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Concentrations of N^{ϵ}-Carboxymethyllysine in Human Breast Milk, Infant Formulas, and Urine of Infants

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Maillard products, such as N^{ϵ}-carboxymethyllysine (CML), are readily formed during the manufacturing of infant formulas. Little has been known, however, about the presence of CML in human breast milk and about the uptake of CML by infants. In this study, CML was measured in the serum and breast milk of 32 healthy mothers by ELISA. CML concentrations in breast milk (137 ± 82.7 ng/mL) were significantly lower than in the serum (399 ± 67.8 ng/mL, $p < 0.001$) and on average 35-fold lower than in infant formulas (4754 ± 4299.5 ng/mL). CML was also measured in the urine of 21 infants, which were fed with breast milk or formulas. Although there was a tendency toward higher urinary CML excretion in infants fed with hypoallergenic formulas compared to breast-fed ones, the differences were not significant. Neonates that were delivered by vaginal birth had significantly higher concentrations of CML compared to those delivered by caesarean section (1306 ± 653 vs 601 ± 220 ng/mL, $p = 0.012$). It is conclued that CML passes from the serum into the breast milk, but the levels are by far lower than in infant formulas. In very young neonates (≤3 days), the mode of delivery has a greater influence on urinary CML excretion than the nutrition.

KEYWORDS: Advanced glycation end-products; breast milk; N^{ϵ}-carboxymethyllysine; infant formulas; Maillard products; newborn; urine

INTRODUCTION

Maillard reaction products are formed by the nonenzymatic reaction of sugars with proteins or amino acids. Processed milk is particularly prone to Maillard reaction because of its relatively high content of lactose and proteins, which can directly interact (*1*). In the first steps, the Amadori product lactuloselysine is formed (*2*, *3*), which is degraded during prolonged heating to a wide range of advanced Maillard products. In heat-treated milk, several free (*4*) and protein-bound products, such as N^{ϵ}-carboxymethyllysine (CML) (*5*, *6*), pentosidine (*7*), pyrraline (*8*), or oxalic acid monolysinylamide (OMA) (*9*), were detected. Compared to regular milk products, milk-based infant formulas show even higher concentrations of Maillard products (*10*). In the latter, the Maillard reaction is promoted by intensive heat treatment to ensure microbiological safety and by supplementation of lactose, iron, and ascorbic acid (*11*). Thus, CML levels in powdered infant formulas were 2.5-fold higher compared to milk powder and in liquid infant formulas 3-fold higher than in similarly processed cow's milk (*10*). Particularly in hypoallergenic infant formulas, which are partially or extensively hydrolyzed prior to heat treatment, very high levels of Maillard products were determined. Thus, increased fluorescence (excitation at 375 nm, emission at 450 nm) and CML formation were measured in hypoallergenic infant formulas compared to regular ones of similar composition (*12*).

The fate of Maillard products after consumption is still not fully revealed. Foerster and Henle showed that protein-bound pyrraline is almost completely bioavailable and renally excreted within 48 h (*13*). On the other hand, the fate of 95% of the ingested Amadori product (fructosyllysine) could not be determined with only 2.68% excretion in urine (*14*), suggesting microbial decomposition in the intestine. CML has first been detected in the urine of infants and children, but a relationship to nutritional intake was not investigated in this study (*15*). Elevated serum levels of Maillard products (advanced glycation end-products; AGEs) were measured after the intake of egg white heated in the presence of fructose. From this study, it was concluded that dietary Maillard products contribute to the overall pool of AGEs in the human organism (*16*). Taken together, resorption and metabolism seem to be largely dependent on the structure of the specific Maillard product.

Only little is known, however, about the transition of Maillard products into human milk and their availability for infants. Fohgelberg et al. detected acrylamide concentrations above the

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Table 1. Characteristics of the Study Groups

	breast milk and serum samples	urine samples
mothers (*n*)	32	19
infants (*n*)	33	21
age of child at sample collection (days; median, min, max)	3, 2, 6	2, 1, 164
age of mother (years; mean ± SD)	31.2 ± 5.7	28.4 ± 6.9
birth wt (g; mean ± SD)	3297 ± 598	3290 ± 567
caesarean section (*n*)	7	11
vaginal delivery (*n*)	25	8
Apgar score[a] (I, II, III; mean)	8.7, 9.6, 9.9	8.9, 9.7, 10
time of delivery (weeks + days; mean)	39 + 3	39 + 3
creatinine concn (mg/dL; mean ± SD)		50.2 ± 43.2

[a] The Apgar score describes the physical status of the infant 1 (I), 5 (II), and 10 (III) min after birth. Values between 9 and 10 are optimal and below 7 indicate a distress syndrome of the newborn.

quantification limit in only 1 of 19 breast milk samples (*17*). In another study, renal excretion of lactulosyllysine and lysinoalanine as well as kidney function was investigated in preterm infants in relation to their nutrition. Lactulosyllysine and lysinoalanine concentrations in the urine of formula-fed infants were significantly higher than those found after breast feeding without obvious influence on the kidney function. These results indicate that the concentrations of lactulosyllysine in human milk were most likely lower than in the formulas (*18*).

