Chapter 3

Materials and Methods

This study was funded from several resources, Prof MC Kruger – grant, Prof M Viljoen – grant, Physiology Department electron microscopy fund, and AM Koorts - NAVKOM grant. The ethical clearance committee number is 116/98. All patients and control subjects gave their informed consent. The intracellular free calcium determinations were performed at the Department of Physiology, UP. The intracellular calcium localisation studies were conducted at the Department of Physiology and the Electron Microscopy unit, UP. The red blood cell membrane lipid composition determinations were performed at the MRC, Cape Town. The vitamin A, vitamin E, vitamin C, PTH, albumin, total serum calcium and serum free calcium were all performed at the Chemical Pathology Department, UP. The blood sampling for all the determinations for one subject was performed on one occasion, but the sampling of the blood for each patient and each control subject was performed on separate occasions, since intracellular free Ca^{2+} determinations could only be performed one sample at a time. MHT blood was drawn predialysis from the arterial side, before any mixing of saline and blood occurred. The procedure for the sampling of the control subject's blood was comparable to that of the patient. The following anticoagulated blood and serum was collected:

1. 7 ml ACD-anticoagulated blood kept at room temperature, for the intracellular free Ca^{2+} determinations, transmembrane Ca^{2+} flux determinations and intracellular calcium localisation studies in the neutrophils.

2. 10 ml EDTA-anticoagulated blood kept on ice in the dark, for red blood cell membrane phospholipid and fatty acid determinations, and vitamin A, E and C determinations on the obtained plasma.
3. 7 ml serum kept on ice, for PTH and albumin determinations.
4. 7 ml serum kept on ice, for total calcium and ionised calcium determinations.

The protocols for the various determinations or sample preparation for storage in order for the determinations to be performed at a later stage are presented in the following sections.

A. The determination of intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes in the neutrophils

Materials

1. Sampling – 7 ml of ACD-anticoagulated blood kept at room temperature. The period between the drawing of the blood and the starting of the procedure was the same for all patients and subjects, i.e., 30 minutes.
2. Histopaque – 1077 Sigma Diagnostics Cat no. 1077-1. For the separation of MNL's, plasma, platelets and PMNL's. Solution of polysucrose and sodium diatrizoate, adjusted to a density of 1.077 ± 0.001 g/ml.
3. Ammonium chloride solution for red blood cell lysis

	mM
NH_4Cl	155
$NaHCO_3$	12
EDTA	0.25

4. Hanks – Highveld Biological PTY. LTD. Cat no. CN 2027-3. Containing: KCl, KH_2PO_4, NaCl, Na_2HPO_4, Glucose, $MgSO_4.7H_2O$, Hepes, $NaHCO_3$, $CaCl_2$, pH 7.4 at 25°C.
5. Bovine serum albumin – Sigma Cat no. A-9647.

6. Turk's Blood

   5ml of a 1% aqueous Gentian violet solution

   1ml glacial acetic acid

   94 ml H_2O (reversed osmosis)

7. Fura-PE3/AM Sigma Cat no. F-0918

   8 mM Fura-PE3/acetoxy methyl ester stock solution

   Dissolve 0.5 mg Fura-PE3/acetoxy methyl ester (FW 1258.1) in 50 µl of DMSO. Keep the stock solutions at -20°C.

8. fMLP N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine Sigma Cat. No. F-3506, 2 mM fMLP stock solution

   Dissolve 10 mg fMLP (FW 437.6) in 11.5 ml DMSO. Keep the stock solutions at -20°C.

9. 1% TritonX-100 (v/v)

10. 0.5M EGTA/3M Tris pH 8.7 at 25°C

Method

Isolation of the neutrophils

1. Pipette 3 ml of the Histopaque-1077 (8°C) in a blue topped conical tube (volume 15 ml).
2. Layer 6 ml of ACD-anticoagulated blood (room temperature) on top of the Histopaque-1077. Take care not to disturb the surface tension of the histopaque layer.
3. Centrifuge at 1800 r.p.m. (12°C) for 25 minutes.
4. Blood cells and plasma are separated in layers.
5. Discard the supernatant, consisting of plasma, platelets and MNL's.
6. Fill the tube with the remaining red blood cells and polymorphonuclear leucocytes with ammonium chloride solution (8°C) and mix well by aspiration.
7. Incubate on ice for 10 minutes.
8. Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.

9. A white pellet is formed consisting of neutrophils, eosinophils and basophils. No further separation of the neutrophils, eosinophils and basophils are necessary since eosinophils contribute only 0.5-6% and basophils only 0-1% to the total amount of circulating white blood cells. If the pellet is still contaminated with red blood cells, the red blood cell lysis step can be repeated.
10. Discard the supernate and gently dissolve the pellet in 0.25% BSA supplemented Hanks (8°C).
11. Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.
12. Discard the supernate and dissolve the pellet in 2 ml 0.25% BSA supplemented Hanks (neutrophil cell suspension).
13. Incubate on ice for 45 minutes.

Counting of the neutrophils

1. Mix 50 µl of the neutrophil cell suspension with 450 µl of the white cell staining fluid (Turk's blood).
2. Fill the hemositometer.
3. Count the neutrophils in the 5 blocks indicated with a W.
4. Calculate the concentration of the neutrophil cell suspension as follows:

   Total amount of neutrophils in all 5 cell chambers = N

   Amount of neutrophils in one cell chamber = N/5

   Volume of one cell chamber = 0.1 mm^3

   Concentration of neutrophils in 1 mm^3 after dilution with Turk's blood solution = N/5 . 10

   Concentration of neutrophils in 1 mm^3 of the neutrophil cell suspension = N/5 . 10 . 10

   Thus the concentration of neutrophils in the neutrophil cell suspension per milliliter = N/5 . 10 . 10 . 10 . 10 . 10

   = N/5 . 10^5

5. Adjust the neutrophil cell suspension to a final concentration of 2 . 10^6 cells/ml.

The optimised protocol for the determination of intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes in the neutrophils by employing fluorescence spectrophotometry is presented in chapter 2, page 105.

B. The localisation of intracellular calcium in the neutrophil

Materials

1. 1 ml neutrophil suspension as obtained in section A.
2. 2.5% Glutaraldehyde (GA) in 0.1 M KPBS, Glutaraldehyde 25% - Cat. No. 16400 Premier Technologies
3. 0.1 M KPBS
4. 1% OsO_4 in 2% $KSb(OH)_6$. $KSb(OH)_6$ – Cat. No. 247286 Sigma, OsO_4 – Cat. No. R1015 Wirsam Scientific. This solution of osmium tetroxide and potassium-pyroantimonate was prepared by the mixing of equal volumes of two aqueous stock solutions – 2% OsO_4 and 4% $KSb(OH)_6$. Dissolving of osmium tetroxide and potassium-pyroantimonate simultaneously may result in spontaneous precipitation. The stock solution of potassium-pyroantimonate was prepared by boiling in water, on cooling to room temperature the solution was restored to its original volume and filtered through a 0.22µm Millipore filter to remove pyroantimonate that may have re-precipitated. (1, 2)
5. 50% ethanol, 70% ethanol, 95% ethanol and 100% ethanol
6. 0.2 M EDTA pH 8.4. The EDTA solution requires the addition of a strong base to reach the pH of 8.4. At this pH EDTA effectively chelates divalent cations. It is important that KOH rather than NaOH be used for this adjustment, otherwise as calcium deposits are dissolved antimonate can re-precipitate with sodium. Use of KOH also avoids problems that can arise when chelators are used

for pretreatment or during fixation of tissues, where high levels of sodium introduced with the chelator could precipitate upon exposure to antimonate.

The optimised protocol for localisation of intracellular calcium in the neutrophil by using transmission electron microscopy is presented in chapter 2, page 128.

C. Red blood cell preparation for membrane phospholipid and fatty acid determinations

1. Centrifuge 10 ml EDTA anticoagulated blood at 2000 r.p.m. 4°C for 10 minutes.
2. Discard the supernatant and buffy coat.
3. Wash 2x with ice cold saline, centrifuge at 2000 r.p.m. 4°C for 10 minutes.
4. Place the washed red blood cells in a cryotube and store at -70°C.
5. Batch all the red blood cell samples for patients and control subjects.

Determinations were performed at the MRC in Cape Town, the method employed involved the use of gas chromatography.

D. EDTA plasma preparation for vitamin A, E and C determinations

1. 10 ml EDTA plasma, kept on ice in the dark and protected from oxidation.
2. Centrifuge at 2000 r.p.m. 4°C for 10 minutes.
3. Remove enough plasma to fill a 1.5 ml dark eppendorff to the top – 1.7 ml for vitamin A and E determinations.
4. Store at -70°C.

5. Remove 500 µl of plasma and add 500 µl of the vitamin C precipitation solution (10% perchloric acid v/v and 1% meta-phosphoric acid w/v).
6. Vortex for 2 minutes.
7. Centrifuge at 4°C, 10000 r.p.m. for 10 minutes.
8. Remove the supernatant and store in a dark eppendorff.
9. Store at -70°C.
10. Batch all the vitamin A, E and C samples for patients and controls.

Vitamin A, E and C determinations were performed at Chemical Pathology UP, all methods involved the use of high performance liquid chromatography.

E. PTH determinations

The PTH determinations were performed at Chemical Pathology UP. PTH determinations were performed with a RIA kit from Nichols Institute Diagnostics. This intact PTH immunoassay is a two-site immunoradiometric assay for the measurement of the biologically intact 84 amino acid chain of PTH. One antibody is prepared to bind only the mid-region and C-terminal PTH 38-84 and this antibody is immobilised onto plastic beads. The other antibody is prepared to bind only the N-terminal PTH 1-34 and this antibody is radio-labelled for detection.

F. Albumin determinations

The albumin determinations were performed at Chemical Pathology UP. Albumin concentrations were determined employing bromcresol purple. Albumin binding to bromcresol purple results in the formation of a purple complex. This system monitors the change in absorbance at 600 nm. The change in absorbance is directly proportional to the concentration of albumin in the sample.

G. Total serum calcium

The total serum calcium determinations were performed at Chemical Pathology UP. The total calcium content in samples was determined by indirect potentiometry utilising a calcium sensitive electrode with a sodium reference electrode. In principle, a calcium-ion selective electrode measures unbound free calcium ions in solution. Total calcium can only be calculated from free calcium ions when the molar ratio between free and total calcium concentrations is constant. This constant molar ratio is achieved by a buffered solution which contains strong calcium complexing agents.

H. Ionised serum calcium

The ionised free calcium determinations were performed at Chemical Pathology UP. The ionised serum calcium was determined by using a calcium sensitive electrode.

References

1) Appleton J, Morris DC. The Use of the Potassium Pyroantimonate-Osmium Method as a Means of Identifying and Localizing Calcium at the Ultrastructural Level in the Cells of Calcifying Systems. The Journal of Histochemistry and Cytochemistry 1979; 27(2): 676-680.

2) Tandler CJ, Libanati CM, Sanchis CA. The Intracellular Localization of Inorganic Cations with Potassium Pyroantimonate. The Journal of Cell Biology 1970; 45: 355-366.

Chapter 4

Results

The results of the technique development and evaluation studies for the fluorescent determination of intracellular free Ca^{2+} in the neutrophil were presented in chapter 2. The present chapter deals with the results obtained by employing this standardised technique in the determination of intracellular free Ca^{2+} in the neutrophils of maintenance haemodialysis patients. As fura-PE3 was identified as the most reliable fluorescent calcium indicator, all intracellular free Ca^{2+} determinations were performed employing this fluorescent calcium indicator. The electron microscopic techniques are likewise based on the results of chapter 2. The non-parametric Mann-Whitney test was applied for statistical comparisons between the MHTtotal patient group and the control group. A p-value less than 0.05 was taken as a significant difference and a p-value less than 0.1 and greater than 0.05 was taken as a non-significant difference. For the statistical comparisons between the MHTnon-epo patient group, MHTepo patient group and the control group the p-value was determined by the application of an ANOVA to the ranks. A p-value less than 0.05 was taken as a significant difference. A p-value less than 0.1 and greater than 0.05 was taken as a non-significant difference. Statistical determinations for correlations between variables were performed by employing Pearson's correlation test.

In order to facilitate the reading of this chapter a summary of the sequence of results to be presented is provided:

A: Tables 1 to 3 provide the relevant information on the maintenance haemodialysis patients group as a whole (MHTtotal) and on occasion

separated into those receiving erythropoietin (MHTepo) and those not receiving erythropoietin (MHTnon-epo)

Table 1: Relevant clinical information of the maintenance haemodialysis patients (page 148)

Table 2: Relevant biochemical values of the maintenance haemodialysis patients, means, standard deviations and deviation from normal (page 150)

Table 3: Circulatory characteristics of the maintenance haemodialysis patients, means, standard deviations and deviation from normal (page 151)

B: Figures 1a to 1w present the real-time recordings of the fluorescent intracellular free Ca^{2+} determinations and transmembrane Ca^{2+} fluxes obtained from the neutrophils isolated from the blood of the patients and control subjects (page 152 - 155) Tables 4 to 6 contain the individual intracellular calcium determinations and statistical comparisons between groups of patients (page 156 - 158).

C and D: Tables 7 to 12 contain the results obtained and the statistical comparison between the groups of patients for the factors that could influence the calcium status and fluxes, including the lipid composition of the membranes, PTH, the oxidative status as indicated by the anti-oxidative vitamins and the free serum calcium (page 164 - 194).

E: Table 13 contains the correlations between variables for MHTtotal patients (page 199 - 200).

F: The last section contains the electron microscopy photographs for the localisation of intracellular calcium in the neutrophil for patients and control subjects (page 202 - 204).

