Synthetic as compared with natural vitamin E is preferentially excreted as α-CEHC in human urine: studies using deuterated α-tocopheryl acetates

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Abstract α-CEHC (2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman) is a urinary vitamin E metabolite with a truncated phytyl tail. To test whether natural and synthetic vitamin E are similarly converted to α-CEHC, 6 humans consumed 150 mg each *RRR*-α-[5-(C^2H_3)]- and *all rac*-α-[5,7($C^2H_3)_2$]-tocopheryl acetates (d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates, respectively). Plasma was collected at 0, 6, 12 and 24 h; urine (24 h) at 0, 1, 2, 3, 4 and 8 days. Following dosing, plasma was enriched with d_3*RRR*-α-tocopherol, while urine was enriched with α-CEHC derived from d_6*all rac*-α-tocopherol. Thus, synthetic compared with natural vitamin E is preferentially metabolized to α-CEHC and excreted.

Key words: Vitamin E urinary metabolite; α-CEHC; γ-CEHC; LLUα; Vitamin E biological activity; Human

1. Introduction

Vitamin E is not just one molecule, but rather there are eight different, naturally occurring forms of vitamin E: α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols; yet only α-tocopherol has high biological activity [1]. Vitamin E is unique because the chemically synthesized form is not identical to the naturally occurring form, *RRR*-α-tocopherol. The synthesized form has eight different stereoisomers and is labeled *all rac*-α-tocopherol. Historically, the rat fetal resorption assay was used to assess the biological activity of naturally occurring non-α-tocopherol vitamin E forms [2]. The use of deuterated tocopherols and the use of chiral columns to examine vitamin E stereoisomers in plasma and tissues [3,4], as well as the isolation and cloning of the α-tocopherol transfer protein to allow direct determination of its binding properties [5] have confirmed that plasma contains few or no 2*S*-α-tocopherol isomers, and that the α-tocopherol transfer protein (α-TTP) does not bind them. Commercially available vitamin E supplements can contain either the natural (*RRR*-α-tocopherol) or the synthetic form (*all rac*-α-tocopherol).

The biological activity of vitamin E has been suggested to be dependent upon the function of α-TTP [5]. However, vitamin E metabolism may also have an important role in this regard. Vitamin E metabolites in human urine include both the metabolite of α-tocopherol (2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman, α-CEHC) [6,7] and that of γ-tocopherol (2,7,8-trimethyl-2-(2′-carboxyethyl)-6-hydroxychroman, γ-CEHC or LLUα) [8,9]. These metabolites result from degradation of the phytyl tail; they are not oxidation products of vitamin E. It is unknown where the metabolites are synthesized, but the central role of the liver in discriminating between vitamin E forms suggests that the liver may be the site of vitamin E metabolism. Previously, α-CEHC was found excreted in the urine only in response to daily vitamin E supplementation with at least 50–150 mg α-tocopherol [6]. However, improvements in the detection limit of α-CEHC excretion have allowed its measurement in the urine of non-vitamin E supplemented individuals (A. Elsner, M. Schultz, J. Brueckner, M.G. Traber, R. Brigelius-Flohé, unpublished). These studies revealed α- CEHC as a normal vitamin E metabolite constitutively present in human urine at low concentrations and it was probably dependent on vitamin E intake.

The relationship between plasma vitamin E and urinary metabolites is unclear. α-CEHC increases in the urine with increasing plasma α-tocopherol concentrations reflecting increasing amounts of vitamin E supplementation [6]. It is unknown what happens to γ-CEHC. Conversion of *all rac*-α-tocopherol to the vitamin E metabolite, α-CEHC, may be increased compared with the conversion of *RRR*-α-tocopherol because less *all rac*-α-tocopherol is exported into plasma [10]. To test this hypothesis, we have used a single dose of two forms of vitamin E labeled with different amounts of deuterium to assess whether the natural and synthetic forms of vitamin E are similarly converted to α-CEHC.

