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Abnormal arachidonic acid content of red blood cell membranes and main lithogenic factors in stone formers

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Abstract

Background. Increased arachidonic acid content in red blood cell membranes of stone formers (SF) has recently been reported and is hypothesized as representing the underlying causal factor for both hyperoxaluria and hypercalciuria. We performed the present study to see whether we could confirm this finding and to test whether any relationship exists between the fatty acid composition of red blood cell membranes and the main metabolic factors involved in stone formation.

Methods. In 21 SF and 40 healthy controls subjects the fatty acid composition of red blood cell membranes was assessed. In addition, the following parameters were evaluated in SF: daily and fasting urinary calcium excretion, fractional intestinal calcium absorption, 1,25-dihydroxy-vitamin D, intact parathyroid hormone, hydroxyproline in fasting urine, daily urinary excretion of oxalate, citrate, urate, electrolytes, urea, sulphate, relative supersaturation for calcium oxalate monohydrate.

Results. The red blood cell membrane of SF had a lower content of arachidonic acid, linoleic acid, and docosahexaenoic acid than that of control subjects. Arachidonic acid content was not correlated with any of the parameters studied. However, when patients were grouped according to the degree of oxalate excretion, hyperoxaluric SF had a higher arachidonic acid content and arachidonic/linoleic acid ratio than SF with normal oxalate excretion.

Conclusions. Our results do not confirm the finding of an increased arachidonic acid content of red blood cell membrane in SF. On the contrary, reduced arachidonic acid levels were found in our patients. However, hyperoxaluric SF had a relatively higher arachidonic acid content than SF with normal urinary oxalate excretion.

Keywords: arachidonic acid; calcium; fatty acids; oxalate; urolithiasis

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Introduction

Changes in the fatty acid content of cell membrane have been related to a large number of cellular functions [1–5]. Baggio *et al.* recently reported an increased concentration of arachidonic acid and arachidonic/linoleic acid ratio in red blood cell membranes in a group of stone formers (SF) [6]. They also found a relationship between the changes in fatty acid composition of red blood cell membranes and both erythrocyte oxalate exchange and oxalate urinary excretion in the patients studied, and suggested a role for increased arachidonate cell membrane content in the mild-hyperoxaluric syndromes.

However, increased arachidonic acid in cell membranes could in theory also affect calcium excretion, by increased production of PGE2, an arachidonate metabolite, which in turn could increase urinary calcium output, intestinal calcium absorption and calcium resorption from bone [7,8]. Against this background Baggio *et al.* have recently suggested a unifying hypothesis according to which, a change in the fatty acid composition of red blood cell membranes in SF may be the defect that underlies the two most common and outstanding metabolic anomalies found in nephrolithiasis: hyperoxaluria and hypercalciuria [9].

Our investigation aimed to test the finding of increased arachidonic acid content in red blood cell membranes of SF and to determine whether any relationship is present between the fatty acid composition of red blood cell membranes and the main chemical and metabolic factors involved in nephrolithiasis.

Patients and methods

Patients

Twenty-one patients (six females), aged 25–63 years, with active stone disease (more than two stones formed in the last 3 years) participated in the study. Primary hyperparathyroidism, medullary sponge kidney disease and tubular acidosis were excluded by our routine evaluation. All patients were

free of any pharmacological treatment for at least the 3 months preceding the study. Their renal functions were within normal range as judged by serum creatinine (<130 μmol/l) and creatinine clearance (>90 ml/min/1.73 m).

The patients were submitted to our routine study protocol for nephrolithiasis, described elsewhere [10], while on their at-home free-choice diet. The supply of the main dietary components was indirectly assessed by daily urinary excretion of the diet-related substances, as described previously [11].

Evaluated parameters

In each of the 21 SF patients the following parameters were assessed. (i) Calcium related parameters: urinary calcium excretion in two 24-h urine collections and in a fasting urine sample; fractional intestinal calcium absorption (ICaA%); serum calcitriol and intact parathyroid hormone (PTH) levels; hydroxyproline excretion in fasting urine, evaluated as the molar ratio with creatinine (OHP/Cr). (ii) Other factors involved in stone formation: oxalate, citrate, magnesium, urate in two 24-h urine samples, collected under 6 M HCl; relative urine supersaturation for calcium oxalate monohydrate (β_{CaOx}). (iii) Diet-related factors: sodium (Na), potassium (K), urea, sulphate, phosphate, body mass index (BMI). The normal values of ICaA% (30.7 ± 5.27%, mean $\pm$ SD) were obtained by the same method in a historical control group of 22 normal subjects (nine males and 13 females, aged 19–57 years). The normal values of oxalate excretion (0.305 ± 0.073 mmol/day, mean $\pm$ SD) were obtained in a historical group of 50 control subjects (32 males and 18 females, aged 21–55 years).