A Clinical and biochemical background of the maintenance haemodialysis patients included in the study

Fourteen CRF patients on maintenance haemodialysis treatment (MHT) at the Pretoria Academic Hospital were suitable for inclusion in this

study. The Ethical Clearance Committee number for the study is 116/98. Patients where doubt existed with regard to relevant medication, where the treatment regimen was altered within the time period of this study, those whose haematocrits were too low, or who were on the program for less than one year, were excluded from the study. Table 1 contains the relevant clinical characteristics. The total patient group (MHTtotal) consisted of 14 patients, 9 male, 5 female, 6 caucasian, 8 black. The mean and the standard deviation for age for the 14 patients are 37.286 and 12.548, and the mean and standard deviation for the years on dialysis treatment are 5.8214 and 6.1005. Nine of the patients (MHTepo) were receiving recombinant human erythropoietin (Eprex), while the remaining five patients have never received recombinant human erythropoietin (MHTnon-epo). In addition the nine patients receiving recombinant human erythropoietin (rHuEPO) also received the calcium channel blocker norvasc (Amlodipine). Only one of the five patients not receiving rHuEPO received the calcium channel blocker. The control group consisted of 10 individuals, 6 male, 4 female, 5 caucasian, 5 black. The mean and the standard deviation for age for the control subjects are 38.100 and 14.685. The MHTepo group included 8 male patients and one female patient, 8 black patients and one caucasian patient, the mean and standard deviation for age of this group are 35.111 and 9.3868, and the mean and standard deviation for the years on dialysis treatment are 3.2778 and 1.1211. Four of the 5 patients of the MHTnon-epo group were female and the group included only caucasians, the mean and standard deviation for age of this group are 41.200 and 17.484, and the mean and standard deviation for the years on dialysis treatment are 10.400 and 8.8133. The unequal distribution between groups will be considered further in the discussion. Suffice to say that the main aim of the study was to investigate the calcium status of all available patients on the Pretoria Academic Hospital Maintenance Haemodialysis Program as it exists.

Table 1. Clinical information of the maintenance haemodialysis patients (MHT)

Patient	Race	Sex	Age	Etiology of CRF	Period on dialysis	Dialysis protocol	EPO	Ca channel blocker
1	Caucasian	Male	61	Chronic glome rulonephritis	16 years	3 x 4h/w	None	None
2	Caucasian	Female	39	Chronic pyelo phritis	8.5 years	3 x 4h/w	None	None
3	Caucasian	Female	54	Polycystic kidney disease	4 years	3 x 4h/w	None	10 mg
4	Caucasian	Female	16	SLE	1 year	3 x 4h/w	None	None
5	Caucasian	Female	36	Gassers	22.5 years	3 x 4h/w	None	None
6	Black	Male	27	Pancreatitis	1.5 years	3 x 4h/w	0.38mlx3/wk	10 mg
7	Black	Male	21	Hypertension	3.5 years	3 x 4h/w	0.55mlx3/wk	10 mg
8	Black	Male	36	Glomerulonephritis	2.5 years	3 x 4h/w	0.25mlx3/wk	10 mg
9	Black	Male	38	Hypertensive ne phropathy	3 years	3 x 4h/w	0.41mlx1/wk	10 mg
10	Black	Male	47	Hypertensive ne phropathy	5 years	3 x 4h/w	0.33mlx3/wk	10 mg
11	Black	Female	34	Glomerulonephritis	4 years	3 x 4h/w	0.43mlx3/wk	10 mg
12	Black	Male	30	Glomerulonephritis	4 years	3 x 4h/w	0.50mlx3/wk	10 mg
13	Caucasian	Male	32	Trauma	2 years	3 x 4h/w	0.35mlx3/wk	10 mg
14	Black	Male	51	Hypertensive ne phropathy	4 years	3 x 4h/w	0.47mlx1/wk	10 mg

The relevant biochemical and circulatory values are presented in Tables 2 and 3. Patients were divided into the two groups determined by the administration of recombinant erythropoietin or not. Tables 2 and 3 contain the individual patient data, mean, standard deviation and the degree to which the patients group deviate from normal. A significant difference between the group of patients receiving rHuEPO (MHTepo) and the group of patients not receiving rHuEPO (MHTnon-epo) is indicated for only serum-creatinin, p-value 0.0234. No significant differences are indicated between the MHTepo and MHTnon-epo patients for serum-ureum, serum-phosphate, serum-ferritin, serum-albumin, HB, HCT, systolic blood pressure, diastolic blood pressure, MAP, temperature, age and the period of dialysis treatment.

B Intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes in the neutrophils of maintenance haemodialysis patients and control subjects

In figures 1a to 1w the real-time determination of intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes of the patients as well as the control subjects are presented. In Table 4 the intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes as determined by the employment of the intracellular fluorescent calcium indicator fura-PE3 are presented. During the initial phases of the study one patient and one control subject were investigated per day as the samples can be fitted into a day. The consistency of the differences between patients and control subjects forced the reconsideration of the possibility that the 30 minutes delay between measurement of the two samples (patient and control subject) may be a factor. The study was repeated and the data presented were obtained one subject per day. Table 5 and 6 contain the statistical evaluation of the various groups of patients.

Table 2. Biochemical values of the maintenance haemodialysis patients (MHT)

Patients	Serum ureum 3.1-7.8mmol/l	Serum creatinin 81-114 mol/l	Serum phosphate 0.87-1.45mmol/l	Serum ferritin 20.0-250.0 g/l	Serum albumin 39.0-50.0 g/l
1	40.6	1310	1.24	6.4	39
2	42.2	1051	3.73	282	38.2
3	31.9	1100	1.55	9	31
4	25.1	918	1.09	936	35
5	36.3	872	1.38	12990	36
MHTnon-epo	n=5 mean=35.22 SD=6.933 5.9SD	n=5 mean=1050.2 SD=172.63 20.9SD	n=5 mean=1.798 SD=1.0933 7.5SD	n=5 mean=2844.7 SD=5684.1 98.9SD	n=5 mean=35.84 SD=3.1509 1.1SD
6	38	1269	1.57	38	34
7	20.2	1380	1.97	52	34
8	41.9	1930	2.36	27.4	38.2
9	36.9	1637	1.19	901	40
10	32.7	1482	1.91	27	36
11	25.8	1261	2.33	144	36
12	21.3	1322	1.83	18	32
13	25.1	1039	2.06	760	31
14	29.8	1406	1.46	306	37.8
MHTepo	n=9 mean=30.189 SD=7.6942 6.5SD	n=9 mean=1414 SD=253.81 30.8SD	n=9 mean=1.8533 SD=0.3904 2.7SD	n=9 mean=252.6 SD=341.95 5.9SD	n=9 mean=35.444 SD=2.9644 1.1SD
MHTtotal	n=14 mean=31.986 SD=7.5815 6.5SD	n=14 mean=1284.1 SD=285.55 34.6SD	n=14 mean=1.8336 SD=0.68 4.7SD	n=14 mean=1178.3 SD=3416.8 59.4SD	n=14 mean=35.586 SD=2.9157 1.1SD

Table 3. Circulatory characteristics of the maintenance haemodialysis patients (MHT)

Patients	HB 13-18g/dl	HCT 0.40-0.52l/l	SYS BP	DIAS BP	MAP	TEMP
1	9.3	0.299	167	98	121	35
2	8.4	0.262	165	92	116	36.3
3	8.9	0.282	139	82	101	36.1
4	10.3	0.311	220	125	157	36.5
5	7.8	0.233	110	70	83	36
MHT non-epo	n=5 mean=8.94 SD=0.945 0.8SD	n=5 mean=0.2744 SD=0.0309 1SD	n=5 mean=160.2 SD=40.666 4.1SD	n=5 mean=93.4 SD=20.611 2.7SD	n=5 mean=115.6 SD=27.474 3.3SD	n=5 mean=35.98 SD=0.5805 1.7SD
6	7.8	0.249	173	92	119	35
7	8.6	0.264	151	97	115	35.5
8	8.8	0.286	159	104	122	35
9	12	0.358	128	78	95	35.6
10	10	0.318	142	91	108	35
11	11.5	0.352	195	121	146	35.2
12	10.6	0.321	182	111	135	35
13	8.9	0.272	152	93	113	36
14	11.4	0.363	152	93	113	36
MHT epo	n=9 mean=9.9556 SD=1.5001 1.2SD	n=9 mean=0.3092 SD=0.0432 1.4SD	n=9 mean=159.33 SD=20.712 2.1SD	n=9 mean=97.778 SD=12.597 1.7SD	n=9 mean=118.44 SD=14.901 1.8SD	n=9 mean=35.367 SD=0.4243 1.2SD
MHT total	n=14 mean=9.5929 SD=1.3837 1.1SD	n=14 mean=0.2979 SD=0.0411 1.4SD	n=14 mean=159.64 SD=27.803 2.8SD	n=14 mean=96.214 SD=15.268 2SD	n=14 mean=117.43 SD=19.258 2.3SD	n=14 mean=35.586 SD=0.5545 1.6SD

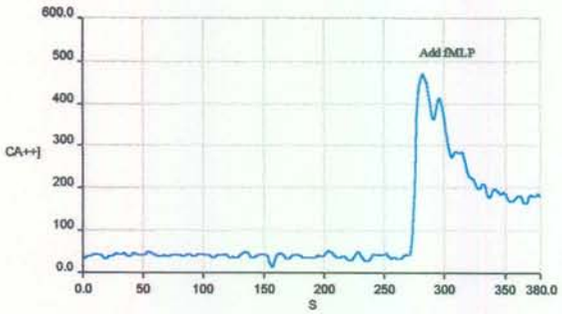

Figure 1a-patient 1

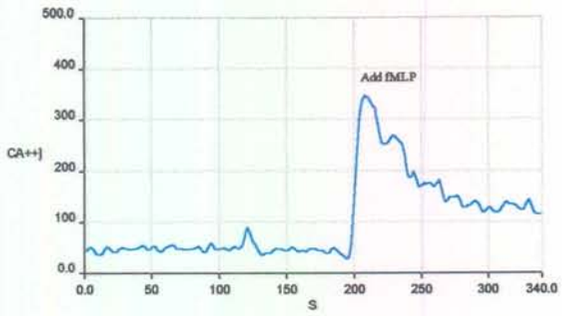

Figure 1b-patient 2

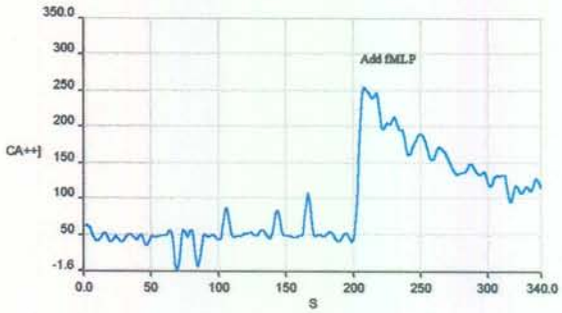

Figure 1c-patient 3

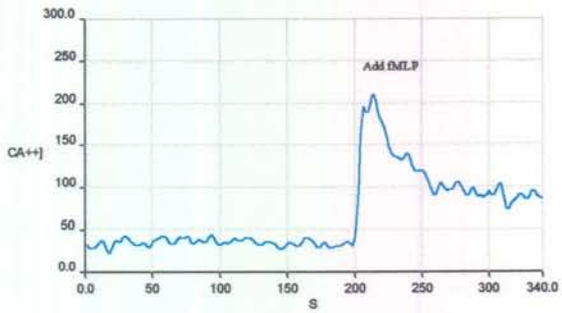

Figure 1d-patient 4

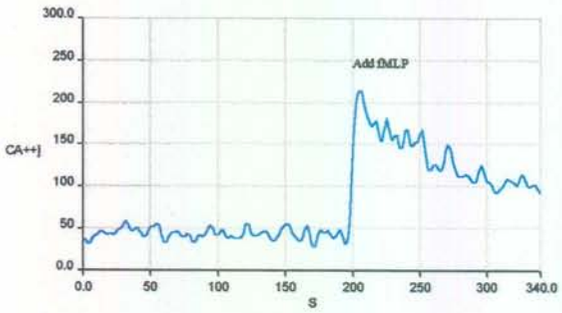

Figure 1e-patient 5

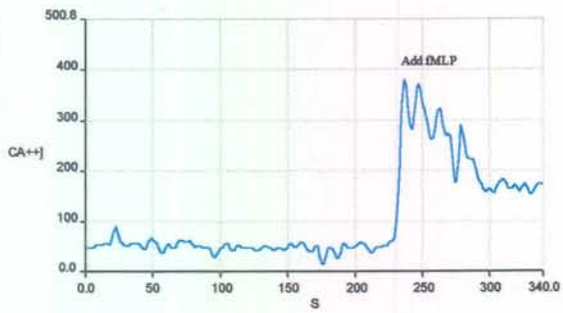

Figure 1f-patient 6

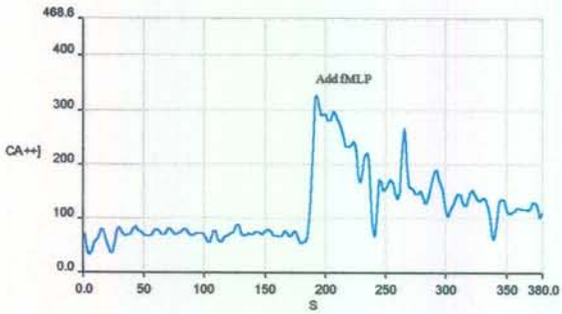

Figure 1g-patient 7

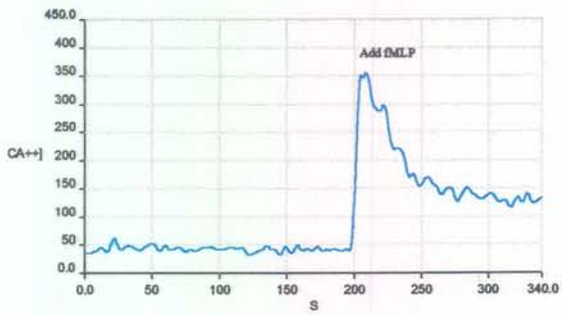

Figure 1h-patient 8

Figure 1. Real-time intracellular free Ca^{2+} determinations for the patients (refer to figures 1a to 1n) and control subjects (refer to figures 1o to 1x)

