Influence of Dietary Fatty Acid, Vegetable, and Vitamin Intake on Etheno-DNA Adducts in White Blood Cells of Healthy Female Volunteers: A Pilot Study[1]

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Abstract

Etheno-DNA adducts such as 1,N^6-ethenodeoxyadenosine (ϵdA) and N^2,3-ethenodeoxycytidine (ϵdC) are formed as result of oxidative stress and lipid peroxidation via reactive alkenals (J. Nair *et al.*, Mutat. Res., *424:* 59–69, 1999). High ϖ-6 polyunsaturated fatty acid intake markedly increased levels of WBCs in female volunteers on a controlled diet (J. Nair *et al.*, Cancer Epidemiol. Biomark. Prev., *6:* 591–601, 1997). In healthy female volunteers we investigated possible correlations between dietary fatty acid intake (by questionnaire), concentration of linoleic acid (LA) and oleic acid (OA) in serum (n = 34), and etheno-DNA adduct levels in WBC (n = 42). Two groups of samples were selected according to dietary intake >15 g (group A) or <5 g (group B) LA/day. Serum samples were analyzed for free OA and LA by gas chromatography-mass spectroscopy and WBC-DNA for ϵdA and ϵdC adducts by immunoaffinity ^{32}P postlabeling. On a group level, serum LA and OA concentrations were higher in group A than group B, whereas the LA/OA ratios were similar. The mean ϵdA and ϵdC levels did not significantly differ in groups A and B, but a third of the individuals had more than twice the mean adduct levels than the rest. Correlation analyses revealed a significant inverse correlation for ϵdA in WBC-DNA and vegetable or vitamin E consumption. We conclude that etheno-DNA adduct levels are not determined by LA intake alone but might depend on the ratio of ϖ-6 polyunsaturated fatty acid:other fatty acids and of antioxidants consumed in the diet. This pilot study also indicated a protective effect of dietary vitamin E and vegetables against miscoding, lipid peroxidation-induced DNA lesions.

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Introduction

Persistent cellular oxidative stress and enhanced LPO,[3] leading to macromolecular damage and disruption of signaling pathways are implicated in the development of human malignancies (1–3). LPO of omega (ϖ)-6 PUFA generates reactive α, β-unsaturated aldehydes such as HNE or malondialdehyde, which can form promutagenic exocyclic DNA adducts in human cells and may, thus, contribute to diet-related cancers (4–6). HNE, one of the major LPO products, is formed by oxidation of LA or arachidonic acid (ϖ-6 PUFAs) and is readily oxidized by fatty acid peroxides to form 2,3-epoxy-4-hydroxynonanal. This bifunctional alkylating agent can react with DNA to yield etheno and other base adducts. Etheno adducts are highly miscoding lesions in mammalian cells and are thought to initiate the carcinogenic process through specific point mutations, as shown for the known carcinogens vinyl chloride and urethane (7–10).

Earlier we have used an ultrasensitive detection method to analyze WBC DNA from volunteers in a carefully controlled dietary study and showed that a high intake of ϖ-6 PUFA (LA in sunflower oil) but not of OA in rape seed oil increased the frequency of etheno-DNA adducts in WBC 40 times in women but not in men (11). These results clearly indicated that the DNA adduction was attributable to consumption of an LA-rich diet, which resulted in increased LPO of membrane lipids. The marked interindividual difference in etheno adduct levels in women on the same high ϖ-6 PUFA diet remains unexplained; it could be related to a synergism between high dietary ϖ-6 PUFA intake and estrogen catabolism as discussed earlier (12, 13).

Taking advantage of the available blood samples from the EPIC Heidelberg study (14), we have investigated possible correlations between dietary fatty acid intake (by questionnaire), concentration of LA and OA in serum, and etheno-DNA adduct levels (ϵdA and ϵdC) in WBC collected from healthy female volunteers. We also investigated fat-soluble antioxidants (vitamin E and β-carotene) and fruit or vegetable consumption on the WBC etheno-DNA adduct levels.

