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Methods of Nutritional Biochemistry

Determination of human plasma and leukocyte ascorbic acid by microtiter plate assay

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Methods for analyzing ascorbic acid (AA) in human blood are often laborious, difficult, expensive, and require the use of large sample volumes. The lack of an inexpensive reliable method is particularly disconcerting for leukocyte analysis. We have overcome these concerns by developing a microtiter plate assay (MPA) to measure the AA dinitrophenylhydrazine (DNPH) derivative (515 nm and 562 nm) in plasma or leukocytes. Plasma and leukocytes were isolated from whole blood by gradient sedimentation using Histopaque 1077 and 1119. The AA contribution from red blood cells was <0.01%. The incubation time for AA DNPH derivative formation was reduced to 50% of the time used by investigators cited in previously published papers with no significant loss in recovery (for 2 hr incubation, >90%). AA values by our MPA method using human subjects agreed with published values or when compared with standard spectophotometic assays. The advantages of the MPA assay over traditional methods are that it can handle small sample volumes and multiple samples simultaneously. In addition, the MPA method requires using smaller volumes of hazardous reagents and can be done in less time with less blood. (J. Nutr. Biochem. 7:179–183, 1996.)

Keywords: ascorbic acid; vitamin C; leukocyte; microtiter; antioxidant

Introduction

Many published techniques and procedures for the determination of ascorbic acid (AA) levels are available.[1–4] In general, the methods used are time-consuming, cumbersome, and require large sample volumes and large amounts of hazardous reagents.[2,3] Many methods deal only with plasma or serum AA levels, which best reflect the recent intake of AA, and have little correlation with tissue stores.[1,5–7] An even more serious drawback is that these methods usually lack the sensitivity to deal with the limited sample sizes that are usually available from subjects for assessment purposes.

We have focused our attention on the leukocyte analysis of AA in an attempt to provide a better understanding of whether it reflects the body's nutritional status

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of the vitamin.[8–13] Therefore, a micromethod with the capability of handling multiple samples would be extremely useful. The advent of the microtiter plate assay (MPA) has provided the capability of multiple sample readings in microliter amounts. We have developed a microtiter assay to measure the AA content in human plasma and leukocytes using the 2,4-dinitrophenylhydrazine (DNPH) reaction with ketonic groups of dehydroascorbic acid. The MPA technique is a convenient method for measuring AA levels in plasma or leukocytes. It provides the sensitivity required when using limited amounts of sample (1 mL of whole blood) and the added advantage of running multiple samples simultaneously. We have also examined the effects of a shortened incubation time and a reduced number of lysis steps on AA recovery. We also compared levels of AA employing the MPA method against the standard spectrophotometric assays usually employed.

Methods and materials

Chemical and reagents

Histopaque 1119 and 1077, used for leukocyte preparation, were obtained from Sigma Chemical Co. (St. Louis, MO USA). Blood collection equipment, chemicals, and reagents were obtained from Fischer Scientific Co. (Santa Clara, CA USA). AA standards were prepared fresh daily.

Instrumentation

Untreated 96-well polystyrene microtiter plates were obtained from Fisher Scientific Co. The Microtiter Plate Reader used was a BIO-TEK, Model El 340 (BIO-TEK Instruments, Inc., Winooski, VT USA). The spectrophotometer was a Model DV-64 (Beckman Instruments, Inc., Fullerton, CA USA), and a Marathron Model 21K/Br table-top refrigerated centrifuge (Fisher Scientific Co.) was also used.

Blood collection and preparation

All work was done in a darkened room to reduce light-induced degradation of AA derivatives. Whole blood was drawn from the antecubital vein of healthy volunteers into 10 mL vacutainer heparinized tubes. Blood was obtained from healthy volunteers using approved procedures outlined in our antioxidant protocol, approved by the Biomedical Human Subjects Committee, University of Nevada, Reno. The volunteers were nonsmokers who had no chronic illnesses and consumed their usual diets prior to venipuncture. One milliliter of blood was removed and saved for the separation of leukocyte fractions. The remaining blood was immediately centrifuged at 1,500 *g* for 15 min at 4°C as illustrated in *Figure 1*. Five milliliters of the plasma layer was transferred to another centrifuge tube with an equal volume of freshly prepared 10% trichloroacetic acid (TCA). Each tube was vortexed for 3 min and centrifuged at 1,500 *g* for 15 min at 4°C. The supernatant was removed, and aliquots were used to compare the AA content using spectrophotometric (SP)[2] and MPA methods.

