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Apolipoprotein E genotype may influence urinary gammacarboxyglutamate (Gla) concentrations in young individuals

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Abstract. Upon degradation of vitamin K-dependent proteins (known as Gla-proteins) the free aminoacid Gla cannot be re-utilized and is excreted in the urine, where it can be used as an overall marker for vitamin K status. We report the urinary Gla excretion values in first morning void urine for healthy young Romanian subjects from birth, childhood and young adulthood. In these subjects we have evaluated age, gender and apo E genotype as potential confounders. The urinary free Gla/creat ratio (Gla/creat, mg/g) was highest in newborns (34.8 ± 19.5; $p < 0.001$), than fell in the group 4 to 48 months old (13.1 ± 11.1) to levels that were not significantly different from the young adult group (18.3 ± 5.5). No gender-related differences were observed in Gla/creat in newborns and young children, but Gla excretion in women was higher than in men (28.6%; $p < 0.029$). Remarkably, Gla excretion in subjects bearing the apo ε_2 + allele was significantly lower (11.9 ± 4.2) than in those bearing combinations of the ε_3 + and ε_4 + alleles (20.3 ± 4.1). The novelty of this study resides in the evaluation of urinary Gla excretion in relation with apo E genotype, suggesting that apo ε2 allele is a risk factor for developing vitamin K insufficiency.

Key words: Apolipoprotein E genotype — Vitamin K — Phylloquinone — Gammacarboxyglutamic acid — Gla proteins

Abbreviations: creat, creatinine; Gla, γ-carboxyglutamate; PIVKA, Proteins Induced by Vitamin K Absence.

Introduction

The result of vitamin K action is the formation of γ-carboxyglutamate (Gla) residues in proteins (Suttie and Preusch 1986). In humans, Gla-containing proteins are synthesized in a wide range of tissues; main contributors to the total Gla production in the body are the liver (clotting factors), bone (osteocalcin), and arteries (matrix Gla-protein). After they have fulfilled their function, Gla-proteins are metabolized; in contrast to the usual aminoacids, the unusual aminoacid Gla cannot be re-utilized anymore, but is excreted as free Gla *via* the urine. In this paper we aim to evaluate the urinary excretion of Gla for its suitability to serve as a marker for overall Gla-protein turnover as a surrogate marker for overall vitamin K status, differentiated for age and gender. Well known examples of Gla-proteins are prothrombin, which is uniquely formed by hepatocytes and contains 10 Gla-residues *per* molecule (Furie and Furie 1988), and osteocalcin which possesses three Gla-residues and is the product of osteoblasts (Hauschka et al. 1989).

During episodes of vitamin K insufficiency abnormal Gla-proteins (also known as undercarboxylated or descarboxy proteins) enter into the circulation. Since the discovery of the chemical structure of Gla (Stenflo et al. 1974), the name Proteins Induced by Vitamin K Absence (PIVKA's) has become obsolete. Assessment of circulating vitamin K is a time-consuming technique, only available in a small number of specialized laboratories (Haroon et al. 1982). Moreover, the relation between circulating and tissue vitamin K, as well as the level of plasma vitamin K at which

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a subject must be regarded as vitamin K-deficient, have not yet been established. An intricated aspect is that apolipoprotein E may play an important role in vitamin K status at tissue level, since the apo E genotype determines the affinity of lipoproteins for the apo E receptor (Bohnet et al. 1996). Subjects bearing the *ε2* allele were shown to take up the lipid complexes slower than those bearing apo E alleles *ε3* and *ε4* (Schiele et al. 2000).

