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SHORT COMMUNICATION

Effects of basal level of antioxidants on oxidative DNA damage in humans

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Abstract *Background* Vitamins A, E and C, and uric acid, which can scavenge free radicals should also protect DNA from the damage. It is reasonable to assume that agents that decrease oxidative DNA damage should also decrease subsequent cancer development. *Aim of the study* A relationship between basal level of antioxidants (vitamins A, C and E and uric acid) and oxidative DNA damage was assessed. For the first time, the broad spectrum of oxidative DNA damage biomarkers: urinary excretion of 8-oxodG, 8-oxoGua and 5HMUra as well as the level of oxidative DNA damage in leukocytes was analyzed in healthy subjects (n = 158). *Methods* Using HPLC prepurification/isotope dilution GC/MS methodology, we examined the amount of oxidative DNA damage products excreted into urine and the amount of 8-oxodG in leukocytes' DNA (with HPLC/EC technique). The level of antioxidant vitamins and uric acid was estimated by HPLC technique with fluorimetric and UV detection. *Results* Analyses of relationship between the most common antioxidants (vitamins A, C, E and uric acid) and oxidative DNA damage products reveal weak, statistically significant negative correlation between retinol and all the measured parameters except 5HMUra. Vitamin C negatively correlates with urinary excretion of 8-oxodG and 8-oxoGua. Uric acid revealed statistically significant negative correlation with 8-oxodG in cellular DNA and urinary excretion of 5HMUra, while α-tocopherol correlates negatively only with 8-oxodG in cellular DNA. Good, significant ($P < 0.0001$), positive correlation ($r = 0.61$) was noted between urinary levels of the base, 8-oxoGua and the deoxynucleoside, 8-oxodG. *Conclusion* Our results suggest that oxidative DNA damage shows limited but significant response to antioxidants analyzed in this study and is more affected by many other cellular functions like antioxidant enzymes or DNA repair enzymes as well as genetics.

Key words oxidative DNA damage – DNA repair – antioxidant vitamins

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Introduction

Many epidemiological studies have reported inverse association between vegetable and fruit consumption and occurrence of cancer and other degenerative diseases [1]. One of the possible mechanisms of this protective effect is by the antioxidative activities of such plant food constituents as vitamins A, C and E. These antioxidant vitamins are effective free radical scavengers; therefore they should protect biomolecules such as DNA from oxidative damage. It is reasonable to assume that agents that decrease oxidative DNA damage should also decrease subsequent cancer development.

The most popular way of exploring antioxidant effects in humans is intervention studies with the detection of 8-oxo-7,8-dihydroguanine (8-oxoGua) of surrogate tissues such as white blood cells (WBC) and/or determination of urinary excretion of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG).

The background level of 8-oxoGua in cellular DNA represents a dynamic equilibrium between the rate of oxidative DNA damage formation, and the rate of repair in the specific tissue/cells studied. Several, highly redundant, repair processes exist to prevent 8-oxoGua persisting in DNA, a clear indicator of the importance this lesion has in disrupting genome stability. Normal metabolic processes can give rise to 8-oxoGua, and as a consequence, levels of this lesion can be detected in cells (so-called background levels). However, controversy surrounds the issue of exactly how much damage is present, not least due to the potential for damage to be formed during extraction of DNA from cells. The European Standards Committee on Oxidative DNA Damage (ESCODD) was formed to resolve the problems associated with the measurement of background levels of oxidative damage to DNA (in particular 8-oxoGua) in human cells. As a result of these endeavors, assays for this damage have become more precise and accurate [2]. Instead of measuring damage in specific cells, with concomitant problems such as artifact formation, a whole body burden of oxidative stress may be assessed by the measurement of urinary excretion of 8-oxoGua, and its deoxynucleoside equivalent 8-oxodG and 5-(hydroxymethyl)uracil (5HMUra) [3].

The analysis of 8-oxoGua in urine presents particular difficulties (i.e poor solubility can cause a loss of the analyte) [3] and until recently there has been no reliable assay for its detection. New techniques, based upon mass spectrometric detection (MS), have been developed which allow for the simultaneous determination of 8-oxodG, 8-oxoGua and 5HMUra in the same urine sample [4]. One such method involves HPLC prepurification followed by gas chromatography with isotope dilution MS detection [4]. In addition to unequivocal identification of the analyzed compounds and high sensitivity, the use of isotopically-labeled internal standards compensates for potential losses of the analytes during sample work-up [3].

