussels, Belgium), and fat (Intralipid 20%, Kabi Pharmacia, Stockholm, Sweden) with addition of electrolytes, oligoelements, and vitamins in quantities adapted to the situation. The patients from group B suffered from a variety of diseases commonly observed in a neonatal intensive care unit; 24 were considered to have had perinatal asphyxia based on the criteria mentioned earlier. The majority of the patients from group B received one or more medications, mainly antibiotics, caffeine, dopamine, and anticonvulsants.

We used a modification of the method described by Morris *et al.* (6). With this method, purine and pyrimidine metabolites are separated by reversed-phase chromatography and identified by their retention time and characteristic UV absorption spectrum. We modified the method in several aspects. The pH of solvent A (see below) was lowered from 5.0 to 4.8 to improve the separation of uric acid and dihydroxyadenine. The gradient profile was changed to reduce analysis time. Automatic injection allowed the unattended processing of the samples during 2 consecutive d. Monitoring at different wavelengths and determination of the UV spectrum of the metabolites was made possible by diode array detection.

The separation was performed on a 250 × 4.6 stainless steel column with 5-μm Spherisorb ODS-1 (Alltech, Laarne, Belgium). The solvents were degassed by ultrasonication and consisted of 30 mM ammonium acetate brought to pH 4.8 with acetic acid and 1% methanol for solvent A. Solvent B consisted of methanol:acetonitrile:tetrahydrofuran (80:10:10). Initially, 100% solvent A was pumped through the column for 10 min. Solvent B was then added, its concentration being augmented linearily to 10% after 15 min and 12% after 20 min. The final concentration of 45% was attained after 30 min. The column was reequilibrated by pumping solvent A for 7 min. The flow rate was kept constant at 1.2 mL/min and back pressure ranged from 130 to 190 bars. The chromatographic system consisted of two model 303 pumps

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Table 1. *Known metabolic disorders of purine with specific urinary metabolites*[2-5]

Defective enzyme	Specific urinary metabolites	Clinical expression
Phosphoribosyl pyrophosphate synthetase superactivity	Uric acid	Gout, urolithiasis, renal failure, nerve deafness, hypotonia
Adenylosuccinate lyase	Succinyladenosine	Severe psychomotor retardation, epilepsy, autism, growth retardation, failure to thrive, muscular wasting
Purine nucleoside phosphorylase	Inosine, guanosine, deoxyinosine, deoxyguanosine, uric acid (decreased)	Deficient cellular immunity, lymphopenia (T cells), anemia, neurologic deficits (50%)
Adenosine deaminase	Adenosine, deoxyadenosine	Severe combined immune deficiency, bone abnormalities
Hypoxanthine-guanine phosphoribosyl transferase	Uric acid, hypoxanthine	Mental retardation, self-mutilation, gout, choreoathetosis, spasticity, urolithiasis
Adenine phosphoribosyl transferase	2,8-Dihydroxyadenine, adenine	Urolithiasis, renal failure
Xanthine oxidase (isolated form)	Xanthine, uric acid (decreased)	Urolithiasis, myopathy, recurrent polyarthritis

Table 2. *Known metabolic disorders of pyrimidine metabolism with specific urinary metabolites*[2-5]

Defective enzyme	Specific urinary metabolites	Clinical expression
Uridyl monophosphate synthase (bifunctional enzyme)		
Type I	Orotic acid	Megaloblastic/hemolytic anemia, psychomotor retardation, failure to thrive, growth retardation, cardiac malformation, strabismus, urolithiasis
Type II	Orotidine, orotic acid	
Dihydropyrimidine dehydrogenase	Uracil, thymine, 5-hydroxymethyl uracil	Psychomotor delay, autism, hypertonia
Dihydropyrimidinase	Dihydrouracil, dihydrothymine (thymine, uracil)	Convulsions (mental retardation)

with washed steel piston pump heads (10 WSC), a model 802 C manometric module, a model 811 Dynamic Mixer, an ASTED autoinjector, all from Gilson (Villiers-le-Bel, France), and an LKB-2140 Rapid Spectral Detector controlled by WSEG software (Pharmacia, Broma, Sweden) on an IBM Personal System/2 model 55 SX computer with a 100-MB hard disk and a color display. The analog outputs of the detector were used to monitor at 225, 254, and 280 nm. Gilson 714 HPLC System Controller software on an IBM Personal System/2 model 30 computer controlled the pump system, the autoinjector, and the detector via a Gilson model 621 interface and integrated the analog outputs of the detector. A Hewlett-Packard Desk Jet printer was used for the data output. Three types of information appeared on the display: *1*) the isogram, which represented a three-dimensional chromatogram (time, wavelength, and absorbance); *2*) the absorption spectrum of a selected compound; and *3*) the chromatogram at a chosen wavelength. Apparently abnormal chromatograms were stored on floppy disks.