Subclinical vitamin K-deficiency is generally assessed by the occurrence of circulating descarboxy prothrombin. Neither commonly used coagulation assays, nor gel immuno-electrophoretic techniques allow the detection of descarboxy prothrombin at levels below 3–5% of the normal prothrombin concentration. If measured in this way, vitamin K insufficiency is rarely seen, even among subjects who are known to have a low dietary vitamin K intake like newborns (von Kries et al. 1988) or elderly women (Knapen et al. 1989). It is well known, however, that due to the poor placental transport all newborns have very low circulating vitamin K levels, and are at risk for developing clinical vitamin K deficiency with gastrointestinal or intracranial hemorrhages. The most sophisticated tests for descarboxy prothrombin presently available are based on monospecific or monoclonal antibodies, but even with these tests the occurrence of trace amounts of descarboxy prothrombin could be demonstrated in not more than 20% of the untreated newborns (Motohara et al. 1985; Widdershoven et al. 1987). It seems, therefore, that at decreasing vitamin K supply undercarboxylated blood coagulation factors are only formed during the more severe stages of vitamin K deficiency. For that reason it must be questioned seriously whether descarboxy prothrombin is an ideal marker to monitor vitamin K-deficiency in an early stage and should be used, for instance, to assess the risk of developing vitamin K deficiency bleeding (VKDB) in the newborn.

Several arguments indicate that poor vitamin K status manifests itself sooner in extrahepatic proteins than in clotting factors (Bügel 2008). The question is relevant, therefore, whether the dietary vitamin K requirement of various tissues is similar. Firstly, it has been reported that substantial fractions of the bone Gla-proteins osteocalcin (OC) and matrix Gla-protein (MGP) may occur as uncarboxylated species (Price 1988). Secondly, it is well known that nutritional vitamin K intake decreases with age, and that – in contrast to prothrombin – circulating uncarboxylated osteocalcin is common in elderly women (Knapen et al. 1989, 1993; Plantalech et al. 1991; Szulc et al. 1993). Since both OC and MGP are synthesized almost exclusively in bone and arteries, respectively, uncarboxylated species of these proteins give information on the vitamin K status of these tissues, but not on overall vitamin K status.

Urinary Gla excretion is an overall test for the turnover of Gla-proteins, and was shown to be associated with vitamin K intake in rats (Crăciun et al. 1997). In humans, reference values have not been reported in the literature. Here we report the levels of urinary free Gla in apparently healthy individuals, from newborns to children and young adults. In one study among young healthy individuals we compared urinary Gla excretion in subjects with different apo E genotypes. These data are indispensible in studies in which overall vitamin K status is linked to disease status in various patient groups.

Materials and Methods

Subjects

Our study included three experiments in which apparently healthy subjects from Cluj County, Romania, were selected. Exclusion criteria were all diseases potentially interfering with vitamin K status including food malabsorption, coagulation disorders, kidney and liver diseases. In all three studies urinary Gla concentrations are expressed as the ratio free Gla/urinary creatinine.

For experiment 1, healthy newborns (n = 29, 15 males and 14 females) were investigated from the 1st Gynecological Clinic, all being born during the month of January. Half of the newborns (n = 14) received intramuscularly 1 mg K_1 (Konakion® 1 mg, Roche, Switzerland) in their first day of life, after collecting their first urine sample. The remaining 15 newborns were exclusively breastfed and did not receive any form of vitamin K_1 supplementation during three days. Urine samples were collected in day 1 and day 3 after birth in all newborns and used for Gla determination.

In experiment 2, healthy institutionalized children (n = 50, 30 males and 20 females), from Cluj County, aged 4 to 48 months old entered the study in January. First void urine samples were collected for free Gla determination. Subsequently, the subjects were divided in two control groups, one re-investigated in March (control 1, n = 23 subjects, 16 males and 7 females) and the second in April (control 2, n = 27 subjects, 14 males and 13 females). Anamnesis at intake included the date of birth, Apgar score, weight and length at birth and at present. In this study we were interested for the variation of free Gla excretion over a period of 2 and 3 months, in small children.