The leukocyte-rich fraction was prepared by using a modification of a previously reported procedure[14] and is illustrated in *Figure 2*. First, 500 μL of Histopaque 1119 was pipetted into the bottom of a 2 mL microcentrifuge tube with a screw top cap. Then 500 μl of Histopaque 1077 was carefully layered on top of the 1119 layer followed by 1 mL of heparinized whole blood (*Figure 2*). The tube was capped and centrifuged at room temperature at 700 *g* for 30 min. One hundred microliters of the plasma layer was removed and extracted with an equal volume of 10% TCA by vortexing for 3 min. This was followed by centrifugation at 10,000 *g* for 20 min at 4°C. One hundred microliters of the resulting supernatant was removed and placed in a microcentrifuge tube, capped, and kept at 4°C in the dark until analysis. The mononuclear/platelet/granulocyte layers were removed together and transferred into a 15 mL plastic centrifuge tube. Three milliliters of 150 mM phosphate-buffered saline containing 20 U/mL of sodium heparin (PBS/heparin), pH 7.2, was added to each tube, mixed gently, and centrifuged at 700 *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 1 mL of PBS/heparin and transferred to a 1.5 mL plastic microcentrifuge tube. The tubes were centrifuged at 700 *g* for 10 min at 4°C, the supernatant discarded, and the pellet resuspended and washed again. Erythrocytes in the leukocyte preparations were lysed by the addition of 900 μL of ice-cold distilled water containing 20 U/mL of sodium heparin. Pellets were resuspended and mixed gently for 10 sec, and 100 μL of 1.5 M NaCl was immediately added to restore isotonicity. The pellets