2. Methods

2.1. Deuterated vitamin E

RRR-α-5-(C^2H_3)Tocopheryl acetate and *all rac*-α-5,7-($C^2H_3)_2$tocopheryl acetate (d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates, respectively) were a gift from the Natural Source Vitamin E Association (NSVEA) and were synthesized by Eastman Kodak, Rochester, NY, USA. The compounds were determined to be 96% *RRR*- and 93% *all rac*-α-tocopheryl acetate by weight. Their isotopic purities at their nominal level of deuteration were 84% (d_0: 4.0%; d_1: 2.0%; d_2: 9.7%) and 86% (d_0, d_1: $<0.1\%$; d_2: 0.1%; d_3: 0.8%; d_4: 1.3%; d_5: 11.2%), respectively. The d_3-*RRR*- and d_6-*all rac*-α-tocopheryl acetates were encapsulated in gelatin capsules as 1:1 mixtures in 150-mg quantities diluted in α-tocopherol stripped corn oil. The *RRR*/*all rac* ratio was determined by GC-MS to be 0.98.

2.2. α-CEHC and γ-CEHC

α-CEHC (2,5,7,8-tetramethyl-2(2′-carboxyethyl)-6-hydroxychroman) was synthesized by Laborat, Berlin, Germany. γ-CEHC (2,7,8-trimethyl-2(2′-carboxyethyl)-6-hydroxychroman, LLUα) was a kind gift from W.J. Wechter (Laboratory of Chemical Endocrinology, Loma Linda University School of Medicine, Loma Linda, CA, USA).

2.3. Study design

The study was approved by the local (Brandenberg, Germany) Research Ethics Committee in accordance with the Helsinki Declaration and by the human subjects committee of the University of California, Davis, CA, USA. The study was carried out at the German Institute of Human Nutrition using 6 normolipidemic adult volunteers aged 27–52 years, one male and 5 female subjects, who gave written in-

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formed consent. They consumed a single dose of encapsulated 150-mg d_3-*RRR*- and 150-mg d_6-*all rac*-α-tocopheryl acetates (8:00 a.m.). Blood samples from non-fasting volunteers were taken prior to supplementation and at 6, 12 and 24 h. 24-h urine was collected (from 7:00 to 7:00 a.m.) a day prior to supplementation, and 1, 2, 3, 4 and 8 days after supplementation.

2.4. Extraction of plasma vitamin E and measurement of non-deuterated and deuterated α-tocopherols and γ-tocopherol

Plasma tocopherols were analyzed essentially as described [11]. Briefly, plasma was isolated from blood (5 ml) drawn into tubes containing EDTA, by centrifugation at 3000 rpm for 10 min at 5°C and transferred into plastic tubes. 100-μl aliquots were stored at −80°C under nitrogen until analysis. Previously, we have found that vitamin E is stable for as long as 2 years under these conditions.

To 100 μl plasma was added 100 μl ethanol (ACROS Organics, Geel, Belgium) and internal standard (α-tocopheryl acetate, 5 μl, 0.2 mM in ethanol). The sample was vigorously mixed for 10 s and extracted with 100 μl *n*-heptane. Then the *n*-heptane phase was separated and evaporated with nitrogen. The tocopherols converted to their trimethylsilyl ethers using 50 μl pyridine p.a. dried (Merck, Darmstadt, Germany) and 25 μl *N,O*-bis(trimethylsilyl)trifluoroacetamide (Fluka, Neu-Ulm, Germany) with 1% trimethylchlorosilane (Merck, Darmstadt, Germany) in an electrically heated block at 65°C in closed vials for 15 min.