In all the 21 SF and in 40 control subjects (C), enrolled in the transfusional unit, a fasting blood sample, collected on the first morning of the study, was used for the evaluation of fatty acids in red blood cell membranes.

Methods

ICaA% was evaluated by means of kinetic methodology, utilizing stable strontium as tracer, as described previously [11]. Strontium is recognized to be a good tracer for calcium kinetics assessment, given that no substantial amount of calcium is simultaneously introduced [12,13]. Briefly, on the first day, after an overnight fast, 1.25 mmol of $SrCl_2$ was given by mouth and blood samples were collected at 0, 30, 60, 120, 180, 240, 360, 480, 680, and 1440 min for the measurement of strontium concentration. The patients were allowed to eat after the 360 min. Two days later, the same amount of $SrCl_2$ was injected i.v. in an antecubital vein over 2 min and blood samples were collected from the controlateral arm at 0, 5, 15, 30, 60, 120, 180, 240, 360, 480, 600, and 1440 min. The ICaA% was calculated according to the deconvolution method described by Tothill *et al.* [14].

The urine saturation index relative to calcium oxalate monohydrate (β_{CaOx}) was calculated by means of an iterative computer method devised by Marangella *et al.* [15].

BMI was calculated as body weight (kg) $\times$ height $(m)^{-2}$.

Analytical and statistical methodology

For the fatty acid content evaluation in red blood cell membranes, a blood sample was collected in EDTA-containing tubes after an overnight fast. Red blood cells were separated (3000 *g* for 5 min), washed three times, resuspended in saline and conserved in the presence of α-tocopherol. Then 200 μl of packed red blood cells were lysed in 400 μl of distilled water and lipids were extracted according to Folch's method [16] with slight modifications. Briefly, total fatty acids were analysed as their methyl esters after transesterification catalysis of lipid extracts with trimethylanilinium hydroxyde (TMAH) and separation was performed by gas-chromatography (DANI 3865, capillary column Omegawax 320, Supelco Inc., Bellefonte, PA, USA). The fatty acid concentration was expressed as % of the total.

Electrolytes, creatinine, uric acid and urea in serum and urine were measured by standard methodology (autoanalyser, absorption spectrophotometry, flame photometry).

Urinary oxalate and citrate were measured by column gas chromatography (Hewlett-Packard 5894 II, Palo Alto, CA, Supelco SBP-5), in urine collected under 6 M HCl as described elsewhere [11].

Strontium in serum was measured by atomic absorption spectrophotometry (Perkin-Elmer Zeeman 5000, Norwalk, CT).

PTH was measured by intact-PTH (i-PTH) immunoradiometric assay (IRMA, Nichols Institute Diagnostic, San Juan Capistrano, CA, USA), with intra-assay and inter-assay coefficients of variation of 2.4 and 5.6%, respectively. The normal range for i-PTH was 5–55 pg/ml.

For 1,25-vitamin D (1,25-vitD) determination, a radioreceptorial method was used (RRA, Nichols Institute Diagnostic), with a recovery of between 60 and 80% (each sample was corrected for its own recovery) and intra- and inter-assay coefficients of variation of 10.0 and 14.0%, respectively. The normal range for 1,25-vitD was 20–45 pg/ml.

Statistics were calculated by STATISTICA software package (StatSoft, Inc., Tulsa, OK, USA), on a 133 Pentium personal computer.

Results

The descriptive statistical values of the main parameters studied in the SF patients are shown in Table 1.

Of the 21 SF participating in the study, four had hypercalciuria only in the fasting urine (Ca/Cr > 0.320 mmol/mmol), three had daily hypercalciuria (uCa/day > 0.1 mmol/kg bw), six had both fasting and daily hypercalciuria, and the remaining eight patients were not hypercalciuric. Seven of the patients had oxalate excretion > 0.450 mmol/day (which represent the mean + 2 SD of the oxalate excretion of the above-mentioned normal population).