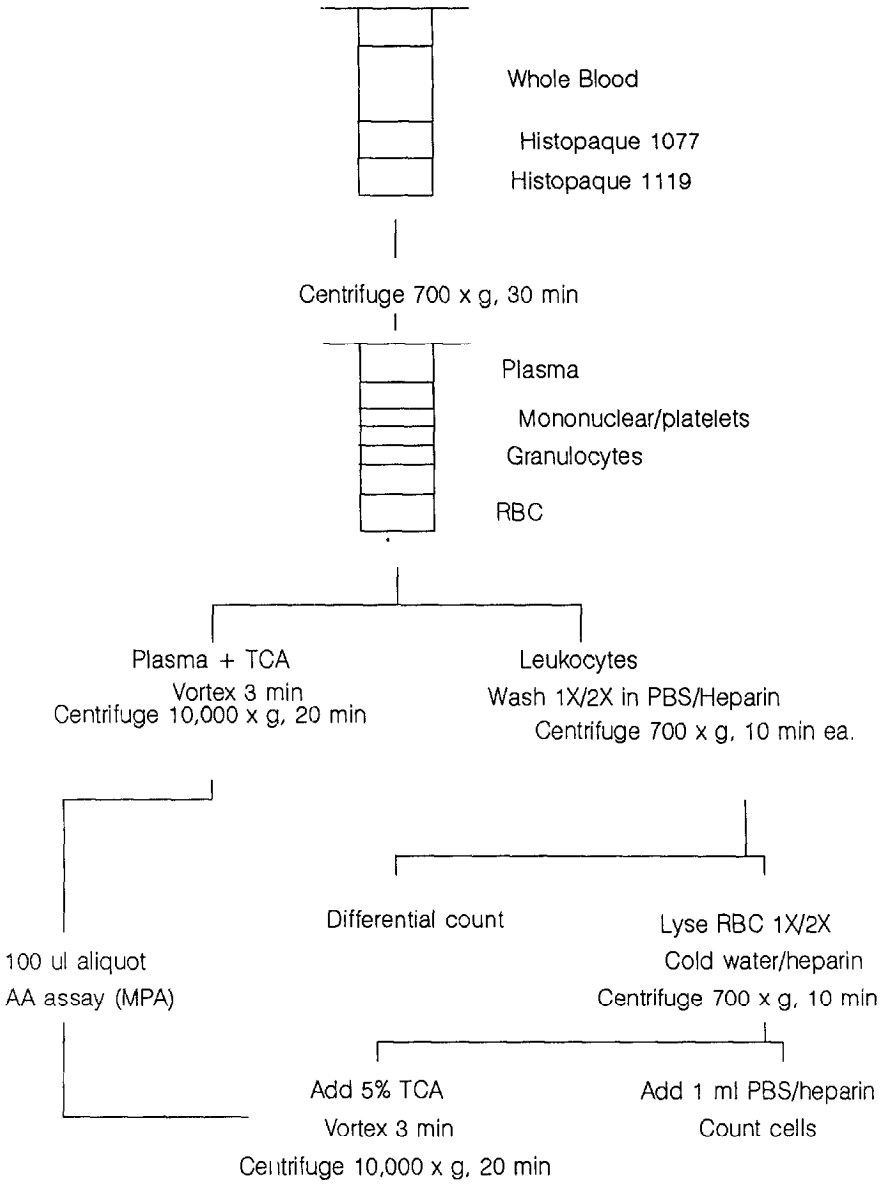

Figure 1 Isolation of leukocytes from human blood.

were then centrifuged at 700 *g* for 10 min at 4°C. To test the effects of multiple lysis steps on AA recovery, the procedure was repeated a second time on duplicate samples. One hundred microliters of 5% TCA was added to the resultant pellets, vortexed for 3 min, and centrifuged at 10,000 *g* for 20 min at 4°C. The supernatant was assayed for AA. In duplicate samples, the pellets were resuspended in 1 mL of PBS/heparin, and 250 μL was transferred to microscope slides, fixed with methyl alcohol, stained using the method of Wright, and WBC differentially counted. The total WBC was counted on 20 μL of resuspended sample using a hemacytometer.

Ascorbic acid analysis

Standard AA solutions varying from 0.0 to 2.0 μg of ascorbic acid/100 μL were determined in triplicate. Either 500 or 100 uL of TCA-stabilized samples were used for the SP or the MPA, respectively. The colorometic assay was a modification of the DNPH assay.[2,15] Briefly, a DNPH/thiourea/copper (DTC) solution was added to all samples: 100 μL to glass test tubes for the spectrophotometric assay and 20 μL to the microplate and leukocyte microcentrifuge tubes. All samples were vortexed gently, covered with aluminum foil to protect them from extraneous light, and placed in a water bath at 37°C for 2 and 4 hr. Samples were vortexed every 30 min during the incubation period. After the incubation period, samples were placed on ice and 750 μL, 150 μL, and 150 μL of ice-cold 65%