Materials and Methods

Study Subjects and Biological Sample Collection. Available buffy coat and serum samples remaining after the main collection protocol for the EPIC (15) study from healthy women from the Heidelberg area (South Western Germany) were used in the

[3] The abbreviations used are: LPO, lipid peroxidation; ϵdA, 1,N^6-ethenodeoxyadenosine; ϵdC, N^2,3-ethenodeoxycytidine; FFQ, food frequency questionnaire; EPIC, European Prospective Investigation into Cancer and Nutrition; HNE, *trans*-4-hydroxy-2-nonenal; LA, linoleic acid; OA, oleic acid; PUFA, polyunsaturated fatty acid.

study. For this purpose venous blood samples had been collected in citrate buffer (0.05 M sodium citrate pH .7) cooled to 4–8°C and centrifuged (2900 rpm, 20 min). Leukocytes (buffy coats) and plasma fractions from the same subject were separated and stored at −80°C for up to 14 weeks. Samples (~1200) were collected during 14 weeks, and 42 samples were selected according to the following dietary LA intake: >15 g LA/day (group A) and <5 g LA/day (group B) based on questionnaire data. Dietary information was obtained from a self-administered FFQ, which was sent to the volunteers, filled out at home, and handed over in the study center during a visit for an additional interview, anthropometric measurements, and for drawing a blood sample (14). The questionnaire asked for average consumption habits during the past 12 months. The questionnaire comprised 12 food groups and 148 single food items. The frequency of consumption was assessed according to frequency categories ranging from "never" to "several times a day." The assessment of portion sizes was supported partially by pictures of differently sized pieces of meat, portions of vegetables, and so forth. (16). Although sample collection was aimed for premenopausal women only, 8 women were identified during the analysis as having entered menopause through questionnaire review. This data set was also analyzed separately from the remaining 34 samples but because etheno-DNA-adduct levels did not differ, they were combined.

Determination of Free Fatty Acids in Serum. Free fatty acids were extracted from 100 μl of available serum samples ($n = 34$) and purified on aminopropyl columns (Supelclean LC-Si; Sigma-Aldrich, Deisenhofen, Germany) by the procedure of Agren *et al.* (17). Deuterated (d_2)-OA (5 μg) was added before the extraction as internal standard. The free fatty acids, LA and OA, were derivatized by diazomethane in ether. The esters were injected into a gas chromatograph (HP 5890; Hewlett Packard, Waldbronn, Germany) equipped with a 0.25-μm OV-101 capillary column, 30 mm × 0.25 mm i.d. mass spectrometer (HP 5970; Hewlett Packard). Quantification of LA and OA was achieved by single ion monitoring mode using d_2-OA as an internal standard. The following ions were used: 224 (d_2-OA), 264 (OA), and 294 (LA).

DNA Isolation and Etheno Adduct Analysis. The isolation of genomic DNA from buffy coats was achieved using the blood and cell culture Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol except with the modification of the supplied pH to 7.4 and the NaCl concentration in the elution buffer to 1.4 M. Some DNA samples were isolated using the hydroxylapatite method modified as described (18), yielding the same results as the Qiagen extraction procedure. εdA and εdC were analyzed in DNA by the immunoaffinity/^{32}P postlabeling method (18). In brief, ~25 μg of DNA was hydrolyzed to nucleotide 3′-monophosphates using micrococcal nuclease and spleen phosphodiesterase (Sigma-Aldrich). Normal nucleotides were quantitated by high-performance liquid chromatography, and the adducts were enriched on immunoaffinity columns prepared from the monoclonal antibodies EM-A-1 (εdA) and EM-C-1 (εdC). The antibodies used in this study were provided by Dr. Manfred Rajewsky (Institute of Cell Biology, University of Essen, Essen, Germany); their characteristics have been reported previously (19). The adducts and the internal standard deoxyuridine 3′-monophosphate was labeled with [γ-^{32}P]ATP (>5000 Ci/mmol) and T4 polynucleotide kinase (Amersham Buchler, Braunschweig, Germany and Pharmacia Biotech, Freiburg, Germany, respectively). The adducts were resolved on polyethyleneimine-TLC plates using two-directional chromatography [D1 = 1 M acetic acid (pH 3.5), D2 = saturated ammonium sulfate (pH 3.5)]. After autoradiogram, the adduct spots and the internal standard were marked, cut, and the radioactivity was measured in a liquid scintillation counter. The absolute adduct levels were quantitated using standards, and the relative adduct level per parent nucleotides were determined with the amount of deoxycytidine (dC) and deoxyadenosine (dA) obtained from high-performance liquid chromatography analysis as described (18).

