Abnormal lipid metabolism in collagen-induced arthritis rat model: In vitro, high resolution NMR spectroscopy based analysis

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Collagen-induced arthritis (CIA) was induced in female Wistar rats by intradermal injection of porcine immunization grade native collagen type II (Chondrex). Development and progression of CIA was monitored by studying histopathological, radiographical and biochemical features of arthritic manifestations in the knee joints, hind limb and blood plasma. In addition, oxidative stress status of arthritic animals was determined by measuring lipid peroxidation and the antioxidant enzymes: catalase, superoxide dismutase and glutathione peroxidase. High resolution proton NMR spectroscopy was employed for the analysis of lipid components in the lipid extracts of the joint tissue and plasma of collagen-induced arthritic and control rats. Triglyceride levels showed significant decreases in plasma (1.7 times) but were unchanged in the joint tissue of CIA rats as compared to control. One-dimensional proton NMR spectra showed a 6.2 times reduction in the quantity of choline-containing phospholipids in the plasma of CIA as compared to control rats. There was a 1.6 times elevation of choline-containing phospholipids in the joint tissue of CIA rats as compared to controls. Induction of arthritis showed a 4.0 times reduction in the level of total cholesterol in the plasma and 1.6 times elevation in the joint tissue of CIA rats as compared to controls. The ratio of saturated fatty acids to unsaturated fatty acids was 1.5 times significantly higher in joint tissue and 2.1 times significantly higher in plasma of CIA rats as compared to controls. The results demonstrated significantly altered lipid patterns in the joint tissue and plasma of collagen-induced arthritic rats as detected by one- and two-dimensional NMR spectroscopy compared with controls.

Keywords: Cholesterol, Collagen-induced arthritis, Lipid extraction, Lipid components, Metabolism, NMR spectroscopy, Oxidative stress, Phospholipids

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial joints and destruction of articular cartilage. The inflamed arthritic joint exhibits a markedly abnormal metabolic profile characterized by low-oxygen tension, decreased glucose concentrations, increased utilization of lipids for fuel, and high level of free fatty acids and ketone bodies. Further the metabolites of synovial fluids may be markedly different from those of serum. Rheumatoid arthritis patients were reported to have decreased concentrations of serum lipids, total serum cholesterol, LDL and HDL cholesterol, free-fatty acids and phospholipids. Rheumatoid arthritis is thus accompanied by alterations in circulating metabolites. Oxidative stress is thought to play an important role in the etiology of RA. Increased levels of lipid peroxidation in the erythrocytes and blood plasma of patients with RA and oxidative-stress associated decrease in phospholipids have been reported in children with chronic arthritis. In an animal model of arthritis (k/BxN mice) abnormal generation of reactive oxygen species and lipolysis have been observed. Decreased levels of cholesterol and phospholipids in the erythrocytes membranes and reduced levels of total lipids and cholesterol in the serum of RA patients have also been found.

The abnormality of lipid metabolism in patients with rheumatoid arthritis has been characterized in several studies. Reduced levels of total lipids, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, apolipoprotein A1 and HDL-cholesterol were observed in the serum of patients with RA as compared to healthy blood donors. Anomalous decreases in the total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides have also been observed in the serum of adjuvant-induced arthritic rats.
Proton NMR spectroscopy ($^1$H NMR spectroscopy) is a versatile technique and permits quick assessment of the nature and levels of a large numbers of biomolecules simultaneously. This technique has been applied in many clinical studies and has major advantages over gas chromatography and liquid chromatography for the analysis of lipid components, and identification of lipid components is almost unequivocal even if a few distinct resonances are resolved.

There have been a number of proton NMR spectroscopy based studies on rheumatoid arthritis. These biochemical and NMR spectroscopy-based studies of the plasma and synovial fluid of arthritic patients and animal models have indicated the possibility of abnormal lipid metabolism in arthritis. However, in these studies, absolute quantification of lipid components: triglycerides (TG), choline-containing phospholipids (PLc), total cholesterol (T.Chol) and the ratio of saturated fatty acids (SFA) to unsaturated fatty acids (USFA) in the plasma as well as in the joint tissue were not made.