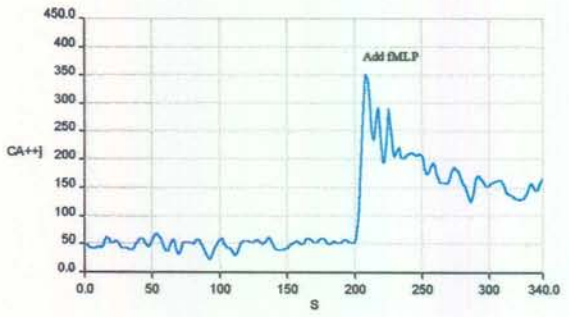

Figure 1i-patient 9

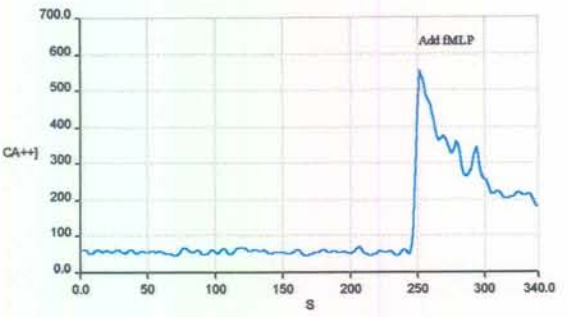

Figure 1j-patient 10

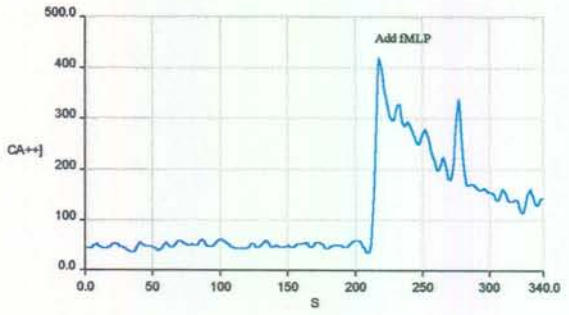

Figure 1k-patient 11

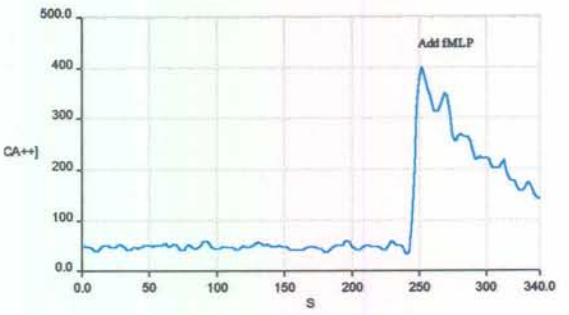

Figure 1l-patient 12

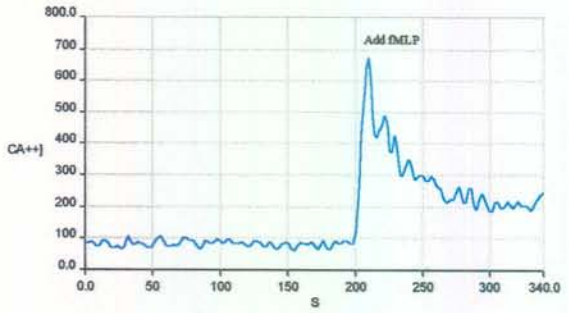

Figure 1m-patient 13

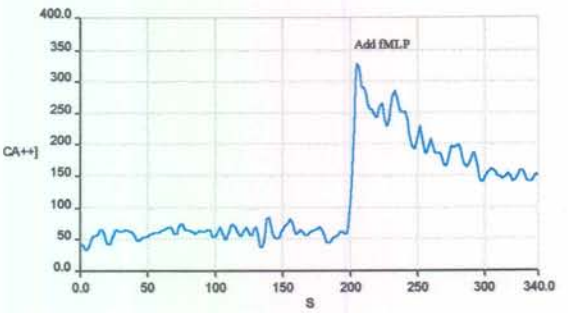

Figure 1n-patient 14

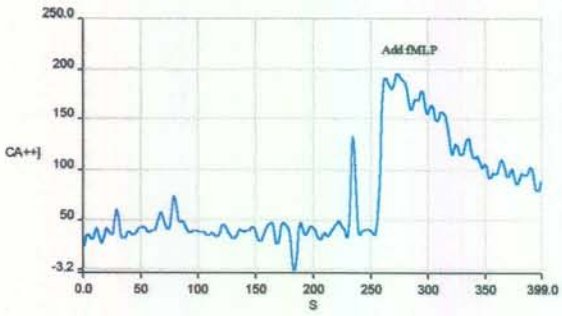

Figure 1o-normal 1

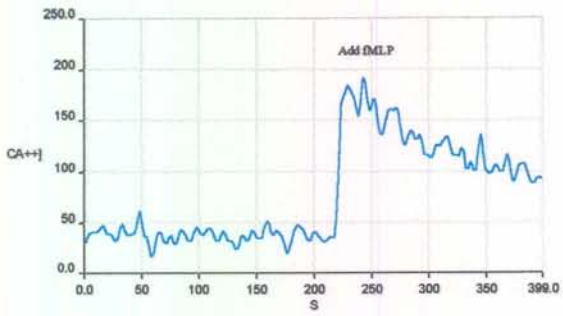

Figure 1p-normal 2

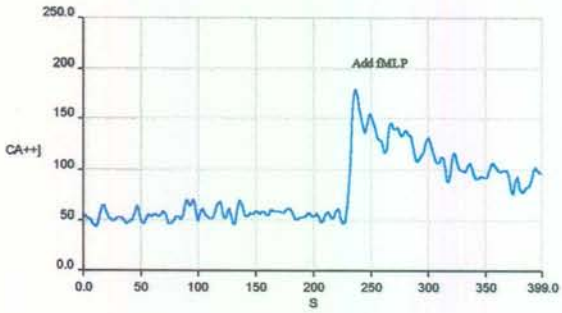

Figure 1q-normal 3

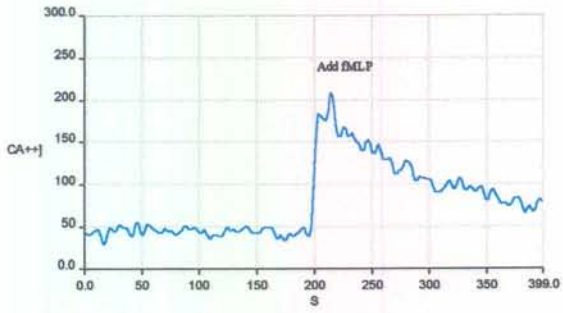

Figure 1r-normal 4

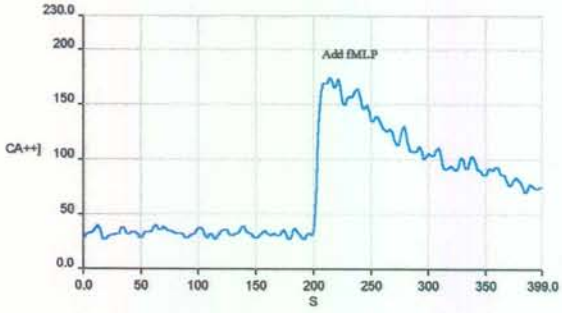

Figure 1s-normal 5

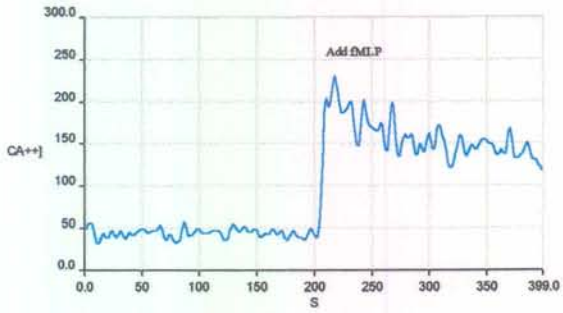

Figure 1t-normal 6

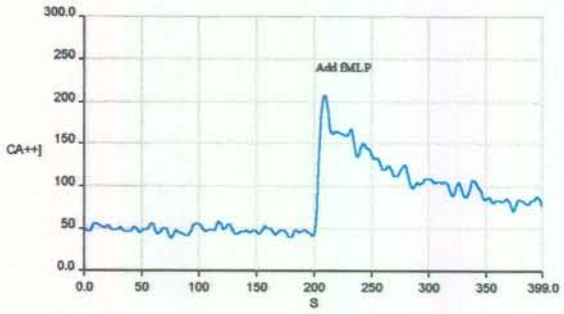

Figure 1u-normal 7

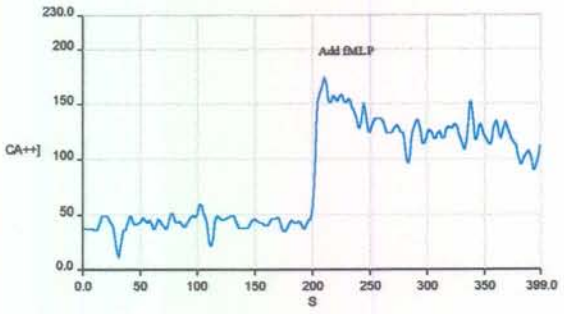

Figure 1v-normal 8

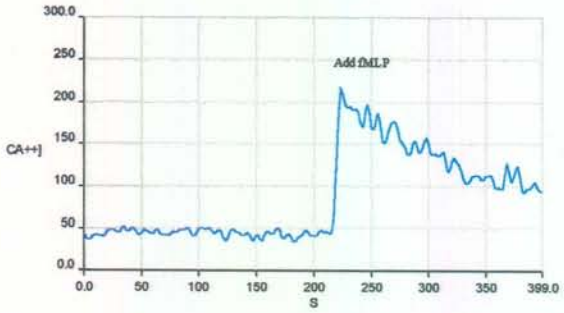

Figure 1w-normal 9

Table 4. Intracellular free calcium and transmembrane calcium fluxes of the patients and control subjects

Patients	Intracellular free calcium	Transmembrane calcium fluxes	Rate lny	Rate y
1	42	473	-0.009	-2.29007
2	49	348	-0.00447	-0.8369
3	49	256	-0.0055	-0.7403
4	36	211	-0.00372	-0.41704
5	45	215	-0.00436	-0.5305
6	51	389	-0.00589	-1.16713
7	71	332	-0.00347	-0.60521
8	44	357	-0.00341	-0.66631
9	51	354	-0.00378	-0.68633
10	58	555	-0.00743	-1.98438
11	51	426	-0.00639	-1.26094
12	49	403	-0.00672	-1.47762
13	86	677	-0.00426	-1.2493
14	61	328	-0.00434	-0.83478
Controls				
1	40	197	-0.00669	-0.8658
2	38	192	-0.00376	-0.47675
3	56	181	-0.00363	-0.42024
4	45	211	-0.00477	-0.54419
5	33	175	-0.00453	-0.51251
6	45	233	-0.00181	-0.29527
7	49	209	-0.00418	-0.48988
8	43	175	-0.00175	-0.22492
9	45	219	-0.0043	-0.60277
10	38	220	-0.00257	-0.32762

Transmembrane calcium fluxes=magnitude of intracellular free calcium increase upon fMLP stimulation
Rate lny=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the ln values of the real-time calcium measurements
Rate y=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the real-time calcium measurements

Table 5. Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
Intracellular free calcium	n	14	10	
	mean	53.071	43.2	* 0.0242
	SD	12.761	6.4601	
Transmembrane calcium flux	n	14	10	
	mean	380.29	201.2	* 0.0002
	SD	126.68	20.335	
Decrease of iCa rate lny	n	14	10	
	mean	-0.0052	-0.0038	# 0.0952
	SD	0.001686	0.001488	
Decrease of iCa rate y	n	14	10	
	mean	-1.0533	-0.476	* 0.0017
	SD	0.5566	0.1811	

Transmembrane Ca flux=magnitude of intracellular free calcium increase upon fMLP stimulation
Decrease of iCa rate lny=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the ln values of the real-time calcium measurements
Decrease of iCa rate y=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the real-time calcium measurements
*=significant difference
#=non-significant difference

Table 6. Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Control	p-value ANOVA applied to ranks	
Intracellular free Ca	n	5	9	10	* 0.0016	e=ne ne=c e>c
	mean	44.2	58	43.2		
	SD	5.4498	13.162	6.4601		
Transmembrane Ca flux	n	5	9	10	* < 0.001	e=ne ne=c e>c
	mean	300.6	424.56	201.2		
	SD	111.03	117.21	20.335		
Decrease of iCa rate lny	n	5	9	10	0.2248	
	mean	-0.00541	-0.005077	-0.003799		
	SD	0.002106	0.001536	0.001488		
Decrease of iCa rate y	n	5	9	10	* 0.0011	c=ne ne=e e> c
	mean	-0.963	-1.1036	-0.476		
	SD	0.7602	0.4544	0.1811		

Transmembrane Ca flux=magnitude of intracellular free calcium increase upon fMLP stimulation
Decrease of iCa rate lny=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the ln values of the real-time calcium measurements
Decrease of iCa rate y=rate of intracellular free calcium decrease toward baseline levels represented as the slope of a line fitted to the real-time calcium measurements
e=MHTepo *=significant difference
ne=MHTnon-epo
c=control subjects

The following significant differences are indicated between the MHTtotal patients and the control subjects

1. A significant difference is indicated for intracellular free Ca^{2+} in the neutrophils of the MHTtotal patients compared to the control subjects, p-value 0.0242. Intracellular free Ca^{2+} is higher in the neutrophils of the MHTtotal patients compared to the control subjects. Refer to figure 2 - Box and Whisker plot.