Vitamins C, E and A are effective antioxidants in vitro, and might be expected to protect against cancer, but several large-scale antioxidant supplementation trials have failed to show any clear evidence for a decrease in cancer risk [5, 6]. On the other hand, numerous small- and medium-scale supplementation trials have focused on the influence of antioxidant supplements on oxidative DNA damage and in many (but not all) cases have shown a decrease in this biomarker [6].

In the present study, a relationship between basal level of antioxidants (vitamins A, C and E and uric acid) and oxidative DNA damage was assessed. Basal plasma levels of antioxidants would provide better estimation of antioxidant status than supplementation data, taking into account not only the consumption, which may reflect a transient state but also the absorption and utilization. To our best knowledge there were no comprehensive studies concerning an association between steady state level of antioxidant vitamins and several markers of oxidative DNA damage in humans.

For the first time, the broad spectrum of oxidative DNA damage biomarkers: urinary excretion of 8-oxodG, 8-oxoGua and 5HMUra as well as the level of oxidative DNA damage in leukocytes, was analyzed. The subjects in the study group had similar dietary habits.

Materials and methods

Subjects

The subjects consisted of 158 healthy male (n = 71) and female (n = 87) individuals, with a median age 56 years (range 26–87 years). The group comprised 71 smokers and 87 non-smokers. The subjects who declared vitamin supplementation were excluded from the study. An additional criterion was reported lack of history of any chronic disease, which may be linked with oxidative stress.

All the subjects were asked to fill up the dietary questionnaire. Interviewers were asked to estimate average frequency of consumption of various dietary items in the year previous to interview. The majority of them reportedly consumed three servings of fruit and vegetables and about 250 g of meat and fat per day. Average body weight of the patients was 75.7 ± 8.34 kg (average Body Mass Index was 24.37 ± 3.91).

The blood and urine were taken before their medical examination. Spot urine samples were collected. The exclusion criteria for the healthy subjects were symptoms of any disease. The study was approved by the medical ethics committee of The L. Rydygier Medical University, Bydgoszcz, Poland, (in accordance with Good Clinical Practice, Warsaw 1998) and all the persons gave informed consent.

Methods

Determination of urinary excretion of 8-oxodG, 8-oxoGua and 5HMUra

Urine sample preparation, HPLC purification and GC/MS analysis were conducted as described earlier [7].

Determination of plasma vitamins A, E, C and uric acid concentration by HPLC

Quantification of vitamin E (α-tocopherol), vitamin A (retinol), vitamin C (ascorbic acid) and uric acid by HPLC technique was described previously [8].

Isolation of leukocytes from venous blood

Venous blood samples from the patients were collected. The blood was carefully applied on top of Histopaque 1119 solution (Sigma-Aldrich, St. Louis, MO) and leukocytes were isolated by centrifugation according to the procedure laid down by the manufacturer.

DNA isolation and 8-oxodG determination in DNA isolates

DNA from leukocytes was isolated as described [2]. Quantification of 8-oxodG and 2′-deoxyguanosine (dG) by the mean of HPLC/UV/ECD technique was described previously [9].

Statistical analysis

All results are expressed as mean ± SD. The STATISTICA (version 6.0) computer software (StatSoft, Inc, Tulsa, OK, USA) was used for the statistical analysis. The correlations were analyzed using the Pearson correlation coefficients (r). Statistical significance was considered at $P < 0.05$.

Results

Analyses of relationship between the most common antioxidants (vitamins A, C, E and uric acid) and oxidative DNA damage products reveal weak, statistically significant, negative correlation between retinol and all the measured parameters except urinary excretion of 5HMUra (Fig. 1A–C). The respective r values were; with 8-oxodG in cellular DNA −0.26 ($P < 0.001$), urinary 8-oxodG −0.29 ($P < 0.02$) and 8-oxoGua −0.35 ($P < 0.001$). Vitamin C correlates significantly, negatively with urinary 8-oxodG and 8-oxoGua with r values −0.37 ($P < 0.001$) and −0.27 ($P < 0.007$) (Fig. 2A,B). Uric acid revealed statistically significant negative correlation with 8-oxodG in cellular DNA and urinary excretion of 5HMUra with respective r values −0.24 ($P < 0.002$) and −0.33 ($P < 0.05$) (Fig. 2C,D), while α-tocopherol correlates significantly, negatively only with 8-oxodG in cellular DNA ($r = -0.24$; $P < 0.002$) (Fig. 1D). There was strong, significant positive correlation between retinol and vitamin E, while relationship between the other antioxidants reveal poor correlations (Table 1).