Table 1 Range, mean, and median levels of etheno-adducts in WBC-DNA and of free fatty acid concentrations in serum of healthy female volunteers on a high (A) and low (B) LA intake; *P*s for group comparison

	High LA intake (>15 g/d) (A)	Low LA intake (<5 g/d) (B)
Age (yrs)		
Range	35–50	36–56
Mean	40.48 ($n = 21$)	45.38 ($n = 21$), $P = 0.02$
εdA/10^9 dA		
Range (*n*)	1–126 (21)	0.9–169 (21)
Median	6	32
Mean ± SD	28.2 ± 40.8	51.0 ± 53.3, $P = 0.08$
εdC/10^9 dC		
Range (*n*)	26–371 (20)[a]	9–671 (21)
Median	79	142
Mean ± SD	110.5 ± 95.4	175.9 ± 171.9, $P = 0.42$
LA intake [g/d] by questionnaire		
Range (*n*)	15.0–50.4 (21)	3.9–5.0 (21)
Median	17.4	4.7
Mean ± SD	19.6 ± 7.7	4.5 ± 0.5
LA in serum [μg/100 μl]		
Range (*n*)	0.8–11.9 (15)[b]	0.5–7.6 (19)[b]
Median	4.6	2.5
Mean ± SD	5.8 ± 4.1	3.1 ± 2.0, $P = 0.06$
OA in serum [μg/100 μl]		
Range (*n*)	2.5–32.1 (15)[b]	1.5–23.3 (19)[b]
Median	8.8	6.9
Mean ± SD	15.2 ± 9.8	9.6 ± 6.2, $P = 0.10$
Ratio LA:OA		
Range (*n*)	0.18–0.47 (15)[b]	0.2–0.56 (19)[b]
Median	0.36	0.32
Mean ± SD	0.37 ± 0.13	0.34 ± 0.09, $P = 0.10$

[a] For εdC, one aberrant value of 1555/10^9 dC was excluded.
[b] Serum samples were available for gas chromatography/mass spectroscopy for $n = 15$ (group A) and $n = 19$ (group B), respectively.

Statistical Methods. Statistical analysis of the data were performed with the SAS program version 6.12 (SAS Institute, Cary, NC). The comparison of groups was done using Wilcoxon's rank-sum test, and the correlation between the parameters was determined using the Kendall's tau b correlation coefficient.

Evaluation of Dietary Questionnaire Data. Women (1200) recruited for EPIC were principally eligible for the present investigation (see "Study Subjects and Biological Sample Collection"). The LA intake was computed from the FFQ with the evaluation program, which was developed specifically for the assessment of the intake of food constituents from the FFQ using the official German food composition data of the "Bundeslebensmittelschlüssel" published by the Federal Office of Health, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (reviewed in Ref. 16). On the basis of these data on LA intake, all of the eligible women with LA intake >15 g/day were selected for the "high intake" group; those with LA intake <5 g/day were selected for the "low intake" group.

Ethical Clearance. EPIC-Heidelberg is a random sample of the population of the Heidelberg area men ages 40–64 years

