血清中燐脂質測定によるアトピー性皮膚疾患の予知と予防の試み

（０７６７０４３２）

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はじめに

高齢化社会の進行に伴い、老化による慢性炎症性疾患を始めとして、アトピー性皮膚炎などの増加も著しい。これらの疾患に共通と思われるのは、免疫機能の異常であり、かつ誘因として食生活、住生活環境の変化や環境汚染の増大等が関与していることも明らかになっている。

アトピー性皮膚疾患は現在の処、原因物質を取り除く予防活動および発生した場合の対症療法とが行われているが、その成立のメカニズムが明確でない為、疾患と如何に上手に付き合うかという事が対策の焦点であり、予防活動には決め手を欠く現状である。

本研究代表者は 1994 年度にフィンランド、ヘルシンキ大学に留学して、アトピー患者の血清、単核球中の脂肪酸測定の技法を学び、それを基にして 1995, 1996 年度科学研究費によって実際の患者に Efamol を投与して見られる症状の改善とそれに伴う血液値の変化について検討を行い、アトピー患者においてはガンマーリノレン酸の経口投与が症状改善をもたらす事、また血清中の脂肪酸の割合を測定することにより、患者、非患者のスクリーニングが可能との感触を得た。

本論では脂肪酸の健康にとっての意義と生体における役割を概説し、アトピー性皮膚炎の小児患者における血清および単核球中脂肪酸組成と疾病発生・治癒との関連について検討を行った。

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必須脂肪酸の定義と働きについて

ガンマーリノレン酸（gamma-linolenic acid：GLA）は必須脂肪酸（essential fatty acid：EFA）のひとつである。必須脂肪酸はビタミン類や必須アミノ酸と同様、生体内で産生されない必須栄養素であり、食物の形で補給されねばならない。

必須脂肪酸には２つのタイプがある。ひとつはリノール酸（linoleic acid：LA）から派生するn-6タイプであり、もうひとつはアルファーリノレン酸（alpha-linolenic acid：ALA）から派生するn-3タイプである。この両者は互換性がない。またこの名前は分子のメチル基近くの二重結合の位置に由来している。n-3系列は、３番目と４番目の炭素原子の間に二重結合があり、n-6系列では６番目と７番目の間にある。

必須脂肪酸は不飽和脂肪酸であり、これらは２～３個の二重結合を持つ。必須脂肪酸においては二重結合の並び方はcisでなくてはならない。ひとつでもtransの位置に二重結合が存在すると、それはもう必須脂肪酸としての力を失う。すべての必須脂肪酸は多価不飽和脂肪酸（polyunsaturated fatty acid：PUFAs）であるが、１個以上のtrans位置の二重結合を含む多くの不飽和脂肪酸は必須脂肪酸とは言えない。栄養に関するアドバイスを与えたり受けたりする際に、この事は多くの混乱を招いた。栄養上の不飽和脂肪酸の働きというのは、それが必須脂肪酸であるような不飽和脂肪酸に限られる。必須脂肪酸でない不飽和脂肪酸は、心筋系に対し望ましい働きをせず、寧ろ逆の効果をもたらすことが多い。

体内で、LA と ALA は交互に生じる一連の不飽和化（２個の水素原子を取り去り１個の余分な二重結合を造る）と鎖の延長（２個の炭素原子を加える）により代謝される。この際、LA と ALA を代謝する一連の酵素系は同一であると広く考えられている。これらは疑いなく非常によく似ているが、完全に同一であるとは言い切れない。

必須脂肪酸として働く為には、LA および ALA は代謝されなければならない。ALA 自身についての特有の働きは知られていない。LA 自身の作用としては、皮膚の水透過性の保持や血管内皮の抗血栓作用がある。双方共、LA の 15-lipoxygenase による C13 の水酸化を通じての 13-hydroxydienoic acid(13-HODE) への変換に依存している。

必須脂肪酸の定義

n-6 系列の必須脂肪酸は、これが食事に入っていないために生じるあらゆる症状を改善することの出来る脂肪酸である

これらのことをどのように証明するかということが最も大切な基本条件といえよう。

この定義に乗っ取り、実験を行うと、LAとGLAとアラキドン酸は必須脂肪酸欠乏症の生化学的、生理学的値を回復させることが出来る。ディホモ・ガンマリノレン酸

(dihomogammalinolenic acid：DGLA) は、GLA と AA の間の中間産物であるが、これがそのような活性を持たないのは驚くべきことである。必須脂肪酸の生化学的・生物学的活性は、LA から AA へと、鎖の下へ行くに従って大きくなる。

リノール酸こそが、食物中に最も多く含まれる故に、n-6 シリーズにおける唯一の必須

脂肪酸であると主張する栄養学者もいる。しかし如何に食物中に多く含まれるか否かで判断出来る根拠は無い。GLAとアラキドン酸は確かに必須脂肪酸欠乏を回復させ、そして少量ではあるが広く食物中に含まれる。アラキドン酸は肉、卵、卵黄、魚油やその他の海産物、そして人の母乳に含まれる。GLAは人の母乳、オーツ、オオムギに含まれ、そして少量であるが広く普通の食物中に存在する。これを大量に含むのは、ある種類の植物の種子である。ことに月見草、菜種、黒すぐり、そしてある種の藻類や菌類の貯蔵油である。

必須脂肪酸の生理学的意義

必須脂肪酸の二つの主要な機能は、細胞膜構造における役割と細胞活性の様々な側面の調節を行う短時間で消滅する中間生成物の合成とに関係している。

必須脂肪酸は膜に対して流動性や柔軟性を付与し、レセプターやATPaseやイオンチャンネルといった膜結合蛋白の行動を調節している。膜活性についての正確な化学については未だ解っていない。それは必ずしも脂肪酸の二重結合の数や鎖の長さに関与している訳ではない。必須脂肪酸は膜において部分的にエステル化されて燐脂質やコレステロールの形で見いだされるが、また遊離酸やトリグリセリドの形でも存在している。燐脂質それ自身は、フォスファチジル・コリン、イノシトール、エタノールアミン、セリン、カーディオリピン、スピンゴーミエリンといったカテゴリーに分けられる。必須脂肪酸がこれらの

要素の中でどのような役割を演じているのかは、その初期についてのみ知られている。リガンドのレセプターへの結合についての研究を通じて、必須脂肪酸の膜での働きが徐々に解明されている。一般にエストロゲンやプロゲスチンの如きステロイドホルモンや、アンギオテンシンのようなペプタイドやオピオイドなどは、レセプターの周囲に必須脂肪酸が十分ある場合にはレセプターに対する親和性が低くなる。別の面から見ると、必須脂肪酸が膜に十分ゆきわたらないと、通常濃度のリガンドの効果が増幅される。

必須脂肪酸は赤血球の流動性にも関与している。赤血球の直径は毛細管の直径よりも大きい為、赤血球が容易に毛細管を通って組織に酸素を送る為には柔軟でなくてはならない。赤血球膜に必須脂肪酸が欠乏すると、普通より固くなり、毛細管を容易に通過出来ず組織に十分な酸素を送れなくなる。

必須脂肪酸の役割としてより大きく注目されているのは、プロスタグランディン、リュウコトリエン、ヒドロキシ酸といった生命の短い調節分子の多くの前駆物質であるということである。これらのことをエイコサノイドと呼ぶこともあり、それはこれらが２０の炭素を含む必須脂肪酸、特にn-6シリーズのAAやDGLA、n-3シリーズのエイコサペンタエイン酸（eicosapentaenoic acid：EPA）から派生してくるからである。これらには、５－リポオクシゲナース酵素系で産生されるリュウコトリエンや、シクロオクシゲナース系で産生されるプロスタグランディンも含まれる。しかしながら、１８個または２２個の炭素原子から成る脂肪酸から１２または１５－リポオクシゲナースやその他の酵素によって作られる化合物が近年重要視されるようになり、エイコサノイドという言葉はこれらの物質

すべてを表すには意味が狭すぎると思われる。

一般的にこれらの物質は、それが必要とされる時に局所的に産生され、そしてしかる後にやはり局所的に速やかに破壊される。これらは非常に数多くの機能調節に使われている。これら必須脂肪酸の派生物のもたらす多くの効果を単純に概説することは容易でないが、いずれにしろ細胞が可能な範囲の反応を十分に普通に発揮する為には必須脂肪酸のレベルが適当であることが必要条件であることは間違いない。

n-6 系列の必須脂肪酸は他に最低２つの機能を持っている。皮膚の水分不透過性を保つのに使われるが、これはLA とGLA だけが行い他の必須脂肪酸は関与していない。これは１３ーハイドロキシの派生物（リノール酸の場合は 13-HODE）へ転換される結果と思われる。13-HODE もまた、血管系の裏壁を丈夫に保つのに重要な役割を果たしている。従って、LAや GLA の 13-ヒドロキシの派生物が体中の壁膜に特有の透過性を調節している可能性がある。

そして、必須脂肪酸のもうひとつの役割として、体中にコレステロールを移動させる働きが挙げられる。コレステロールは通常、脂肪酸を伴ったエステルの形で移送される。飽和脂肪酸を伴うエステルは、不飽和脂肪酸を伴うエステルと比較すると、溶けにくく、また移動性も低い。例えば、様々なコレステロール・エステルを皮膚表面に付着させると、不飽和脂肪酸によって作られたエステルの方が容易に除去出来るのである。

体のあらゆる組織は、その正常な機能を発揮するために、必須脂肪酸を必要とする。従って、必須脂肪酸の欠乏が、皮膚、神経組織、免疫系や炎症系、心筋血管系、消化器

系、内分泌系、腎臓、呼吸器系、生殖器系などの不調を引き起こすのは当然のことである。体のどの組織も、必須脂肪酸不足によって生じる不調から逃れることは出来ない。この点において必須脂肪酸は他の必要な栄養素があらゆる細胞に存在する代謝経路において重要な役割を果たしているのと同様である。

n-6 と n-3 の必須脂肪酸の重要性の比較

近年、魚油に含まれる EPA やドコサヘキサエン酸（docosahexaenoic acid：DHA）に多大な関心が寄せられ、GLA のような n-6 系列の必須脂肪酸を凌駕する勢いである。これは実験的には根拠の薄いものであり、必須脂肪酸の両系列の相対的な重要性の問題である。考慮すべきは次のポイントである。