Derivatized tocopherols were analyzed by GC-MS with a SSQ 710 MAT from Finnigan MAT (Bremen, Germany) with a Varian 3400 Gas Chromatograph. A 30 m×0.25 mm I.D., 0.25-μm film thickness DB-5MS fused-silica column was used for sample separation. The temperature program was 2 min 180°C, 10°C/min to 280°C and finally 20 min 280°C. The injector temperature was set to 260°C and the transfer line temperature at 300°C. The carrier gas was helium. The injection volume was 1 μl. EI mass spectra were recorded at an ionization energy of 70 eV and an ion source temperature of 150°C. In the selected ion-monitoring mode detected masses of the silyl ethers were as follows: 430 mass units for internal standard α-tocopherol acetate, 488 mass units for γ-tocopherol and 502 (d_0), 505 (d_3), 508 (d_6) mass units for α-tocopherols. The concentrations of γ-tocopherol, d_0-, d_3- and d_6-α-tocopherol were calculated from the peak area of the corresponding molecular ions in the mass spectra relative to the fragment ion at *m/z* 430 of the α-tocopheryl acetate internal standard (molecule ion at *m/z* 472 is of low intensity). The measurements of d_3- and d_6-α-tocopherol were corrected for the contribution of the natural abundance isotopes (M+3) present in the non-deuterated and d_3-α-tocopherol silyl ether, respectively. Therefore 2.4% of the peak area of the isotopomer d_0-α-tocopherol and d_3-α-tocopherol were subtracted from the peak of the isotopomers d_3-α-tocopherol and d_6-α-tocopherol, respectively. The results are means of measurement of two samples of each plasma collection.

2.5. Extraction of the urine samples and measurement of non-deuterated α- and γ-CEHC and deuterated α-CEHC

Aliquots of 24-h urine collections were lyophilized and the powders were stored under nitrogen at −80°C. Three samples of 100 mg were taken from each urine powder. To one sample 10 μg (3.6 mM in ethanol) Trolox (2,5,7,8-tetramethyl-2(2′-carboxymethyl)-6-hydroxychroman, Aldrich, Steinheim, Germany) as internal standard and to the others 2 μg Trolox were added. The samples were extracted with 5 ml methanol 3 times and evaporated. Then enzymatic hydrolysis of the CEHC conjugates was carried out in a mixture of 600 μl water, 1600 μl sodium acetate buffer (0.1 M, pH 4.5) and 800 μl enzyme solution, consisting of 8 mg β-glucuronidase/sulfatase (133 U sulfatase, 2700 U glucuronidase (Sigma G0751, Sigma, Deisenhofen, Germany)) in acetate buffer. The tubes were flushed with argon, closed and incubated in a shaking water bath at 37°C for 4 h, then were extracted 4 times with 5 ml diethylether, and the ether collected and evaporated under nitrogen. The CEHC compounds were converted to their silyl ether esters using 100 μl hexane, 80 μl *N,O*-bis(trimethylsilyl)trifluoroacetamide (Fluka, Neu-Ulm, Germany), 16 μl *N,O*-bis(trimethylsilyl)acetamide (Aldrich, Steinheim, Germany), 4 μl trimethylchlorosilane (Merck, Darmstadt, Germany) in an electrically heated block at 50°C for 2 h [8,9].

Derivatized deuterated and non-deuterated CEHCs were analyzed by GC-MS with a SSQ 710 MAT from Finnigan MAT (Bremen, Germany) with a Varian 3400 Gas Chromatograph. The same 30 m×0.25 mm I.D., 0.25-μm film thickness DB-5MS fused-silica column as for tocopherol analysis was used. The initial temperature was 100°C for 0.5 min, followed by an increase of 30°C/min to 280°C and held for 10 min. The injector temperature was 280°C. The carrier gas was helium and the transfer line temperature was set at 300°C. The injection volume was 2 μl. Electron ionization mass spectra were recorded at an ionization energy of 70 eV and an ion source temperature of 150°C. Selected masses for the silyl ether esters monitored were: 394 mass units for internal standard Trolox, 408 mass units for γ-CEHC and 422 (d_0), 425 (d_3), 428 (d_6) mass units for α-CEHC silyl derivatives. The concentrations of γ-CEHC, d_0-, d_3- and d_6-α-CEHC were calculated from the peak area of the corresponding molecule ions relative to the peak area of the molecular ion 394 of the internal standard Trolox and the response factor. Corrections were made for the contribution of the naturally silicone isotopes (M+3) present in the CEHC silyl ether esters differing by three atomic mass units. It was calculated by subtraction of 2.9% of the peak area of d_0-α-CEHC (*m/z* 422) from d_3-α-CEHC (*m/z* 425) and of d_3-α-CEHC (*m/z* 425) from d_6-α-CEHC (*m/z* 428), respectively. The results are means of the analysis of three samples of each urine collection.