Eight patients had citrate excretion < 1.8 mmol/day; five patients had uric acid excretion > 4.5 mmol/day. These metabolic patterns are representative of an average common population of stone-forming patients. One of these patients had serum PTH levels above the normal range for our laboratory (patient = 81.9 pg/ml, laboratory normal = < 65 pg/ml); his serum calcium concentration was in the lower normal range (2.2 mmol/l), with a substantially increased calcium excretion in his fasting urine (Ca/Cr = 0.648 mmol/mmol). By these data primary hyperparathyroidism was excluded and the patient was classified as a renal hypercalciuric SF.

The fatty acid content of red blood cell membranes in SF and C is reported in Table 2.

Table 1. Descriptive statistics of the main parameters evaluated in the 21 studied SF patients

	Mean	Standard deviation	Range
Calcium related parameters			
Urinary calcium mmol/day	7.39	3.6	2.47–15.8
Fast calcium/creatinine mmol/mmol	0.374	0.273	0.087–1.128
ICaA%	42.2	17.1	16–74
1,25-vitamin D pg/ml	35.5	8.6	21.1–48.8
Parathyroid hormone pg/ml	33.1	19.2	15.2–81.9
Hydroxyproline/creatinine μmol/mmol	18.2	10.9	6.73–48.9
Lithogenesis-related parameters			
Urinary oxalate mmol/day	0.395	0.191	0.171–0.855
β_{CaOx}	7.93	4.40	1.23–17.9
Urinary urate mmol/day	4.65	4.27	2.49–22.9
Urinary citrate mmol/day	2.14	1.62	0.25–7.2
Urinary magnesium mmol/day	4.29	1.16	2.62–6.84
Diet-related parameters			
Urinary sodium mmol/day	187	65	81–321
Urinary potassium mmol/day	62	24	32–121
Urinary urea mmol/day	433	84	316–591
Urinary sulphate mmol/day	23.9	13.7	6.1–70.7
Urinary phosphate mmol/day	27.7	10.8	7.1–54.9
Body mass index	25.0	4.74	20.4–40.4

ICaA%, fractional intestinal calcium absorption.

Table 2. Main fatty acid content of red blood cell membranes in 21 SF and 40 C subjects

PA	SA	OA	LA	AA	EPA	DPA	DHA	POL/SAT	AA/LA	
16:0	18:0	18:1 $n=9$	18:2 $n=6$	20:4 $n=6$	20:5 $n=3$	22:5 $n=3$	22:6 $n=3$			
SF	29.95 ±4.2	17.6 ±2.6	17.3 ±2.1	11.5* ±2.0	9.2** ±3.0	0.44 ±0.15	1.82 ±1.9	2.6* ±1.6	0.58** ±0.18	0.83* ±0.36
C	28.4 ±4.3	17.4 ±1.9	17.1 ±2.2	12.5 ±1.6	11.3 ±2.2	0.57 ±0.32	1.54 ±0.65	3.4 ±1.0	0.713 ±0.1	0.91 ±0.19

PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; POL/SAT, polyunsaturated/saturated acid ratio. The values are expressed as % of total fatty acids; mean ± SD; *$P<0.05$, **$P<0.001$.

SF were characterized by lower values of linoleic, arachidonic, and docosahexaenoic acids in red blood cell membranes when compared with C. As a consequence, both polyunsaturated/saturated fatty acid and arachidonate/linoleate ratios were reduced.

The values of arachidonic acid content were inversely related to palmitic ($r=-0.607$, $P=0.005$), stearic (r–0.509, $P=0.02$) and oleic acid (r–0.473, $P=0.03$) content and directly related to docosahexaenoic ($r=0.619$, $P=0.004$) and the docosapentaenoic acid ($r=0.754$, $P=0.0001$) content of red blood cell membranes.

The mean oxalate excretion in urine of SF patients was significantly higher than that found in our historical control group (0.395 ± 0.191 *vs* 0.305 ± 0.073 mmol/day, $P<0.01$).

When we grouped the SF according to the levels of oxalate excretion (greater or less than 0.450 mmol/day, 0.450 mmol/day being the upper limit of the normal range in our normal population), the seven SF patients with oxalate excretion >0.450 mmol/day, had a higher arachidonate content (Figure 2A) and a higher arachidonate/linoleate ratio (Figure 2B) than the 14 SF with oxalate excretion <0.450 mmol/day.