The standards used were the metabolites of purine and pyrimidine metabolism, which could be expected to appear in the urine of normal individuals or patients with known inherited disorders of this metabolism. Creatinine was also added. Standard stock solutions of all compounds except dihydroxyadenine were obtained as follows. Uric acid (250 mg) was dissolved in 250 mL of water containing 300 mg of lithium carbonate by stirring at 60°C; after cooling, the solution was diluted to 500 mL. Forty mg of xanthine, guanosine, hypoxanthine, adenosine-5′-succinate, and guanine were dissolved in 20 mL of 0.05 M sodium hydroxide each. All other standards were dissolved in 20 mL of water. Dissolution was facilitated in some instances by ultrasonication. Creatinine (80 mg) was dissolved in 250 mL of water to which 0.5 mL of 37% hydrochloric acid was added. Working standard solutions containing metabolites that are specific for different errors of purine and pyrimidine metabolism were prepared by diluting with water 2 mL, 2 mL, and 0.5 mL of the stock solution of creatinine, uric acid, and each of the concerned metabolites, respectively, up to a volume of 10 mL. For dihydroxyadenine, a saturated solution in water was used. Fractions of these standard solutions were stored at −20°C. Uric acid and hypoxanthine were from Merck (Darmstadt, Germany); all other purines and pyrimidines and their analogues were from Sigma (St. Louis, MO). Solvents were from Carlo Erba (Milan, Italy). Only water obtained from a Milli-Q system (Millipore, Bedford, MA) was used. For examination of the urine, the samples were thawed, then incubated at 37°C for 1 h and diluted with an equal volume of water. Particulate matter was removed by centrifugation. Twenty-five μL were injected onto the column.

The peaks of the standards were well separated on the chromatogram. Their retention time and UV absorption maximum are listed in Table 3. Identification of the metabolites in the urine samples was based on the comparison of their retention times and UV spectra with those of the standard solution. The validation of the method for different metabolites is represented in Table 4. The interassay and intraassay reproducibility was calculated after adding the metabolite to pooled urine from neonates, except for 2,8-dihydroxyadenine and adenine, for which urine from a patient with adenine phosphoribosyltransferase deficiency was used. Ten urine samples were assayed for the calculation of the intraassay coefficient of variation and five for the interassay coefficient of variation and the recovery. For statistical analysis, the Wilcoxon rank sum test and the *t* test were used.

RESULTS

Figure 1 represents a chromatogram of the urine from a neonate belonging to group A. Only a limited number of purine and pyrimidine metabolites, namely pseudouridine, uric acid, and 7-methylguanine, appeared as easily discernible peaks. The peaks of xanthine and hypoxanthine were much lower or even

Table 3. *Retention time and UV maximum of purine and pyrimidine metabolites (and creatinine) excreted in the urine in normal and in pathologic conditions*

Orotidine	2.1	263
Orotic acid	2.5	275
Pseudouridine	3.8	260
Dihydrouracil	4.2	220
Uracil	4.2	255
Uric acid	5.9	281
Dihydroxyadenine	7.0	293
Hypoxanthine	7.3	247
Dihydrothymine	7.8	220
Thymine	8.2	262
Xanthine	9.0	265
Inosine	13.2	246
Guanosine	15.7	252
Deoxyinosine	17.4	245
Deoxyguanosine	19.2	249
7-Methylguanine	19.4	264
Adenosine	21.4	257
Adenine	21.5	258
Deoxyadenosine	23.8	257
Succinyladenosine	25.0	257
Creatinine	4.4	222

absent, differing from one sample to another. Because of interference with other compounds that were eluated early, only very high concentrations of orotic acid appeared as isolated peaks. Some chromatograms in group A showed a number of unidentified peaks. One of these peaks (compound 5) with a retention time of 8.5 min and a maximal UV absorption at 249 nm was observed in many chromatograms. Its molecular weight, as determined by mass spectrometry, was 109. This peak can easily be confused with the peak representing thymine. The chromatographic pattern, including the unknown peaks, was essentially identical in all the neonates from this group, whether they were breast fed or received formula feeding.