The aim of the present study was to investigate the occurrence of the Maillard product CML in breast milk and to monitor CML levels in the urine of young infants in relation to their nutrition.

MATERIALS AND METHODS

Sample Collection. Exclusion criteria were a reported disease of mother or child as well as complications during delivery. Breast milk and serum samples were collected from mothers several days after full-term deliveries (39 ± 2 weeks) with the exception of one preterm delivery (34th week) by caesarean section or vaginal birth. The collection period was 24 days. The characteristics of the study group are summarized in **Table 1**. Blood was collected by venous puncture on the same day as breast milk was obtained. Blood was centrifuged, and the serum, which was separated from cellular components and the fibrin clot, was stored for further examinations. Breast milk samples were collected using clean polypropylene bottles (50 mL; Greiner, Solingen, Germany) and later aliquoted in 5 mL tubes.

Urine samples were collected from young infants, which were fed with breast milk or infant formulas. The collection period was 34 days. The characteristics of the study group are summarized in **Table 1**. Urine was collected with pediatric urine collection bags (U-Bag, Hollister, Libertyville, IL) and transferred to 5 mL tubes. All samples were stored at −20 °C before CML analysis. Approval for the study was obtained from the ethical committee of the local faculty of medicine.

Infant Formulas. Eight different formulas from five suppliers, which are representative for the German market, were included in the study. Five of the formulas were hypoallergenic (IF HA), and the other three were regular infant formulas (IF reg).

CML Analysis. CML was measured by an ELISA provided from MicroCoat Biotechnologie GmbH (Bernried, Germany) as described before (*10*, *19*, *20*). (Unless otherwise noted, all chemicals used in the ELISA including the antibody were obtained from MicroCoat Biotechnologie GmbH.) Briefly, streptavidin-coated microtiter plates were incubated with biotinylated BSA−AGE, and a horseradish peroxidase-labeled monoclonal CML−antibody was added either with the sample or with the standard [6-(*N*-carboxymethylamino)caproate]. Antibody binding was visualized by the addition of 2,2′-azinobis(3-ethylbenzthiazoline sulfonate (ABTS, Roche Diagnostics, Mannheim, Germany).

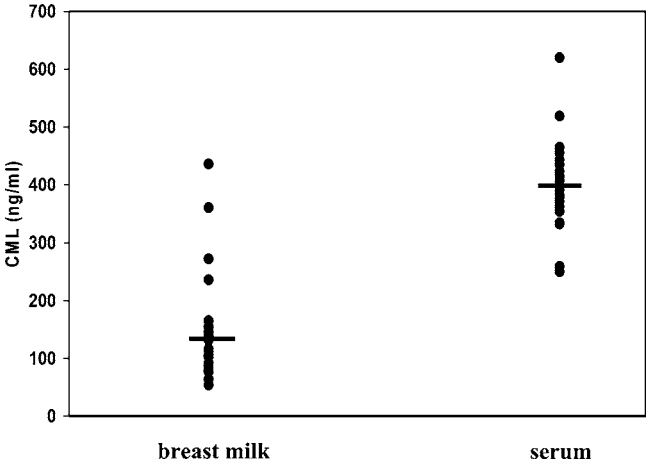

Figure 1. CML concentrations in breast milk and serum of 32 healthy mothers. The difference is significant ($p < 0.001$); the horizontal line indicates the mean.

Serum samples were digested by proteinase K prior to analysis, whereas urine and milk samples were applied directly after dilution. For proteinase K digest, 10 μL of the serum and 100 μL of proteinase K (Roche Biochemica, Mannheim, Germany; 1.1 mg/mL) were incubated for 2 h at 37 °C. Proteinase K was inactivated by heating for 10 min at 80 °C (Thermocycler), and after cooling to room temperature, the samples were directly used for ELISA. Thus, the serum was diluted 1:10 by this procedure, whereas urine was diluted 1:20 and breast milk 1:10 and 1:20 (analysis in quadruplicates) in assay buffer. Powdered infant formulas were suspended in water as described by the manufacturers and diluted. Liquid formulas were diluted directly in assay buffer as necessary to measure in the linear range of the assay. CML concentrations of the powdered formulas are given for the ready-to-use drink. All samples were analyzed in duplicate or quadruplicate with a coefficient of variation of ≈2%. Further validation of the assay has been described elsewhere (*19*). For milk samples, an assay sensitivity of 5 ng/mL and intra- and interassay precisions of 4 and 5%, respectively, were reported (*12*). The CML concentrations of the different groups are given as mean ± SD.