In experiment 3, healthy young adults (n = 100, 41 males and 59 females) of 22.8 ± 1.2 years old entered the study after a local call addressed to medical students of the University of Medicine and Pharmacy Cluj-Napoca. Venous blood was collected for apolipoprotein E genotyping from all subjects and urinary Gla concentrations were measured in the urine of 32 subjects (13 males and 19 females), covering all genotypes identified in the Romanian population, the

rest of 68 subjects (28 males and 40 females) bearing all the apo E3/3 genotype. Urinary Gla was determined in the first void urine.

All studies received the approval from the Medical Ethics Committee of the University Hospital Cluj and carried out in accordance with declaration of Helsinki. Informed consent was obtained from all participants or from their parents. This work was supported by the Romanian National Research Council, grant 1262/2005.

Samples

Urine samples were collected from the first morning void urine. In newborns urinary bags were used and first urine was collected within the first day of life of the infant. Samples were stored at –80°C until serial testing. For apolipoprotein E genotyping blood was taken by venipuncture on EDTA BD vacutainers®. All blood samples were stored at –80°C until serial testing.

Biochemical determinations

Urinary Gla detection: The urine samples were diluted with distilled water between 2 and 16 times, and the diluted samples were analyzed according to the procedure of Kuwada and Katayama (1983), using a Separations high precision HPLC pump (model 300), a Kratos Spectraflow 980 detector and a Nucleosil 100-5 SB anion exchange resin (5 μm particles, Macherey-Nagel, Düren, Germany) at a flow rate of 1 ml/min. Reference curves were prepared from authentic Gla (Sigma, St. Louis, MO, USA). The sensitivity of the assay was 0.3 μM. The intra- and interassay coefficients of variation were 2.2 and 6.4%, respectively.

Urinary creatinine concentrations were determined on an automatic analyzer Cobas Mira plus (Hoffman La Roche, Switzerland) and using a commercial kit for creatinine determination (Unimate 7 Creatinine, Roche Nederland BV). Intra- and interassay coefficients of variation for creatinine were 2.8 and 4.3%, respectively.

Apolipoprotein E genotyping: The apolipoprotein E genotype was determined according to the method described by Main (1991). DNA was extracted from 200 μl integral blood, (DNeasy Blood and Tissue kit, Qiagen, USA) containing approximately 400 ng DNA, which was used to perform the PCR technique. Taq polymerase, primers F4 (5'-TAA GCT TGG CAC GGC TGT CCA AGG A-3') and F6 (5'-GTG TAC CAG GCC GGG GCG AAT TCT GT-3'), restriction enzyme Hha I, and markers for 82bp, 66bp, 48 bp were used from Gibco BRL Life Technologies, Germany. The separation of the DNA fragments was performed in 5% metaphor agarose in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA. All chemicals were obtained from Sigma (St. Louis, MO, USA).

Statistical analysis

The SPSS/PC+ software package version 16.0 (SPSS Inc., Chicago, USA) was used for statistical analysis, and Slide-Write 7 (Rancho Santa Fe, CA, USA) was used for graphical representations. All data were expressed as mean values ± SD, and 95% confidence intervals for normal distributed values; otherwise the median was used. Unless stated otherwise, urinary Gla concentrations are expressed as the Gla/creatinine (Gla/creat, mg/g) ratio throughout this paper.

Metric data were summarized as mean and standard deviation whenever data proved to be normally distributed. Otherwise the median was used to summarize variables. The Kolmogorov-Smirnov test at a significance level of 5% was used to test the normality. The Student *t*-test was used to compare means of two groups whenever data proved to be normally distributed. The means between more than two groups for normally distributed variables were tested by ANOVA; and the post-hoc Tukey test was applied to compare two groups. Non-parametric tests (Mann-Whithney, Kruskall-Wallis, Median test) were used for comparisons whenever metric data proved to be non-normally distributed. The Cohen test (d) was applied for evaluation of the effect size when significant differences in small size groups were observed.