ICaA% in SF was significantly higher than the mean values obtained in our control population (42.2 ± 17.1 *vs* $30.7 \pm 5.27\%$, $P<0.01$). However, ICaA% was significantly correlated with both the arachidonate/linoleate ratio (Figure 1A), and docosapentaenoic acid content (Figure 1B) by a direct relationship.

Discussion

It has been widely demonstrated that varying fatty acid content of cell membrane can affect a great number of cellular functions, such as enzyme activity, transport systems, hormone binding, and cellular growth [1–5,17,18].

Baggio *et al.* recently reported an increased arachidonic acid content and a higher arachidonate/linoleate

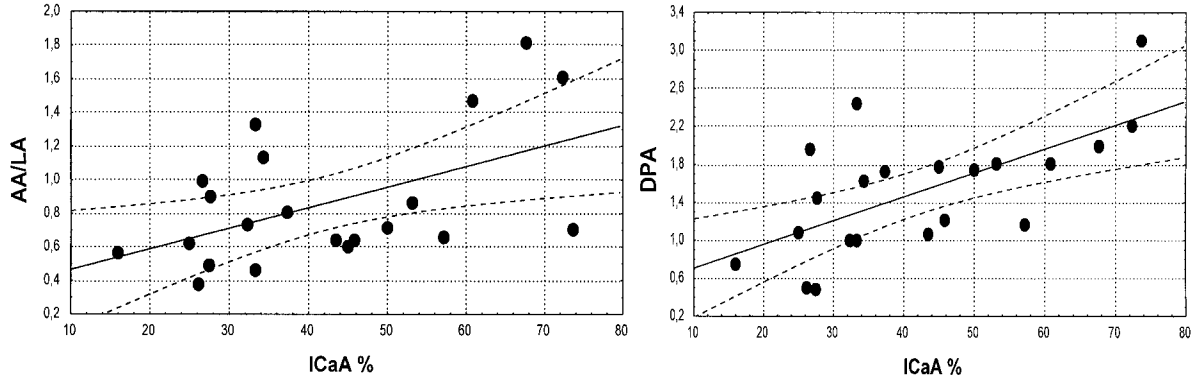

Fig. 1. (**A**) The linear relationship between fractional ICaA and AA/LA ratio (AA/LA = 0.343 + 0.0123 × ICaA; $r = 0.521$, $P = 0.015$). (**B**) The direct relationship between ICaA and DPA content (DPA = 0.454 + 0.025 × ICaA; $r = 0.645$, $P = 0.002$).

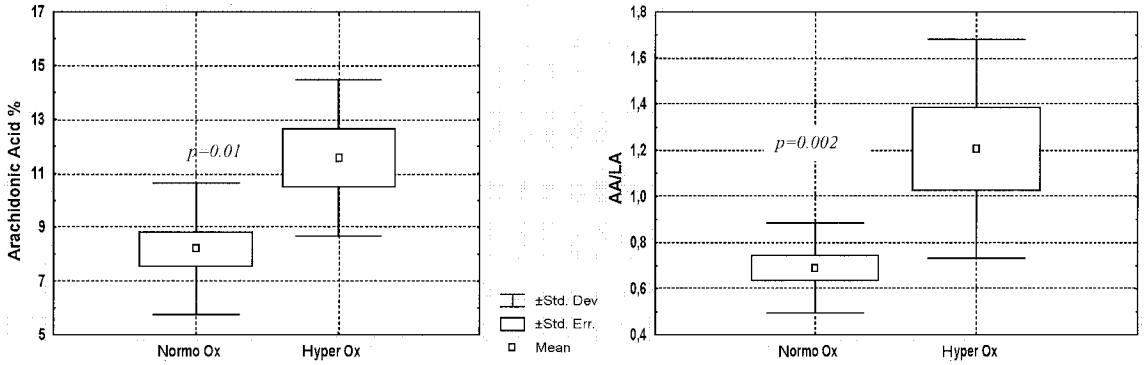

Fig. 2. AA content (**A**) and AA/LA ratio (**B**) in red blood cell membranes of the SF studied, grouped according normal oxaluria (Normo Ox) or hyperoxaluria (Hyper Ox) (see text). Hyperoxaluric SF were characterized by significantly higher values of both parameters when compared with SF with normal oxalate excretion.

ratio in the red blood cell membranes of SF [6]. Furthermore, these authors found that the increased arachidonate levels correlated with the erythrocyte oxalate exchange [6], probably due to an increased phosphorylation of the band-3 anion transporter, which is recognized as the main transport pathway for oxalate [19]. In the same study, the authors demonstrated an increased excretion of PGE2 in urine collected from the same patients, suggesting that increased arachidonate content of the cell membranes, by means of consequent increased prostanoid production, might be responsible for increased calcium excretion and possibly increased intestinal calcium transport. Based on these data the authors proposed that the increased arachidonic acid content in cell membrane might be the unifying causal factor for the main metabolic findings characteristic of SF, namely hyperoxaluria and hypercalciuria [9].