Creatinine Analysis. Urinary creatinine concentrations were measured with an enzymatic assay (Creatinine-PAP AU, BIOMED, Oberschleissheim, Germany).

Statistics. Excel and SPSS were used for statistical evaluation. ANOVA with Bonferroni correction was used to compare between groups; *t* test was additionally used to compare within groups (urine).

RESULTS

In the first part of the study, the transition of CML from the serum into human breast milk was investigated. Therefore, blood and breast milk were collected on the same day from 32 healthy donors, and CML concentrations were measured by ELISA. The characteristics of the donors are summarized in **Table 1**. CML was detected in all breast milk samples, but the concentrations were significantly lower compared to the CML content in serum (137 ± 82.7 ng/mL, $n = 32$, vs 399 ± 67.8 ng/mL, $n = 32$, $p < 0.001$; **Figure 1**). A significant correlation between the serum and breast milk levels of the individual donors was not observed (correlation coefficient = 0.03, $p = 0.85$). The serum levels of the mothers were not different from those of control women between 20 and 35 years of age (400−440 ng/mL; reference values provided by MicroCoat Biotechnologie GmbH).

Furthermore, CML levels were measured in eight different types and brands of infant formulas, which are representative for the German market (**Table 2**). The mean CML content of the infant formulas was on average 35-fold higher than that of breast milk (4754 ± 4299.5 ng/mL, $n = 8$, vs 137 ± 82.7 ng/mL, $n = 32$, $p = 0.001$; **Table 3**).

Table 2. CML Concentrations in Eight Different Infant Formulas Administered to the Infants

	CML (ng/mL)
manufacturer 1 HA	11372
manufacturer 2 HA	4738
manufacturer 3 HA	4942
manufacturer 4 HA	2746
manufacturer 5 HA	11035
manufacturer 2	702
manufacturer 3	1982
manufacturer 4	514

Table 3. CML Concentrations in Breast Milk, Serum of Mothers, Hypoallergenic Infant Formulas (IF HA), Regular Infant Formulas (IF Reg), and Urine of the Infants

	CML concn (ng/mL)					
	mean	SD	min	max	median	*n*
breast milk	137	82.7	54	436	114.5	32
serum	399	67.8	250	620	395.5	32
IF HA	6967	3963.5	2746	11372	4942	5
IF reg	1066	798.8	514	1982	702	3
urine	887	646.5	132	2534	631	21

In the second part of the study, the CML burden of very young infants was determined in relation to their nutrition. For this purpose, urine was collected from 21 infants [2 (median), 1 (min), and 164 (max) days after delivery]. The characteristics of the study group are summarized in **Table 1**. Twelve of the infants had been given one of the formulas, described in **Table 2**. The other nine infants were breast fed. CML concentrations were then measured in the urine by ELISA. The mean CML concentration in the urine samples was 887 ± 646.5 ng/mL ($n = 21$). The urine of breast-fed children did not differ significantly from that of children fed by infant formula, when compared without division into subgroups. The CML in the urine of infants fed by hypoallergenic infant formulas ($n = 9$) was 3 times higher than that of infants fed by regular (not hypoallergenic) infant formula ($n = 3$) and twice as high as that of breast-fed infants. These differences were significant using the *t* test, but not significant using ANOVA with Bonferroni correction. Also, breast-fed babies who additionally had free access to tea (fennel baby tea supplemented with maltodextrin, $n = 3$) showed elevated CML levels in urine compared to those who were only breast fed ($n = 6$, **Figure 2**).

The influence of the delivery mode on the infant's CML levels in the first 3 days after birth was also determined. In vaginally delivered neonates ($n = 6$), urinary CML levels were >2-fold higher compared to neonates delivered by caesarean section ($n = 9$) (1306 ± 653.9 vs 601 ± 220.6 ng/mL, $p = 0.012$; **Figure 3**).