Good, significant ($P < 0.0001$) positive correlation ($r = 0.61$) was noted between urinary levels of the base, 8-oxoGua and the deoxynucleoside, 8-oxodG (Table 1).

The mean levels of all the measured antioxidant vitamins were significantly lower in smokers in comparison with non-smokers. The mean levels of the analyzed oxidative DNA damage markers were significantly higher in smokers when compared with non-smokers, except urinary excretion of 5HMUra. (Table 2).

Discussion

There is considerable circumstantial evidence that oxidative DNA damage may be used as a marker predictive of cancer development later on (reviewed in [10, 11]). However, it should be remembered that most of these data come from case-control studies where more oxidative damage is found in cancer patients and could be an effect rather than a cause of disease. If we assume that oxidative DNA damage contributes to cancer development then agents that decrease the level of this damage should decrease the risk of cancer. Therefore, it is important to know factors which may influence oxidative DNA damage. It is also important to know which biomarker of oxidative DNA damage can be used to address the above mentioned points.

Antioxidant components like vitamins A, E and C, and uric acid, which can scavenge free radicals should also protect DNA from the damage. The most popular way of exploring antioxidant effects in humans is supplementation trials. However, a summary of these kinds of studies reported in a review of Moller and

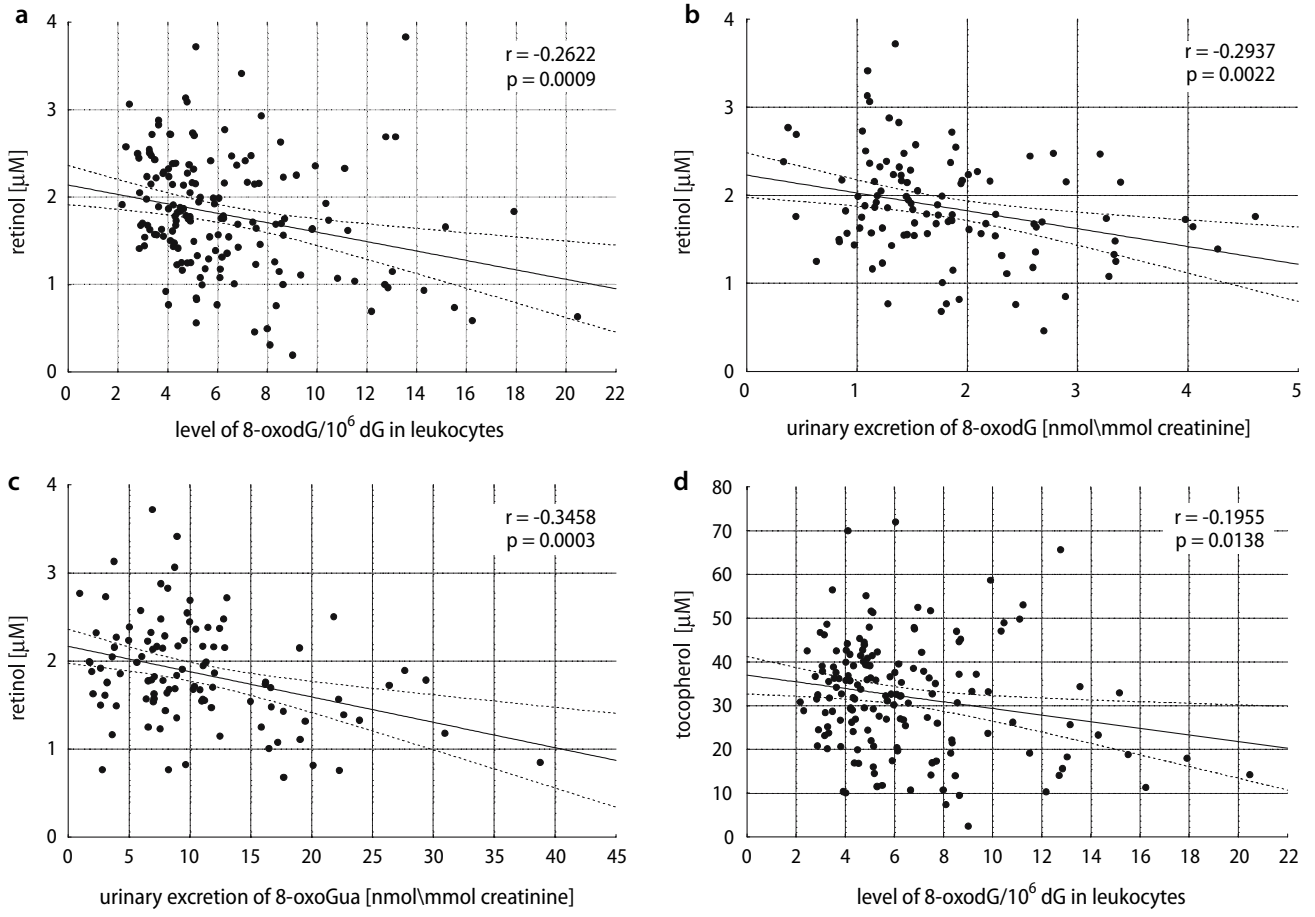

Fig. 1 Relationship between plasma antioxidant concentrations: retinol versus 8-oxodG in cellular DNA (**a**); retinol versus urinary excretion of 8-oxodG (**b**); retinol versus urinary excretion of 8-oxoGua (**c**) and tocopherol versus 8-oxodG in cellular DNA (**d**)