1）n-6 系列の必須脂肪酸の欠乏状態に置かれた動物は、殆どすべての主要な組織において生化学・生理学的な異常が見られる。これに対して、n-3 系列の必須脂肪酸のみの欠乏においては、動物に何らかの生物学的な異常を見いだすことは困難である。極めて最近になってから、n-3 必須脂肪酸のみの欠乏による網膜や脳の機能異常や生育の異常を、非常に繊細なテクニックを用いて、動物で示せるようになった。

2）n-3 及び n-6 双方の脂肪酸の欠乏によって、あらゆる異常が容易に動物に引き起こされるが、これらの異常は n-6 脂肪酸のみを与えることによっても回復する。それに対して n-3 だけを食事に与えると、障害は全く回復しないどころか、毛細管や皮膚の障害で

は増悪さえ見られる。n-3 が効果的であるとしても、それは適当な量の n-6 が存在する場合のみである。

3）生体の多くの組織において、 n-6 の n-3 に対する割合は３：１～９：１の範囲にある。これは例えばキリンのような動物においてもそうであり、これは草から ALA の形で摂取する n-3 必須脂肪酸しか取っていないにも拘わらず、である。従って、食事における必須脂肪酸の多くが n-3 であっても、どん欲かつ選択的に n-6 の必須脂肪酸を摂取しようと努める。

以上の事柄により、ほ乳類の器官にあっては、 n-6 が圧倒的に n-3 より重要であると言うことが出来る。これは n-3 の、特定の状況下における重要性を否定するものではない。

GLA 投与の原理

アトピー性皮膚炎への投与

これは　6 番目の不飽和化の割合が遺伝的に低いことにより生じると説明されている。普通かそれ以上の濃度の LA と ALA が存在する時、それらの代謝物は、臍帯血や、子供及び大人の血漿や赤血球、母乳その他の脂肪組織において低い濃度となる。このような異常がこの疾病の仕組みを解く鍵であるならば、失われた n-6 及び n-3 必須脂肪酸投与が症状の改善に繋がる筈である。

今日に至るまで、Epogam の形で GLA を投与する試みが広範になされてきた。そして非常に高い有意性をもって、症状の改善、特に痒みの改善が見られたとの報告が多い。そして局所に使用するステロイド剤を 1/3 以下に抑えた。またステロイド剤や抗ヒスタミン剤や抗生物質の患者に経口投与する量を減らしたり中断したり出来る効果があったと報告されている。いずれにしろ、アトピー性皮膚炎患者の多くが GLA 投与により症状が改善しているという報告は多い。

しかしながら、多くのアトピー患者が、時には重篤な者でも、GLA を摂取している間は快方に向かうことを経験する一方で、ある者については全く効き目がないか、あっても部分的でしかなかったりする。これにはいくつかの理由が考えられる。第一に、EPO は、n-6 の必須脂肪酸しか供給しないのに、n-3 と n-6 の両方の欠乏があるからだということ。Efamol Marine （80% の EPO と 20% の魚油の混合物）を使った予備実験においては、EPO 単独投与よりも良好な結果が得られたと述べている。第二に、他の不飽和化のステップが異常であるからということ。そうすると、例えばアラキドン酸とアドレニン酸の欠乏が生じる。これは GLA 投与のみでは改善されないからである。第三に、必須脂肪酸の代謝の最初の部分に異常があり、これが　免疫系の非可逆的変化を引き起こすということ。この変化が一度生じると、足りない必須脂肪酸を投与しても元に戻らない。例えて言うならば、石油が無くなってエンジンが停止した時、石油を与えれば再度エンジンは動くことが出来るが、オイル不足の為にエンジンが停止してしまった時には、オイルを供給してもエンジンは再び機能しないということ。必須脂肪酸欠乏のある種類のものは必須脂

肪酸を投与することにより元に戻るが、特に免疫系などにおける欠乏などは、非可逆的である。もし上記の事実があるならば、予防こそが鍵となる。第四に、　アトピー性皮膚炎は他の多くの疾病と同様、１つ以上の原因によって生じる臨床的症状群であり、その原因のひとつが必須脂肪酸の異常であるに過ぎないということ。

　では、予防を如何に行うか？　母乳哺育がアトピー疾患を予防するという報告がこれまでに根強く存在し、その最も典型的な例はベルギーで行われた前向き研究である。臍帯血の検査により高レベルの IgE が存在する乳児をアトピーに罹りやすい児と同定した。そうすると、高いIgE 値を持ち、母乳哺育された児のアトピーに罹る危険性は、人工栄養の児に比較して劇的に低くなった。この母乳哺育による予防効果は、蛋白抗原と抗体とで説明されているが、母乳中に含まれる必須脂肪酸の存在も同じように、或いはそれ以上に重要であろう。母乳には普通、十分な量の６ー不飽和化脂肪酸（GLA, DGLA, AA, EPA, DHA）は含まれているわけではない。牛乳または植物油から作られた人工栄養ミルクには LA と ALA のみが含まれる。従って母乳で育てられる乳児には、必須脂肪酸の不飽和化を行う必要がないのに、人工栄養の乳児は　不飽和化を自ら行わなければならない。この事は通常は大した問題とはならないが、必須脂肪酸代謝がアトピー的である乳児においては、６ー不飽和化脂肪酸の欠如によってアトピー性皮膚炎やその他のアトピー症状が出現することになる。何故なら、アトピー性皮膚炎を持つ母親の母乳には、そうでないものに比べてより少ないレベルの６ー不飽和化脂肪酸しか含まれていないからである。だから母親がアトピーである場合には、母乳哺育は必要な必須脂肪酸供給の為にさして役立たな

いことになる。６ー不飽和化脂肪酸の前駆物質を投与することにより乳児にアトピー疾患発生を予防しようとする試みもある。これは人工栄養ミルクにGLAのような必須脂肪酸を添加すればよい。日本では市販ミルクの３０％にこのような添加が既に行われている。第二に、特にアトピーを持つ母親の母乳哺育では、母乳中の６ー不飽和化脂肪酸レベルを、GLAをEPOの形で母親に消費させることにより、増加させることが出来る。これによってミルクに含まれるエネルギー量を上げ、またミルクの流れをも上げることが可能である。

今日、EPOのアトピー疾患への効力は疑問視されている。これは恐らく、n-6とn-3の両方が必要だからか、投与量が適当でないか、または必須脂肪酸の欠乏によって始まったアトピーであっても病状が既に必須脂肪酸投与では回復不能となっているか、或いは、必須脂肪酸が含まれて居ないか、によるものであろう。

DETERMINATION FOR FATTY ACID PROPORTIONS OF SERUM AND LYMPHOCYTE PHOSPHOLIPIDS

Yutaka Nasu (Nagano College of Nursing)

1.Separation of Blood Samples to Mononuclear Cells and Serum

Equipment and Reagents needed

Equipments

1) falcon tubes (sample number X 3)
2) pipettes of 10 or 20ml (sample number X 3)
3) pasteur pipettes
4) glass tubes top covered with black cap (sample number)

Reagents Preparation

1) Phosphate Buffer Saline
2) Limphoprep

Preparation and Actions

1) Put blood samples into the falcon tube.
2) To 12ml of blood samples add the same amount of PBS (Phosphate Buffer Saline) and mix using the pipet.
   (To make the total amount of 25ml is preferable.)
3) Add carefully at the bottom of the sample 3ml of Limphoprep with pasteur pipet.
4) Centrifuge at 1,600 r.p.m. for 40 minutes.
5) After separation suck up the lymphocytes at the middle phase very carefully with pasteur pipet and put it to another falcon tube.
6) Suck up the serum phase (the upper phase) and stock in the freezer.
7) Add the lymphocytes so much of Buffer (PBS), mix using the same pipet, and spin at 800 r.p.m. for 10 minutes.
8) Flow away the Buffer and and add newly, and spin again.
9) Repeat 8) at least one more time.
10) Lymphocyte should be washed into the glass tube with water just before the extraction.

2.Lipid Extraction

Principle

By making contact with organic solvent, lipid fraction of the serum moves to solvent phase.

Equipment and Reagents needed

Equipment

1) glass tubes top covered with black cap [sample No. X 3]
2) 1 ml pipet [sample No. X 2], 5 ml pipet [5]
3) thin pipet (Pasteur Pipettes) [sample No. X 2}
4) beaker 50ml [5]
5) mess cylinder 50ml [1], 100ml [1]
6) mess flask 100ml [1], 25ml[1]
7) centrifuger

Reagents Preparation

1) chloroform
2) distilled water
3) Methanol
4) The mixture of chloroform:methanol=1:1
5) nitrogen gas

Preparation and Actions

1) Put 1ml of serum sample into the glass tube and add at first 2.5ml of methanol and then 1.25ml chloroform. This should be a clear one phase system.
2) Add 10μl of antioxygen with Hamilton syringe, and after washing the syringe, add 55μl of standard phospholipid.
3) Wait and mix gently for 10 to 15 minutes.
4) Add 3.75ml of chloroform and 0.9ml of water (if sample volume is 0.5ml, then add 1.4ml water), and spin the tube at 3,000 r.p.m., for 10 minutes. AMPS should not go over 9.
5) After the spin if the mixture separates clearly to two phases holding protein between phases, suck up the chlorophorm phase (the lower phase) with thin pipet and put it to another tube.
6) If the separation is not clear, for example the protein settles on the bottom, suck up the chloroform phase with thin pipet, put it to another

tube and add 1ml of chloroform and 1ml of water, and again make the spin.

7) After the separation and chloroform phase sucked up, make the injection with nitrogen gas for the evaporation of chloroform and continue till chloroform completely goes away.
(This is to be made within almost 15 minutes. But be careful not to touch the tip for gas to the liquid.)

8) As soon as the injection is over, add 0.05ml (50μl) of chloroform and methanol (1:1) mixture and bring the test tube to the freezer.