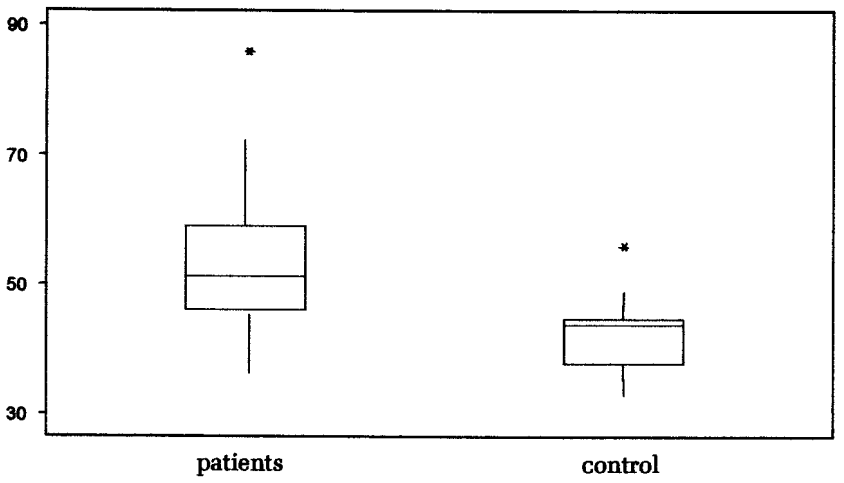

Figure 2. Box and Whisker Plot – intracellular free calcium (nM)

2. There is a significant difference in the magnitude of the highest level of intracellular free Ca^{2+} attained upon fMLP stimulation in the neutrophils of the MHTtotal patients compared to the control subjects, p-value 0.0002. The magnitude of the transmembrane Ca^{2+} flux is increased in the MHTtotal patients. Refer to figure 3 – Box and Whisker plot.

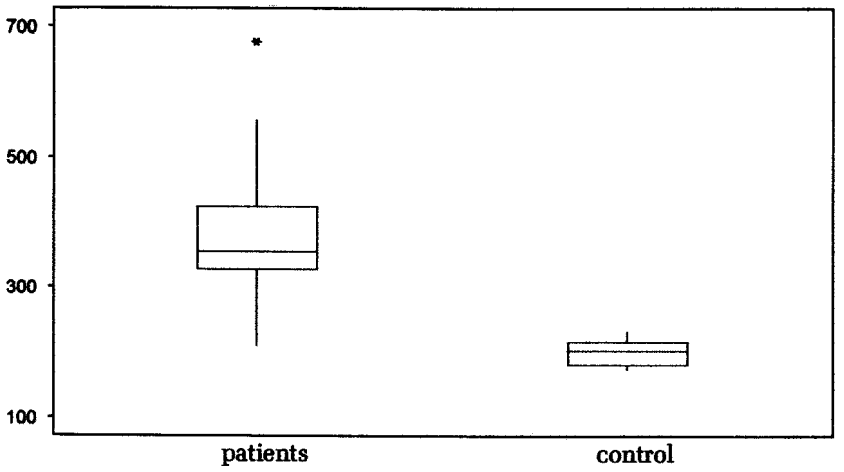

Figure 3. Box and Whisker Plot – transmembrane calcium flux (nM)

3. The rate of decrease of intracellular free Ca^{2+} toward baseline levels was determined as the slope of a line fitted to the real-time Ca^{2+} measurements - rate y. The decrease of intracellular free Ca^{2+} toward baseline levels occurs at a faster rate for the MHTtotal patients compared to the control subjects, p-value 0.0017. Refer to figure 4 – Box and Whisker plot.

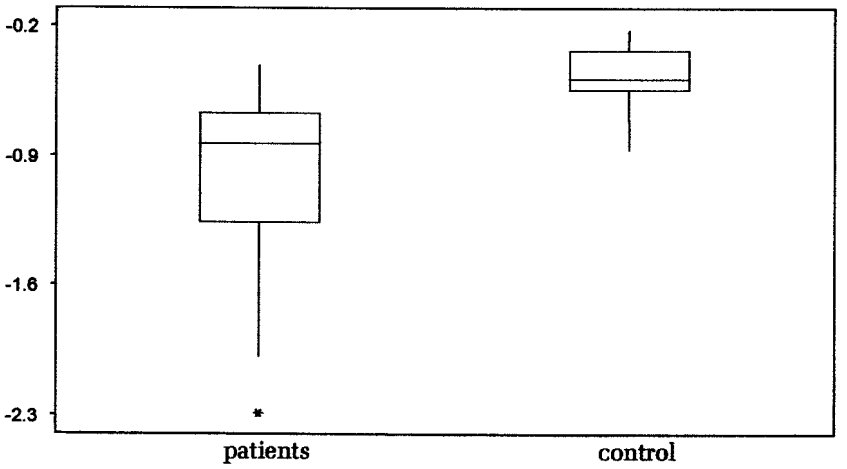

Figure 4. Box and Whisker Plot – rate of intracellular free calcium decrease (nM/s)

The following significant differences are indicated between the MHTnon-epo patients and the MHTepo patients

1. No significant difference for intracellular free calcium is indicated between the MHTnon-epo patients and the MHTepo patients. Refer to figure 5 – Box and Whisker plot.

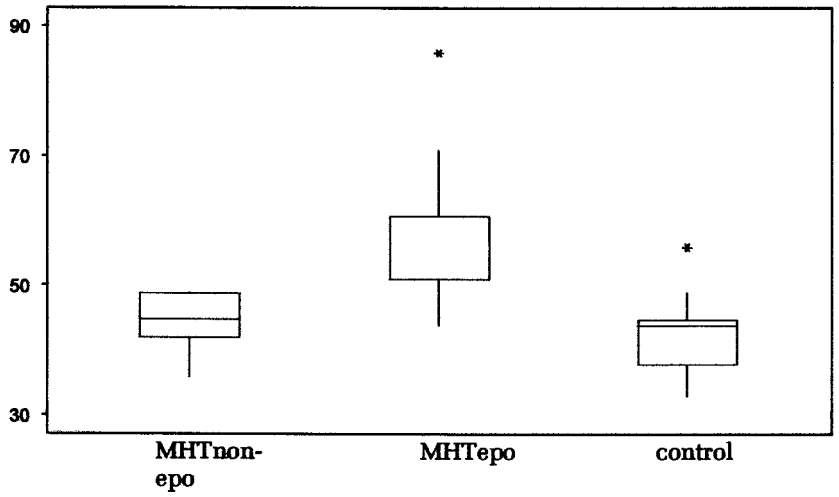

Figure 5. Box and Whisker plot – intracellular free calcium (nM)

However, the presentation of the measured values on a scatter plot, refer to figure 6, seems to indicate a difference for intracellular free calcium between MHTnon-epo patients and MHTepo patients. Performing a Mann-Whitney test between only the MHTnon-epo and MHTepo groups to indicate significance resulted in a p-value of 0.0164, a value smaller than 0.0167 indicates a significant difference between groups.

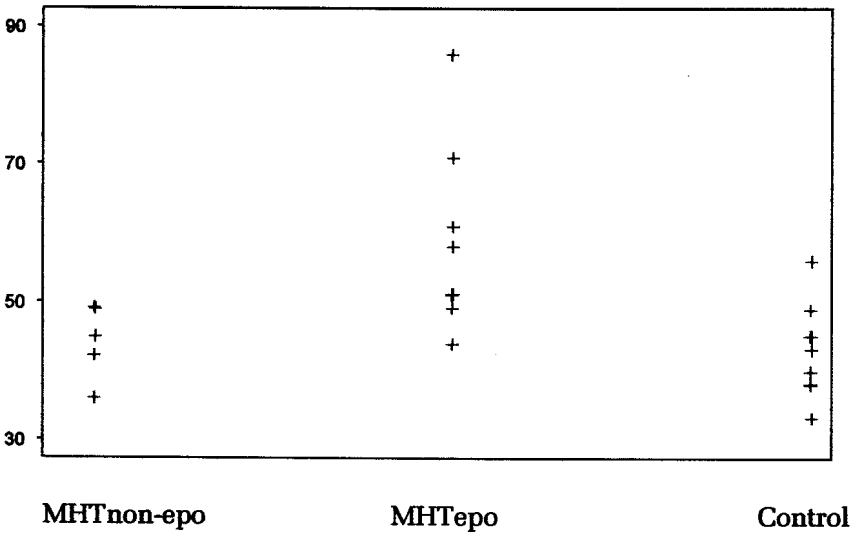

Figure 6. Scatter plot – intracellular free calcium (nM)

2. No significant differences are indicated between the MHTnon-epo and MHTepo patients for transmembrane Ca^{2+} flux and the rate of decrease of intracellular free Ca^{2+}.

C Erythrocyte membrane fatty acid and phospholipid composition of the patients and control subjects

It is known that the fatty acid and phospholipid composition of cell membranes may have a profound influence on intracellular calcium homeostasis. Due to the number of determinations, the anaemia of the patients, and the volumes of blood necessary to obtain enough neutrophils for lipid analysis, it was decided to use the erythrocyte membranes as an indication of the composition of neutrophil membranes. This is fairly common practice. (1 - 3) By doing this no

extra blood has to be collected as the red blood cells are usually discarded in neutrophil determinations. The determinations included five phospholipid classes; sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. The red blood cell membrane fatty acids determined included various saturated fatty acids (SFA's), mono-unsaturated fatty acids (MUFA's), and poly-unsaturated fatty acids (PUFA's). The SFA's determined included; 14:0, 16:0, 18:0, 20:0, 22:0, and 24:0. The MUFA's determined included; 16:1, 18:1, n-9 20:1, n-9 22:1, and n-9 24:1. The PUFA's determined included; n-6 18:2, n-6 18:3, n-3 18:3, n-6 20:2, n-6 20:3, n-6 20:4, n-3 20:3, n-3 20:5, n-6 22:2, n-6 22:4, n-6 22:5, n-3 22:5 and n-3 22:6.

Tables 7 to 9 contain the individual data and statistical comparison between the different groups.

Table 7. Erythrocyte membrane fatty acid and phospholipid composition of patients and control subjects

Patients	TPL	SM	PC	PEA	PI	PS	F14:0	F16:0	F16:1	F18:0	F18:1	Fn-6 18:2
1	2446.79	564.25	577.5	499.23	179.4	184.15	0.93	24.13	0.87	18.35	14.72	9.62
2	2820.14	503.87	765	529.42	109.02	180.34	0.22	19.34	0.21	16.21	14.16	11.65
3	2500.13	445.82	592.5	594.43	215.28	278.13	0.43	22.46	0.19	15.64	14.04	10.23
4	2153.44	329.72	480	394.74	110.4	278.13	0.44	21.79	0.05	17.5	13.15	11.43
5	2266.78	332.05	777.5	417.96	107.64	207.01	0.31	22.08	0.2	16.02	12.86	13.38
6	2580.13	478.33	612.5	568.89	124.2	246.38	0.26	21.25	0.18	15.8	12.63	10.52
7	2286.78	471.37	577.5	482.98	107.64	256.54	0.27	26.44	0.09	19.27	13.59	7.88
8	2538.98	499.23	777.5	568.89	111.78	224.79	0.47	25.92	0.17	18.6	13.03	7.49
9	2313.45	508.52	537.5	575.86	216.66	226.06	0.26	18.37	0.14	16.6	13.36	13.95
10	2220.11	427.25	700	554.96	131.1	215.9	0.2	19.13	0.17	16.57	14.65	13.47
11	2428.03	678.02	572.5	501.55	104.88	229.87	0.27	25.07	0.04	21.35	11.46	7.47
12	2333.45	376.16	655	489.94	317.4	254	0.13	23.75	0.12	18.5	12.68	8.29
13	2121.26	434.21	627.5	431.89	103.5	226.06	0.35	21.63	0.2	16.31	13.3	10.76
14	2273.45	387.77	640	422.6	111.78	191.77	0.3	21.28	0.17	16.21	13.15	9.36
Controls												
1	2386.79	406.73	567.5	506.2	106.26	231.14	0.33	22.32	0.18	14.61	11.09	13.45
2	2266.78	360.73	602.5	401.71	107.64	189.23	0.55	22.58	0.04	18.02	12.96	11.19
3	2206.78	389.78	607.5	359.91	106.26	265.43	0.41	21.01	0.1	16.9	12.97	10.43
4	2293.45	508.41	655	517.81	161.46	232.41	0.56	20.34	0.13	16.62	13.01	12.16
5	2166.78	370.41	577.5	413.32	107.64	184.15	0.44	20.59	0.16	16.65	12.61	10.41
6	2633.47	351.05	590	452.79	107.64	158.75	0.23	21.8	0.17	14.87	11.96	13.83
7	2593.46	305.05	660	399.38	106.26	236.22	0.26	21.11	0.18	17.44	12.33	9.47
8	2113.44	370.41	610	541.03	114.54	184.15	0.33	19.9	0.16	16.55	13.39	12.72
9	2153.44	397.04	645	392.42	111.78	195.58	0.42	21	0.43	17.26	10.17	12.98
10	2620.13	484.2	655	529.42	154.56	204.47	0.41	21.44	0.18	17.46	11.79	14.47

Table 7. (continue) Erythrocyte membrane fatty acid and phospholipid composition of the patients and control subjects