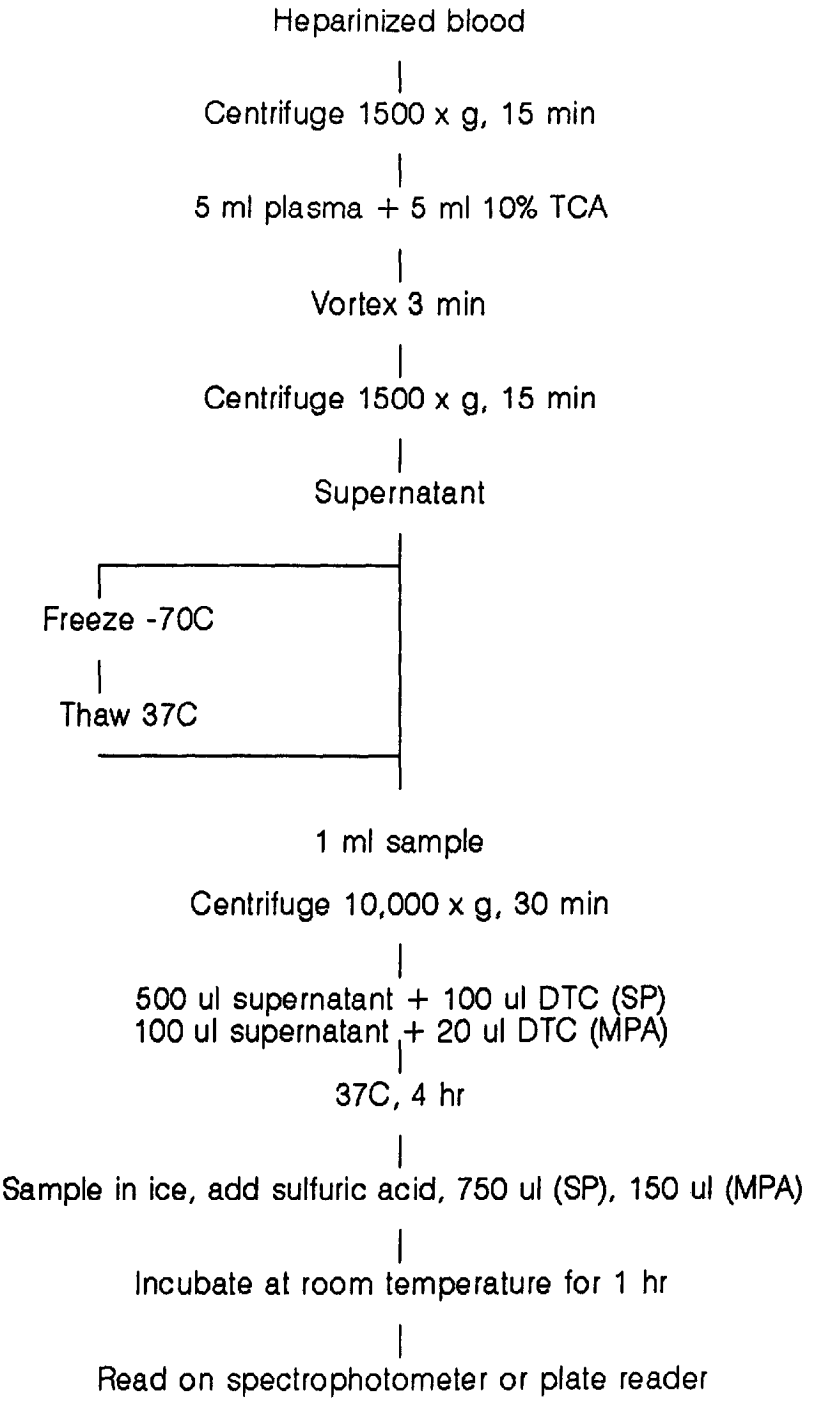

Figure 2 Preparation of blood fractions for AA analysis.

H_2SO_4 were added to glass test tubes for SP assay, MPA, and leukocyte microcentrifuge tubes, respectively. All samples were vortexed gently and placed in the dark at room temperature for 1 hr with gentle vortexing after the first 30 min. Samples for the spectrophotometric assay were read on a Beckman DU-64 spectrophotometer at 515 nm. The samples for microplate assay and leukocyte samples were transferred to a 96-well microtiter plate and then read on a BIO-TEK, Model EL340, microtiter plate reader at 515 nm and 562 nm (*Figure 1*).[16]

Statistical methods

The data in this study are presented as mean ± standard deviation (SD), coefficient of variation (CV) in percent, or as linear regression values (with differences considered statistically significant at $P < 0.05$ level).[17]