Loft [6] showed that half the works reported protective effects, while the rest showed no effect. It is hard to explain why in some intervention studies beneficial effects were reported whereas in others no effect was described. (It is important to note, however, that in none of the studies reviewed were harmful effects detected). It is possible that the main reason for the discrepancies may be that the effects of antioxidants are too short-lived to be detected during intervention studies [6]. It should also be remembered that supplementation of the individuals who are deficient in antioxidants might be beneficial. On the other hand the same supplementation might not show any effect in the case of individuals who have "sufficient" level of the micronutrient [6, 12].

Despite the fact that study subjects have similar dietary habits (according to dietary questionnaires), substantial differences in the vitamin concentrations among study subjects were observed. The range of plasma concentration for α-tocopherol (2.5–72 μM) ascorbic acid (3.7–139 μM) and retinol (0.2–3.8 μM), comprise sufficient span representing close to deficiency and saturation. It is possible that the questionnaires do not fully reflect the diet. However, it may only partially explain the substantial differences in vitamin level.

The differences in antioxidant levels are likely to reflect more the balance between absorption and tissue secretion. Therefore, they may be, at least partially, genetically determined [13–15].

Despite the fact that vitamin C and uric acid are the strongest determinants of plasma antioxidant capacity, their effect on oxidative DNA damage biomarkers was similar to that of vitamins E and A which contribute to a much lower extent to antioxidant potential. It is possible that other food components which can accompany vitamins A and E like polyphenols and phytochemicals may be partially responsible for the observed effects. Surprisingly vitamin A with the lowest antioxidant potential has the strongest effect