3.Fractionization of Lipid with Thin Layer Chromatography

Equipment and Reagents needed

Equipment

1) Hamilton syringe
2) thin layer
3) chromatography chamber
4) thin pipet
5) thin spatula
6) 1ml of pipet
7) glass tube [sample No. X 2]
8) centrifuger

Reagents Preparation

1) the solvent for thin layer chromatography
hexane:diethyl ether:acetic acid=50:50:1
2) chloroform
3) iodine
4) the mixture of chloroform-methanol-water-acetic acid

chloroform	100
methanol	100
distilled water	20
acetic acid	0.25

5) nitrogen gas
6) PE standard

Preparation and Actions

1) Activate the thin layer at 110 ℃ for 2 hrs.
2) Prepare the solvent mixture in the chamber and make a few minutes evaporation.
3) Prepare chloroform for washing Hamilton syringe.
4) Bring the standard and the sample from the freezer.
5) Draw the lines for spotting with a pencil on the thin layer.
6) Spotting
   (1) Wash Hamilton syringe with chloroform several times.
   (2) Make spots along the left lane with 5 μl of standard.
   (3) Wash syringe with chloroform several times.
   (4) Make spots along the centre lane with 5 μl (for example) of sample.
   (5) Make spots along the right lane with 50 μl (for example) of sample.
   Do not touch the syringe to ssilica and make spot slowly as the solvent never spread too widely.
7) Put the thin layer into the chamber and keep it straight up,and do not make only one side touch solvent earlier than another side. And be careful the lanes should be a little higher than solvent level.
   (With this fractionization, (1)neutral lipids, (2)free fatty acids, (3)free cholesterol, and (4)phospholipids separate.
   We want to get the 4th one. It would be colored with yellow.)
8) After the solvent are absorbed into thin layer to its top, put it out and dry.
9) After drying, stain only standard lane with iodine.
   Staining would damage phospholipid. So to stain with iodine only standard lane keep other lanes covered with something.
   The staining should be made by blowing nitrogen gas through glass tube holding iodine inside among cotton phases.
10) Draw the square surrounding each sample lane with a pencil and cut the sillica along the pencil line with needle to isolate each band.
11) Blow water to each square using thin pipet.
12) Cut the wet part of cillica with thin spatula and put the cutted cillica into the glass tube.
13) Add 1ml of chloroform-methanol-water-acetic acid mixture and shake a couple of minutes.
14) Spin and down the sillica with 3,000 r.p.m. for 5 minutes.
15) Extract silica one more time with same solvent.
16) Take up the solvent and make evaporation with nitrogen gas.
17) Stock in -20 ℃ after adding 50 μl of chloroform-methanol

complex.

4.Transmethylation of Phospholipids

Equipment and Reagents needed

Equipments

1)Incubator
2)glass tube
3)Centrifuger
4)pasteur pipettes

Reagents

1)Methanol containing 1% sulfuric acid
2)Hexane
3)Distilled water
4)Absolute EtOH

Preparations and Actions

1) Evaporate the sample into dryness.
2) Add 1ml of Methanol containing 1% sulfuric acid.
3) Keep the sample in black capped tubes overnight at 50 ℃.
4) Let the tubes cool, and add 1ml H2O+2ml Hexane,
vortex, or turn around about 50 times.
5) Centrifuge at 2,000 rpm for 5 minutes.
6) Suck the lower phase with pasteur and discard it.
(If the silica pieces are floating, then suck the upper phase and put it to another tube. Always better to take this way.)
7) Evaporate the upper phase (which is left) and add 1ml of absolute ethanol.
8) Evaporate this and add 20-30 μl of Hexane.

5.The Methyl Esters of Fatty Acids Analysis in a HP 5890 Gas Chromatograph Using OV 351 Sillica Capillary Column

Equipment and Reagents needed

Equipments

1)microsyringe

2)gas chromatograph (Oven, Electrometer, Programmer, Injector)

3)integrator (3390A INTEGRATOR HEWLETT*PACKARD)

4)gas cyrinders of nitrogen, hydrogen and air

Reagents

1)chloroform

2)hexane 3)ethanol

Preparations and Actions

I.Setting of Machine

1) Close the oven door.

2) Open the gas cock of Hydrogen gas and Air gas cylinder, but never touch Nitrogen Gas cylinder.

[1] main cock

[2] sub cock: Adjust Hydrogen to 2.9, and Air to 2, as indicated on the meter with magic ink.

3) Open the gas tube over the oven. (Take off the cock.)

4) Put the "cool" switch of MODE off.

5) Put the main switches of MODE, PROGRAMMER and ELECTROMETER on, and also recorder on. Then recorder indicates "ready".

6) Put injector lever to the arrowed place(=275), and when the injector lamp begins "on and off" successively, everything is ready for beginning gas chromatograph.

7) Adjust the Hydrogen gas pressure of FRACTOVAP to 1 and Air to 0.8.

8) Put the Ignission switch of ELECTROMETER down, keeping it down TILL THE TOP HAT OF CENSOR JUMPS UP, and then automatically it will be recovered. (Sometimes it bombs).

9) Increase Air pressure to 1.
Decrease Hydrogen pressure slowly to 0.5.

10) Programme is already set and never touch them.
Wait for a time setting the integrator till the injector lamp begins on and off.

II.Setting of Integrator

1) LIST, LIST

2) ATTENUATER2, 0, ENTER

3) CHART SPEED, 1, ENTER

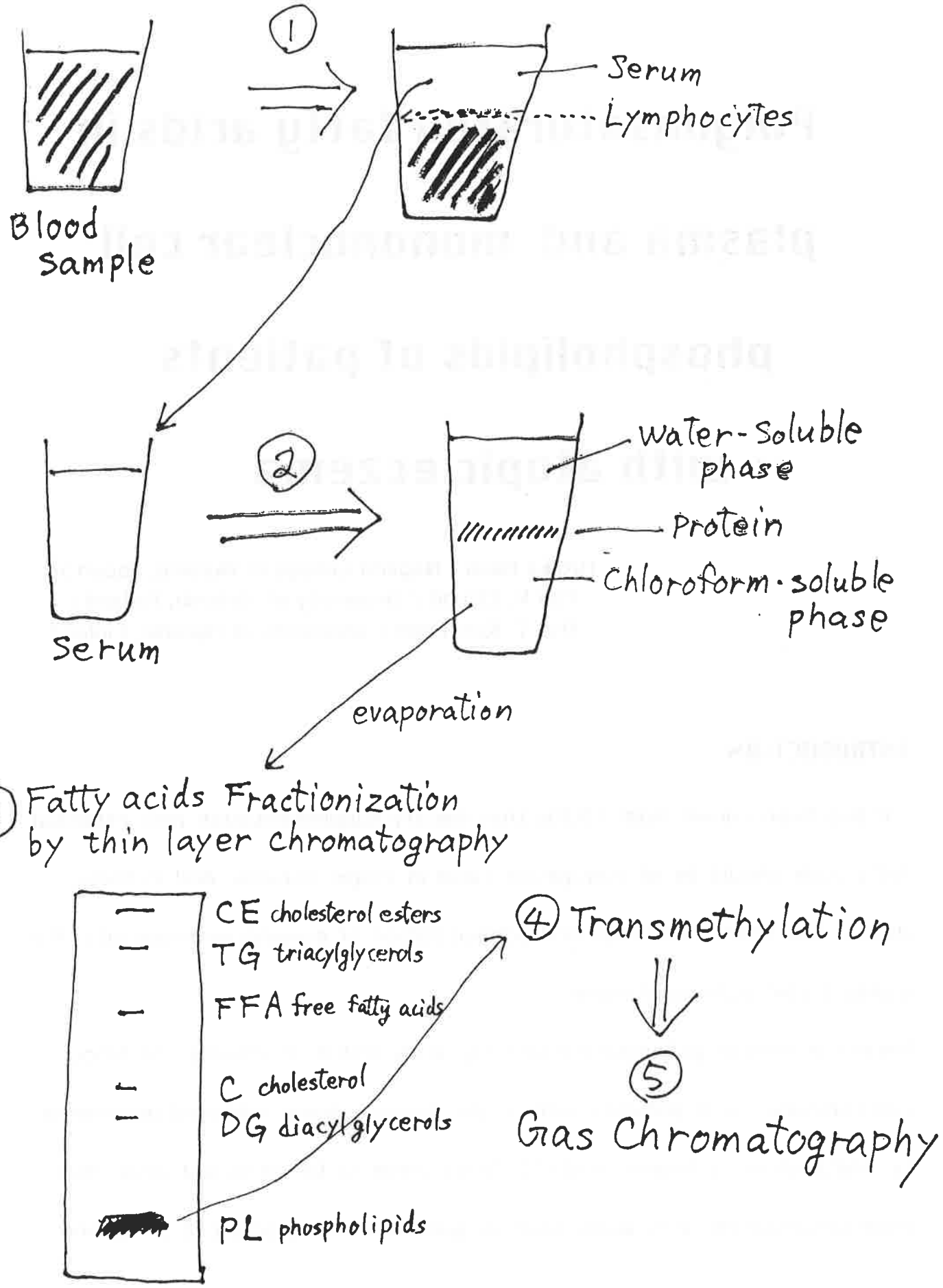
4) PEAK WIDTH, 0.1, ENTER
5) THRESHOLD, 0, ENTER
6) AREA REJECTION, 0, ENTER
7) LIST, LIST
8) LIST, TIME, ENTER
9) INTG, 9, TIME, 0, ENTER
10) INTG, -9, TIME, 2, ENTER
11) PK WD, 0.1, TIME, 5, ENTER
12) PK WD, 0.3, TIME, 15, ENTER
13) STOP, TIME 25, ENTER
14) LIST TIME

III. Operating the Machine

1) Before injection wash syringe with chroloform or absolute EtOH 10 times.
2) Make injection of the sample. (5ul just enough.)
Injection must be made carefully and rapidly. All sample liquid in the syringe must be injected at a time.
3) Make start of the [1] PROGRAMMER , [2] INTEGRATOR just after the injection.
4) Write away on the margin of the chart all your knowledges about working sample.

IV. Ending of Gas Chromatograph Work

1) ELECTROMETER main switch off.
2) PROGRAMMER main switch off.
3) "cool" switch of MODE ON.
4) Open the even door.
5) INJECTOR off.
6) Hydrogen pressure to 0.
Air pressure to 0.
7) Close the main cocks and sub cocks of each gas cylinder, but never touch the nitrogen gas cylinder.
8) Integrator off.
9) Wait at least 15 minutes.
10) Close the gas tube.
11) Main switch of MODE off.


Blood Sample
1
Serum
Lymphocytes
Serum
2
water-Soluble phase
Protein
Chloroform-soluble phase
evaporation
3 Fatty acids Fractionization by thin layer chromatography
CE cholesterol esters
TG triacylglycerols
FFA free fatty acids
C cholesterol
DG diacylglycerols
PL phospholipids
4 Transmethylation
5
Gas Chromatography

Polyunsaturated fatty acids in plasma and mononuclear cell phospholipids of patients with atopic eczema

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Kari K. Eklund (University of Helsinki, Finland)
Yrjo T. Konttinen (University of Helsinki, Finland)

INTRODUCTION

It has been known from 1930s that dietary supplementation with essential fatty acids should be of therapeutic value in atopic eczema. And in these days it has been found that oral administration of evening primrose oil (EPO) makes atopic eczema improve.