Patients	Fn-6 18:3	Fn-3 18:3	F20:0	Fn-9 20:1	Fn-6 20:2	Fn-6 20:3	Fn-6 20:4	Fn-3 20:3	Fn-3 20:5	F22:0	Fn-9 22:1	Fn-6 22:2	Fn-6 22:4
1	0.04	0.2	0.37	0.22	0.24	1.22	14.95	0	0.2	1.16	0.12	0.09	2.76
2	0.03	0.19	0.41	0.33	0.39	1.38	17.07	0.04	0.31	1.54	0.23	0.08	4.19
3	0.03	0.16	0.35	0.3	0.42	1.75	14.81	0.03	0.14	1.94	0.18	0.06	3.95
4	0	0.1	0.96	0.28	0.7	0.68	14.3	0	0.22	1.8	0.08	0.22	3.85
5	0.04	0.23	0.36	0.26	0.43	1.36	14.67	0.04	0.29	1.63	0.15	0.12	3.95
6	0.03	0.29	0.32	0.38	0.49	1.18	16.92	0.03	0.3	1.78	0.12	0.2	5
7	0.08	0.11	0.48	0.24	0.25	0.95	12.96	0.13	0.06	2.77	0.13	0.1	3.49
8	0	0.25	0.91	0.54	0.27	1.18	14.16	0	0.23	1.76	0.05	0.18	3.31
9	0.13	0.11	0.3	0.16	0.35	1.21	15.99	0	0.24	1.61	0.09	0.07	4.65
10	0.08	0.12	0.49	0.43	0.28	1.35	14.36	0.1	0.17	2.22	0.09	0.11	3.47
11	0.03	0.19	0.45	0.31	0.38	1.21	12.24	0.03	0.15	2.44	0.09	0.09	3.75
12	0.04	0.08	0.48	0.66	0.56	1.17	13.97	0.07	0.15	2.07	0.06	0.12	3.76
13	0.04	0.21	0.39	0.28	0.36	2.27	14.41	0.04	0.22	1.85	0.18	0.08	4.67
14	0.07	0.11	0.41	0.35	0.99	1.6	16.5	0.04	0.15	1.89	0.07	0.06	3.4
Controls													
1	0.08	0.08	0.47	0.24	0.68	1.56	17.6	0	0.27	1.28	0.04	0.16	4.11
2	0	0.13	0.4	0.23	0.19	1.77	15.22	0	0.22	1.47	0.02	0.06	3.02
3	0.05	0.12	0.42	0.27	0.56	2.71	15.32	0.02	0.18	1.71	0.02	0.04	3.46
4	0.02	0.23	0.44	0.26	0.32	1.8	13.67	0.01	0.27	1.67	0.04	0.06	3.78
5	0.03	0.1	0.44	0.23	0.38	1.77	16.25	0	0.31	1.82	0.12	0.1	3.79
6	0.04	0.1	0.27	0.22	0.41	1.27	17.34	0.06	0.49	1.24	0.08	0.08	3.13
7	0.04	0.12	0.34	0.14	0.39	1.47	17.91	0.06	0.29	1.63	0.09	0.2	4.03
8	0.02	0.17	0.33	0.23	0.43	1.17	15.92	0.05	0.3	1.54	0.09	0.22	3.96
9	0.03	0.1	0.38	0.2	0.41	1.39	16.21	0.04	0.18	1.51	0.08	0.17	4.13
10	0.02	0.12	0.35	0.2	0.42	1.33	13.79	0.06	0.31	1.55	0.06	0.04	3.58

Table 7. (continue) Erythrocyte membrane fatty acid and phospholipid composition of the patients and control subjects

Patients	Fn-6 22:5	Fn-3 22:5	Fn-3 22:6	F24:0	Fn-9 24:1	TPL-SUM	SM-P	PC-P	PEA-P	PI-P	PS-P	SFA's	MUFA's
1	0.53	1.84	3.41	2.09	1.93	2004.53	28	29	25	8.9	9.2	47.03	17.86
2	0.19	1.61	4.48	2.84	2.91	2087.65	24	37	25	5.2	8.6	40.56	17.84
3	1.15	0.97	3.99	3.4	3.39	2126.16	21	28	28	10.1	13.1	44.22	18.1
4	0.27	1.51	3.64	3.84	3.18	1592.99	21	30	25	6.9	17.5	46.33	16.74
5	0.19	1.48	4.73	2.64	2.62	1842.16	18	42	23	5.8	11.2	43.04	16.09
6	0.3	1.46	4.25	3.34	2.94	2030.3	24	30	28	6.1	12.1	42.75	16.25
7	1.4	0.92	1.83	3.72	2.83	1896.03	25	30	25	5.7	13.5	52.95	16.88
8	0.81	1.25	2.61	3.96	2.84	2182.19	23	36	26	5.1	10.3	51.62	16.63
9	0.29	1.66	4.23	3.71	2.52	2064.6	25	26	28	10.5	10.9	40.85	16.27
10	0.57	1.43	3.3	4.37	2.85	2029.21	21	34	27	6.5	10.6	42.98	18.19
11	1.53	0.99	3.56	3.98	2.92	2086.82	32	27	24	5	11	53.56	14.82
12	1.01	1.4	3.41	4.29	3.22	2092.5	18	31	23	15.2	12.1	49.22	16.74
13	0.33	1.66	4.55	2.95	2.97	1823.16	24	34	24	5.7	12.4	43.48	16.93
14	0.85	1.5	4.42	3.99	3.12	1753.92	22	36	24	6.4	10.9	44.08	16.86
Controls													
1	0.76	1.34	4.08	2.8	2.48	1817.83	22	31	28	5.8	12.7	41.81	14.03
2	0.77	1.42	3.44	3.5	2.78	1661.81	22	36	24	6.5	11.4	46.52	16.03
3	0.74	1.38	3.78	4.15	3.24	1728.88	23	35	21	6.1	15.4	44.6	16.6
4	0.27	1.71	5.2	4	3.43	2075.09	25	32	25	7.8	11.2	43.63	16.87
5	0.65	1.64	4.35	3.5	3.7	1653.02	22	35	25	6.5	11.1	43.44	16.82
6	0.65	2.05	5.21	2.47	2.04	1660.23	21	36	27	6.5	9.6	40.88	14.47
7	0.9	1.63	3.3	3.72	2.93	1706.91	18	39	23	6.2	13.8	44.5	15.67
8	0.92	1.66	4.68	3.1	2.16	1820.13	20	34	30	6.3	10.1	41.75	16.03
9	1.06	1.32	4.36	3.49	2.71	1741.82	23	37	23	6.4	11.2	44.06	13.59
10	0.76	1.49	4.2	3.37	2.6	2027.65	24	32	26	7.6	10.1	44.58	14.83

Table 7. (continue) Erythrocyte membrane fatty acid and phospholipid composition of the patients and control subjects

Patients	PUFA's	PUFA's/SFA's	n-3	n-6	n-6/n-3	D5-DESATURASE	D6-DESATURASE
1	35.1	0.746332128	5.65	29.45	5.212389	12.25409836	0.004158004
2	41.61	1.025887574	6.63	34.98	5.276018	12.36956522	0.002575107
3	37.69	0.852329263	5.29	32.4	6.124764	8.462857143	0.002932551
4	36.92	0.796891863	5.47	31.45	5.749543	21.02941176	0
5	40.91	0.950511152	6.77	34.14	5.042836	10.78676471	0.002989537
6	40.97	0.958362573	6.33	34.64	5.472354	14.33898305	0.002851711
7	30.16	0.569593957	3.05	27.11	8.888525	13.64210526	0.010152284
8	31.74	0.614877954	4.34	27.4	6.313364	12	0
9	42.88	1.049694002	6.24	36.64	5.871795	13.21487603	0.009318996
10	38.81	0.902978129	5.12	33.69	6.580078	10.63703704	0.005939124
11	31.62	0.590365945	4.92	26.7	5.426829	10.11570248	0.004016064
12	34.03	0.691385616	5.11	28.92	5.659491	11.94017094	0.00482509
13	39.6	0.910763569	6.68	32.92	4.928144	6.348017621	0.003717472
14	39.05	0.885889292	6.22	32.83	5.278135	10.3125	0.007478632
Controls							
1	44.17	1.056445826	5.77	38.4	6.655113	11.28205128	0.005947955
2	37.43	0.804600172	5.21	32.22	6.184261	8.598870056	0
3	38.79	0.869730942	5.48	33.31	6.078467	5.653136531	0.004793864
4	39.5	0.905340362	7.42	32.08	4.32345	7.594444444	0.001644737
5	39.78	0.915745856	6.4	33.38	5.215625	9.18079096	0.002881844
6	44.66	1.092465753	7.91	36.75	4.646018	13.65354331	0.002892263
7	39.81	0.894606742	5.4	34.41	6.372222	12.18367347	0.004223865
8	42.22	1.011257485	6.86	35.36	5.154519	13.60683761	0.001572327
9	42.38	0.961870177	6	36.38	6.063333	11.6618705	0.002311248
10	40.59	0.910497981	6.18	34.41	5.567961	10.36842105	0.00138217

Table 7. (continue) Erythrocyte membrane fatty acid and phospholipid composition of the patients and control subjects

TPL=measured total phospholipids
SM=sphingomyelin
PC=phosphatidylcholine
PEA=phosphatidylethanolamine
PI=phosphatidylinositol
PS=phosphatidylserine
TPL-SUM=summation of the different phospholipid classes
SM-P=percentage of sphingomyelin of TPL-SUM
PC-P=percentage of phophatidylcholine of TPL-SUM
PEA-P=percentage of phosphatidylethanolamine of TPL-SUM
PI-P=percentage of phosphatidylinositol of TPL-SUM
PS-P=percentage of phosphatidylserine of TPL-SUM
SFA's=F14:0+F16:0+F18:0+F20:0+F22:0+F24:0
MUFA's=F16:1+F18:1+Fn-9 20:1+Fn-9 22:1+Fn-9 24:1
PUFA's=Fn-6 18:2+Fn-6 18:3+Fn-3 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+Fn-3 20:3+Fn-3 20:5+Fn-6 22:2+Fn-6 22:4+ Fn-6 22:5+Fn-3 22:5+Fn-3 22:6
n-3=Fn-3 18:3+Fn-3 20:3+Fn-3 20:5+Fn-3 22:5+Fn-3 22:6
n-6=Fn-6 18:2+Fn-6 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+Fn-6 22:2+Fn-6 22:4+Fn-6 22:5
D5-DESATURASE=Delta-5 Desaturase activity=Fn-6 20:4/Fn-6 20:3
D6-DESATURASE=Delta-6 Desaturase activity=Fn-6 18:3/Fn-6 18:2

Table 8. Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
Phospholipids				
TPL	n	14	10	
	mean	2377.4	2343.5	0.6605
	SD	188.81	203.39	
SM	n	14	10	
	mean	459.76	394.38	# 0.0841
	SD	92.907	60.91	
PC	n	14	10	
	mean	635.18	617	0.8836
	SD	91.391	34.335	
PEA	n	14	10	
	mean	502.38	451.4	# 0.0895
	SD	66.102	66.675	
PI	n	14	10	
	mean	146.48	118.4	0.2535
	SD	36.487	21.108	
PS	n	14	10	
	mean	228.51	208.15	0.2535
	SD	31.581	32.159	
Fatty acids				
F14:0	n	14	10	
	mean	0.3457	0.394	0.1977
	SD	0.1933	0.1097	
F16:0	n	14	10	
	mean	22.331	21.209	0.1877
	SD	2.489	0.8473	
F16:1	n	14	10	
	mean	0.2	0.173	0.6819
	SD	0.2005	0.1008	
F18:0	n	14	10	
	mean	17.352	16.638	0.7035
	SD	1.6535	1.1021	
F18:1	n	14	10	
	mean	13.341	12.228	* 0.0092
	SD	0.8647	0.9999	

Table 8. (continue) Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
Fn-6 18:2	n	14	10	
	mean	10.393	12.111	# 0.0841
	SD	2.2057	1.6661	
Fn-6 18:3	n	14	10	
	mean	0.0457	0.033	0.334
	SD	0.0344	0.0216	
Fn-3 18:3	n	14	10	
	mean	0.1679	0.127	0.1688
	SD	0.0645	0.0435	
F20:0	n	14	10	
	mean	0.4771	0.384	0.2659
	SD	0.203	0.0617	
Fn-9 20:1	n	14	10	
	mean	0.3386	0.222	* 0.0034
	SD	0.1316	0.0365	
Fn-6 20:2	n	14	10	
	mean	0.4364	0.419	0.7697
	SD	0.2035	0.1303	
Fn-6 20:3	n	14	10	
	mean	1.3221	1.624	* 0.0465
	SD	0.3736	0.4414	
Fn-6 20:4	n	14	10	
	mean	14.808	15.923	# 0.1073
	SD	1.404	1.4697	
Fn-3 20:3	n	14	10	
	mean	0.0393	0.03	0.8836
	SD	0.0385	0.0267	
Fn-3 20:5	n	14	10	
	mean	0.2021	0.282	* 0.0326
	SD	0.0707	0.0883	
F22:0	n	14	10	
	mean	1.89	1.542	* 0.0109
	SD	0.3981	0.1813	

Table 8. (continue) Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
Fn-9 22:1	n	14	10	
	mean	0.1171	0.064	* 0.0139
	SD	0.0522	0.0334	
Fn-6 22:2	n	14	10	
	mean	0.1129	0.113	0.6395
	SD	0.0515	0.0683	
Fn-6 22:4	n	14	10	
	mean	3.8714	3.699	0.7474
	SD	0.6037	0.3949	
Fn-6 22:5	n	14	10	
	mean	0.6729	0.748	0.5195
	SD	0.4572	0.2104	
Fn-3 22:5	n	14	10	
	mean	1.4057	1.564	0.3641
	SD	0.2789	0.2228	
Fn-3 22:6	n	14	10	
	mean	3.7436	4.26	0.2188
	SD	0.8128	0.6533	
F24:0	n	14	10	
	mean	3.5086	3.41	0.6395
	SD	0.6663	0.5129	
Fn-9 24:1	n	14	10	
	mean	2.8743	2.807	0.578
	SD	0.3554	0.5328	
Calculations				
TPL-SUM	n	14	10	
	mean	1972.3	1789.3	* 0.0092
	SD	166.71	150.71	
SM-P	n	14	10	
	mean	23.286	22	0.4124
	SD	3.7092	2	
PC-P	n	14	10	
	mean	32.143	34.7	# 0.0841
	SD	4.504	2.4967	