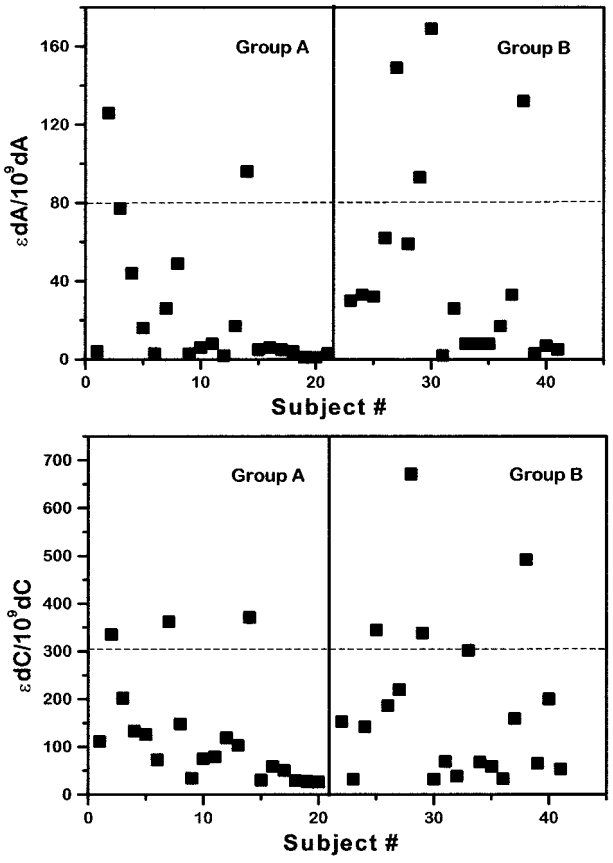

Fig. 1. Scatter diagrams of ϵdA and ϵdC levels in WBC-DNA of healthy female volunteers ($n = 41$). Several subjects had adduct levels more than twice the mean (----), *i.e.*, >300 ϵdC/10^9 dC ($n = 7$) and >80 ϵdA/10^9 dA ($n = 6$). *Group A*, >15 g LA/d; *Group B*, <5 g LA/d. One subject with an ϵdC level of 1555×10^{-9} dC was excluded as outlier; ϵdA level in this subject was 118×10^{-9} dA.

and women ages 35–64 years, respectively, who were invited to participate in a research project on diet and cancer. The participants agreed and signed a consent form to fill out a food frequency questionnaire and give a blood specimen for future biochemical investigations (14).

Results

Mean and median ϵ-DNA adduct levels and LA and OA concentration in serum of women in the high (A) and low (B) LA intake (yearly average) group are summarized in Table 1. In group A mean adduct levels were 28 ϵdA/10^9 dA and 111 ϵdC/10^9 dC; in the low intake group B the corresponding values were 51 and 176, revealing no statistically significant differences in adduction.

When analyzed by linear regression, ϵdA levels were positively correlated with those of ϵdC ($r = 0.71$; $P < 0.001$; $n = 41$). However, the mean ϵdC adduct level (178 ϵdC/10^9 dC) was significantly higher than that of ϵdA (40/10^9 dA). About one-third of all of the 41 samples had more than twice the mean adduct level than the rest, *i.e.*, was above 300 ϵdC/10^9 dC and 80 ϵdA/10^9 dA (Fig. 1). The highest adduction values were seen in 6 of the subjects (Fig. 1), but these levels were not correlated with either high LA daily intake or LA serum concentration or with any of the other parameters analyzed (Table 2).

Table 2 Dietary intake data of healthy female volunteers, classified according to high (>15 g/d) and low (<5 g/d) LA consumption (groups A and B, respectively)

Data were extracted from the EPIC questionnaire.	Means and *P*s are listed.		
	High LA intake (>15 g/d) ($n = 21$) (A)	Low LA intake (<5 g/d) ($n = 21$) (B)	*P*
Body mass index (kg/m^2)	28.1	23.5	$P = 0.02$
Energy intake (kJ/d) per kg	14484.2	5381.7	$P = 0.0001$
Total fat (g/d)	145.4	42.6	$P = 0.0001$
Saturated fatty acids (g/d)	53.7	17.7	$P = 0.0001$
Monounsaturated fatty acids (g/d)	47.0	13.7	$P = 0.0001$
α-Linolenic acid (g/d)	2.46	0.79	$P = 0.0001$
Polyunsaturated fatty acids (g/d)	23.5	5.7	$P = 0.0001$
Potato	110.3	56.5	$P = 0.008$
Vegetable	219.9	110.1	$P = 0.002$
Fruits	136.8	112.7	$P = 0.7$
Juice	163.4	114.2	$P = 0.2$
Vitamin C	177.2	79.8	$P = 0.0001$
Vitamin E	27.0	5.8	$P = 0.0001$
β-Carotene	6320.4	1852.8	$P = 0.0001$

Because of the possible role of hydroxylated estrogen metabolites as redox cycling agents that could increase oxidative damage and LPO, as hypothesized earlier (13, 20, 21), we have compared the 8 WBC-DNA samples from postmenopausal with those from premenopausal women ($n = 34$); no significant difference was seen between the mean ϵ-adduct levels in WBC DNA of both groups (data not shown).