Collagen-induced arthritis (CIA) in the rat is an autoimmune-based disease and in many ways resembles clinical rheumatoid arthritis. The collagen-induced arthritis rat model has thus been considered a relevant animal model of rheumatoid arthritis and has been used in many studies to investigate the pathogenesis of inflammatory arthritis and to evaluate the pharmaco-therapeutic potential of antiarthritic medication.

The main objective of the present study is thus to make a comparative analysis and quantitative estimation of lipid components in the plasma and joint tissue of CIA rats by high resolution $^1$H NMR spectroscopy with a view to provide more precise data on altered metabolism of lipids in arthritis.

**Materials and Methods**

**Induction of CIA**—Female Wistar rats (5-7 weeks of age, weighing 150-180 g) were used. Rats were housed separately in cages, fed with standard rodent chow and water *ad libitum* and maintained at 12:12 h L:D cycles at 25±2 °C and 42±5%, RH. All experimental protocols were approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) and the Institutional Animal Ethical Committee (IAEC number 18/2009). Animals were randomly divided into 2 groups of 10 animals each. One group served as control and another group comprised CIA rats. The induction of arthritis in animals was performed as per Sharma *et al*. Rats were allowed to acclimatize for 7 days before initiating the experiment. Briefly, porcine immunization-grade native collagen type II (cII) (Chondrex) was dissolved overnight (2 mg/mL in 0.05 M acetic acid). Collagen solution was emulsified with an equal volume of complete Freund’s adjuvant (Sigma). Each rat was immunized with a dose of 200 µg intradermally at multiple sites followed by a booster dose with the same antigen preparation on day 7. The gradual onset of arthritis started approximately 2 weeks after primary immunization and rats were observed daily for clinical signs of arthritis. Control rats were injected with saline intradermally at multiple sites.

**Paw diameter measurement and arthritic index**—Using a dial thickness gauge, hindpaw edema of the rats was monitored every 3rd day throughout the experiment. The arthritis Index (AI) was calculated by using the formula described by Coelho *et al*.

\[
\text{AI} \% = \left( \frac{\text{Hind paw diameter day (x)} - \text{Hind paw diameter on day (0)}}{\text{Hind paw diameter on day (0)}} \right) \times 100
\]

**Macroscopic scoring of CIA**—Clinical score, a composite index of disease severity and the number of limbs affected was assessed every 4 days to monitor disease progression. The scores from each of the 4 limbs were then added to obtain an arthritic score. Since, CIA is more pronounced in the hind-limbs, the scoring of rat hind-limbs only has been presented here (data of arthritic score of all the 4 limbs are not shown). Two independent observers, blinded to the experimental groups, performed the scoring.

**Body weight examination**—Body weight was measured every 3rd day. The change in body weight (%) of each individual animal after the onset of arthritis on day 12 was calculated as follows:

\[
\text{Change in body weight} \% = \left( \frac{\text{Body weight on day (x)} - \text{Body weight on day (12)}}{\text{Body weight on day (12)}} \right) \times 100
\]

On the 30th day after arthritis induction, blood plasma was drawn from CIA and control rats by retro-orbital puncture. Rats were sacrificed by cervical dislocation and the joints were excised, trimmed of the excessive fat and connective tissue. Joint tissues were snap-chilled in liquid nitrogen and stored at -80 °C till further use.
Radiological evaluation of CIA—After sacrificing the rats, the hind limbs were removed and the right hind leg was fixed in 10% buffered formalin for radiological evaluation of arthritic manifestations. All radiographs were taken with X-ray film (Kodak Diagnostic Film, Ready-Pack, Kodak, Rochester, NY, U.S.A.) using MBR-1505R (Hitachi Medical Corporation, Tokyo, Japan). Settings for radiography were 5 mA, 40 kV and 1 sec exposure. Films were placed 60 cm below the X-ray source.

Histological assessment of arthritis damage—Knee joint was excised from the fixed hind leg, its skin and muscular parts were trimmed off and subjected to decalcification in 5% HNO₃ for 7-10 days, processed for paraffin embedding, sectioned at 10 µm followed by staining with haematoxylin-eosin and studied using light microscopy.