Recent studies of polyunsaturated fatty acids (PUFA) in plasma and blood cell components of patients with atopic diseases have indicated disordered fat metabolism as linoleic acid (18:2n-6) tends to be increased while the more unsaturated fatty acids, such as gamma-linolenic acid (18:3n-6) and

arachidonic acid (20:4n-6) are present in decreased amounts (Fig 1). In atopic eczema patients, the delta-6-desaturase function should be impaired, then the amount of linoleic acid should be increased, on the other hand those of the linoleic acid metabolites should be decreased. And so when the direct metabolite of linoleic acid, gamma-linolenic acid, is administered for the patients, then the symptoms of this disease should be disappeared.

It has also found that such an abnormality may not be restricted to patients with atopic dermatitis but can also be found in cases of respiratory allergy and other diseases.

On the other hand, Berth-Jones et al. indicates that essential fatty acid supplmentation has no effect for the improvement of atopic dermatitis according to their placebo-controled trial.

We had a chance to have several infant patients with atopic eczema and to make them the treatment with Efamol. At the same time of this treatment, we tried to measure the fatty acid components in the serum and the mononuclear cells phospholipid, and also tried to get some blood data concerning about the inflammation, and tried to make clear the effect of evening primrose oil and the change of metabolism of essential fatty acids.

MATERIALS AND METHODS

Patients and samples

10 patients with atopic eczema aged from 4,7 to 13,7 (mean age 8,03) were entered into a trial of evening primrose oil in the treatment of atopic eczema.

The evening primrose oil used was Efamol, the seed oil from specially bred strain of Oenothera biennis which yields oil of a constant consumption.

Blood samples were drawn into EDTA-treated tubes, separated within hours of collection and stored at -20 ℃ until analysis.

To investigate phospholipid fatty acids in mononuclear cells (MNC), 20ml blood was drawn into an equal amount of Hank's balanced salt solution. MNC were isolated by gradient centrifugion in Ficoll-Hypaque (Pharmacia).

8 children were nominated as control group (aged from 6,5 to 12,4), and they were all free of acute or chronic illness, denied taking either medicinal or drugs and had no personal or family history of atopy.

Assays

All blood samples were taken after an overnight fast. The specimens for

trace elements and vitamin analyses were collected simultaneously with the laboratory tests needed for control of drug therapy and to monitor the disease activity.

The blood samples for trace element determination were drawn using stainless steel needles (Venoject, Terumo, Belgium). Serum samples were stored in polyethene tubes and kept at -20 ℃ until analyzed. Zinc and copper concentrations were determined by flame atomic absorption spectrophotometry (AAS)(Perkin Elmer 300 AAS) using a method developed by Salmela et al (1984). Selenium concentrations were determined with a flameless AAS (Perkin Elmer 5000, HGA 400) employing the method described by Alfthan and Kumpulainen (1985). All the trace element measurements were made in the same laboratory.

Fatty acid compositions of MNC and serum phospholipids were determined using a method developed by William W. Christie:

One ml of pure serum was extracted with 3,75ml of chloroform/methanol (1/2), washed with 3,75 ml of chloroform and 0,9ml of water, centrifuged for 10 minutes and the lower phase (the chloroform phase) was separated and evaporated by nitrogen gas.

After the evaporation 0,05ml of methanol/chloroform(1/1) was added, and fractioned with thin layer chromatography (Kieselgel 60, Merck, Germany)

into neutral lipids, free fatty acids, free cholesterol and phospholipids by using hexane/diethyl ether/acetic acid (50/50/1) as a solvent. Phospholipid fraction was scraped into black capped tubes and transmethylated in 1% H_2SO_4 in dry methanol at 50 ℃ overnight. The resulting methyl esters of fatty acids were separated and measured using a Hewlett Packard 5880 gas chromatography with a 6-foot column packed with 10% silar on chromosorb WAW 106/230.

The carrier gas was hydrogen (0,5kg/㎠). Initial temperature of the oven being 170 ℃, final temperature 240 ℃. Retention times and peak areas were automatically computed by a HP 3390A integrator. Peaks were identified by comparison with standard fatty acid methyl esters from Nuchek Prep. Inc. (Elysian, Minnesota, U.S.A.). Figure 2 shows an example of gas chromatographic chart from certain patient's serum identified each fatty acid.

Evaluation of the results

The hardware used in this study was a .... computer at University of Helsinki. Data were listed into DATABASE version. All statistical analysis were performed using Biomedical dataprocessing program library (BMDP, version 1994).

In before/after comparisons, the statistical significance was tested using paired t-test. Standard deviation (SD) was used to indicate dispersion of results.

RESULTS

Fatty acid concentration

Table 1 shows the mean values of each fatty acid proportion per total fatty acids in MNC, including samples from control groups, from patients before treatment and from patients after treatment. We could be available 4 control samples, 8 patients before treatment and 5 patients after treatment.

No significant differences were seen between control and patients before treatment. By the treatment of EFA, dihomogamma-linolenic acid (20:4n-6) was significantly decreased and behenic acid (22:1n-9?) significantly increased.

Linoleic acid (18:2n-6) of patients looked like to be increased compared with control group and decreased after treatment,though not significantly. And more unsaturated fatty acids, such as homogamma-linolenic acid and

arachidonic acid looked decreased after treatment. Lindskov and Hoelmer has reported that in eczema patient MNC, the ratio of 20:4n-6/20:3n-6 in total lipids (b) had been reduced. In this study, however, there could not find any significant differences of this ratio between control group and patient group before treatment.(Table 1)

Table 1 also shows the mean values of each fatty acid proportion per total fatty acids in serum on the same groups as above. The significant differences were observed on 20:3n-6 between control and patients before, and also between patients before and patients after. Another significant difference was found on 20:4n-6 between patients before and patients after. The ratio of (18:3n-6+20:3n-6)/18:2n-6 (a) in patient before was significantly reduced compared with control, and looked reduced again after the treatment.

With matched t-test about serum value between patients before treatment and those after treatment, significant differences were found on 18:1, 20:3n-6, 20:4n-6, (18:3n-6+20:3n-6)/18:2n-6 and 20:4n-6/18:2n-6. Fig4 shows the change of 20:3n-6 percentage to total fatty acids on each patient, Fig5 about 20:4n-6, Fig6 about (18:3n-6+20:3n-6)/18:2n-6, Fig7 about 20:4n-6/18:2n-6. Also in Fig8 about 18:2n-6. In many cases it has been said that linoleic acid (18:2n-6) concentration increases in the serum of atopic

eczema patients, but in our study no significant tendency was observed on 18:2n-6.

DISCUSSION

Atopic eczema is said to be the condition in which an inherited slow rate of 6-desaturation has been best documented. Normal or elevated concentrations of LA and ALA are associated with reduced concentrations of their metabolites in umbilical cord blood, in the plasma and red cells of children and adults, in milk and in adipose tissue.

From our results we could not find any difference about linoleic acid in the serum between control and patients with atopic eczema.

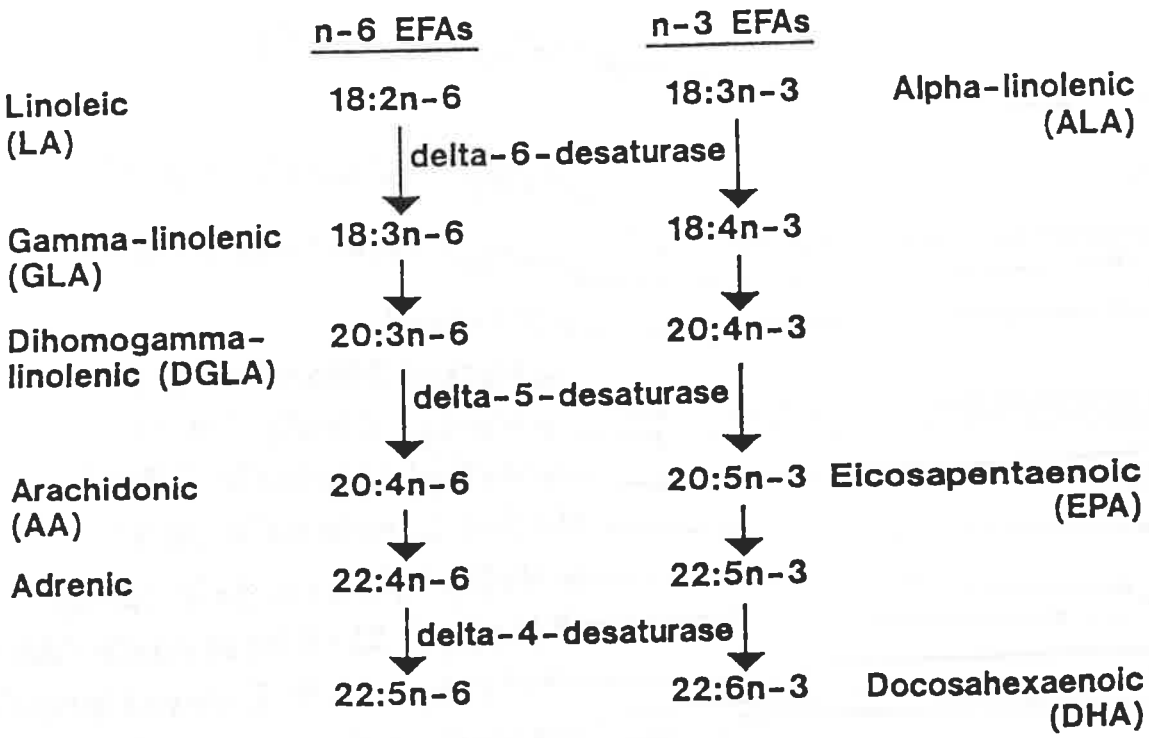
Schalin-Karrila et al. reported in 1987 that the baseline fatty acid composition of plasma phospholipids from the patients with atopic eczema (young adults) did not differ significantly from that in the healthy subjects, except for a slightly lower level of oleic acid (18:1n-9).