Table 8. (continue) Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
PEA-P	n	14	10	
	mean	25.357	25.2	0.8376
	SD	1.7805	2.6583	
PI-P	n	14	10	
	mean	7.3643	6.57	0.6187
	SD	2.8817	0.636	
PS-P	n	14	10	
	mean	11.671	11.66	0.8836
	SD	2.1606	1.8124	
SFA's	n	14	10	
	mean	45.905	43.577	0.4292
	SD	4.352	1.6851	
MUFA's	n	14	10	
	mean	16.871	15.494	* 0.0065
	SD	0.9126	1.1897	
PUFA's	n	14	10	
	mean	37.221	40.933	* 0.0376
	SD	4.0832	2.351	
PUFA's/SFA's	n	14	10	
	mean	0.8247	0.9423	* 0.0371
	SD	0.1597	0.0884	
n-3	n	14	10	
	mean	5.5586	6.263	0.1514
	SD	1.0391	0.8952	
n-6	n	14	10	
	mean	31.662	34.67	* 0.0376
	SD	3.2149	2.0548	
n-6/n-3	n	14	10	
	mean	5.8446	5.6261	0.93
	SD	0.9996	0.7728	

Table 8. (continue) Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
D5-DESATURASE	n	14	10	
	mean	11.961	10.378	0.3055
	SD	3.346	2.6152	
D6-DESATURASE	n	14	10	
	mean	0.004354	0.002765	0.1599
	SD	0.00302	0.00179	

TPL=measured total phospholipids
SM=sphingomyelin
PC=phosphatidylcholine
PEA=phosphatidylethanolamine
PI=phosphatidylinositol
PS=phosphatidylserine
TPL-SUM=summation of the different phospholipid classes
SM-P=percentage of sphingomyelin of TPL-SUM
PC-P=percentage of phophatidylcholine of TPL-SUM
PEA-P=percentage of phosphatidylethanolamine of TPL-SUM
PI-P=percentage of phosphatidylinositol of TPL-SUM
PS-P=percentage of phosphatidylserine of TPL-SUM
SFA's=F14:0+F16:0+F18:0+F20:0+F22:0+F24:0
MUFA's=F16:1+F18:1+Fn-9 20:1+Fn-9 22:1+Fn-9 24:1
PUFA's=Fn-6 18:2+Fn-6 18:3+Fn-3 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+ Fn-3 20:3+Fn-3 20:5+Fn-6 22:2+Fn-6 22:4+Fn-6 22:5+Fn-3 22:5+Fn-3 22:6
n-3=Fn-3 18:3+Fn-3 20:3+Fn-3 20:5+Fn-3 22:5+Fn-3 22:6
n-6=Fn-6 18:2+Fn-6 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+Fn-6 22:2+Fn-6 22:4+ Fn-6 22:5
D5-DESATURASE=Delta-5 Desaturase activity=Fn-6 20:4/Fn-6 20:3
D6-DESATURASE=Delta-6 Desaturase activity=Fn-6 18:3/Fn-6 18:2
*=significant difference
#=non-significant difference

Table 9. Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks
Phospholipids					
TPL	n	5	9	10	0.8249
	mean	2437.5	2344	2343.5	
	SD	255.08	147.94	203.39	
SM	n	5	9	10	0.1554
	mean	435.14	473.43	394.38	
	SD	103.98	89.619	60.91	
PC	n	5	9	10	0.9739
	mean	638.5	633.33	617	
	SD	128.73	72.64	34.335	
PEA	n	5	9	10	0.1825
	mean	487.16	510.84	451.4	
	SD	81.805	59.406	66.675	
PI	n	5	9	10	0.4907
	mean	144.35	147.66	118.4	
	SD	50.02	72.761	21.108	
PS	n	5	9	10	0.4576
	mean	225.55	230.15	208.15	
	SD	49.069	20.208	32.159	

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
Fatty acids						
F14:0	n	5	9	10		
	mean	0.466	0.2789	0.394	0.1014	
	SD	0.2478	0.0944	0.1097		
F16:0	n	5	9	10		
	mean	21.96	22.538	21.209	0.42	
	SD	1.7224	2.9068	0.8473		
F16:1	n	5	9	10		
	mean	0.304	0.1422	0.173	0.1662	
	SD	0.3231	0.0509	0.1008		
F18:0	n	5	9	10		
	mean	16.744	17.69	16.638	0.5862	
	SD	1.1379	1.8536	1.1021		
F18:1	n	5	9	10		ne=e
	mean	13.786	13.094	12.228	* 0.0101	e=c
	SD	0.7646	0.8547	0.9999		ne>c
Fn-6 18:2	n	5	9	10		
	mean	11.262	9.91	12.111	0.1446	
	SD	1.4515	2.4736	1.6661		

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
Fn-6 18:3	n	5	9	10		
	mean	0.028	0.0556	0.033	0.256	
	SD	0.0164	0.0384	0.0216		
Fn-3 18:3	n	5	9	10		
	mean	0.176	0.1633	0.127	0.3378	
	SD	0.0493	0.074	0.0435		
F20:0	n	5	9	10		
	mean	0.49	0.47	0.384	0.4351	
	SD	0.2637	0.1789	0.0617		
Fn-9 20:1	n	5	9	10		ne=e
	mean	0.278	0.3722	0.222	* 0.0036	ne=c
	SD	0.0415	0.154	0.0365		e>c
Fn-6 20:2	n	5	9	10		
	mean	0.436	0.4367	0.419	0.7558	
	SD	0.1662	0.2313	0.1303		
Fn-6 20:3	n	5	9	10		
	mean	1.278	1.3467	1.624	0.1139	
	SD	0.3876	0.387	0.4414		

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
Fn-6 20:4	n	5	9	10		
	mean	15.16	14.612	15.923	0.2144	
	SD	1.0948	1.5759	1.4697		
Fn-3 20:3	n	5	9	10		
	mean	0.022	0.0489	0.03	0.5163	
	SD	0.0205	0.0437	0.0267		
Fn-3 20:5	n	5	9	10		
	mean	0.232	0.1856	0.282	0.0515	
	SD	0.0691	0.0698	0.0883		
F22:0	n	5	9	10		
	mean	1.614	2.0433	1.542	* 0.002	ne=e
	SD	0.297	0.3734	0.1813		ne=c
						e>c
Fn-9 22:1	n	5	9	10		
	mean	0.152	0.0978	0.064	* 0.0092	ne=e
	SD	0.0572	0.0402	0.0334		e=c
						ne>c
Fn-6 22:2	n	5	9	10		
	mean	0.114	0.1122	0.113	0.8909	
	SD	0.0631	0.0482	0.0683		

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks
Fn-6 22:4	n	5	9	10	0.9301
	mean	3.74	3.9444	3.699	
	SD	0.562	0.6462	0.3949	
Fn-6 22:5	n	5	9	10	0.1823
	mean	0.466	0.7878	0.748	
	SD	0.407	0.464	0.2104	
Fn-3 22:5	n	5	9	10	0.4547
	mean	1.482	1.3633	1.564	
	SD	0.3192	0.2642	0.2228	
Fn-3 22:6	n	5	9	10	0.3087
	mean	4.05	3.5733	4.26	
	SD	0.5542	0.9103	0.6533	
F24:0	n	5	9	10	0.0509
	mean	2.962	3.8122	3.41	
	SD	0.6785	0.4481	0.5129	
Fn-9 24:1	n	5	9	10	0.8142
	mean	2.806	2.9122	2.807	
	SD	0.5687	0.1974	0.5328	

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
Calculations						
	n	5	9	10		e=ne
TPL-SUM	mean	1930.7	1995.4	1789.3	* 0.0182	ne=c
	SD	218.05	140.39	150.71		e>c
	n	5	9	10		
SM-P	mean	22.4	23.778	22	0.4522	
	SD	3.7815	3.8006	2		
	n	5	9	10		
PC-P	mean	33.2	31.556	34.7	0.1866	
	SD	6.0581	3.678	2.4967		
	n	5	9	10		
PEA-P	mean	25.2	25.444	25.2	0.9687	
	SD	1.7889	1.8782	2.6583		
	n	5	9	10		
PI-P	mean	7.38	7.3556	6.57	0.6047	
	SD	2.0729	3.3683	0.636		
	n	5	9	10		
PS-P	mean	11.92	11.533	11.66	0.9802	
	SD	3.5871	1.0452	1.8124		

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
SFA's	n	5	9	10		
	mean	44.236	46.832	43.577	0.6468	
	SD	2.6046	4.9676	1.6851		
MUFA's	n	5	9	10		ne=e
	mean	17.326	16.619	15.494	* 0.0096	e=c
	SD	0.8688	0.8803	1.1897		ne>c
PUFA's	n	5	9	10		
	mean	38.446	36.54	40.933	# 0.0948	
	SD	2.7467	4.6756	2.351		
PUFA's/SFA's	n	5	9	10		
	mean	0.8744	0.7971	0.9423	0.1778	
	SD	0.1136	0.1805	0.0884		
n-3	n	5	9	10		
	mean	5.962	5.3344	6.263	0.2004	
	SD	0.6874	1.1663	0.8952		
n-6	n	5	9	10		
	mean	32.484	31.206	34.67	# 0.0981	
	SD	2.194	3.7057	2.0548		

Table 9. (continue) Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks
n-6/n-3	n	5	9	10	
	mean	5.4811	6.0465	5.6261	0.5977
	SD	0.4452	1.1815	0.7728	
D5-Desaturase	n	5	9	10	
	mean	12.981	11.394	10.378	0.5506
	SD	4.7673	2.4121	2.6152	
D6-Desaturase	n	5	9	10	
	mean	0.002531	0.005367	0.002765	# 0.095
	SD	0.001535	0.003226	0.00179	

TPL=measured total phospholipids
SM=sphingomyelin
PC=phosphatidylcholine
PEA=phosphatidylethanolamine
PI=phosphatidylinositol
PS=phosphatidylserine
TPL-SUM=summation of the different phospholipid classes
SM-P=percentage of sphingomyelin of TPL-SUM
PC-P=percentage of phophatidylcholine of TPL-SUM
PEA-P=percentage of phosphatidylethanolamine of TPL-SUM
PI-P=percentage of phosphatidylinositol of TPL-SUM
PS-P=percentage of phosphatidylserine of TPL-SUM
SFA's=F14:0+F16:0+F18:0+F20:0+F22:0+F24:0
MUFA's=F16:1+F18:1+Fn-9 20:1+Fn-9 22:1+Fn-9 24:1
PUFA's=Fn-6 18:2+Fn-6 18:3+Fn-3 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+ Fn-3 20:3+Fn-3 20:5+Fn-6 22:2+Fn-6 22:4+Fn-6 22:5+Fn-3 22:5+Fn-3 22:6
n-3=Fn-3 18:3+Fn-3 20:3+Fn-3 20:5+Fn-3 22:5+Fn-3 22:6
n-6=Fn-6 18:2+Fn-6 18:3+Fn-6 20:2+Fn-6 20:3+Fn-6 20:4+Fn-6 22:2+Fn-6 22:4+ Fn-6 22:5
D5-DESATURASE=Delta-5 Desaturase activity=Fn-6 20:4/Fn-6 20:3
D6-DESATURASE=Delta-6 Desaturase activity=Fn-6 18:3/Fn-6 18:2
e=MHTepo
ne=MHTnon-epo
c=control subjects
*=significant difference
#=non-significant difference

The following significant differences are indicated between the MHTtotal patients and control subjects

1. The total red blood cell membrane phospholipids are significantly higher for the MHTtotal patients compared to the control subjects, p-value 0.0092. Refer to figure 7 – Box and Whisker plot.

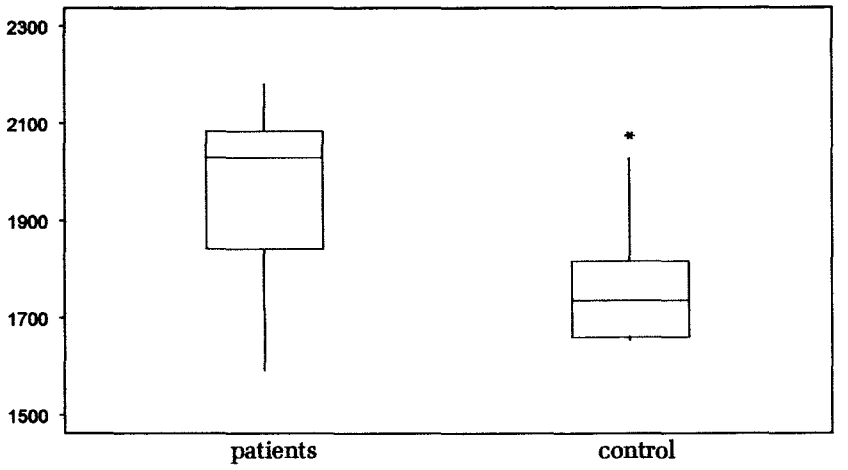

Figure 7. Box and Whisker plot – total phospholipids (µg/ml packed cells)

2. Of the five different classes of membrane phospholipids that were determined; sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, three of the classes; sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine displayed differences, although not statistically significant, between the MHTtotal patients and the control subjects.

Sphingomyelin levels were greater in the MHTtotal patients compared to the control subjects, p-value 0.0841. Refer to figure 8 – Box and Whisker plot.