2.6. Statistical analyses

The statistical significance of the concentrations of the isotopes in plasma and in the urine was determined using ANOVA with repeated measures. Comparisons were made using least square means analysis. Statistical analyses were carried out with SuperAnova (Berkeley, CA, USA). Results were considered to be significant at the 95% confidence level ($P < 0.05$). Reported are the means ± S.E.

3. Results

3.1. Plasma vitamin E concentrations

Measurements of plasma vitamin E concentrations following supplementation with natural and synthetic vitamin E labeled with deuterium were made for the first 24 h following dosing. As seen in Fig. 1A, unlabeled plasma α-tocopherol at time 0 decreased from 23 ± 2 to 21 ± 2 μM ($P < 0.009$) at 12 h

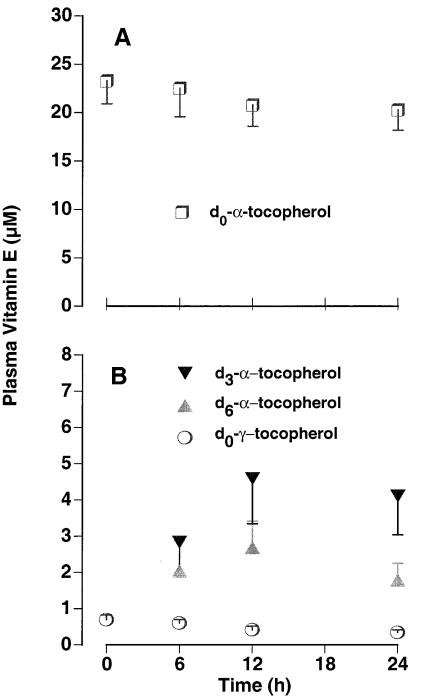

Fig. 1. (A) Plasma unlabeled (d_0) α- and (B) γ-tocopherols and labeled (d_3 and d_6) α-tocopherols (means ± S.E., $n = 6$) following administration of a single dose containing 150 mg each d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates.

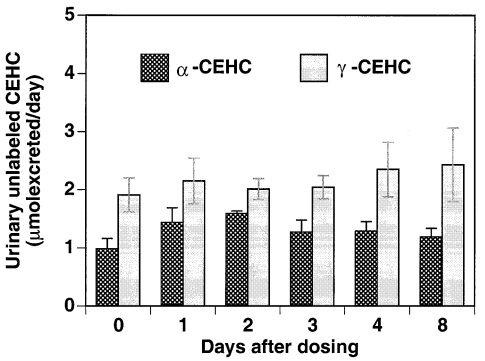

Fig. 2. Urinary unlabeled α- and γ-CEHC (means ± S.E., $n = 6$) in 24-h urine collections before (0) and following administration of a single dose containing 150 mg each d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates.

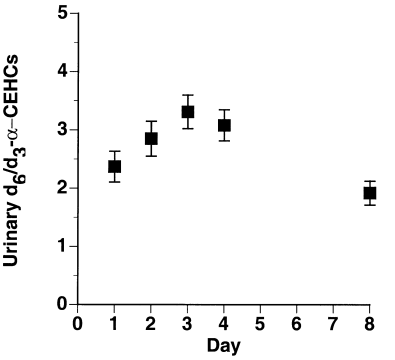

Fig. 4. Ratio of urinary d_3-α- to d_6-α-CEHCs (means ± S.E., $n = 6$) in 24-h urine collections following administration of a single dose containing 150 mg each d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates.

and to 20 ± 2 μM ($P < 0.002$) at 24 h. Similarly, plasma γ-tocopherol decreased from 0.7 ± 0.1 at 0 h to 0.4 ± 0.1 μM ($P < 0.001$) at 12 h and to 0.3 ± 0.1 μM ($P < 0.0002$) at 24 h (Fig. 1B). Six h after the dose both d_3-α- and d_6-α-tocopherols attained similar plasma concentrations; however, by 12 h the concentration of d_3-α-tocopherol (4.6 ± 1.2 μM) was significantly ($P < 0.002$) greater than that of d_6-α-tocopherol (2.7 ± 0.7 μM); similar differences were found at 24 h (d_3α-tocopherol (4.1 ± 1.1 μM) vs. d_6-α-tocopherol (1.8 ± 0.5 μM, $P < 0.0004$) (Fig. 1B).