On the other hand, Manku et al.(1984) had reported that the major dietary n-6 EFA, linoleic acid, was significantly elevated but all its metabolites were significantly reduced in the adult patients'plasma. Beforehand Manku

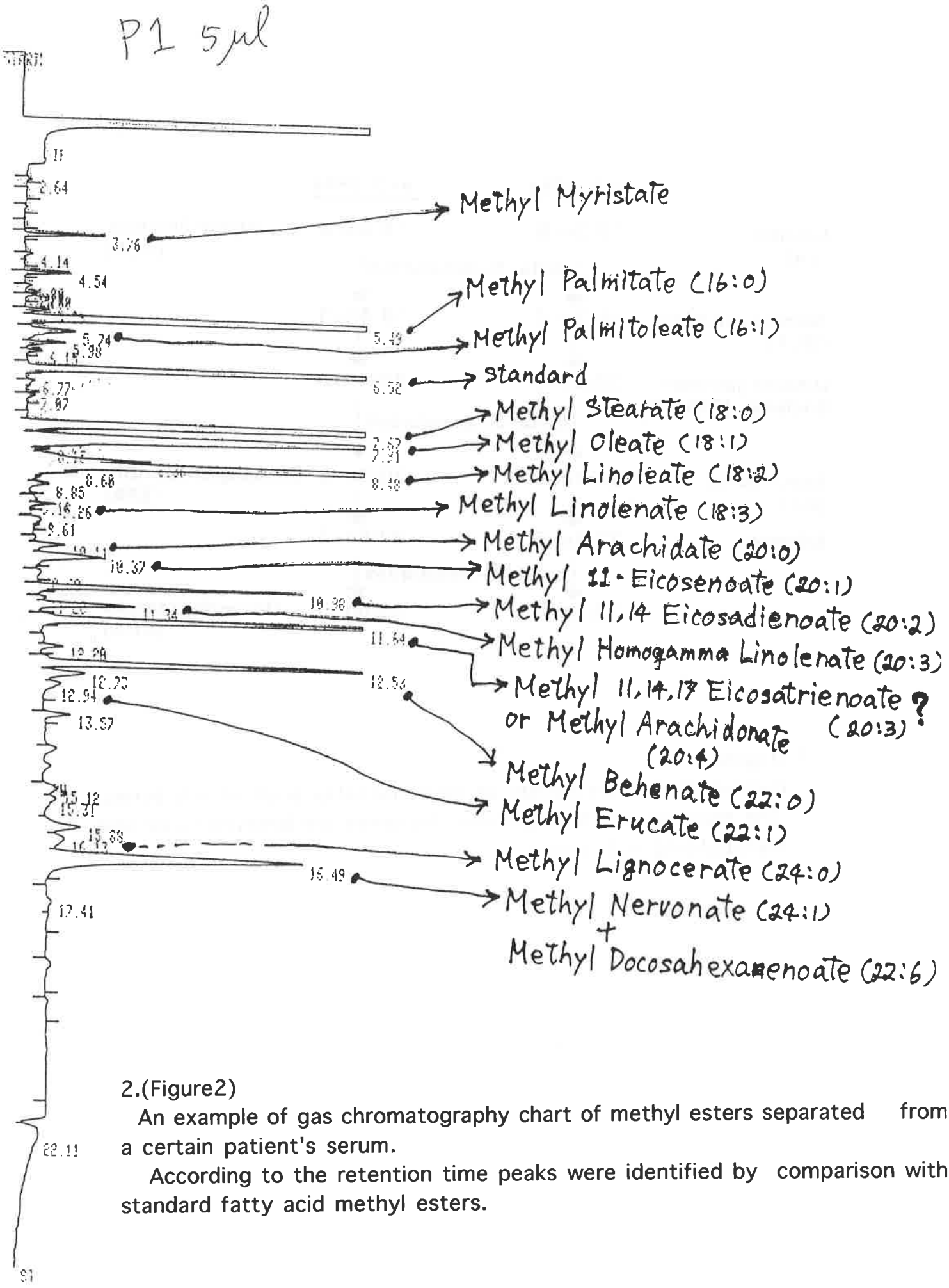
et al. had made clear about the fatty acid composition in plasma in normal humans.

Strannegard et al. (1987) : The fatty acid composition of serum lecithin from children with atopic dermatitis was found to be abnormal, characterized by significantly increased proportion of linoleic acid and reduced levels of metabolites of this fatty acid.

Lindskov et al. (1992) : Examined the PUFA content in phospholipids derived from plasma, red blood cells and mononuclear cells (MNC) in patients with atopic dermatitis. (adults). In plasma no significant differences were found between patients and controls. The most significant findings in eczema patient MNC were reduced ratios of 20:4n-6/20:3n-6 in total lipids and in phosphatidyl ethanolamine (PE) and of 20:4n-6/18:2n-6 in both total phosphatidyl choline (PC) and PE.



1.(Figure1)
Outline of the metabolism of essential fatty acids of n-6 series. The enzymes which metabolise this series are believed to be also metabolising n-3 series.



2.(Figure2)

An example of gas chromatography chart of methyl esters separated from a certain patient's serum.

According to the retention time peaks were identified by comparison with standard fatty acid methyl esters.

Standard 68 A
5 µl

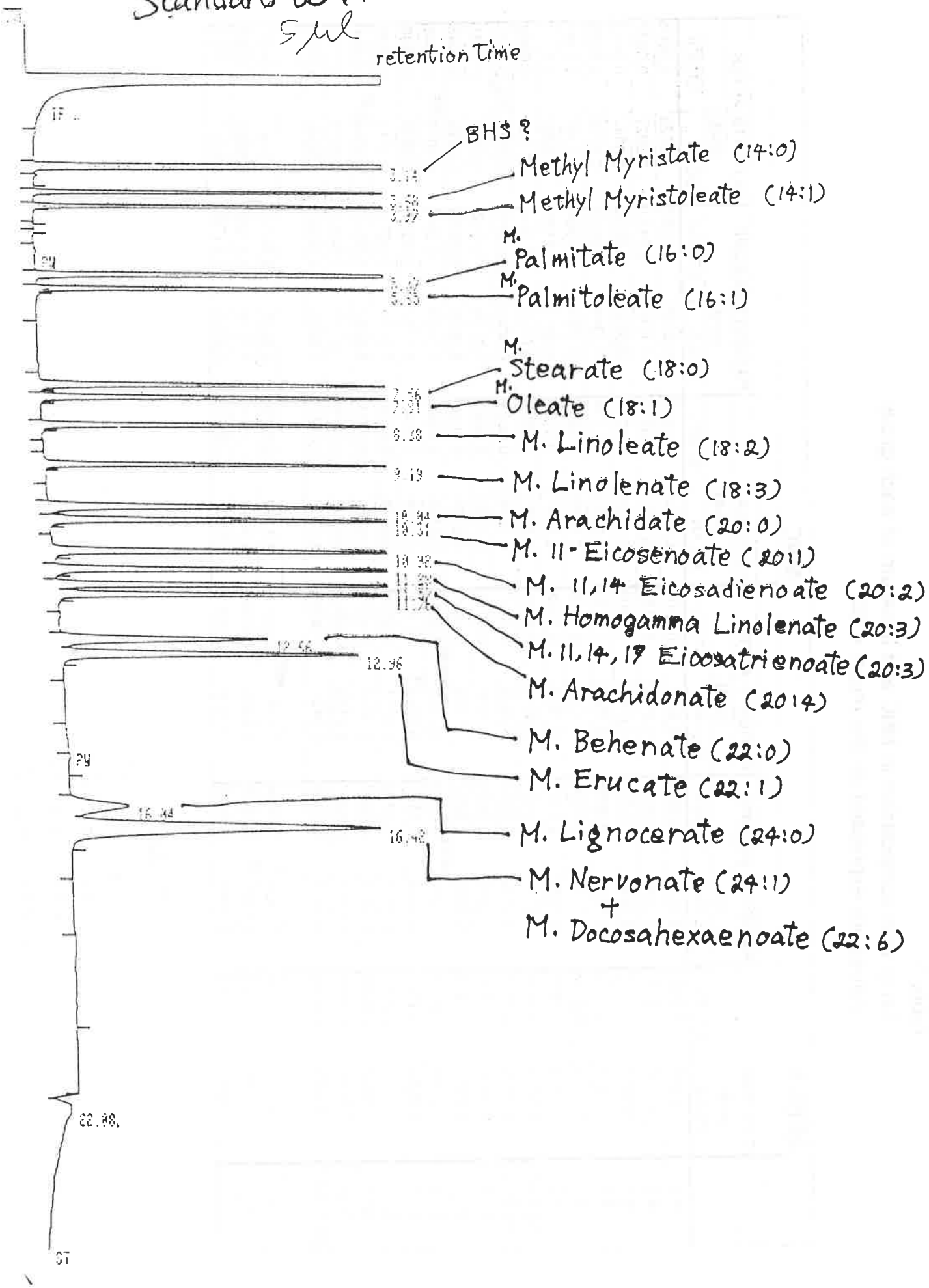


Table 1

Fatty acid composition in MNC and in serum of each group.

Values are expressed as the mean + SD.