Results

Urinary Gla in newborns and the effect of vitamin K_1 supplementation

In their first day of life, newborns excreted 34.8 ± 19.5 mg Gla/g creatinine and no gender-related differences were observed. Remarkably, Gla excretion had not increased in the vitamin K_1-supplemented group at two days after 1 mg vitamin K_1 intake (Table 1), suggesting that shortly after birth the response to intramuscular injection of vitamin K is relatively slow.

Table 1. Gla excretion in newborns with present or absent vitamin K_1 supplementation

Group	Urinary Gla/creat (mg/g)		*p*-value	*n*
	Day 1	Day 3		
K_1	36.9 ± 13.5	42.4 ± 18.9	0.07	14
Breastfed	32.6 ± 24.1	39.7 ± 22.3	0.11	15

Children in the K_1 group received vitamin K_1 both by intramuscular injection immediately after birth and in bottle milk (formula food). Children in the second group were exclusively breastfed. The values represent means ± SD; *p*-value is related to the difference between Day 1 and Day 3.

Table 2. Urinary Gla excretion in young healthy individuals

Age group	Urinary Gla/creat (mg/g)	n
Newborns, day 1	34.8 ± 19.5	29
males	35.2 ± 18.3	15
females	34.4 ± 20.7	14
Children, 4–48 months	10.51 (0.97–47.67)	50
males	9.98 (0.97–47.66)	30
females	11.30 (2.72–40.89)	20
Young adults, 20–23 years	17.8 ± 5.75	30
males	15.01 ± 5.3*	12
females	19.6 ± 5.4	18

Data are presented as mean ± SD when distribution was normal or median (min-max) when distribution was non-Gaussian. *The difference in Gla/creat ratio between men and women in the young adult group was statistically significant at $p < 0.029$.

Urinary Gla excretion in young healthy subjects

Urinary Gla excretion was expressed as ratio with urinary creatinine. Creatinine excretion was lowest in children and higher in newborns and young adults (see Figure 1).

Table 2 presents the urinary Gla excretion in young healthy subjects of different age groups. In newborns the Gla/creat ratio was 2–3 fold higher than in the older age groups ($p < 0.001$). Obviously, the relatively low Gla/creat ratios in young adults compared to newborns mainly originate from their high creatinine excretion, not from low Gla excretion. Likewise, adult women had higher Gla/creat ratios than men ($p < 0.03$) because of the substantially higher creatinine excretion by men.

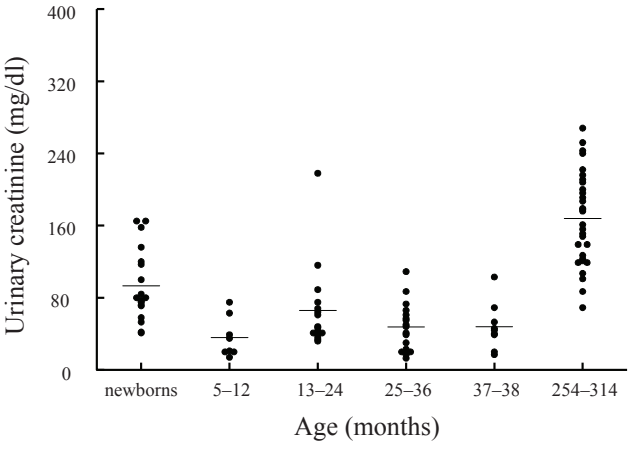

Figure 1. Urinary creatinine concentration (mg/dl) in different age groups. In newborns we found significantly higher creatinine excretion in comparison with small children, but significant lower compared to the young adult group. Creatinine excretion was lowest in the group 4–12 months ($p = 3.2 \cdot 10^{-8}$) and highest in the adult group; ($p = 5.2 \cdot 10^{-8}$, $p = 9.6 \cdot 10^{-7}$, $p = 2.3 \cdot 10^{-8}$, $p = 6.9 \cdot 10^{-8}$ if compared to each group from children 4–48 months old).