Biochemical estimations in plasma and joint tissue—Blood was collected in EDTA-coated vials followed by centrifugation at 2000 rpm for 10 min. Clear plasma was aliquoted and stored at -80 °C for biochemical studies. To prepare tissue homogenate, knee joint was excised, trimmed off excessive fat and muscular tissue, and homogenized in 0.05 M Tris-HCl buffer (pH 7.4) with protease inhibitor cocktail (3 µL/10 mL buffer) to yield a 10% (w/v) homogenate. The homogenate was centrifuged at 1000 g for 10 min. The resultant supernatant was transferred into pre-cooled centrifugation tubes and centrifuged at 12,000 g for 30 min. The supernatant (cytosolic fraction), after discarding any floating lipid layer was used for the estimation of lipid peroxidation (LPO) and superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities. Homogenates were kept at -80 °C until the experiments were performed.

Estimation of lipid peroxidation—Thiobarbituric acid-reactive substance (TBARS), an index of lipid peroxidation was estimated by the method of Ohkawa et al.²⁴. The amount of TBARS was determined spectrophotometrically at 532 nm and expressed as nM of malondialdehyde (MDA)/mg protein. Tetramethoxy propane (TMP) was used as a standard.

Determination of superoxide dismutase, catalase and glutathione peroxidase activities—Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activity was determined spectrophotometrically at 37 °C as per Singh et al.²⁵. Enzyme activity was expressed as units/mg protein.

Lipid extraction from the joint tissue and plasma of collagen-induced arthritic rat—Lipid extraction was performed from the joint tissue and plasma (0.5 mL) specimens of CIA rats as well as control rats by using methanol and chloroform sequentially in 1:3 ratio.²⁶ All dried and lyophilized lipid extract samples were redissolved in 0.5 mL cold (due to high volatility of chloroform) CDCl₃ and taken in 5 mm NMR tube (Wilmad no. 528, USA). The ¹H-NMR experiments were performed using a Bruker Avance 400 MHz spectrometer (Bruker Biospin, Zurich, Switzerland) at 25 °C.

¹H NMR spectroscopy parameters—One-dimensional proton NMR experiments using single pulse sequence were performed. Spectral width used was 8012 Hz with time domain data points of 32 K. Flip angle of the radio frequency pulse was 458 and total relaxation delay was 7.5 s (relaxation delay 5 s and acquisition time 2.5 s). Typically, 128 scans were accumulated for each sample and the resultant data were Fourier transformed after multiplying by exponential window function using a line broadening function of 0.3 Hz and an FT size of 32 000 points.

Sealed and reusable glass capillary with known concentration of TSP [3-(trimethylsilyl) propionic-2, 3, 3-d4 acid, sodium salt] in D₂O was used as a standard reference. The NMR spectra for all the samples were recorded under uniform conditions.

Assignment of the lipid components in the lipid extract of joint tissue and plasma—Assignments of the lipid components in the lipid extract of joint tissue and plasma were completed with the help of one-dimensional single pulse sequence and two-dimensional (2D) total correlation spectroscopy (TOCSY) employing the Bruker’s standard pulse program library. Assignments were also confirmed by literature and spectra of standard lipid components.²⁶-²⁸

Two-dimensional (2D) total correlation spectroscopy (TOCSY) parameters—Total correlation spectroscopy (TOCSY) experiment was recorded by using following parameters: Spectral width = 8012 Hz, time domain data points= 2048; number of scans = 24; dummy scan = 16; relaxation delay = 1.5 second and spin lock time = 70 ms.