DISCUSSION

The formation of advanced Maillard products during the manufacturing of infant formulas is well established (*10*). The manufacturing process as well as the typical composition of formulas promotes glycation, glycoxidation, and oxidation of the milk proteins. Particularly high concentrations of Maillard products are present in hypoallergenic formulas, in which the proteins are hydrolyzed before heat treatment (*12*). In contrast, hardly anything is known about the presence of Maillard products in human breast milk. In this study, we measured CML levels in the serum and breast milk from 32 healthy mothers between 2 and 6 days after delivery. In all breast milk samples,

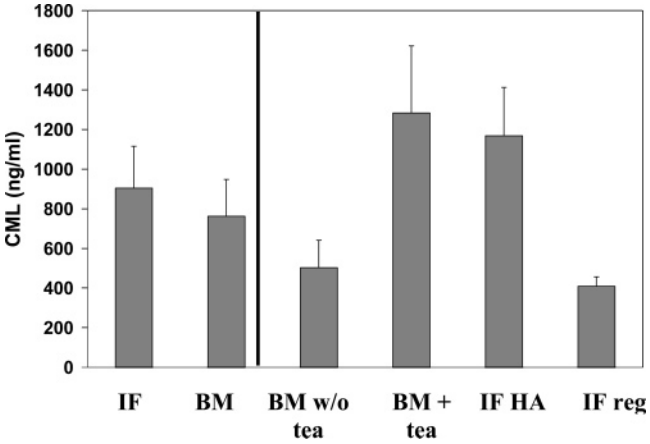

Figure 2. CML concentrations (mean ± SEM) in urine of infants [age 2 (median), 1 (min), 164 (max) days] fed with infant formula (IF, $n = 12$) or breast milk (BM, $n = 9$). Both groups were further subdivided into infants fed with breast milk without any supplementation (BM w/o tea, $n = 6$), breast milk and additional maltodextrin sweetened fennel tea (BM + tea, $n = 3$), hypoallergenic infant formula (IF HA, $n = 9$), and normal infant formula (IF reg, $n = 3$).

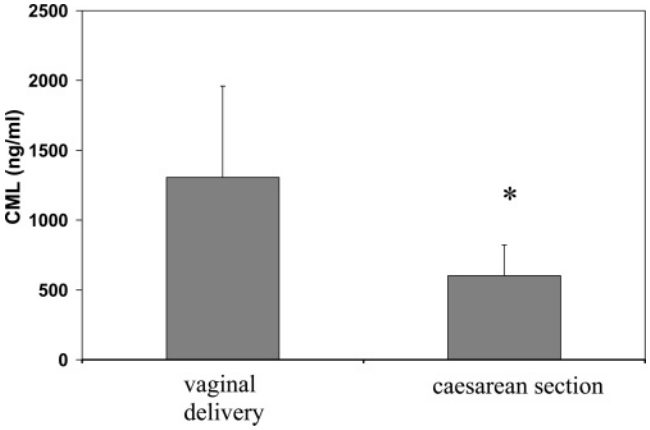

Figure 3. CML concentrations in urine of neonates (age ≤ 3 days) delivered by caesarean section or vaginal birth (*, $p = 0.012$).

CML was detectable in concentrations between 54 and 436 ng/mL. The concentrations were significantly lower than in the sera of the mothers. CML is formed in human serum by endogenous glycation of serum proteins. A second source of Maillard products in the serum is the uptake from nutrition. Glycated amino acids are—at least partially—absorbed from ingested food, increasing the serum levels, before they are renally excreted.

CML in breast milk may be derived from the incorporation of in vivo glycated serum proteins into the milk or by transition of nutrition-derived glycated amino acids from the serum into the milk. In this study, a correlation between CML concentrations in serum and breast milk was not observed, indicating that there are individual differences between both mechanisms. Further work is, therefore, needed to investigate the influence of nutrition on the levels of Maillard products in human breast milk.

The physiological and toxicological relevance of CML in vivo is still under debate. Elevated CML serum levels were detected in patients with diabetes or renal failure (*19*, *21*) and were associated with disease-related pro-inflammatory complications (*22*). In patients with chronic liver disease, serum levels of CML reflected closely the loss of liver function (*23*). Consequently, a diet with a low content of Maillard products reduced several

circulating inflammatory markers in diabetic patients (*24*). In animal studies, an AGE-reduced diet led to a decrease in neointimal formation after arterial injury in hypercholesterolemic mice and to improved insulin sensitivity in diabetic mice (*25*, *26*). On the other hand, two healthy subpopulations with slightly, but significantly, different CML plasma levels due to different nutritional habits had similar levels of the inflammation marker C-reactive protein (*27*). Also, in hemodialysis patients, C-reactive protein did not correlate with CML levels, and patients with high CML plasma concentrations had even a significantly better survival rate than patients with low ones (*28*). Information about the effects of AGEs in infants is rare.