Variation of urinary Gla in children 4 to 48 months old over time

Figure 2 shows the excretion of urinary Gla in children over a period of 3 months. No significant changes in the Gla/creat ratio were observed during the first 2 and 3 months ($p < 0.6$ and $p < 0.5$, respectively). As shown in Figures 2A and 2B, the intra-individual and seasonal variations are low.

Gla excretion in young healthy adults with different apolipoprotein E genotype

The apolipoprotein E genotype was assessed in a subset of the reference population. The majority of the subjects were of the apo E 3/3 genotype (73%, 29 males and 44 females), followed by E3/4 genotype (16%, 9 males and 7 females), and

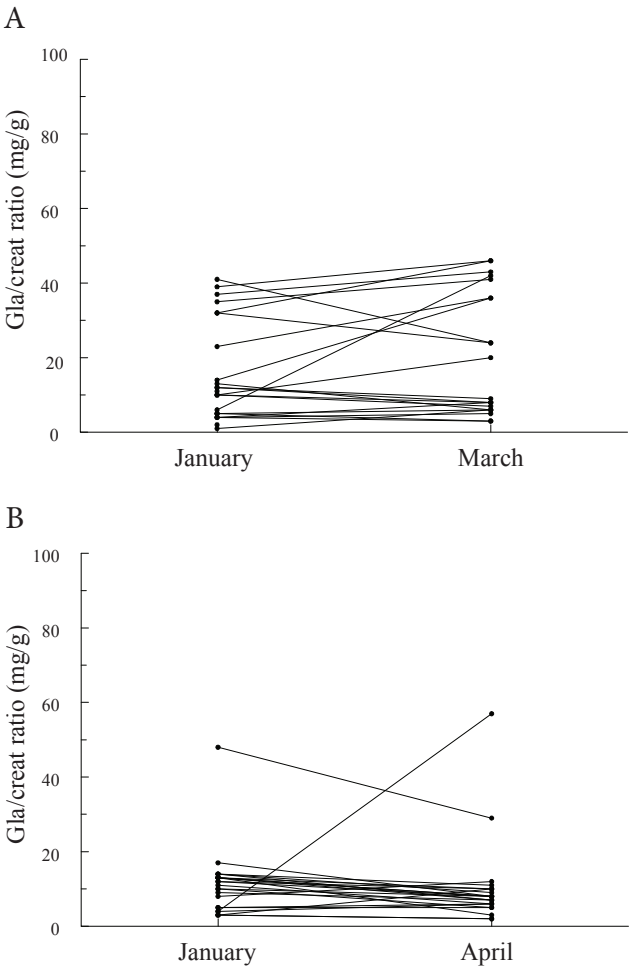

Figure 2. Gla/creat ratio variation over a period of 2 months (**A**) and 3 months (**B**) in children 4–48 months old.

the lesser expressed genotypes apo E 2/3 (9%, 6 males and 3 females) and apo E2/4 (2%, 2 females). From the total study population, selecting all subjects bearing at least one apo ε2 allele (the apo $E2^+$ group consisting of apo E 2/2, 2/3 and 2/4) and subjects of the apo E 3/3, 3/4 and 4/4 genotype (apo $E2^-$ group), we have observed that subjects bearing the *ε2* allele secreted significantly lower Gla than others ($p < 0.001$, Table 3), suggesting that their vitamin K status may be suboptimal. As is shown in Table 3, urinary Gla/creat values were lower in men than in women ($p < 0.029$).

Discussion

After discovery of vitamin's K role in bone and vascular health, apart from its role in blood coagulation, it has become increasingly important to find new tools for establishing vitamin K status and to define (subclinical) insufficiency in patients or healthy subjects. Taking into consideration that Gla-residues are the final result of vitamin K action, our purpose was to measure urinary Gla-excretion in apparently healthy subjects of different ages and to investigate whether and to which extent this marker may vary intra-individually or during time.