	SERUM						MNC					
	CONTROL (n=8)		PATIENTS BEFORE (n=10)		PATIENTS AFTER (n=9)		CONTROL (n=4)		PATIENTS BEFORE (n=8)		PATIENTS AFTER (n=5)	
	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV	MEAN	STDEV
14:0	0,003	0,0007	0,0035	0,0009	0,0031	0,0008	0,0033	0,0016	0,0025	0,0007	0,0025	0,0008
16:0	0,2543	0,0127	0,2543	0,0096	0,2532	0,0155	0,1781	0,0317	0,1781	0,0403	0,2027	0,0337
16:1	0,0055	0,0017	0,0063	0,001	0,0062	0,0009	0,0055	0,0017	0,0041	0,0018	0,0047	0,0012
18:0	0,1554	0,0122	0,1536	0,0098	0,1526	0,0123	0,2319	0,034	0,2344	0,0617	0,2716	0,0375
18:1	0,1054	0,0135	0,1136	0,0206	0,1023	0,0178	0,1019	0,0461	0,1063	0,0364	0,1387	0,0346
18:2	0,1914	0,0391	0,1872	0,0177	0,1853	0,0268	0,0387	0,0131	0,0447	0,0187	0,0287	0,0117
18:3n-6	0,0015	0,0006	0,0015	0,0004	0,0015	0,0004	0,0023	0,0005	0,0023	0,001	0,002	0,0009
18:3n-3	0,0025	0,0008	0,0032	0,0012	0,0029	0,0017	0,0016 (n=2)	0,0008	0,0045 (n=4)	0,0073	0,0005 (n=2)	0,0001
20:0	0,0055	0,0006	0,0056	0,0008	0,0056	0,0011	0,0158	0,0043	0,0136	0,0052	0,0176	0,0031
20:1	0,0035	0,0012	0,0033	0,0007	0,003	0,0007	0,0068	0,0026	0,0076	0,0033	0,0099	0,0048
20:3	0,0199	0,0027	0,0246 ***	0,0029	0,0305 ***	0,0041	0,0103	0,0049	0,0139	0,004	0,0072 **	0,0049
20:4	0,0664	0,0126	0,0665	0,0101	0,0749 *	0,011	0,1169	0,0643	0,1197	0,0646	0,0836	0,0698
22:0	0,0116	0,0015	0,0109	0,0019	0,0111	0,0018	0,0178	0,0038	0,0176	0,0075	0,0213	0,0036
22:1	0,002	0,0006	0,0019	0,0007	0,0018	0,0007	0,0087	0,0034	0,0052	0,0022	0,0094 **	0,004
24:0	0,0148	0,0044	0,0161	0,0037	0,0173	0,0044	0,0204	0,0076	0,018	0,0042	0,0218	0,0105
24:1+22:6	0,045	0,0094	0,0413	0,0078	0,0431	0,0085	0,0207	0,0111	0,0234	0,0096	0,0245	0,0101
a	0,117	0,0234	0,1406 *	0,0194	0,1763 ***	0,0371	0,3199	0,0222	0,3418	0,0877	0,3162	0,0602
b	3,35	0,5421	2,7291 **	0,4991	2,4865	0,4686	10,9084	1,5504	9,7236	2,8109	9,8868	3,9587
c	0,3641	0,1036	0,3586	0,0688	0,414	0,0983	2,807	0,8031	2,5955	0,9176	2,5207	1,5031

T-Test

* : $p < .10$

** : $p < .05$

*** : $p < .02$

**** : $p < .01$

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ORIGINAL ARTICLE

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Alpha-linolenic acid in the treatment of rheumatoid arthritis. A double-blind, placebo-controlled and randomized study: flaxseed vs. safflower seed

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Abstract In rheumatoid arthritis various pro-inflammatory metabolites of arachidonic acid (AA), such as leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), contribute to tissue destruction and pain. In contrast to AA, which is an omega-6 fatty acid, the omega-3 fatty acids, after having been liberated from the cell membrane phospholipids, are further converted into the non- or anti-inflammatory eicosanoids LTB_5 and PGI_3. AA concentration is an important regulatory step in the synthesis of both prostanoids and leukotriens. Dietary supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has therefore been used to decrease the ratio of AA to EPA or DHA to obtain beneficial clinical effects. EPA and DHA are found in animal fat and are quite expensive compared to their precursor alpha-linolenic acid (alpha-LNA) found in flaxseed oil. We, therefore, performed a placebo-controlled trial with alpha-LNA in 22 patients with rheumatoid arthritis, using a linoleic acid preparation as a placebo. After a 3-month follow-up, the treatment group showed an increased bleeding time, but the clinical, subjective (global assessment, classification of functional status, joint score index, visual analogue scale, pain tendereness score) and laboratory parameters (haemoglobin, erythrocyte sedimentation rate, C-reactive protein) did not show any statistical alterations. AA, EPA and DHA did not change either in spite of a significant increase in alpha-LNA in the treatment group. Thus, 3-month's supplementation with alpha-LNA did not prove to be beneficial in rheumatoid arthritis.

Key words Dietary intervention · Alpha-linolenic acid Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is an inflammatory disease involving multiple synovial joints. RA is associated with the classical signs of inflammation, including vasodilatation, oedema, pain in movement and joint tenderness. These symptoms are probably to some extent due to the local production of pro-inflammatory eicosanoids, in particular prostaglandins (PG) such as PGE_2 [1] and leukotrienes (LT) such as LTB_4 [2]. Accordingly, non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of RA. One important mechanism of action of these NSAID-type drugs is believed to be a direct enzymatic inhibition of the PGH_2 synthetase enzyme, which catalyses two subsequent reactions: first, 5-cyclo-oxygenazation of arachidonic acid (AA) into a labile intermediate PGE_2 immediately followed by conversion into PGH_2; PGH_2 is further metabolized into pro-inflammatory prostanoids such as PGE_2. Steroids, administered intra-articularly or systemically, are even more efficient anti-inflammatory drugs. This seems to be due to induction of lipomodulin, which binds to cell membrane phospholipids and prevents the action of the enzyme phospholipase A_2, which otherwise would be able to cleave AA from cell membrane phospholipids. Diminished production of AA decreases not only the production of the above-mentioned prostanoids, but also the production of alternative AA products, namely various leukotrienes such as LTB_4. These are usually formed from AA by the enzyme 5-lipoxygenase, which converts AA into 8R, 15S-di-hydroxyeicosatetraenoic acid, which is further metabolized into pro-inflammatory leukotrienes such as LTB_4 [1].

Because AA concentration is an important regulatory step in the synthesis of both prostanoids and leukotrienes [3], dietary supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been used to decrease the ratio of AA to EPA and DHA. In contrast to AA, EPA and DHA, after having been liberated from the cell membrane phospholipids, are further converted into the non- or anti-inflammatory eicosanoids LTB_5 and PGI_3. The clinical effects are beneficial and are well documented [4–9]. Because EPA and DHA are found in animal fat, they are quite expensive compared to their precursor alpha-linolenic acid (alpha-LNA), which is found in flaxseed oil. Therefore, nutritional supplementation by flaxseed oil could be a rational alternative. It has been speculated that alpha-LNA could be beneficial by two different means: first, by being elongated into long-chain EPA- and DHA-type fatty acids (and thus decreasing the AA "load") and second, by competing with linoleic acid (LA) for the Δ^6-desaturase enzyme, which would lead to formation of AA from LA, and thus, diminish AA and inflammation caused by metabolites therof. Furthermore, alpha-LNA could have a steroid-like effect by decreasing production of both pro-inflammatory prostanoids and leukotrienes [1–3]. Therefore, we decided to perform a double-blind, randomized study comparing alpha-LNA to "placebo" (flaxseed to safflower seed).

Patients and methods

The study was performed at the outpatient clinic of the Fourth Department of Medicine, Helsinki University Central Hospital. The 22 patients of both sexes [mean ages; treatment group: 51 years (range 34–62); placebo group: 53 years (range 40–72)] with RA (fulfilling the 1987 ARA criteria [10]) were randomly enrolled from a group that had previously participated in another nutritional study [11]. All participants gave their informed consent in writing. The entire study protocol was approved by the ethics committee prior to initiation. Patients were told not to change their dietary habits nor antirheumatic medication during the study period of 3 (3.2 ± 0.8) months.

Flaxseed and safflower powders were kindly provided by Dr. Paul Stitt (ENRECO, Essential Nutrient Research Company, Manitowoc, Wisconsin, USA). Patients were randomized into two groups. The treatment group ingested 30 g of flaxseed oil (32% alpha-LNA) per day for 3 months, while the control group received 30 g of safflower oil (33% linoleic acid). The safflower oil and flaxseed oil powders were made identical in taste and appearance. The dosage recommendations followed those given by the manufacturer. A larger dosage has been shown to produce diarrhoea, and some patients experienced loose stools in the present study.

Clinical assessment {patient's and investigator's five-scale global assessment, classification of functional class [12], Kaarela's joint score index (measuring joint involvement by swelling or limitation of motion in proximal interphalangeal, metacarpophalangeal, wrist, elbow, shoulder, toe, metatarsophalangeal, subtaloid, talocrural, knee and hip joints on each side and cervical spine; each joint scoring one and the maximum joint score being 23) [13], subjective visual analogue scale for pain [14]} and the measurement of the laboratory values reflecting the activity of disease [sedimentation rate, C-reactive protein (CRP), haemoglobin] were performed by the chief investigator (DN) at the beginning and end of the treatment period (Table 1). Soft tissue tenderness was also measured using a dolorimeter. The tender points used were those proposed by Yunus et al. [15], except that we measured the lower lumbar and cervical spine bilaterally. Pressure was increased gradually by the physician and removed as soon as the patient indicated discontent verbally. The pain threshold levels at different sites were normalized and summed up for pain tenderness scores [16]. In addition, the bleeding time (by the method of Duke) was measured to assess patient compliance.

The fatty acid profile (percentage change in AA, EPA, DHA, alpha-LNA and LA; Table 2) of serum phospholipids was measured using the method developed by Metsä-Ketelä et al. [11]. Lipids were extracted with chloroform/methanol (2:1) and fractionated with thin-layer chromatography (Kiselgel G, Merck, Germany) into natural lipids, free fatty acids, free cholesterol and phospholipids by using petroleum ether/diethyl ether/acetic acid (80/20/1) as a solvent. The phospholipid fraction was scrapped into glass-stopped tubes and transmethylated in 1.4 *M* HCl in dry methanol at +85 °C for 2 h. The methyl esters of fatty acids were analysed in a HP 5890 gas chromatography (Hewlett Packard Company, Avondale, USA) using an OV-351 silica capillary column (Nordion OY, Helsinki, Finland). The peaks were quantified with a flame ionization detector and HP3393A integrator.