Therefore, in the second part of this study, we investigated the influence of the nutrition on the CML burden of 21 infants. Nine infants were breast fed, whereas the other 12 infants had been given commercially available infant formulas (9 hypoallergenic and 3 regular formulas). The mean CML level in the hypoallergenic infant formulas was 50 times higher and that in regular formulas almost 8 times higher than the CML level in breast milk. Because serum from healthy babies is not readily available, the CML burden was measured by its excretion with the urine. It has been documented that free plasma CML is efficiently excreted with the urine, so that urine CML levels should mirror the plasma burden (*16*, *29*, *30*).

Despite the high differences in the CML content of the nutrition in both study groups, CML excretion was only slightly, but not significantly, higher in the formula-fed infants compared to the breast-fed ones. When the formula group was divided into infants fed with hypoallergenic formulas and those fed with regular formulas, the CML concentration in the HA group was significantly higher compared to the non-HA group (directly compared with the *t* test). However, due to the small sample size in the non-HA group ($n = 3$), this differentiation must be considered as preliminary. It was also striking that the two infants with the by far highest CML concentrations (1745 and 1479 ng of CML/mL) in the breast milk group had free access to fennel tea supplemented with maltodextrin. Therefore, we defined also a subgroup of babies who were exclusively breast fed. The infants who were exclusively breast fed had significantly lower CML excretion than the infant formula group (directly compared with the *t* test). However, the differences in the four subgroups were not significant, applying ANOVA with Bonferroni adjustment. Because of the small sample size in the breast milk/tea group, the influence of tea supplementation on CML concentrations in neonates requires further investigations. CML was not detectable in the tea itself.

In adult populations, urinary solutes are often related to the creatinine concentration to normalize for urine concentration. However, in neonates creatinine seems not to be a good reference. First, urinary creatinine concentrations are dependent on the lean body mass (*31*), which may have a greater influence in this study group. More important, in the first postnatal week, the values and variability of urinary creatinine concentrations are much higher compared to those of older children or adults. Also, the ratio between urinary creatinine and osmolality shows high variations, indicating that the former is not a good standard for urine concentrations in this study group. From these data it was concluded that urinary creatinine should not be used to standardize the excretion of solutes in urine during the first week of life (*32*). The creatinine concentrations that were measured in this study were similar to those determined by Matos et al. (*32*) and confirmed high values and variation during the first week of life (**Table 1**). Because most urine samples included in this study were from infants younger than 1 week, CML excretion was not related to urinary creatinine concentrations.

CML is formed by glycoxidation of proteins and amino acids, requiring the presence of sugars as well as oxidative conditions. Therefore, CML formation in vivo is favored not only by elevated glucose concentrations (e.g., in diabetes mellitus) but also by oxidative stress (*33*). The influence of nutrition was only marginal in the studied population comprising mostly neonates. Therefore, we also investigated the influence of the delivery mode on CML excretion by neonates, because the labor process leads to elevated levels of oxidative stress in the fetus (*34*). In the group of neonates, who were not more than 3 days old, a >2-fold significant increase in renal CML excretion was measured after vaginal delivery compared to caesarean section (1306 ± 653 vs 601 ± 220 ng/mL, $p = 0.012$). Interestingly, all of the infants delivered by caesarean section were fed infant formulas, whereas this was the case for only 33% of the spontaneously delivered babies. Thus, it can be hypothesized that during the first days after birth, urinary CML excretion rather mirrors endogenous oxidative stress than nutritional uptake, so that differences between breast-fed and formula-fed infants are overcompensated. On the other hand, it cannot be ruled out that lack of difference between both nutrition groups is caused by a lower uptake of protein-bound CML from formulas or by differences in the metabolism, glomerular filtration rate, or urine concentration between both groups. These factors may also have an influence on urinary CML concentrations after vaginal delivery or caesarean section. To clarify the role of the different factors, further studies are required that differentiate between free and protein-bound CML in breast milk, infant formulas, maternal serum, and infantile urine.

In summary, these data indicate that CML, one of the best-characterized Maillard products/AGEs, passes from the maternal blood into the breast milk. However, CML concentrations were much lower in breast milk compared to infant formulas. In very young babies, the CML excretion is rather dependent on the labor process than on nutritional CML intake. Further studies are now required to investigate if the nutritional CML content has an impact in older infants, for whom labor effects are not relevant.

ABBREVIATIONS USED

AGE, advanced glycation end-product; CML, N^{ϵ}-carboxymethyllysine; IF HA, hypoallergenic infant formula; IF reg, regular infant formula; OMA, oxalic acid monolysinylamide.

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