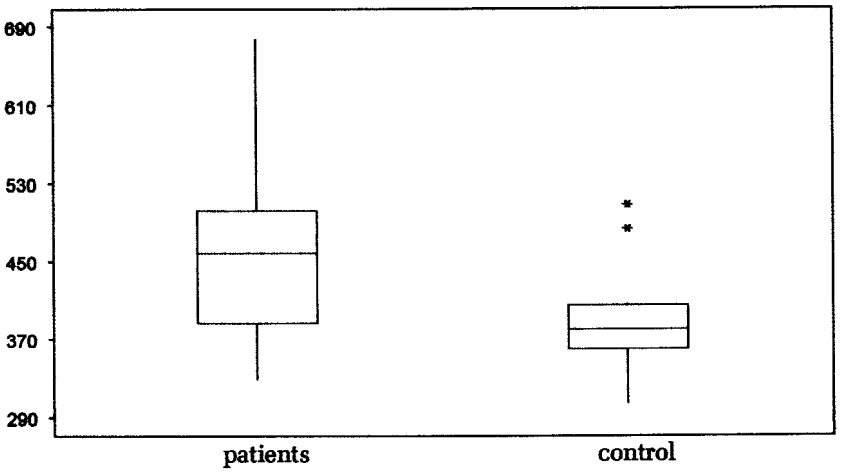

Figure 8. Box and Whisker plot – SM (µg/ml packed cells)

Phosphatidylethanolamine levels, the same as for sphingomyelin levels, were greater in the MHTtotal patients compared to the control subjects, p-value 0.0895. Refer to figure 9 – Box and Whisker plot.

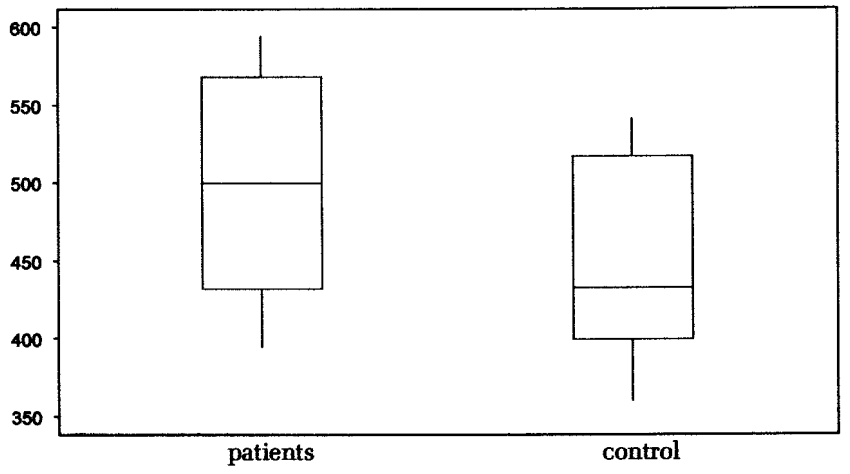

Figure 9. Box and Whisker Plot – PEA (µg/ml packed cells)

The percentage of phosphatidylcholine of the total phospholipids is reduced in the MHTtotal patients compared to the control subjects, p-value 0.0841. Refer to figure 10 – Box and Whisker plot.

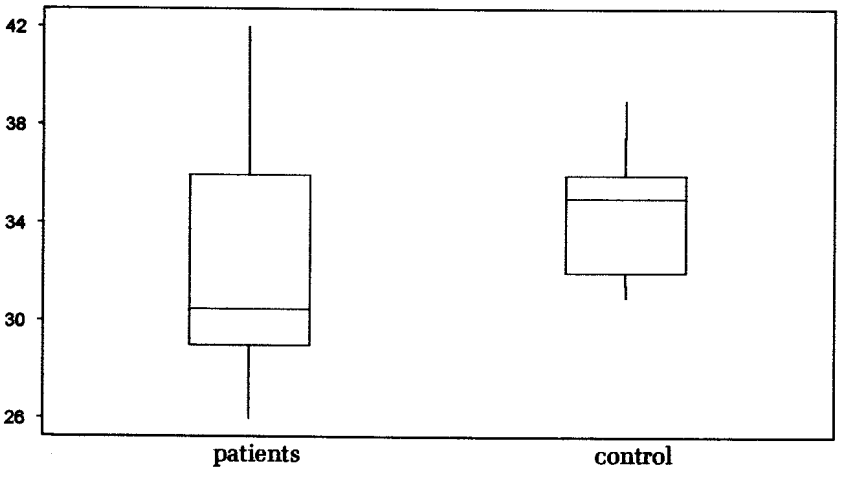

Figure 10. Box and Whisker Plot – PC (%)

3. For only one of the SFA's a significant difference is indicated between the MHTtotal patients and the control subjects. The content of 22:0 in the red blood cell membranes is increased in the MHTtotal patients compared to the control subjects, p-value 0.0109. Refer to figure 11 – Box and Whisker plot.

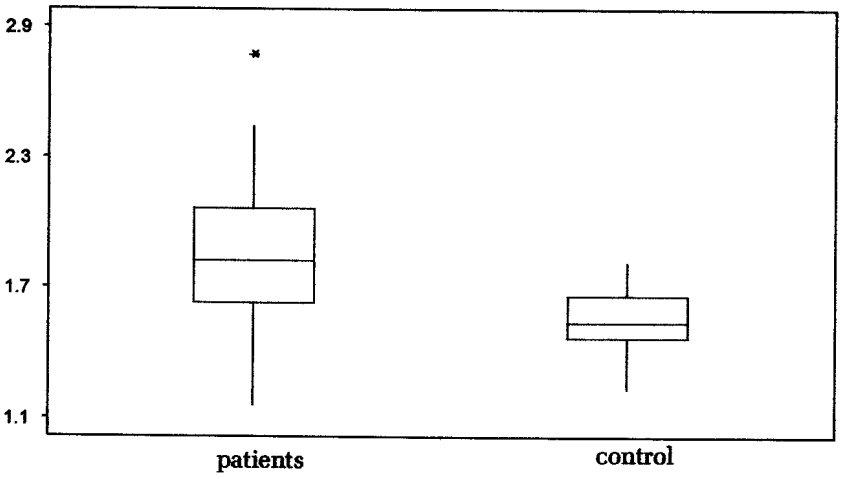

Figure 11. Box and Whisker Plot – 22:0 (weight %)

4. The MUFA content in the red blood cell membranes of the MHTtotal patients are significantly higher compared to the control subjects, p-value 0.0065. Refer to figure 12 – Box and Whisker plot.

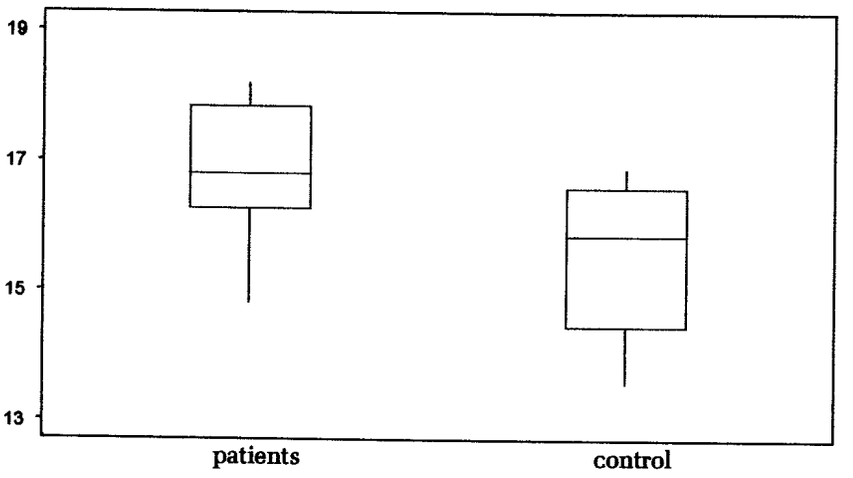

Figure 12. Box and Whisker Plot - MUFA's (weight %)

5. The following MUFA's are significantly different between the MHTtotal patients and the control subjects. The content of 18:1 is higher in the MHTtotal patients compared to the control subjects, p-value 0.0092. Refer to figure 13 – Box and Whisker plot.

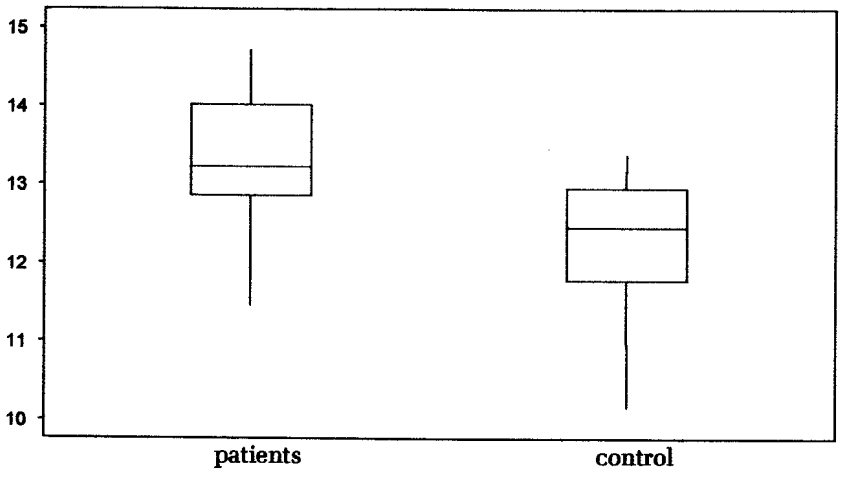

Figure 13. Box and Whisker Plot – 18:1 (weight %)

The content of n-9 20:1 is significantly higher in the patient group compared to the control group, p-value 0.0034. Refer to figure 14 – Box and Whisker plot.

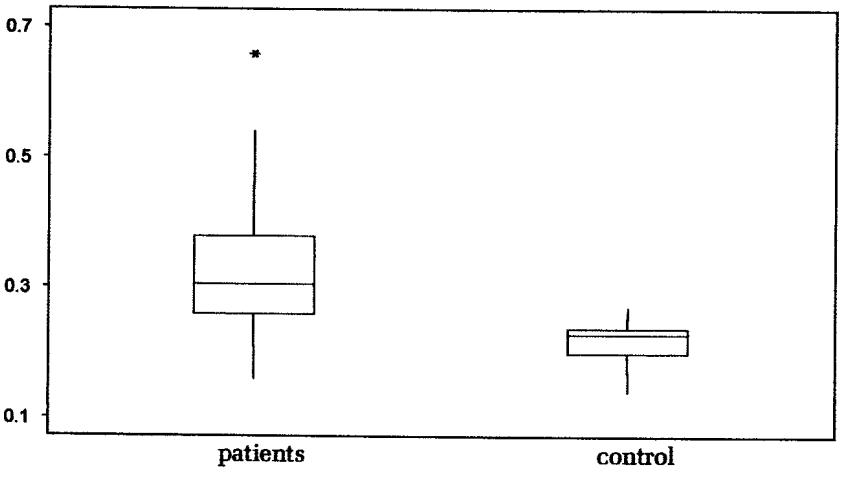

Figure 14. Box and Whisker plot – n-9 20:1 (weight %)

The content of n-9 22:1 is significantly higher in the patient group compared to the control group, p-value 0.0139. Refer to figure 15 – Box and Whisker plot.

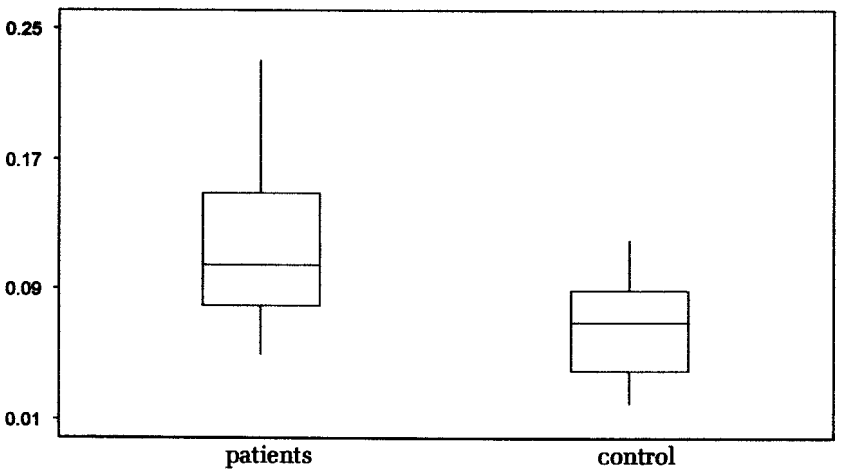

Figure 15. Box and Whisker Plot – n-9 22:1 (weight %)

6. The total content of PUFA's is significantly decreased in MHTtotal patients compared to the control subjects, p-value 0.0376. Refer to figure 16 – Box and Whisker plot.