Quantification of lipid components (TG, PLC, TChol and SFA/ USFA) in joint tissue and plasma lipid extract—One-dimensional ¹H NMR spectra was used for the quantification of the lipid components in lipid extract of joint tissue and plasma. Total triglycerides (including triacylglycerol and diacylglycerol) were estimated by taking the integral area of – CH₂ proton of glycerol backbone at 4.14 ppm. In plasma, there was an overlap of phospholipid
glycerol backbone signal to the –CH2 proton of glycerol backbone at 4.14 ppm and accurate quantity of triglycerides was measured by subtraction of phospholipid quantity from the actual measured triglyceride quantity with overlapped signal. Integral area of N’(CH3)3 signals of phospholipids at 3.30 ppm and C18 methyl signal of cholesterol at 0.68 ppm were obtained, relative to the TSP [3-(trimethylsilyl) propionioic-2, 2, 3, 3-d4 acid, sodium salt] (a known quantity of TSP was dissolved in deuterated water and a definite volume was taken in a reusable sealed capillary tube) signal at 0.0 ppm, for the quantitative estimation of choline-containing phospholipids (PLc) (including phosphatidylcholine, sphingomyelin, plasmalogen, and platelet-activating factors) and total cholesterol (T.Chol) (including cholesterol and cholesterol esters) respectively. Using the integrals, the quantities of PLc and T.Chol were calculated by using computerized program for the quantification of lipids26-28,32,33. Ratio of saturated fatty acids (SFA) to unsaturated fatty acids (USFA) was calculated by taking the division of the sum of the integral areas of all the saturated fatty acid signals to the sum of all the unsaturated fatty acid signals.

Statistical analysis—Mean levels of TG, PLc and T.Chol in CIA and control rats were compared by Student’s t-test for independent groups. The P values < 0.05 were considered significant. Mean level of the ratio of SFA/USFA in CIA and control rats were compared by Mann-Whitney ‘U’ test for independent groups. The data management and analyses were performed by using the statistical software SPSS version 15.0.

Results

Severity of CIA in Wistar rats—Clinical signs of arthritis (mild periarticular erythema and edema) accompanied by the redness and swelling of the joints first appeared in hind paws approximately 10 days post challenge. The disease progression increased in frequency and severity in a time-dependent manner and maintained a plateau of the peak of CIA response from day 15 to 30 (mean AI of 125.01) (Fig. 1a). Control rats showed no increase in hind paw diameter over time. Hind paw swelling and erythema also showed a time-dependent increase in severity with maximum arthritis scores of ~8 observed between days 12 and 30 in rats immunized with CII (Fig. 1b). There was no macroscopic evidence of either hind paw erythema in the control rats.

Change in body weight—Body weight was recorded every 3rd day during the experimental period of 30 days. The onset of arthritis was associated with a drastic body weight loss that continued till the end of the experimental period (Fig. 1c). Rats in the control group showed a gradual increase in their body weight with time.

Histopathological and radiographic analyses—On day 30, histological evaluation of the joints in the CIA animals revealed marked characteristic features of severe arthritis like synovial hyperplasia, massive mixed (neutrophil, macrophage, and lymphocyte) infiltration along with articular cartilage and bone erosion. No such arthritic features of inflammation and tissue destruction were evident in the joints of healthy control groups (Fig. 2a). On day 30, the...
radiographic analysis of hind paws from CIA rats revealed soft tissue swelling, bone matrix resorption and the joint space narrowing (Fig. 2b). There was no evidence of pathology in the control rats.

Lipid peroxidation—The lipid peroxide levels were significantly increased 2.8- ($P < 0.0001$) and 2-fold ($P < 0.0001$) in joints and plasma of CIA rats, respectively (Fig. 3a).

Antioxidant enzymes status of the joint tissue and plasma—The activities of the antioxidant enzymes, total superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were higher in the joints and plasma of CIA rats compared to control. Catalase showed 2.87- fold increase in the arthritic rat joints followed by 2.22- and 1.65-fold increases in the activity of SOD and GPx respectively. The joint tissue showed higher levels of antioxidant enzymes than the plasma (Fig. 3b-d).

Qualitative analysis of lipid components in joint tissue and plasma of rats—Lipid components were assigned in the lipid extract of joint tissue and plasma of rats. The assigned lipid components were fatty

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Fig. 2—(a) Histopathological features of (a) control rat showing the smooth and monolayer synovial cells lining (indication with arrow), and (b) CIA rats showing the hyperplastic synovial cell, erosion and disruption of synovial lining. Representative radiographs of the hind limbs showing the tibiotarsal and tibiofemoral joints of rats from (c) control and (d) CIA rats. Arrows indicate the bone damage and space narrowing in both tibiotarsal and tibiofemoral joints. The encircled areas grossly show the severe pathological conditions of the hind limbs, such as swollen joints and edema.
acids; saturated and unsaturated fatty acids (FA), triglycerides (TG), choline-containing phospholipids (PLc) and total cholesterol (T. Chol) (Fig. 4 a and b).