In the urine from the neonates from group B, the same chromatographic pattern was observed as in group A, but compound 5 was found in a much higher percentage of the neonates (33% compared with 2.3% in group A). Also in group B, the type of diet did not result in a different chromatographic pattern, including the unidentified peaks, nor did the parenteral nutrition. In this group, different peaks were identified as metabolites of the medication administered to the neonate: the location of caffeine and its metabolites and phenobarbital on the chromatogram was identical to that mentioned in an earlier publication (6); we also located cefotaxime, which had a retention time of 28.7 min and a UV absorption maximal at 239 nm.

Pseudouridine excretion followed a normal distribution in group A. This was not the case in group B, where higher values were found more frequently. As can be seen in Table 5, however, the median and 97th percentile values are identical in both groups. A highly significant linear correlation was found between pseudouridine and creatinine: $y = 0.29x - 4.97$; $r = 0.94$; $t = 27.45$; $p < 0.001$ (group A); and $y = 0.37x + 1.07$; $r = 0.86$; $t = 15.48$; $p < 0.001$ (group B). Uric acid excretion followed a normal distribution in group A but not in group B. The median and 97th percentile values were lower in group B than in group A; this difference is statistically significant ($p < 0.005$). In agreement with the literature (7), the excretion ratio of uric acid on creatinine (mmol/mmol) was very high in both populations.

The chromatogram from one female neonate from group A showed a markedly increased peak of uracil (Fig. 2). Orotic acid, which was not detected in her chromatogram, was measured with a colorimetric method and was shown to be markedly increased (2.43 μmol/mol creatinine; normal upper value 0.31 μmol/mol creatinine). Orotidine, uridine, and thymine were absent in the chromatogram. After an oral loading test with 1 g protein/kg body weight at the age of 6 wk, orotic acid in the urine increased from undetectable to 1.93 μmol/mol creatinine; uracil was no longer detected. Clinical investigation in this girl was normal. Family history revealed that a maternal uncle died of convulsions at the age of 4 mo. All the females of this family, who were also asymptomatic, were challenged with allopurinol (8, 9). The maternal grandmother, the mother, two maternal aunts, and one maternal niece excreted orotic acid quantities under allopurinol that were in the range observed in carriers for OCT transferase deficiency. DNA analysis will be performed in this family.

Finally, hypoxanthine was found in increased amounts in the urine from one of 24 neonates from group B who were considered to have suffered from perinatal asphyxia. Plasma hypoxanthine levels were not measured in these neonates. No known disorders of purine and pyrimidine metabolism were detected in the 614 newborns.

Table 4. *Validation of method* *

		Intraassay reproducibility		Interassay reproducibility				
Compound	λ (nm)	Mean (mmol/L)	CV (%)	Mean (mmol/L)	CV (%)	Recovery (%)	Sensitivity (μmol/L)	Linear range (mmol/L)
Orotidine	257	0.437	0.9	0.385	2.2	96	1.6	1.5
Orotic acid	275	1.86	0.5	1.98	1.6	88	1.7	7.0
Pseudouridine	257	1.202	2.3	1.199	3.8	99	1.6	4.7
Dihydrouracil	225	4.23	5.2	4.62	9.5	127	11	14.2
Uracil	257	2.06	2.8	2.22	1.1	95	1.5	10.6
Uric acid	257	3.150	3.1	3.150	3.7	95	3.4	8.0
Dihydroxyadenine	293	0.0105	14.7	0.010	7.3	90	1.3	0.014
Hypoxanthine	247	1.94	3.4	1.97	1.5	89	1.2	8.7
Dihydrothymine	225	2.32	3.5	2.66	1.8	97	24	12.8
Thymine	257	1.85	2.4	2.02	0.8	93	1.8	9.4
Xanthine	257	2.05	1.2	2.12	0.9	101	1.4	8.5
Inosine	247	0.752	2.2	0.849	2.7	99	1.2	4.2
Guanosine	257	0.787	2.6	0.796	2.3	97	1.3	3.8
Deoxyinosine	247	0.778	2.5	0.751	7.3	96	1.0	3.6
Deoxyguanosine	247	0.540	2.3	0.556	8.4	97	4.9	2.7
Adenosine	257	0.821	2.4	0.925	2.3	95	0.8	4.4
Adenine	257	0.080	10.0	0.078	8.5	98	11.8	7.2
Deoxyadenosine	257	0.801	2.3	1.015	1.1	98	0.8	5.0
Succinyladenosine	257	0.805	1.0	0.796	1.5	100	0.8	4.0

* CV, coefficient of variation.