At this time no interventional clinical studies have been published in children in whom the effect of increased vitamin K intake on bone health is monitored. Population-based studies have shown an inverse association between vitamin K status (monitored by osteocalcin carboxylation) and bone health in children (Kalkwarf et al. 2004; van Summeren et al. 2004). Moreover, animal experiments in young rats and lambs have clearly shown that the negative effects of vitamin K antagonists on bone strength in growing animals are manifold stronger than in adult ones (Pastoureau et al. 1993). The strong impact of vitamin K status on bone health in young creatures is probably related to the high bone turnover and there are no reasons to expect that human bone metabolism differs from them in this respect growth (Hall et al. 1980). Especially in young children bone turnover is very high, resulting in a doubling of their height during the first year of life. It is well known that they are at high risk of vitamin K-deficiency since during the pre- and perinatal stage vitamin K availability through the placenta is low and the gut is sterile. Therefore we expected that in newborns urinary Gla would be lower in newborns than in other age groups. Also, in our study, newborns receiving vitamin K_1 supplementation additionally received formula milk (90 µg K_1/100 g), which had a much lower contribution to vitamin K status than i.m. administration of vitamin K_1 (1 mg). Although the vitamin K_1 supplementation was substantial in this group, urinary Gla excretion measured at the third day after birth was not significantly different from that in the non-supplemented group or from their first urine. This is accordance with an another study in newborns, showing a shift in carboxylated osteocalcin appears only five days after vitamin K administration and increased intake in breast milk or formula (Shimizu et al. 2002). Unfortunately, in our study no samples were taken after longer treatment periods. The fact that urinary ratio Gla/creat was significantly increased in newborns may be the result of an immature glomerular filtration rate (Hoseini 2012) and to an increased metabolic rate and high degradation rate of Gla containing proteins at birth (Topp 1998). In this case, a distinct reference values interval for urinary Gla in newborns should be established. In the group between 0 and 4 years of age no differences related to gender were observed in urinary Gla excretion. Another observation was that despite the high bone turnover during the first year of life, the urinary Gla/creat ratio did not change significantly during the first four years of life. Sokol and Sadovski (1990) found in the population aged 18–85 years that urinary Gla/creat ratios increase with age in both males and females. In that study women had 20% higher ratios than in men during the entire life span. This gender-related difference in Gla excretion

Table 3. ApoE genotype and urinary Gla excretion in healthy individuals.

	Gla/creat (mg/g)				*p*	Cohen's d (r)
	Apo $E2^+$		Apo $E2^-$			
	Median (min–max)	*n*	Median (min–max)	*n*		
Men	9.03 (8.3–10.5)	5	19.6 (15.9–21.6)	7	<0.001	6.9 (0.96)
Women	13.88 (11.5–21.4)	4	19.6 (13.3–29.2)	14	0.04	1.19 (0.51)
All	10.53 (8.3–21.4)	9	19.6 (13.3–29.2)	21	<0.001	2.02 (0.71)

Urinary Gla and creatinine concentrations were assessed in a subset ($n = 30$, aged 22.8 ± 1.2 years) of healthy individuals. Apo $E2^+$ stands for subjects of the apo E2/2, apo E2/3 and apo E 2/4 genotypes, whereas apo $E2^-$ stands for subjects of the apo E3/3, apo E 3/4 and apo E 4/4 genotypes. Data are presented as median (min–max). The *p*-value refers to the difference between the apo $E2^+$ and apo $E2^-$whitin groups (men, women and all) whereas the Cohen test was applied for evaluation of the effect size when significant differences in small size groups were observed.

was confirmed by our study as well, with values that were 28.6% higher in women than in men, in the young adult group, but not significant different at birth and in the 0–48 months old age groups.

Another observation is that Gla/creat ratio decreased to half in young adult, while creatinine concentration approximately doubled in comparison with the moment of birth. As is shown in Table 2, the inter-individual variation of the Gla/creat ratio is smaller in young adults than in children or newborns, which may reflect a more stabilized Gla protein metabolism in the adult group. These observations lead to the conclusion that Gla excretion in newborns is at least similar to that in adults, which is consistent with the finding that total Gla excretion *per* 24 hours and *per* body weight does not depend on age (Sann et al. 1984).