The ratio of natural to synthetic (d_3- to that of d_6-α-tocopherol) in the plasma increased from approximately 1 in the administered dose to 1.5 ± 0.1 at 6 h, 1.8 ± 0.1 at 12 h and 2.1 ± 0.1 at 24 h.

3.2. Urinary vitamin E metabolite concentrations

Unlabeled α- and γ-CEHCs were measured in 24-h urine collections prior to and after deuterated vitamin E dosing. As shown in Fig. 2, despite the low concentrations of γ-tocopherol in the plasma, the urine contained higher concentrations of γ-CEHC than of α-CEHC (main effect $P < 0.02$, daily comparisons except on day 2 were statistically significant, $P < 0.01$). Although γ-CEHC was excreted in relatively constant daily amounts, α-CEHC varied in response to the 300 mg of deuterated α-tocopheryl acetate administered. Unlabeled α-CEHC excretion increased from 1.0 ± 0.2 μmol on day 0 to 1.4 ± 0.2 μmol on day 1 ($P < 0.004$) to 1.6 ± 0.1 μmol ($P < 0.003$) on day 2 and back to 1.3 ± 0.2 μmol ($P < 0.04$) on day 3; subsequent days were not statistically different from day 0. Thus, we observed increased unlabeled α-CEHC excretion in response to deuterated vitamin E supplementation. These data are in accordance with the observation that new vitamin E replaces the circulating [12,13], and that excess vitamin E is metabolized [6].

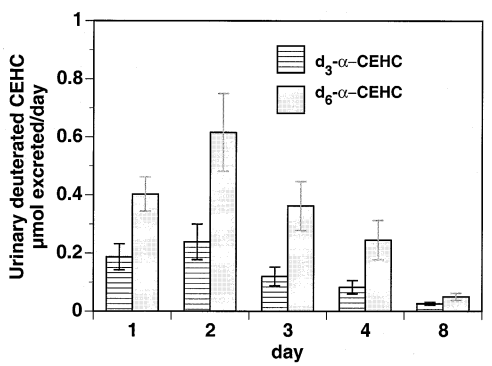

Fig. 3. Urinary d_3-α- and d_6-α-CEHC (means ± S.E., $n = 6$) in 24-h urine collections following administration of a single dose containing 150 mg each d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates.

The major purpose of this study was to evaluate excretion of labeled α-CEHC following administration of d_3*RRR*-α- and d_6*all rac*-α-tocopheryl acetates. As shown in Fig. 3, metabolites from both forms of vitamin E were readily detected in urine up to 8 days following dosing. Remarkably, more d_6α-CEHC than d_3α-CEHC was excreted daily during the first 4 days of the study ($P < 0.0001$ daily, except day 4, $P < 0.0002$; day 8, not significant). This greater excretion of synthetic vitamin E derived metabolite is more obvious in Fig. 4, where the ratio of metabolite derived from synthetic to natural vitamin E is shown. The d_6/d_3 ratio in the administered material was 1 and in contrast to the plasma where the natural vitamin E form was preferentially retained (ratio equals about 2 in favor of d_3*RRR*-α- compared with d_6*all rac*-α-tocopherol), in the urine the synthetic was preferentially excreted. The ratio of synthetic to natural increased from 2.4 on day 1 to 3.3 on day 3 before decreasing back to 1.9 on day 8. The ratio was significantly greater ($P < 0.002$) than the administered dose ratio of 1 on all days.

The total amount of deuterated α-CEHC excreted was estimated by summing the daily values. The total d_6-α-CEHC excreted (1.68 ± 0.30 μmol) was on average about 2.7 times that of d_3-α-CEHC (0.65 ± 0.12 μmol, $P < 0.002$), once again documenting that more of the d_6-*all rac*-α-tocopherol was converted to metabolite.