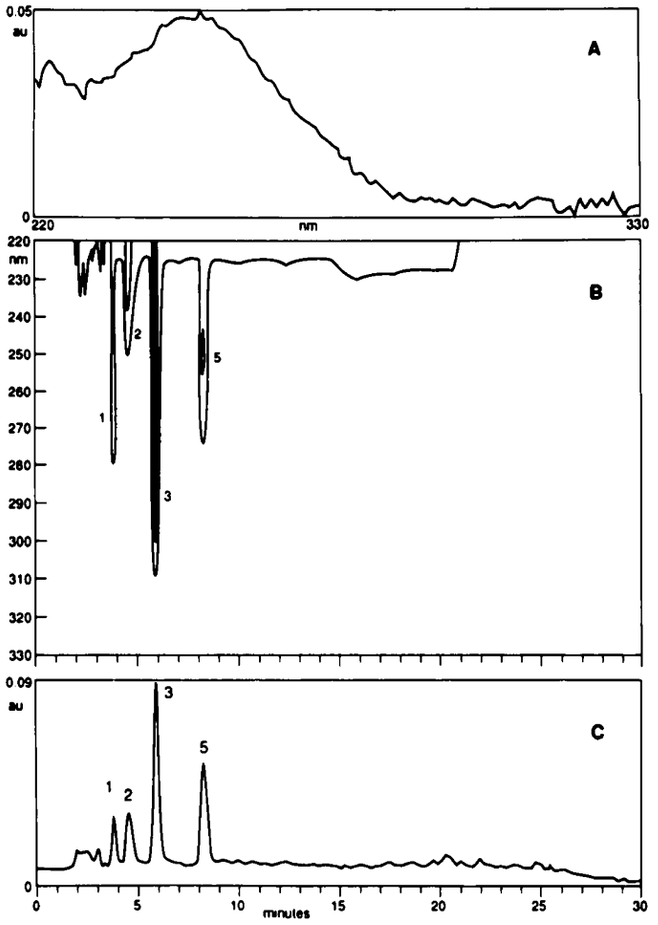

Fig. 1. Printout of a urinary chromatogram from a newborn belonging to group A. *A*, selected UV spectrum (in this instance, UV spectrum of pseudouridine). *B*, isogram. *C*, chromatogram at 263 nm (*1*, pseudouridine; *2*, creatinine; *3*, uric acid; *5*, unidentified compound).

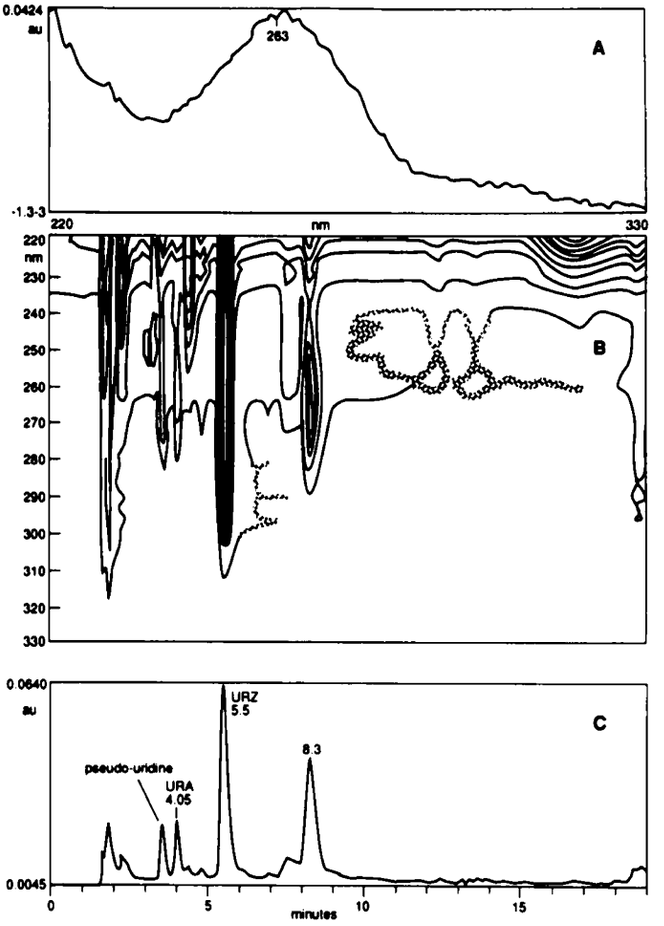

Fig. 2. Printout of the chromatogram from a newborn heterozygous for OCT deficiency. *A*, UV spectrum of uracil. *B*, isogram. *C*, chromatogram at 255 nm (*URA*, uracil; *URZ*, uric acid; 8.3 min = compound 5 in Fig. 1 = unidentified compounds).