Although genotyping was only possible in a sub-group of our study population, it was found that the apolipoprotein E genotype may substantially influence vitamin K status, and as result urinary Gla excretion is different in subjects bearing the *ε2* allele. Of the six possible genotypes we have identified four more frequent ones in the Romanian population. It is known that apo E2 has a low affinity for the apo E receptor (Bohnet 1996; Siest 1998), which may affect vitamin K transport. The three common apo E isoforms have different affinities for the LDL-receptor. Apo E3/3 and E4/4 have same affinity for this receptor, whereas apo E2/2 shows defective binding activity corresponding to 1% of that of the two other isoforms (Siest 1998). Apo E2/2 has been associated with increased remnants in plasma caused by the reduced lipoprotein receptor-binding activity of apo E2/2 (Davignon 1988). Consistently, we found that subjects bearing the apo ε2 allele (E2/2, E2/3, E2/4) had about 50% lower urinary Gla excretion than subjects not bearing the apo ε2 allele. Studies in which the potential association between urinary Gla-excretion and (extra-hepatic) Gla-protein carboxylation (osteocalcin, matrix Gla-protein, Gla-rich protein) is tested will demonstrate whether the apo ε2 allele is a risk factor for developing vitamin K insufficiency.

This study has several limitations. First, the number of subjects in the different age groups was low; therefore the levels of urinary Gla/creatinine are only indicative and cannot be used to define the reference biological interval for the individuals. Second, we had blood samples from only a limited number of participants so that apo E genotyping could only be accomplished in part of the population. Third, the absence of seasonal variations could only be confirmed for a period of 4 months; new studies will have to reveal whether the Gla/creat excretion is independent of seasonal variations. Finally, it may be debated whether the first void urine sample is the most suitable sample to be used. Obviously, when assessing a metabolite in the urine it is logical to obtain a sample in which the accumulation of the metabolite is maximal over a specific period of time possible. Therefore, we have chosen the first void urine in order to measure Gla and creatinine. The WHO criteria, for an acceptable sample of first void urine for adults, should have creatinine concentration between 30 mg/dl to 300 mg/dl. On the other side, one study among 196 young children has found that in 20.8% of the first morning void urines creatinine concentrations were lower than 50 mg/dl and in 0.6% above 300 mg/dl (O'Rourke et al. 1999). In conclusion, creatinine in the first void urine of healthy young subjects may include, to some extent, some "unacceptable" samples, with lower levels in children under 6 years old (up to over 20%) and higher levels in young adult (3.3% or more) (Barr 2005). To our knowledge, no reference studies performed in small children regarding the valid creatinine concentration of the first morning void urine sample have been published. In our studies all samples were considered as valid, with the remark that in newborns urine creatinine had a normal distribution, with all concentrations above 30 mg/dl, whereas in young children (4 to 48 months old) about 26% of the urine samples contained less than 30 mg/dl (maybe it was not a real first void urine, urination during night). In the young adult population two of the 32 samples had creatinine concentrations above 300 mg/dl (one male and one female, both apo E3/3), and were excluded. But excluding the outriders in our study did not significantly influence the Gla/creat ratios, or our conclusion that this ratio was not related to age.

The most important finding in this paper was that the apo E genotype is a major determinant in urinary Gla excretion, which is consistent with observations that also serum vitamin K levels (Yan et al. 2005) and the extent of osteocalcin carboxylation are strongly dependent on the apo E genotype (Beavan et al. 2005; Pilkey et al. 2007).

In conclusion, we have shown that the urinary Gla/creat ratio turned out to be remarkably stable, notably in small children in whom a non-invasive marker for vitamin K status is mostly wanted. Therefore, urinary Gla excretion may become a valuable, non invasive tool for assessing overall Gla protein metabolism and indirectly vitamin K status.

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