4. Discussion

Synthetic vitamin E (*all rac*-α-tocopherol), which contains 8 different stereoisomers resulting from 3 chiral centers in the phytyl tail, is less biologically potent than the naturally occurring form, *RRR*-α-tocopherol [14]. Because the α-CEHC metabolite arises from the truncation of the phytyl tail and its excretion increases in response to 'excess' vitamin E, we hypothesized that *all rac*-α-tocopherol might be metabolized to α-CEHC more readily than the natural form. By 24 h after supplementation with 150 mg each d_3*RRR*-α- and d_6*all rac*-α-

tocopheryl acetates, the plasma ratio of natural to synthetic (d_3 to d_6) was about 2, while the opposite was found in the urine – nearly three times as much d_6-α-CEHC (derived from synthetic vitamin E) was excreted!

Increased excretion of the metabolite arising from d_6*all rac*-α-tocopherol might be a result of the preference of the metabolizing system for non-*RRR*-forms. Or more specifically α-TTP preferentially sorts out the 2*R*-forms resulting in the plasma preference ratio of 2, natural to synthetic. In this way the liver becomes enriched with the synthetic (2*S*-forms) which are then degraded together with excess vitamin E (both *RRR*- and other 2*R*-α-forms, as well as other vitamin E forms). α-CEHC excretion then reflects the vitamin E concentrations in the liver. Plasma and tissue data reported by Burton et al. [12] are in accordance with this interpretation.

The lack of change in γ-CEHC excretion in response to decreases in plasma γ-tocopherol following vitamin E supplementation shown here suggests that γ-CEHC synthesis is possibly regulated independently of plasma γ-tocopherol concentrations. Alternatively, the small decreases in plasma γ-tocopherol result in undetectable increases in γ-CEHC. In response to deuterated vitamin E supplementation, plasma unlabeled α- and γ-tocopherol concentrations both decreased (at least in the first 24 h after dosing; Fig. 1). Thus, as reported previously, newly absorbed α-tocopherol replaces the circulating α-tocopherol [12,13], and causes a decrease in circulating γ-tocopherol [15–17]; as shown in Fig. 2, it causes an increase in α-CEHC excretion [6], but does not further increase γ-CEHC excretion.

The present study was carried out in Germany, where plasma α- to γ-tocopherol ratios were about 20:1, in contrast to previous reports of subjects studied in the United States who have α- to γ-tocopherol ratios about 5:1 [18,19]. Despite the high plasma α- to γ-tocopherol ratio in the present study, twice as much γ-CEHC compared with α-CEHC was excreted in the urine. High γ-CEHC excretion levels were also presented in a preliminary report; Swanson et al. [20] suggested that all of the γ-tocopherol consumed is converted to γ-CEHC. Consistent with these observations is the hypothesis that CEHC metabolites are synthesized from excess liver vitamin E. In the case of γ-tocopherol, little is needed by the body as evidenced by its rapid turnover [18,21], so γ-CEHC is readily made from 'excess' γ-tocopherol and is increased in response to increased consumption of γ-tocopherol. Thus, German subjects have less γ-CEHC in their urine than Americans because they consume less γ-tocopherol. Following this logic, less of the synthetic vitamin E is recognized by α-TTP [5], therefore, like excess γ-tocopherol, less is exported to the plasma and more synthetic vitamin E is converted to the metabolite.

The lower biological activity of synthetic vitamin E cannot be quantitatively attributed to its higher rate of metabolism. Although nearly 3 times as much metabolite was made from synthetic compared with natural vitamin E, the amounts of the metabolite produced and excreted in the urine account for only a few percent of the deuterated vitamin E consumed. This low level of urinary metabolite excretion is consistent with the urinary excretion of radioactivity following ^{3}H-α-tocopherol administration to humans carried out by MacMahon and Neale [22] over 30 years ago. Because only urine was examined, other routes of CEHC excretion cannot be ruled out. The amount of CEHC in the bile has not yet been examined, so the importance of vitamin E metabolism in determining biological activity and the extent of vitamin E catabolism to CEHCs remains to be investigated. It is clear that these metabolites of tocopherols are not oxidation products because they are derived from unoxidized tocopherols and their synthesis apparently is dependent upon vitamin E being present in excess. The practical application of CEHC measurements are not yet clear; potentially they can be used as a measure of adequacy of vitamin E status, as was suggested by Schultz et al. [6].

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