Table 5. *Comparison between pseudouridine and uric acid excretion in both groups*

	Group A (n = 510)		Group B (n = 104)	
	Median	97th percentile	Median	97th percentile
Pseudouridine (mmol/L)	0.16	0.55	0.16	0.53
Uric acid (mmol/L)	4.01	10.26	3.24	7.37
Pseudouridine/creatinine ratio (mmol/mmol)	0.12	0.16	0.16	0.25
Uric acid/creatinine ratio (mmol/mmol)	3.03	4.71	3.33	6.53
Creatinine (mmol/L)	1.33	4.60	0.97	2.52

DISCUSSION

The aim of the present work was to screen neonates for metabolites of purine and pyrimidine metabolism. For this purpose, a semiquantitative method that detects the metabolites that are specific for the known inherited disorders of this metabolism was developed. Such disorders were not detected in the 614 neonates whose random urine samples were investigated. The pattern that was commonly found consisted of a restricted number of purine and pyrimidine metabolites and a number of peaks that remain to be identified. The pattern was identical in term neonates and in premature or dysmature neonates, except that in the latter two more unidentified peaks, especially compound 5, were present. An important finding was that the pattern was not influenced by the type of feeding or by i.v. nutrition, which would greatly diminish the value of a systematic screening. However, metabolites of medications that are commonly administered to ill neonates appeared on the chromatogram with an identical location as described earlier (6); this should be taken into account when screening is performed in neonates.

Pseudouridine and uric acid showed peak heights that differed qualitatively between both populations studied. For this reason, the excretion of these two metabolites was quantified (Table 5). The excretion of pseudouridine was not significantly different in both groups of neonates. This could suggest that the production of pseudouridine, which is an end product of transfer RNA (10), is not influenced by such factors as gestational age or body weight. Furthermore, in both groups we found a highly significant correlation between the pseudouridine and the creatinine excretion. A possibility that has to be considered is that the pseudouridine excretion parallels the glomerular filtration rate: this would be in agreement with the finding of high pseudouridine levels in the cerebrospinal fluid of patients with chronic renal failure (11). We will investigate the value of urinary pseudouridine as an indicator of glomerular filtration rate in another study. Similar quantitative studies of other metabolites, although feasible (Table 4), were not performed.

For a correct interpretation of uric acid excretion, a 24-h urine collection and calculation of the fractional excretion is needed and the clinical situation of the patient has to be considered. In the present study, only random urine samples were investigated; definite conclusions about uric acid excretion in neonates there-

fore can not be made, and this was outside the scope of our study. Nevertheless, it can be stated that the absolute values of the uric acid excretion were higher in the term neonates compared with the premature and dysmature neonates and that the uric acid/creatinine molar ratio was markedly elevated in all neonates, as has been shown before (7).

With the present method, orotic acid is eluated at the very beginning of the chromatogram together with numerous other metabolites and must be increased markedly, as in uridyl monophosphate synthase deficiency, before it can be detected. An increased excretion of uracil has been reported in the literature in five heterozygotes for OCT deficiency (12). Orotic acid and, less frequently, uridine were also increased in these carriers. With the present screening method, OCT deficiency should be suspected when increased amounts of uracil, without increased thymine, are found on the chromatogram.

In only one of 24 neonates who had suffered from hypoxia was a markedly increased hypoxanthine peak observed. Hypoxanthine levels in umbilical cord blood have been shown to be a reliable indicator of perinatal hypoxia (13, 14), but there are no data on the value of urinary hypoxanthine in this respect. Because the blood levels rapidly decrease after the hypoxia occurs, it is unlikely that elevated urinary hypoxanthine values will be observed on the 4th d of life. Quantitative measurement of hypoxanthine in a urine sample taken shortly after birth may be a better indicator of perinatal hypoxia.

We believe that the method described here is sensitive enough to detect inborn errors of purine and pyrimidine metabolism. The use of an automatic injection system allows the unattended processing of a great number of samples and therefore permits the screening of large populations. Nevertheless, expertise is required for the recognition of some metabolites that appear in small amounts and the work load should not be underestimated. No hereditary disorders of purine and pyrimidine metabolism were detected in the 614 neonates in this study; a screening on a larger scale that will also allow calculation of the cost/benefit ratio of such a screening is planned.

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