When the free fatty acids were determined in serum by gas chromatography-mass spectroscopy (using d_2-OA as internal standard), the mean LA concentration (μg/100 μl) was different, amounting to 5.8 and 3.1 in groups A and B, respectively ($P = 0.06$). Thus, the questionnaire data on daily LA intake in groups A and B (as averaged over 1 year) predicted the actually measured LA serum concentration on a group but not at an individual level. Also, the serum OA concentration tended to be higher in the A group although not significantly (15.2 *versus* 9.6 μg/100 μl; $P = 0.1$). However, the ratios of LA:OA levels in serum were 0.35 ± 0.11 (all samples) and did not differ between the A and B groups (Table 1).

For exploratory analyses data on dietary intake and demographic data were extracted from the EPIC questionnaire and are tabulated (Tables 2 and 3). For a final statistical evaluation, the data from all of the subjects ($n = 42$) were included except for serum measurements ($n = 34$). In group A, where women were on average 5 years younger than in B ($P = 0.02$), the mean daily intake of the total calories was three times, saturated fatty acids and monounsaturated fatty acids was 3.5 times, and α-LA (an ϖ-3 PUFA) even 4 times higher than in group B. Whereas in the low LA intake group (B), the mean body mass index (23.5 kg/m^2) was in the normal range, group A women reached a mean value of 28 kg/m^2, which is already indicative of a health risk. A difference of energy intake expressed in kJ/kg body weight between groups A and B was observed and also statistically highly significant. The intake of vegetables, vitamins C and E, and β-carotene but not of fresh fruits was significantly different between groups A and B. None of the variables in Table 2 (except vitamin E and vegetable intake) had an apparent influence on group ϵ-adduct levels in WBC nor did smoking habits (analyzed on $n = 15$ smokers; $n = 14$

Table 3 Association of vegetable, fruit, and vitamin consumption with ε-DNA adduct levels in WBC of healthy female volunteers

Kendall's tau b correlation coefficients (significance level) and number of samples are listed.

Adduct level in WBC	Frequency of intake						
	Vegetables	Fresh fruits	Fresh juices	Potatoes	Vitamin C	Vitamin E	β-Carotene
εdA	−0.25706	−0.07160	−0.07421	−0.17724	−0.18227	−0.21950	−0.14203
	(0.0175)	(0.5080)	(0.4940)	(0.1013)	(0.0960)	(0.0424)	(0.1892)
	$n = 42$	42	42	42	41[a]	42	42
εdC	−0.20648	−0.06231	−0.04292	−0.11362	−0.09756	−0.18693	−0.10874
	(0.0576)	(0.5667)	(0.6940)	(0.2961)	(0.3758)	(0.0856)	(0.3174)
	$n = 41$	41	41	41	40[a]	41	41

[a] One outlier for εdC and one for vitamin C were excluded.

nonsmokers, data not shown) reveal an effect. From the FFQ, the intake of vegetables, fresh fruits, vitamins E and C, and β-carotene was calculated and ranked for women in groups A and B ($n = 40–42$). Using the Kendall's tau b correlation, an inverse correlation between ϵdA levels in WBC-DNA and vegetables or vitamin E consumption was observed (Table 3). A similar trend was seen for ϵdC but not quite reaching statistical significance. Fresh fruit, vitamin C, and β-carotene intake did not show an obvious relationship with DNA adduction.

Discussion

Miscoding ϵ-DNA adducts are formed through LPO of PUFAs, notably from ϖ-6 PUFAs, such as linoleic and arachidonic acid, yielding 4-hydroxyalkenals as intermediates. On epoxidation they can react with nucleic acid bases to form exocyclic etheno-base adducts, *e.g.*, ϵdA and ϵdC. For their quantitation in WBC-DNA we have applied our ultrasensitive detection method involving immunoaffinity clean-up and ^{32}P-postlabeling analysis (18). Using this method in a previously conducted, strictly controlled dietary study in female and male volunteers, we found unexpected high levels of ϵdA and ϵdC in WBC-DNA of females but not in males when they were on a 7-week high-sunflower oil diet (LA intake: >35 g/day = 12% energy). Adducts were not elevated in either gender when the diet was rich in rape seed oil containing mainly OA (11).