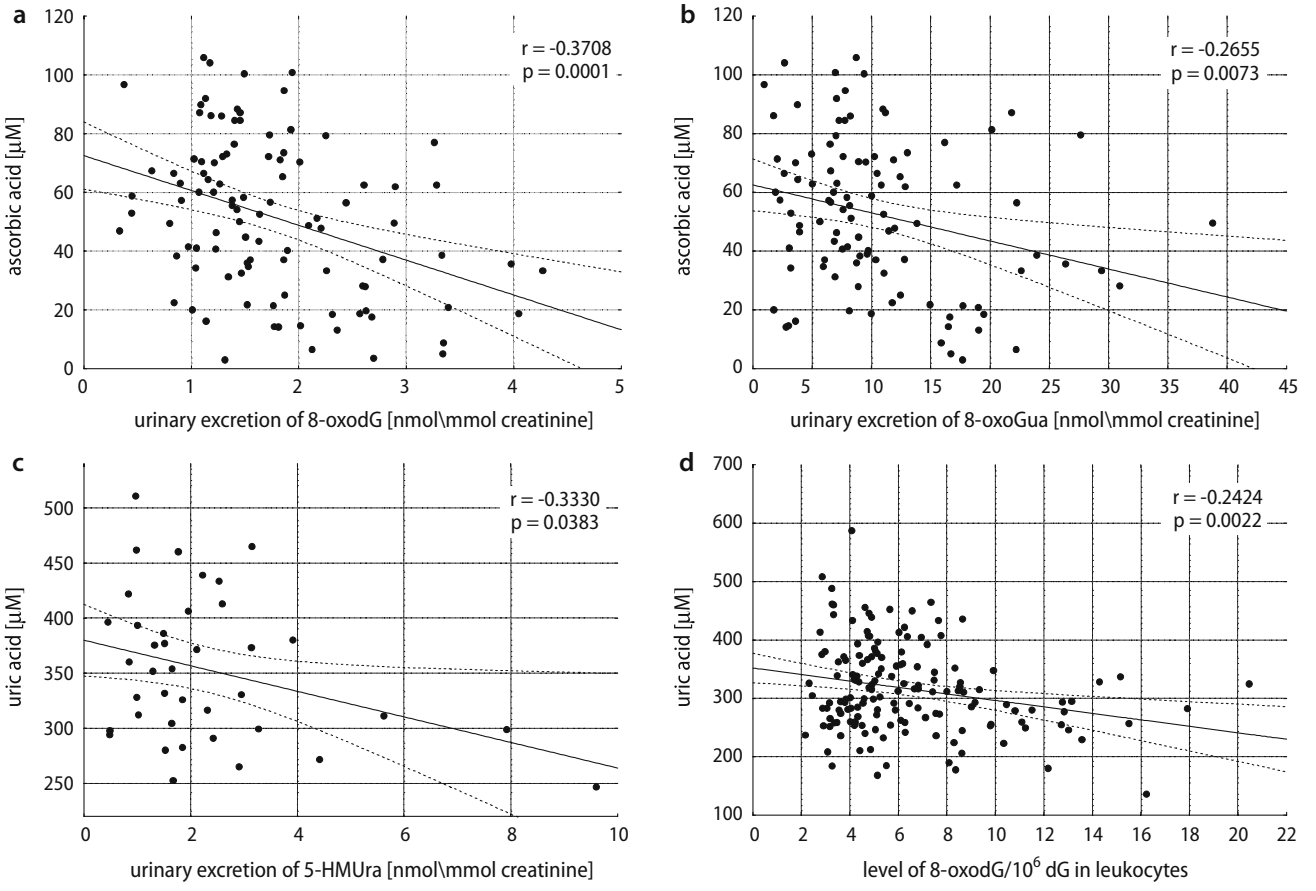

Fig. 2 Relationship between plasma antioxidant concentrations: ascorbic acid versus urinary excretion of 8-oxodG (**a**); ascorbic acid versus urinary excretion of 8-oxoGua (**b**); uric acid versus urinary excretion of 5-HMUra (**c**) and uric acid versus 8-oxodG in cellular DNA (**d**)