The present investigation aimed to test the finding of an increased arachidonate content of red blood cell membranes in SF and possibly to demonstrate a relationship with the major calcium-related metabolic parameters and other main factors involved in the lithogenic process.

In fact, we were not able to confirm the finding of an increased arachidonate content in red blood cell membranes of a group of SF who had heterogeneous metabolic patterns that may be considered representative of an average SF population. On the contrary, we found reduced levels of both $\omega 6$ and $\omega 3$ fatty acids in the red blood cell membranes of these SF when considered as a group. There is no clear explanation for these quite divergent findings when compared with data presented by Baggio *et al.* except that in the study by Baggio *et al.* fatty acids were assessed in the red blood cell membranes of only 10 of their SF and this may have limited the representative value of their results.

As regards the explanation of our data, a reduced linoleic acid content of the diet cannot be considered as the cause of the reduced arachidonate content, due to the fact that in this condition the arachidonate/linoleate ratio is expected to be increased or at least unchanged, but not reduced as is the case with our patients. Increased $\omega 3$ fatty acids could be an alternative causal factor of reduced arachidonate production, due to competition for desaturase activity between $\omega 6$ and $\omega 3$ fatty acids [20]. However, in our SF, $\omega 3$ fatty acid content in red blood cell membranes was reduced

as well and this finding makes the competition hypothesis untenable. An increased consumption of arachidonic acid in prostanoid generation cannot be considered as a possible explanation of our results, since it is well established that at least in red blood cells the prostanoid metabolic pathway is not present.

Arachidonic acid content was inversely related to the unsaturated and monosaturated fatty acid contents of red blood cell membranes, which might be partially explained by varying fatty acid contents of diets. It was not possible to have detailed data about the fatty acid content of the diets consumed by our patients. No relation was found between fatty acid composition of red blood cell membranes and the urinary diet-related parameters. Finally, we cannot exclude the possibility that some unexplored genetic and/or acquired factor(s) might be involved in these changes.

It is worth noting that in the patients studied hyperoxaluria was matched with relatively increased arachidonate content and arachidonate/linoleate ratio values in red blood cell membranes. The link between these metabolic alterations is not clear. We found that the ICaA% values were correlated with both arachidonate/linoleate ratio and docosapentaenoic acid content in red blood cell membranes. In a previous study [11], we found that oxalate excretion was related to ICaA%. We did not measure ICaA% in the subjects who were checked for fatty acid composition of red blood cell membranes. Therefore, we cannot draw definitive conclusions about whether the relationship between intestinal absorption of calcium and fatty acid content in cell membrane is specific to SF or if it can be found in the normal population. To the best of our knowledge, no study has examined this issue. However, there is some indirect experimental evidence that suggests a possible link between arachidonic acid content of the intestinal cells and calcium transport [21,22]. It may be hypothesized that if a relatively higher arachidonate/linoleate ratio in cell membrane induces, by some as yet unknown mechanism, increased intestinal calcium transport, the result may be an increase in oxalate absorption. If true, this finding might partially support Baggio's hypothesis of a dependence of both hyperoxaluria and hypercalciuria on cell membrane fatty acid composition, at least in the subgroup of SF with increased oxaluria. Our data does not allow any conclusion on this point. It is clear that a definite pattern of arachidonic acid content of red blood cell membranes cannot be considered a hallmark of urinary stone disease, due to the discrepancy between the results of the present study and those of Baggio *et al.*

In conclusion, the finding of a reduced arachidonate content in red blood cell membranes of our SF and the lack of correlation with any of the studied parameters, directly or indirectly related to stone formation, casts doubt on the possibility that increased arachidonic acid content might be the common alteration underlying the lithogenic process. Nevertheless, it is possible that, at least in those SF presenting with increased oxalate excretion, the changes in fatty acid composition of cell membranes might be associated, in an as yet undefined way, with this lithogenic condition. More extensive data are needed to draw definitive conclusions.

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