Quantitative analysis of lipid components in joint tissue and plasma rats—Quantitative analysis of lipid components in the lipid extract of the joint tissue was performed. Triglyceride levels were unaltered in the joint tissue of CIA rats. There was a 1.6 times significant increase in the T. Chol and PLc in CIA rats (P< 0.05). The SFA/USFA ratio were 1.5 times significantly higher in the joint tissue (Table 1 and Fig. 5). Quantative analysis of lipid components in the lipid extract of plasma showed a 1.7 times decrease in TG level (P<0.05). There was a 4.0 times decrease in T. Chol as well as a 6.2 times decrease in PLc in CIA rats (P< 0.05). The ratios of SFA/USFA were 2.1 times higher in plasma of CIA rats (Table 1 and Fig. 6).

Discussion

The results of the present study demonstrated a highly significant elevation in lipid peroxidation in the joint tissue as well as in the plasma of CIA rats indicating increased oxidative stress in arthritis. The

<table>
<thead>
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<th>Table 1—Quantitative comparison of lipid components in lipid extract of the joint tissue and plasma of CIA and control rats</th>
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<tr>
<td>[Values are mean ± SD of 10 animals per group. P values: *&lt;0.05, **&lt;0.01, ***&lt;0.0001].</td>
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<thead>
<tr>
<th>Female Wistar rats</th>
<th>Joint tissue</th>
<th>Plasma</th>
</tr>
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<tbody>
<tr>
<td>TG (Triglycerides)</td>
<td>(µM/g)</td>
<td>PLc (Choline-containing phospholipids) (µM/g)</td>
</tr>
<tr>
<td>Control rats</td>
<td>163.14±84.81</td>
<td>15.48±1.84</td>
</tr>
<tr>
<td>CIA rats</td>
<td>149.71±69.54*</td>
<td>24.59±4.26**</td>
</tr>
<tr>
<td>P values: *&lt;0.05; **&lt;0.01; ***&lt;0.001</td>
<td><strong>Student ‘t’ test</strong></td>
<td>Mann-Whitney U test</td>
</tr>
<tr>
<td>Control rats</td>
<td>25.03+ 2.23</td>
<td>1.36+0.59</td>
</tr>
<tr>
<td>CIA rats</td>
<td>15.00+3.41**</td>
<td>0.22+0.07**</td>
</tr>
<tr>
<td>P values: *&lt;0.05, **&lt;0.01; ***&lt;0.001</td>
<td>Student ‘t’ test</td>
<td>Mann-Whitney U test</td>
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antioxidant enzymes activities were, however, found elevated and this would obviously seem to be a compensatory response insufficient to counter the oxidative stress-causing processes. Increased activity of SOD with decreased activities of CAT and GPx has been observed in arthritic patients. Even the present data showed that the increase in the activity of GPx was much lower compared with the increase in that of SOD and CAT.

The major finding of the present study emerging from the $^1$H NMR-based analysis of the lipid components in the joint tissue and plasma of CIA rats is that there is a quantitative alteration in the lipid components (TG, T.Chol, PLc and SFA/USFA) of the blood plasma and the joint tissue.

TG slightly decreased in the joint tissue of CIA rats as compared to control, but the decrease was not statistically significant. A significant decrease was observed in the plasma of CIA rats as compared to control. Ratio of SFA/USFA showed a marked elevation in both plasma and joints of CIA rats as compared to control. This again indicates that unsaturated fatty acids are reduced in both plasma and joint tissue of CIA rats. Synthesis of prostaglandin is known to be enhanced during inflammation, and this may be responsible for the higher consumption of unsaturated fatty acids and these unsaturated fatty acids may come from the degradation of the triglycerides in the joint as well as in the blood. Therefore, elevated synthesis of prostaglandin may cause reduction in the levels of triglycerides and unsaturated fatty acids in the blood.