Table 1 Correlation of oxidative DNA damage and plasma antioxidants

	8-oxoGua in urine	8-oxodG in urine	5HMUra in urine	Vitamin C	Uric Acid	Vitamin E	Vitamin A
8-oxodG in leukocytes	*r* = 0.21 *N* = 97 *P* = 0.0392	*r* = 0.20 *N* = 98 *P* = 0.0423	*r* = 0.07 *N* = 38 *P* = 0.6937	*r* = −0.08 *N* = 154 *P* = 0.351	*r* = −0.24 *N* = 157 *P* = 0.002	*r* = −0.19 *N* = 158 *P* = 0.014	*r* = −0.26 *N* = 158 *P* = 0.001
8-oxoGua in urine		*r* = 0.61 N = 95 *P* = 0.0001	*r* = 0.12 N = 38 *P* = 0.4725	*r* = −0.26 N = 101 *P* = 0.0071	*r* = −0.16 N = 103 *P* = 0.108	*r* = −0.19 N = 105 *P* = .0053	*r* = −0.35 N = 105 *P* = 0.0001
8-oxodG in urine			*r* = 0.17 *N* = 36 *P* = 0.3077	*r* = −0.37 *N* = 101 *P* = 0.0001	*r* = −0.01 *N* = 104 *P* = 0.9131	*r* = −0.16 *N* = 106 *P* = 0.103	*r* = −0.29 *N* = 106 *P* = 0.0021
5HMUra in urine				*r* = 0.06 *N* = 39 *P* = 0.7072	*r* = −0.33 *N* = 39 *P* = 0.0381	*r* = −0.02 *N* = 40 *P* = 0.8961	*r* = −0.09 *N* = 40 *P* = 0.5692
Vitamin C					*r* = −0.19 *N* = 154 *P* = 0.0181	*r* = 0.10 *N* = 154 *P* = 0.1986	*r* = 0.14 *N* = 154 *P* = 0.0893
Uric Acid						*r* = 0.23 *N* = 156 *P* = 0.0039	r = 0.28 *N* = 156 *P* = 0.0004
Vitamin E							*r* = 0.56 *N* = 158 *P* = 0.0001

Table 2 Levels of oxidative DNA damage and serum antioxidants (average and SD)

	All subjects			Non-smokers			Smokers			Non-smokers versus smokers (Student *t*-test)
	Average	S.D.	*N*	Average	S.D.	*N*	Average	S.D.	*N*	*P*
8-oxodG in leukocytes [8-oxodG/10^6 dG]	6.34	3.2798	158	5.49	2.4735	87	7.39	3.8211	71	0.00023
8-oxoGua in urine [nmol/mmol creatinine]	10.43	7.0499	97	8.08	4.8279	59	14.08	8.3612	38	0.00002
8-oxodG in urine [nmol/mmol creatinine]	1.77	0.8187	98	1.49	0.5191	57	2.15	0.9929	41	0.00004
5HMUra in urine [nmol/mmol creatinine]	2.42	1.8787	38	2.35	1.9836	29	2.63	1.5762	9	0.69916
Vitamin C [μM]	53.54	26.3317	154	61.16	25.9462	86	43.89	23.6700	68	0.00003
Uric Acid [μM]	316.76	75.3882	156	324.20	77.6960	86	307.62	71.9502	70	0.17269
Vitamin E [μM]	32.20	12.6828	158	35.45	11.0415	87	28.21	13.4801	71	0.00029
Vitamin A [μM]	1.79	0.6753	158	1.94	0.6336	87	1.61	0.6834	71	0.00173

on all the measured biomarkers with the exception of 5HMUra. It is likely that the effect is exerted via regulation of antioxidant enzymes like SOD1 and SOD2 [16], which can neutralize ROS and protect DNA from oxidative damage. Similarly Collins et al. [17] reported a significant negative correlation between basal concentration of total serum carotenoids and oxidative DNA damage measured as endonuclese III sensitive sites, in human lymphocytes. They did not find similar association with concentrations of vitamin C. It is possible that vitamin A is just a particularly good indicator of total intake of antioxidants, or of fruit and vegetables.

Our data indicate that oxidative DNA damage excretion in urine is a better marker to study the protective effect of antioxidant than background level of oxidative DNA damage in leukocytes. It is consistent with the overall assessment of the effects of antioxidant supplementation on urinary excretion of 8-oxodG which demonstrated that in majority of studies protective effect was reported (although it should be remembered that so far antioxidant intervention studies have only been performed with urinary 8-oxodG) [6, 18]. Good correlation between urinary 8-oxodG and 8-oxoGua (Table 1) supports the hypothesis that the lesions may be a products of DNA repair [3] (although the repair system which yields 8-oxodG has not yet been identified). In agreement with earlier reports (for review see [19]) our data demonstrated that tobacco smoking is responsible for oxidative stress/oxidative DNA damage.

Summing up, our results suggest that oxidative DNA damage show limited but significant response to antioxidants analyzed in this study. It is likely that the damage is more affected by many other cellular functions like antioxidant enzymes or DNA repair enzymes as well as genetics.

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