Table 1 Clinical data and outcome results in alpha-linolenic acid treatment and linoleic acid placebo groups

	Treatment group (11 patients) (before/after treatment values)	Placebo group (11 patients) (before/after treatment values)
Clinical data		
Age (years)	51 (range 34–62)	53 (range 40–72)
Disease duration (years)	9.6 (range 4–20)	13.8 (4–36)
Global assessment (patient)	3.0 ± 0.8/2.9 ± 0.9	2.7 ± 0.5/2.7 ± 0.5
Global assessment (doctor)	2.8 ± 0.9/2.8 ± 0.9	2.3 ± 0.5/2.4 ± 0.5
Functional class	2.1 ± 0.7/1.9 ± 0.7	1.9 ± 0.3/1.9 ± 0.3
Joint score index	9.3 ± 7.9/9.1 ± 7.5	11.5 ± 4.2/9.5 ± 4.3
Visual analogue scale (pain)	4.0 ± 2.7/4.0 ± 3.3	4.4 ± 1.8/4.6 ± 2.2
Laboratory parameters		
Sedimentation rate (mm/h)	30.9 ± 24.1/ 35.7 ± 27.1	34.2 ± 20.4/ 32.5 ± 20.5
C-reactive protein (mg/l)	17.2 ± 9.6/ 20.3 ± 12.4	21.7 ± 11.9/ 21.8 ± 16.8
Haemoglobin (g/l)	128.6 ± 12/ 127.2 ± 11.1	129.8 ± 9.5/ 132.2 ± 10.8
Bleeding time (min)	3.1 ± 1.2/4.2 ± 1.3	3.1 ± 1.1/2.2 ± 1.5
Medication	*n*	*n*
NSAID	11	11
Corticosteroids orally	3	4
Sulphasalazine	2	4
Gold (intramuscular/oral)	5/1	3/2
Azathioprine	0	1
Penicillamine	1	1
Methotrexate	1	0
Hydroxychloroquine	1	1

Table 2 Change in fatty acid profile in alpha-linolenic acid treatment and linoleic acid placebo groups

Fatty acid	Treatment group (before/after treatment)		Placebo group (before/after treatment)	
	(%, SD)	(*P* value)	(%, SD)	(*P* value)
Alpha-linolenic acid	0.24 ± 0.29	0.04*	0.01 ± 0.13	0.84
Linoleic acid	1.13 ± 3.89	0.41	2.4 ± 2.42	0.008*
Arachidonic acid	−0.04 ± 0.73	0.88	0.08 ± 0.93	0.78
Eicosapentaenoic acid	0.02 ± 0.38	0.87	−0.75 ± 1.31	0.09
Docosahexaenoic acid	0.45 ± 1.30	0.43	−0.15 ± 1.53	0.74

* $P \leq 0.05$

The hardware used in the study was an IBM PC/AT. All the statistical analyses were performed using Biomedical's data processing program (BMDP, version 1993). In before/after comparisons, the statistical significance tests were performed using paired *t*-test (BMDP3D) of variances. Standard deviation (SD) was used to indicate dispersion of results and a *P* value of ≤0.05 or less was considered statistically significant.

Results

After 3.2 ± 0.8 months of supplementation, the serum alpha-LNA acid concentration (0.24 ± 0.29, $P = 0.04$) and the bleeding time (1.03 ± 3.13, $P = 0.35$) were increased in the flaxseed oil group, and the LA concentration (2.4 ± 2.42, $P = 0.008$) was increased in the safflower oil group (Tables 1, 2), confirming overall patient compliance. None of the clinical parameters nor the condition of the patients improved in the flaxseed oil group (Table 1). Some of the parameters reflecting disease activity improved in the safflower oil group (difference in before/after values; flaxseed vs. safflower; haemoglobin, CRP). The pain tenderness score assessed by the dolorimeter did not change in either group.

The trace element status reflecting nutritional potential for desaturation (zinc) and for antioxidation (selenium, zinc) was as follows. The serum zinc was 12.2 ± 1.6 μmol/l; five patients had low serum zinc levels (< 11 μmol/l) [11]. The serum selenium was 116.2 ± 21.2 μg/l. Copper concentrations may indicate inflammatory activity; the mean value of 23.7 ± 4.2 μmol/l was somewhat higher than the upper limit of normal values of 22 μmol/l [11]. Eleven patients exceeded this upper limit value.

Discussion

Inflammation in RA is in part caused by metabolites of AA and, therefore, it might be expected that a decreased load of AA could be beneficial. To this end RA patients were supplemented wiht either alpha-LNA or LA in a double-blind and randomized manner. Participation in the study was voluntary and patient compliance seemed excellent. This was supported by the change, during the course of the dietary intervention, in the serum phospholipid fatty acid profile; after the codes were opened, it was shown that patients on alpha-LNA specifically had significantly increased alpha-LNA serum values (with no changes in LA levels) and, vice versa, patients on LA had significantly increased LA values only.

Not only did the dietary intervention have an objective effect on the serum phospholipid fatty acid profile (Table 2), but also the bleeding time was affected, being lengthened in patients on alpha-LNA. This has also been reported by others [17, 18] and has been considered to be due to the rapid turnover of the cell membrane phospholipids in the nuclear platelets and thus a rapid change in lipid mediators, including the pro-aggregatory thromboxane A_2, released upon platelet activation induced by surface contact [18]. This welcomed effect of alpha-LNA has also been used successfully in the secondary prevention of coronary heart disease [18].

Contrary to expectations, there was no clinical improvement in RA patients on alpha-LNA (or LA; Table 1). In particular, it was of interest that there was no change in the pain threshold in alpha-LNA-supplemented patients. It is known that PGE_2, PGI_2 and 8R, 15S-diHETE (an intermediate in the LTB_4 metabolism) sensitize the primary afferent nociceptive fibres to subsequent stimuli including mechanical pressure [19, 20]. If there had been a favourable effect on the prostaglandin metabolism, this could have led to a higher pain threshold in the soft tissues also. However, this was not the case.

Increased intake of (omega-3) polyunsaturated fatty acids (PUFA) without adequate antioxidant protection could result in increased free radical formation and lipid peroxidation. Thus, when (omega-3) PUFAs are used to reduce the inflammatory events of RA, their possible adverse effects should be considered and prevented [21]. It has been shown that in patients with inflammatory joint disease the synovial fluid concentration of alpha-tocopherol is significantly lower relative to that of paired serum samples [22]. This indicates the consumption of alpha-tocopherol within the inflamed joint, perhaps due to its role in the termination of lipid peroxidation. PUFAs are easily oxidized and, therefore, the antioxidant protection should be properly acknowledged. Normally the vegetable oils provide lipid-soluble antioxidants in addition to fatty acids.

Alpha-LNA and LA are the most abundant PUFAs in Western diets, with LA body levels usually exceeding those of alpha-LNA. It is therefore possible that a short-term supplementation for 3 months only was not enough to significantly increase the alpha-LNA : LA ratio. If that were the case, supplementation with alpha-LNA would not change the EPA and DHA profile. This was supported by the present observations, because alpha-LNA supplementation increased the serum alpha-LNA levels ($P = 0.04$) but had no effect whatsoever on EPA, DHA, AA or other fatty acids (Table 2). Although minor effects on prostaglandin levels might be possible [23], this was not reflected in the clinical status; clinical and laboratory disease activity parameters were unaltered in patients receiving alpha-LNA. On the other hand, LA supplemen-

tation, which might have been expected to lead to an increased AA load, did not increase the serum AA levels (but did, however, decrease EPA and DHA levels; Table 2) not did it change disease activity scores. Adam et al. [23] have shown that PG synthesis, but not the conversion of LA to AA is suppressed by alpha-LNA ingestion in man.

Some studies have indicated possible clinical benefits of supplementation by both gamma-linolenic acid of the omega-6 series and by fish oils of the omega-3 series in the treatment of RA [24, 25]. Conversion of alpha-LNA to EPA and DHA in man has been suspected for some time; recent findings suggest that such a conversion, initiated by the Δ^6-desaturase, is indeed possible. However, the extent of such a change is an open question and may be dependent on zinc. The utilization, metabolism and lipid peroxidation of fatty acids depend, in addition to vitamin E, on other nutrients such as zinc. In particular, zinc controls Δ^6-desaturase activity, catalysing the first step from linolenic acid to EPA and LA to gamma-linolenic acid [26]. A low intake of zinc may impair desaturation and elongation of essential fatty acids. Low zinc levels apparently increase oxidation of alpha-LNA [27]. It was, therefore, of interest that the RA patients in this study had low serum zinc values. Low conversion of alpha-LNA to EPA and DHA, together with a low alpha-LNA:LA body ratio, might explain why a short-term alpha-LNA supplementation did not alter the RA disease activity.

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REDOX BALANCE IN RHEUMATOID ARTHRITIS: TRACE ELEMENTS AND VITAMINS VERSUS DIET, DISEASE ACTIVITY AND MEDICATION

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Key words: zinc, copper, selenium, superoxide dismutase, glutathione peroxidase, retinol, alpha-tocopherol, retinol binding protein, nutrition, disease modifying anti-rheumatic drugs, sulphasalazin, rheumatoid arthritis

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INTRODUCTION

The interactions between the interphase of chronic inflammation, medication and diet are not known. We tackled this problem by combining new tools, Setti-N nutritional database developed at National Health Institute in Finland and multifactorial statistical analysis, with careful clinical and biochemical characterization. RA provides a good model for such purposes and was subjected to a detailed study.

Abbreviations used: RA = rheumatoid arthritis; RF = rheumatoid factor; GSHpx = glutathione peroxidase; SOD = superoxide dismutase; RBP = retinol binding protein; IL-1 = interleukin-1; DMARD = disease modyfying anti-rheumatic drug; RDA = recommended daily allowance; ROS = reactive oxygen species

PATIENTS AND METHODS

Clinical characterization: Patients were recruited from outpatient clinic of the Helsinki University Central Hospital. The extent of joint inflammation was recorded by using Kaarela's joint score index, based on the presence of joint swelling. Sixteen extra-articular tender points were studied using a force gauge dolorimeter; after transformation of the read outs to z-units, all were added to a single tenderness score index. General inflammatory disease activity was assessed using Hb, ESR, CRP and Waaler-Rose test for RF. Various demographic characteristics recorded included age, sex, weight and height. Drug treatment and disease duration were recorded as well.

Biochemical tests: Blood samples were taken after overnight fast. Serum zinc and copper were determined by flame atomic absorption spectrophotometry and selenium with a flameless AAS. Plasma GSHpx was measured using 7 mM t-butyl hydroperoxide as substrate and H_2O_2 as reaction initiator,

whereafter NADPH to NADP conversion was followed by absorption spectrophotometry. Erythrocyte SOD activity was analyzed by following superoxide production in xanthine oxidase system in the presence of NBT. Vitamin A (retinol) was quantitated after HPLC spectrophotometrically by measuring absorbance at 205 nm whereas vitamin E (alpha-tocopherol) fluorescence intensity was measured using excitation at 292 nm and emission at 324 nm. Serum RBP and ceruloplasmin were measured by immunodiffusion.

Dietary assessment: Nutritional therapist or physician gave detailed oral guidance to patients for a throughout one-week dietary recording in three periods during three weeks. Patients were also provided with detailed written instruction with examples. The portion sizes were assessed using a booklet including information of standards in Finland. Supplementation and home-cooking recipes were recorded. Transformation of nutrients was done suing Setti-N database covering all food items available in Finland.