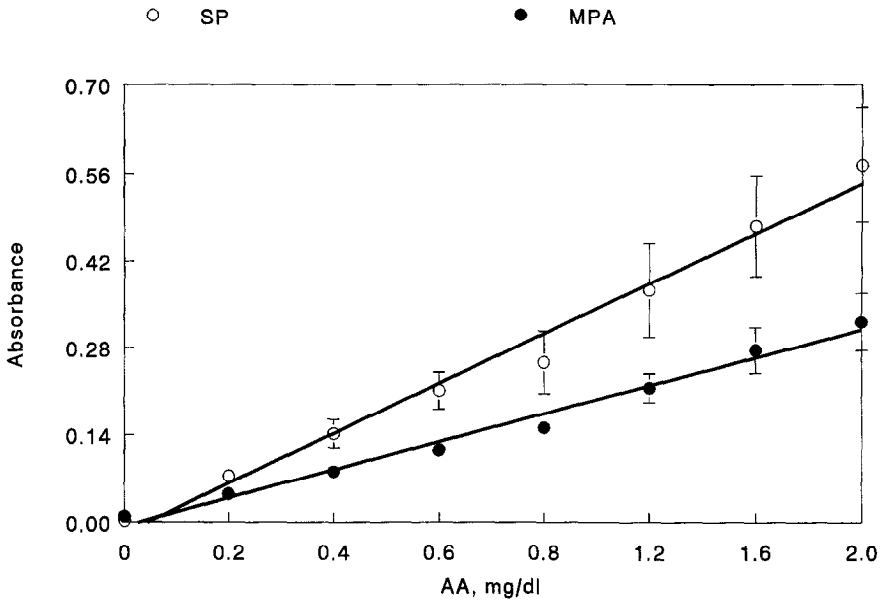

Figure 3 Relationship between absorbance and AA concentration using the SP or MPA method (SP: $r^2 = 0.999998$, $P < 0.001$; MPA: $r^2 = 0.999527$, $P < 0.001$).

Results

Figure 3 illustrates the linear relationship between absorbance and an increasing concentration of AA in pooled human plasma as determined by either the SP or the MPA methods. Both methods were linear over the range of AA concentrations tested (0 to 2 mg/dL). Linear correlation between absorbance and the AA concentration using the SP method was $r^2 = 0.999998$, $P < 0.001$. Linear correlation between absorbance and the AA concentration using the MPA method was $r^2 = 0.999527$, $P < 0.001$.

Comparisons between the SP assay and the MPA for recovery of AA in plasma are documented in *Table 1*. The range of recovery for the SP assay was 95 to 114%, while the range of recovery for the MPA was 95 to 123%. We found similar agreement for experiments examining the recovery of a lower AA concentration of 0.1 to 2 mg/dL. Within subjects, the AA content using the spectrophotometric assay and the microtiter plate assay was very similar, although they tend to be approximately 10% lower in the microplate method which reflects the differences of the optics of the two methods, i.e., dual wavelength versus single wavelength.[18]

The recovery of AA in isolated leukocytes as determined by the MPA method is shown in *Table 2*. Recoveries were between 81% and 118%, and there did not seem to be any relationship between the monocyte/granulocyte ratios and AA recovery.

There was no significant effect using the shorter incubation time on the accuracy of AA analysis for the range of AA concentrations tested. Differences be-

Table 1 Recovery of AA in plasma: Comparison in SP assay and MPA

Method	AA addition (mg/dl)	Recovery (%)		Range of CV within samples
		Range	Mean ± SD	
SP	25	95 to 107	101.8 ± 4.38	0.8 to 7.5
	50	99 to 114	105.8 ± 5.59	0.5 to 5.0
MPA	25	95 to 117	103.2 ± 8.38	1.4 to 5.0
	50	97 to 123	106.0 ± 9.95	1.9 to 7.8

Abbreviations: Spectrophotometric method using DNPH (SP); microtiter plate (MPA) method; ascorbic acid (AA); coefficient of variation (CV); standard deviation (SD). $n \leq 5$/set.

Table 2 Recovery of AA in isolated leukocytes using the MPA

Sample	AA addition (ng/dl)	Percent monocytes/ granulocytes	Recovery %	Recovery CV
1	100	86/14	99	5.8
1	200	86/14	81	2.0
2	100	90/10	99	11.0
2	200	90/10	101	7.9
3	100	93/7	100	16.1
3	200	93/7	97	5.6
4	100	86/14	100	13.6
4	200	86/14	92	4.4
5	100	90/10	118	15.0
5	200	90/10	93	10.5

Pooled samples		
AA addition (ng/dl)	Recovery (%)	
	Range	Mean ± SD
100	99 to 118	103.2 ± 8.29
200	81 to 101	92.8 ± 7.5

Abbreviations: Microtiter plate (MPA) method; ascorbic acid (AA); coefficient of variation (CV); standard deviation (SD).

tween the AA concentration after 2 and 4 hr of incubation were within 10%, and the recovery of AA was above 90%.

The effects of washing and erythrocyte lysis are shown in *Tables 3* and *4*. Washing the sample twice dramatically reduced erythrocyte contamination, and there was no significant loss of AA with repeated washings. Lysing of erythrocyte was optimal after one lysis step, whereas further lysing resulted in less AA. The combination of two washes and one lysis resulted in a maximum reduction of erythrocyte contamination and a minimal loss of white blood cells (WBC).