Comparison of results from the strictly controlled dietary study with those of our present study on female volunteers led us to the following conclusions that require confirmation in a larger cohort: (*a*) on the basis of the EPIC questionnaire data, the calculated mean daily intake of LA (averaged over 1 year) correlated with the LA concentration in serum on a group level but not on an individual level (Table 1); (*b*) although women in groups A and B differed significantly in their daily LA intake and LA serum concentration, no obvious difference in mean ϵ-adduct levels in WBC-DNA was apparent (Table 1); and (*c*) about one-third of the women in this study showed twice the mean background adducts level (Fig. 1); these high values were within the range of adduction, seen in the previous controlled dietary study; (*d*) healthy female volunteers from the Heidelberg area had an estimated mean daily LA intake of 19.6 g (group A) and 4.5 g (group B), which led to a higher mean serum LA concentration in group B (Table 1); (*e*) calculated mean daily intake of OA was also higher in group A than in B, which was reflected by the serum OA concentration; however, the LA:OA ratios in serum did not differ in groups A and B ($P = 0.3$) and were on average 0.35; and (*f*) because in our earlier controlled study volunteers had a high LA but low OA intake with a LA:OA ratio in serum of 1.12, we assume that this ratio of LA (or other ϖ-6 PUFAs) over OA (or other monounsaturated acids or ϖ-3 PUFAs) that is consumed daily is determining, at least in part, the ϵ-adduct levels in WBC-DNA on a free diet. Indeed, none of the dietary intake data or the other variables (Table 2) showed an apparent positive correlation with high DNA-adduction.

A recently completed feeding experiment in rats supports assumption (*f*): gavage of LA (but not of OA) led to high levels of ϵ-adducts in WBC-DNA of females but to little increase in males (22). Thus, these experimental results confirmed our findings in the controlled diet study in human volunteers, whereby the females seemed to be more susceptible to LPO-induced DNA damage, possibly as a consequence of interactions with hydroxylated estradiol metabolites; some may undergo redox-cycling, thereby causing reactive oxygen species production and increased LPO of membrane fatty acids (1, 13, 20). Therefore, it is conceivable that the outliers with the highest ϵ-DNA adduct levels (Fig. 1) are caused by variations in the individual levels and patterns of 2- to 4-hydroxylated metabolites.

There is supportive evidence for assuming a mechanistic link between oxidative stress/LPO-induced DNA damage, diet, and cancer risk, as summarized recently (1). For example, normal tissue adjacent to breast tumors from breast cancer patients had a significantly higher level of putative malonaldehyde-DNA adducts than those from cancer-free controls (23). Malonaldehyde, another major LPO-product, forms miscoding exocyclic deoxyguanosine adducts (2, 7, 24) as does HNE. Serum autoantibodies that recognize 5-hydroxymethyl-2′-deoxyuridine, an oxidized DNA base, served as predictive markers for breast and colorectal cancer risk in women (25). A Phase II chemoprevention trial (26) using the same serum marker demonstrated an inverse relationship between α-tocopherol plasma levels of female volunteers after a short-term dietary vitamin E supplementation. Another study in women on a Mediterranean diet showed no protection against 8-oxodG in WBC-DNA by plasma α-tocopherol and carotenoids (27). However, results from our pilot study in women indicated an inverse correlation of ϵdA levels in WBC with vitamin E or vegetable consumption calculated from the questionnaire (Table 3).

Taken together these results from studies with biomarker incorporation infer that a "mixture" of chemopreventive agents may be needed for full protection of the DNA against various types of oxidative and LPO-associated damage. Such a mechanism may apply for the Mediterranean diet, rich in cereals, fresh vegetables, and olive oil (mostly OA), that has been associated with lower risk of cancers of the colon, breast, bladder, and prostate (28, 29). We have described recently the presence of a range of powerful in part novel antioxidants in

olive oil (30, 31). Several studies now support the notion that the composition of fatty acids is more critical to colon cancer risk than is the total ingested dietary amount of fat (32). Our two studies in healthy women who ingested dietary fatty acids with different LA:OA ratios seem to support that the latter ratio is a partial determinant of LPO-derived DNA damage. This assumption is now being tested in a larger EPIC-related cohort.

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