Statistical analysis: Univariate linear regression was used for comparision of two variable. If variables were skewed after logarithmic and square root transformations for Poisson-distributed variables or Arcus sin transformation for variables presented as proportions, Spearman's rank correlation was used instead.

For comparision between groups, t-test was used for normally distributed variables and Mann-Whitney test for skewed distributions. If there were more than two groups, one-way analysis of variance and Kurskal-Wallis test, respectively, were used.

To study the independent effects of various recorded variables on continuous variables, such as the concentration of a trace element or vitamin, multiple linear stepwise regression analysis in a forward manner was applied. To adjust the material, if necessary, for e.g. age and sex, these were forces into the model first.

In instead the independent effects of various recorded variables, including concentrations of various trace elements and vitamins, on categorial variable were assessed, multiple stepwise logistic regression was applied. The categorial variable thus studied was the treatment with various types of drugs (NSAID, chloroquine, aurotiomalate, sulfasalazine, corticosteroids; yes/no).

RESULTS AND DISCUSSION

Serum zinc concentration was low in RA (10.2 2.0 vs 11.9 1.8 umol/l, $p < 0.001$). Linear multiple regression disclosed e.g. that joint score index ($r=-0.403$, 39%, $p<0.01$), Hb ($r=0.575$, 51%, $p<0.001$), CRP ($r=0.409$, 55%, $p<0.01$) and RF titer ($r=-0.379$, $p<0.05$) explained 59% of the variation of serum zinc in a age and sex adjusted model. Food intake did not affect serum zinc and serum serum zinc did not predict medical treatment. These results suggest that disease activity determines serum zinc whereas nutrition and medication are unimportant as explanatory factors.

In animal models of arthritis, zinc accumulates in liver due to induction of metallothionein.

Interestingly, this metallothionein is regulated by IL-1 (=catabolin, mononuclear cell factor), a cytokine reponsible for many local and systemic inflammatory events. Infusion of recombinant IL-1 causes a decrease in serum zinc. This biochemical background and our observations suggest that, in RA, low serum zinc is caused by IL-1.

Low serum zinc did not compromise erythrocyte Cu,Zn-SOD, which was somewhat increased in RA (4138 562 vs 3704 342 U/g Hb, $p<0.001$). Interestingly, there was a correlation between plasma vitamin A and serum zinc ($r=0.383$, $p<0.005$; $n=61$). We believe that this novel statistical association has its pathophysiological explanation in that the synthesis of RBP in liver is regulated by zinc. Accordingly, when we measured RBP in 30 of our patients, we found a correlation between vitamin A and RBP ($R=0.942$, $p<0.001$) as expected, but also between RBP and serum zinc ($r=0.440$, $p<0.02$). This supports the above mentioned hypothesis and implies a marginal deficiency in functional zinc.

Serum copper was high in RA (23.7 5.3 vs 17.6 4.9 umol/l, $p<0.001$), except in patients over 65 years of age. The raw correlations of serum copper to disease activity parameters (ESR ($r=0.323$, $p=0.01$), CRP ($p=0.363$, $p<0.005$), Hb ($r=-0.272$, $p<0.05$) were significant but the multiple regression picked up joint score index as the only significant, independent predicting parameter ($r=0.383$, 33%, $p<0.01$) in age- and sex-adjusted model. Interestingly, serum copper was high in patients on DMARD ($p<0.01$), which drugs were not commonly used in patients over 65 ($p<0.01$).

Serum copper had the highest correlation with cerulolasmin, which suggests this as the major reason for high serum copper in RA. Interestingly, ceruloplasmin is part of the above mentioned IL-1/IL-6-mediated acute phase response. The same stimulus may thus be responsible for low serum zinc and high serum copper in RA. Association with ceruloplasmin assumes even more significance because of the role of ceruloplasmin as one of the major antioxidants in the aqueous body humours.

It is also noteworthy that the cytoplasmic Cu,Zn-SOD is more sensitive to the availability of copper than of zinc and accordingly, erythrocyte SOD activity was not decreased but in fact increased. Cu,Zn-SOD gene has been sequenced and at least in rat lung is induced in response to hyperoxia (=oxidative stress), which may suggest that the increase in erythrocyte Cu,Zn-SOD is reactive (erythroid precursors in the bone marrow).

Serum copper was also influenced by nutritional factors, namely zinc intake. This is strongly suggested by a correlation between plasma copper/copper intake ratio on zinc intake ($r=-0.638$, $p<0.001$) but not on plasma zinc.

Serum selenium was low in RA and the extent of this decrease was associated with the extent of joint inflammation. Selenium resides at the four active sites of anti-oxidant enzyme GSHpx in a catalytically active selenocysteine residue at amino acid 47. This is the only firmly established role of selenium in man. However, plasma GSHpx was similar in RA and controls (308.6 4.2 vs 308.0 10.5 U/l). Again, recent isolation and characterization of GSHpx gene made it possible to study gene regulation using a promyelocytic human HL-60 cell line. Human GSHpx gene appears to be regulated

post-transcriptionally, probably co-translationally, in reponse to selenium availability. This together with our findings may imply that selenium status in not compromized.

Serum vitamin A was low in RA (2.043 0.671 vs 2.336 0.597 umol/l, $p<0.05$). There was a significant correlation between serum vitamin A and Kaarela's joint score index in Spearman's rank correlation test ($r=0.330$, $p<0.01$). However, when the above mentioned correlation between serum zinc (-RBP) and vitamin A was taken into multiple linear regression, disease activity lossed its explanatory value. We extrapolate our findings so that vitamin A is mainly regulated by Zn-dependent RBP synthesis in liver.

Fat soluble vitamin E is intercalated in biological lipid bilayer membranes so that the hydrophobic tail anchors the molecule in such an orientation as to position the reactive, anti-oxidant hydroxyl group at the polar hydrocarbon interphase of the membrane. Here vitamin E excerts is role as the major lipid peroxidation chain-breaking anti-oxidant. Vitamin E was low in RA (17.7 8.2 vs 25.3 5.4 umol/l, p<), also if vitamin E/(total cholesterol + triglycerides) was used as a variable. However, disease activity parameters were poor predictors. Due to its lipophilia, vitamin E stores are mainly in fat tissue. These vitamin E stores are large compared to RDA. With these vitamin E stores as a "buffer", serum vitamin E and disease activity parameters are likely to get out of pace i.e. we interprit our findings as increased consumption of vitamin E by RA-associated lipid peroxidation in vivo.

When vitamin E reacts with fatty acid peroxyl radical, tocopheroxyl radical and fatty acid hydroperoxide are formed. Tocopheroxyl radical can be reduced back to vitamin E by water soluble anti-oxidants such as ascorbate or glutathione (recycling of vitamin E) and the seleno-enzyme GSHpx catalyzes the reduction of unsaturated lipid hydroperoxide to lipid hydroxyacid. Thus, vitamin E and GSHpx act in concert in termination of lipid peroxidation. However, vitamin E is an essential nutrient,whereas GSHpx may be regulated at gene level as discussed above. This may be an explanation of the present finding on low serum vitamin E en face of normal plasma GSHpx.

Sulfasalazin acts as a superoxide scavenger. We found that GSHpx was high in patients on sulfasalazine compared to other RA patients (343.4 48.2 vs 299.6 32.9, $p<0.002$). Also in multivariate analysis sulfasalazine treatment was the best predictor of GSHpx. These findings taken together suggest that in RA there may be an increased consumption of GSHpx combined with increased synthesis, a situtation unmasked in sulfasalazine treated patients.

ROS formation in RA has been in particular associated with two different generator systems: NADPH oxidase in the plasma membrane of neutrophils and monocyte/macrophages (inflammation) and xanthine oxidase/hypoxanthine system activated by ischcemia/reperfusion. ROS, in particular OH, can have deletary effects on unsaturated lipis, proteins and DNA. although site specific production and production in the bulk phase may also serve useful purposes, such as enzyme activation and microbicidal effects. Oxidant stress seems to cause consumption of anti-oxidants like vitamin E, vitamin A, GSHpx etc even under physiological conditions - if strenuous physical

exercises can be regarded as such. Our observations in RA are in line with the earlier observations on the effect of oxidant stress in e.g. exercise and inflammation. In some conditions the harmful effects of oxidant stress can be recuperated or prevented by antioxidant treatment, so e.g. retrolental fibroplasia in premature babies with respiratory distress syndrome, who are exposed to high oxygen. Whether oxidant damage in RA is primary or secondary or whether it can be modified by e.g. dietary changes or supplementation regims, is an open but interesting question.

SUMMARY

The interactions at the interphase of nutrition, disease activity and medication in RA were studied. In linear multiple regression, low serum zinc was predicted to 66% by six disease activity parameters in age- and sex-adjusted model. Low zinc is most likely due to IL-1-driven, metallothionein-mediated sequestration in liver. Also high serum copper is explained by the same driving stimulus in that the highest correlation was on serum ceruloplasmin, which being an acute phase reactant, is also regulated by IL-1/IL-6. Accordingly, Cu,Zn-SOD, particularly sensitive to the availability of copper, was not decreased. In contrast, high erythrocyte Cu,Zn-SOD suggests a reactive synthesis in erythroid precursors, perhaps by O_2 driven gene activation. Serum selenium was slightly lowered, whereas plasma GSHpx activity was normal, although human GSHpx gene is regulated by the availability of selenium.

In patients on sulfasalazine, a superoxide scavenging DMARD, GSHpx was high, unmasking an underlying increase in GSHpx consumption in RA. This is in accordance with low serum vitamin E (17.7 8.2 vs 25.3 5.4 umol/l, $p<0.05$) intepreted to be a result of its consumption at the major lipid peroxidation chain-breaking anti-oxidant acting in concert with GSHpx. Vitamin A was also slightly lowered (2.043 0.671 vs 2.336 0.597 umol/l, $p<0.05$) but this was best explained by the effect of low zinc on RBP synthesis in liver.

Assessment of food intake using Setti-N nutritional database disclosed that serum vitamin A was also affected by intake. The only other nutritionally affected process was absorption of copper, which was decreased by high zinc intake, probably due to induction of metallothionein in intestinal epithelial cells. In addition to sulfasalazin effect on GSHpx, logistic model showed that DMARD medication was associated with high serum copper. Otherwise medication and diet did not affect serum levels of the substances studied.

It is not known at present, whether some of the key antioxidants and trace elements are just markers of underlying pathophysiological processes or whether they are consumed to the degree as to cause harm and motivate therapeutic intervention. Reactions and treatment potentials involved, however, are highly complex.

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