The present data showed that T.Chol and PLc were significantly reduced in the plasma of arthritic rats whereas they were elevated in their joint tissue. Weljie et al. showed the occurrence of abnormal lipolysis in the serum of arthritic K/BxN mice and Lazarevic et al. and other authors reported that RA patients had significantly reduced level of total lipids and cholesterol in the serum. Results of these studies are thus consistent with the findings of the present study from CIA rats. Reduction in T.Chol level in the plasma of CIA rats may be due to a reduction of cholesterol in LDL and HDL as reported earlier.

Oxidative stress-associated decrease in phospholipid fraction has been reported in children with juvenile chronic arthritis. It is known that there are differences between the metabolic profile of the rheumatoid joint and the serum of arthritic patients. Oxidative stress associated changes were reported in animal models of arthritis also. A previous study on NMR-detectable differences between the synovial fluid and serum of rheumatoid arthritis patients showed the presence of very high levels of fatty acids and their increased utilization in the intra-articular tissue compared with that in the serum. However, there is no report about alterations in choline-containing phospholipids and total cholesterol in rheumatoid arthritis. Weljie et al. showed abnormal generation of reactive oxygen species in the serum of arthritic K/BxN mice and this may be responsible for decreased lipid levels. Since phospholipids are more susceptible to lipid peroxidation, decreased Plc in CIA rats may result from increased rate of lipid peroxidation.
Reduction in T. Chol in the plasma of CIA rat as observed in the present study can also be a consequence of elevated inflammation and enhanced lipid peroxidation. Mature RBCs exchange the cholesterol and phospholipids from plasma as they lack capability for the de novo synthesis of cholesterol and phospholipids. In a biochemical study, decreased levels of cholesterol and phospholipids were observed in the erythrocyte membranes of patients with RA. This is consistent with the NMR-based data on T.Chol and PLc levels seen in the present study on CIA rat.

Inflammation is associated with enhanced activity of the phospholipids-degrading or -hydrolyzing enzymes such as phospholipase A2 (EC 3.1.1.4), phospholipase C (EC 3.1.1.4), phospholipase D (EC 3.1.4.4), and sphingomyelinase (ceramide). Prostaglandin synthesis from unsaturated fatty acids is enhanced during inflammation. Local and systemic elevation of inflammatory cytokines promotes lipolysis, and the consequent systemic release of free fatty acids contributes to the dyslipidemia in RA. Lipolysis, and the consequent systemic release of free fatty acids enhances the elevation of inflammatory cytokines promotes lipolysis, and the consequent systemic release of free fatty acids contributes to the dyslipidemia in RA. Lipolysis, and the consequent systemic release of free fatty acids contributes to the dyslipidemia in RA. Lipolysis, and the consequent systemic release of free fatty acids contributes to the dyslipidemia in RA. Lipolysis, and the consequent systemic release of free fatty acids contributes to the dyslipidemia in RA.

Phospholipids and cholesterol are the major structural components of the cell membrane and the cell proliferation process uses phospholipids. In this regard, cholesterol and phospholipids are also transported from the liver via blood to the joint. Free cholesterol is required for the proliferation process may be further responsible for lowering the amount of total cholesterol and phospholipids in the blood and may increase their amount in the joint tissue of CIA rats.

In conclusion, the present study describes NMR-detectable abnormality in lipid metabolism in the joint tissue and plasma of CIA rats and provides novel data on these parameters. NMR spectroscopy has been extensively applied for clinical purposes such as in inherited disorders of lipid metabolism: Smith–lemli–opitz syndrome, cerebrotendinous xanthomatosis, sitosterolemia and refsum disease through the analysis of whole or lipid extract of blood and plasma. NMR spectroscopy has been extensively applied for clinical purposes such as in inherited disorders of lipid metabolism: Smith–lemli–opitz syndrome, cerebrotendinous xanthomatosis, sitosterolemia and refsum disease through the analysis of whole or lipid extract of blood and plasma. Therefore, in the light of all these studies and the present results from collagen-induced arthritis indicate that NMR spectroscopic based results may be of diagnostic significance in clinical cases.

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