Discussion

We have employed the MPA method extensively in our laboratory in the analysis of plasma and leukocyte specimens. Results from using the MPA method were comparable to the well-established DNPH SP assay and correlate well with AA values found using an HPLC method (unpublished data) for reduced AA in biologic samples. Sample recoveries are in the 90% range for sample-to-sample variation and are no greater than 10%. We have also shown that the incubation time for the MPA method can be reduced from 4 to 2 hr within the range of 0.1 to 2 mg/dL.

The microtiter plate assay for AA is linear over the range from 0 to 2 mg/dL, which is adequate for blood plasma or isolated leukocyte analyses. Once AA has

Table 3 Effect of washing on leukocyte AA content

Treatment	AA content* (μg/ml)
Wash 1	0.361 ± 0.098
Wash 2	0.425 ± 0.130
Wash 3	0.405 ± 0.168

*Mean ± SD (n = 5). There were no significant differences within the three washes.
Abbreviation: ascorbic acid (AA).

Table 4 Effect of hypotonic lysis on leukocyte AA content and WBC and RBC count

Treatment	AA content		WBC	RBC
	μg/ml	μg/10^8	$\times10^4$/ml	$\times10^4$/ml
None	0.506 ± 0.115*	65 ± 30	70 ± 19*	84 ± 37*
Lysing 1	0.401 ± 0.135†	58 ± 26†	59 ± 16†	9 ± 7*†
Lysing 2	0.137 ± 0.077*†	35 ± 20*†	41 ± 7*†	2 ± 2*†

Mean value ± SD ($n = 5$). Abbreviations: ascorbic acid (AA), white blood cells (WBC), red blood cells (RBC). Mean value with identical superscripts are significantly different ($P < 0.05$).

been extracted, samples are stable for several months frozen at −70°C[1] and for at least 8 hr at room temperature.[17]

AA is an essential nutrient for humans and other animals.[1,8,9,19] Its importance in scurvy has long been recognized; however, its exact biochemical role still remains to be determined. AA has a role in collagen synthesis and has been implicated in other cellular reactions and processes including iron absorption and metabolism, steroidal synthesis, carnitine metabolism, and drug metabolism. In addition, AA is an excellent antioxidant and seems to be inversely correlated with the incidence of several serious diseases related to oxidative damage, e.g., cardiovascular disease, cancer, and immune system dysfunction. Therefore, it is important to be able to have an easy, relatively fast, and inexpensive method for assessing AA status both for health-related functions and perhaps for determining the optimal requirements of the vitamin.

The AA content of plasma and leukocyte has been studied in healthy and unhealthy populations.[1,3,8,9] It is thought that the plasma or serum AA content reflects recent intake of the nutrient rather than body stores.[5–7] Because of the simplicity and reliability of plasma AA determinations, plasma AA is preferable for identifying individuals at risk of AA deficiency due to chronic low AA intakes; but plasma AA is less useful for defining the AA status of individuals consuming adequate or high AA intakes. Plasma AA levels reflect the metabolic turnover of the vitamin; however, correlations between leukocyte and plasma AA levels suggest that the leukocyte content reflects the amount of AA available for storage.[1,7,19] Therefore, measuring the leukocyte AA content is a valuable tool for that nutrient's assessment.

A few studies have demonstrated the distribution of AA in various cellular or extracellular constitutents of the blood.[6,7] The AA content of cells was higher in mononuclear leukocytes and granulocytes than in platelets or erythrocytes. Leukocyte AA generally correlated very well with body pool or the tissue content of AA during depletion and repletion.[20] Erythrocytes have a limited ability to concentrate AA over the normal range of plasma AA. The AA contents of granulocytes, platelets, and erythrocytes correlate positively with plasma concentrations of AA in the supplementation or depletion ranges. These studies point to the need for further investigation. In addition, we intend to examine whether it is logistically feasible to run AA incubations with reagent directly in the microtiter plate wells, thereby decreasing other laboratory glassware requirements and labor.

The MPA for plasma and leukocyte AA is sensitive, accurate, and a useful tool for assessing the vitamin's status. In addition, we have reduced the incubation by 50% and have found that the total sampling number is only limited to the number of wells in the microtiter plate. We will be using this method to examine the impact of dietary AA on leukocyte AA in future population studies and to elaborate on the findings from limited studies in this area.

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