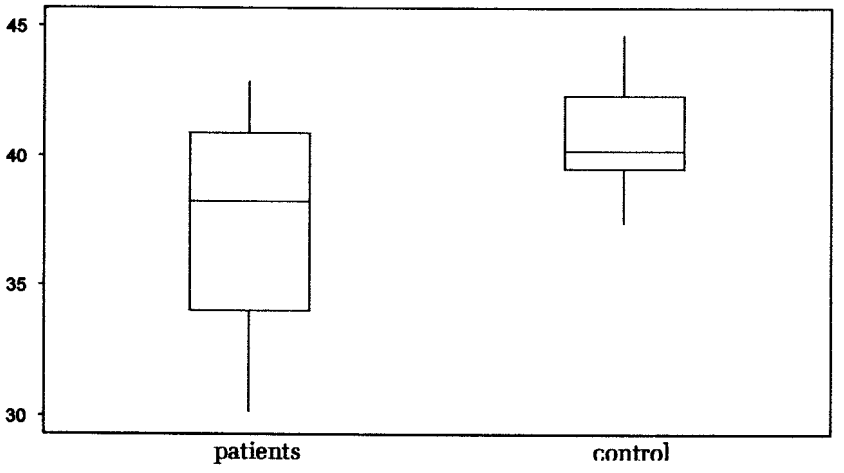

Figure 16. Box and Whisker Plot – PUFA's (weight %)

7. The n-6 fatty acids are significantly decreased in the MHTtotal patients compared to the control subjects, p-value 0.0376. Refer to figure 17 – Box and Whisker plot

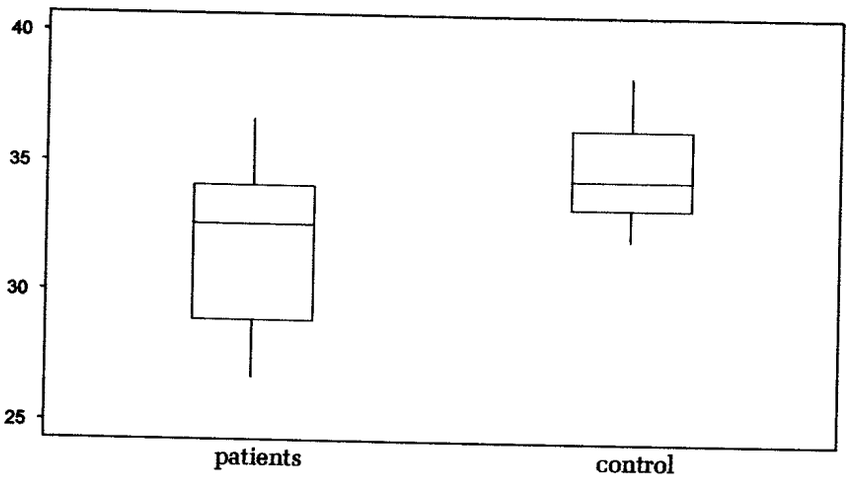

Figure 17. Box and Whisker plot – n-6 fatty acids (weight %)

8. For three of the n-6 fatty acids differences are indicated, although not all differences are significant, between the MHTtotal patients and the control subjects.

The content of n-6 18:2 is non-significantly reduced in the MHTtotal patients compared to the control subjects, p-value 0.0841. Refer to figure 18 – Box and Whisker plot.

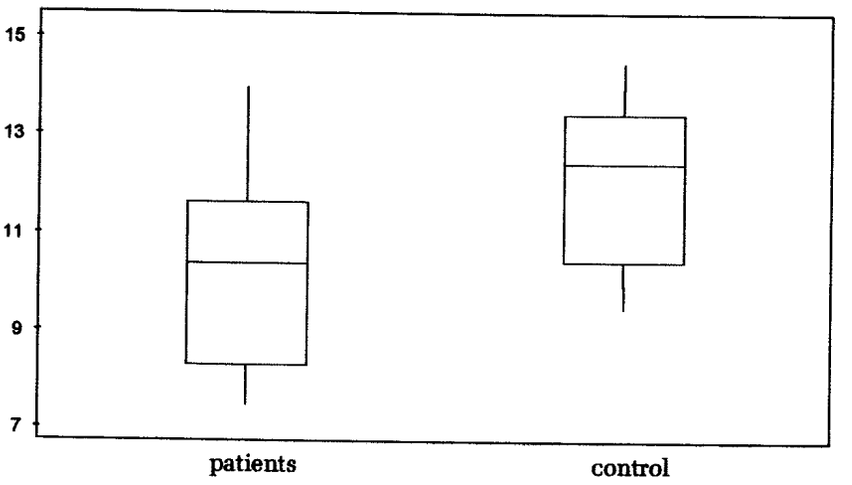

Figure 18. Box and Whisker plot – n-6 18:2 (weight %)

The content of n-6 20:3 is significantly reduced in the MHTtotal patients compared to the control subjects, p-value 0.0465. Refer to figure 19 – Box and Whisker plot.

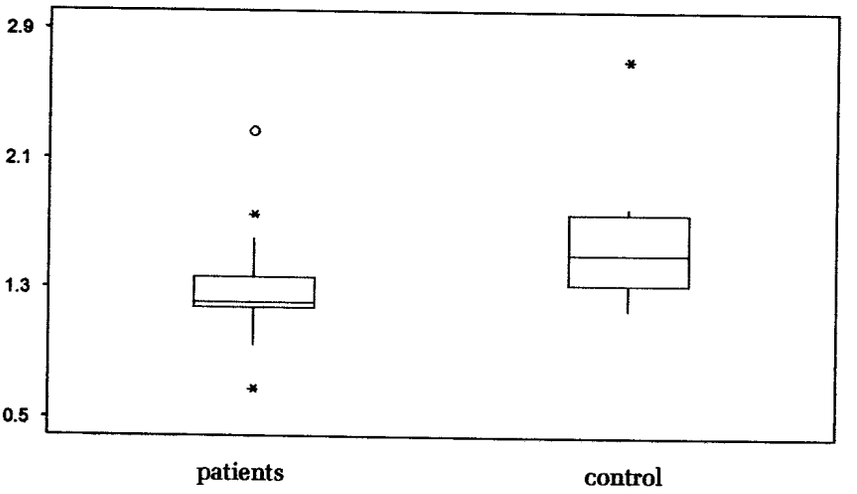

Figure 19. Box and Whisker plot – n-6 20:3 (weight %)

The content of n-6 20:4 is non-significantly reduced in the MHTtotal patients compared to the control subjects, p-value 0.1073. Refer to figure 20 – Box and Whisker plot.

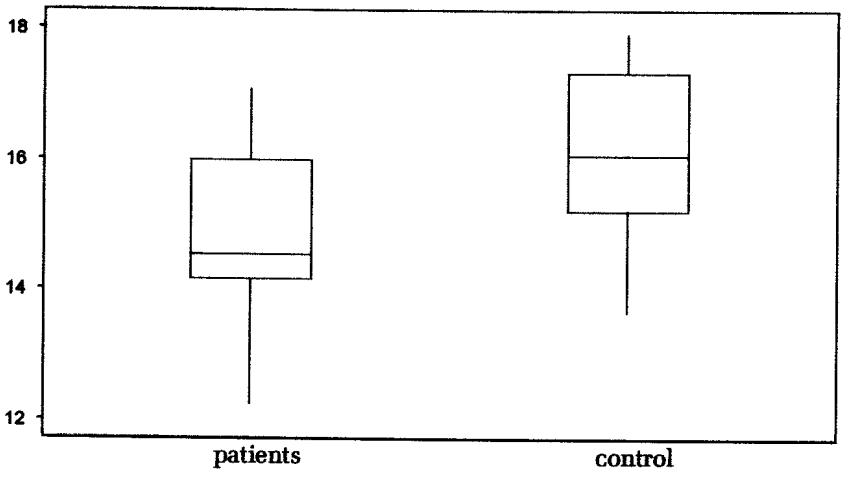

Figure 20. Box and Whisker Plot – n-6 20:4 (weight %)

9. Only one of the n-3 fatty acids determined was significantly decreased for the MHTtotal patients compared to the control subjects, n-3 20:5, p-value 0.0326. Refer to figure 21 – Box and Whisker plot.

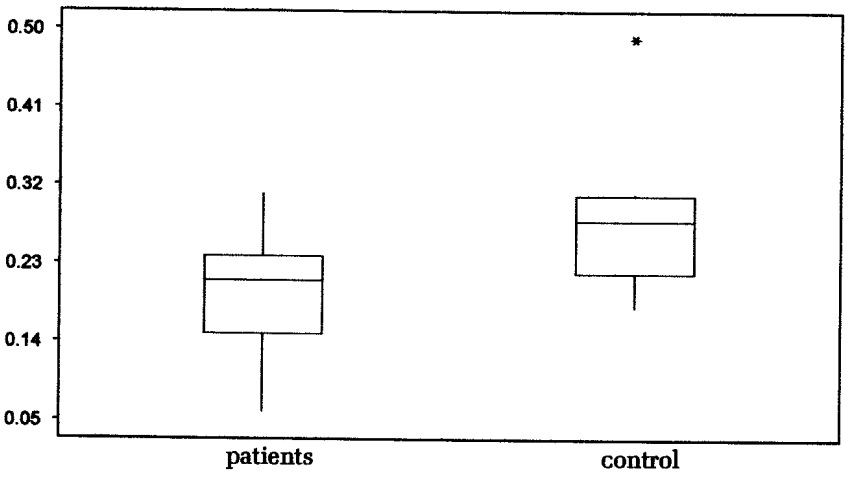

Figure 21. Box and Whisker plot – n-3 20:5 (weight %)

10. The ratio of PUFA's to SFA's indicates a decrease, although not significant, for the MHTtotal patients compared to the control subjects, p-value 0.0741. Refer to the figure 22 - Box and Whisker plot.

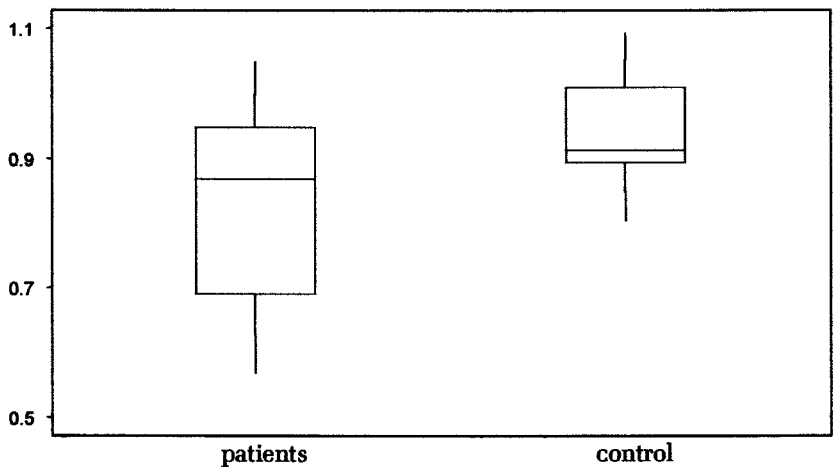

Figure 22. Box and Whisker plot – PUFA's/SFA's

No significant differences are indicated for any of the membrane phospholipid and fatty acid determinations between the MHTnon-epo and the MHTepo patients.

D Other biochemical parameters which may influence the calcium status of neutrophils

The following biochemical parameters may influence the calcium status in the neutrophils; PTH, the oxidative status as indicated by the anti-oxidant vitamins - vitamin A, E and C, and free serum calcium.

Tables 10 to 12 contain the individual data and statistical comparisons between the groups of patients.

Table 10. Other biocemical parameters which may influence the calcium status of neutrophils

Patients	PTH	Vitamin C	Vitamin A	Vitamin E	Serum free Ca
1	314.8	133.2	7.3	19.4	1.2
2	37.5	179.6	9.5	39.9	1.09
3	1320	82.1	9.5	54.1	1.31
4	258.7	44.7	12	32.7	1.23
5	61.9	95.9	14.3	75.9	1.11
6	727.3	46.2	15.1	28.6	1.13
7	951	15.3	6.8	25.9	1.16
8	1624.6	51.8	11	35.3	1.18
9	267	90	24.2	36.6	1.25
10	340	19.5	10.8	35.2	1.18
11	417	48.5	12.3	37.7	1.26
12	1001	15.2	5.8	34.5	1.19
13	633.4	11.1	10.2	30.7	1.18
14	325	68.3	16.4	38.8	1.25
Controls					
1	24	54.5	4.1	29.4	1.2
2	24.1	69.3	5	38.8	1.22
3	21.1	66.1	4.3	36.4	1.2
4	27.6	79.2	4.7	36.3	1.25
5	21.9	81.5	2.4	23.6	1.25
6	30	44.7	3.4	38.6	1.24
7	36	27	2.7	22.8	1.22
8	13.2	57.5	4.7	40.5	1.26
9	31.4	26.9	4.8	29	1.28
10	20.5	46.8	3.4	25.3	1.27

Table 11. Statistical comparison between the MHTtotal patients and control subjects

Variable		MHTtotal	Controls	p-value Mann-Whitney
Serum PTH	n	14	10	
	mean	591.37	24.98	* < 0.001
	SD	477.16	6.4965	
Vitamin C	n	14	10	
	mean	64.386	55.35	1
	SD	48.675	19.344	
Vitamin A	n	14	10	
	mean	11.8	3.95	* < 0.001
	SD	4.7099	0.9229	
Vitamin E	n	14	10	
	mean	37.521	32.07	0.4823
	SD	13.554	6.7962	
Serum free Ca	n	14	10	
	mean	1.1943	1.239	* 0.0404
	SD	0.0615	0.0281	

*=significant difference

Table 12. Statistical comparison between the MHTnon-epo patients, MHTepo patients and control subjects

Variable		MHTnon-epo	MHTepo	Controls	p-value ANOVA applied to ranks	
Serum PTH	n	5	9	10	* < 0.001	e=ne e+ne>c
	mean	398.58	698.48	24.98		
	SD	528.99	440.38	6.4965		
Vitamin C	n	5	9	10	* 0.0204	ne=c e=c ne>e
	mean	107.1	40.656	55.35		
	SD	51.434	27.449	19.344		
Vitamin A	n	5	9	10	* < 0.001	e=ne ne+e>c
	mean	10.52	12.511	3.95		
	SD	2.6892	5.5533	0.9229		
Vitamin E	n	5	9	10	0.5078	
	mean	44.4	33.7	32.07		
	SD	21.613	4.3549	6.7962		
Serum free Ca	n	5	9	10	0.1096	
	mean	1.188	1.1978	1.239		
	SD	0.0901	0.0452	0.0281		

e=MHTepo
ne=MHTnon-epo
c=control subjects

*=significant difference

The following significant differences are indicated between the MHTtotal patients and the control subjects

1. Parathyroid hormone levels are significantly higher in the MHTtotal patients compared to the control subjects, p-value < 0.001. Refer to figure 23 – Box and Whisker plot.

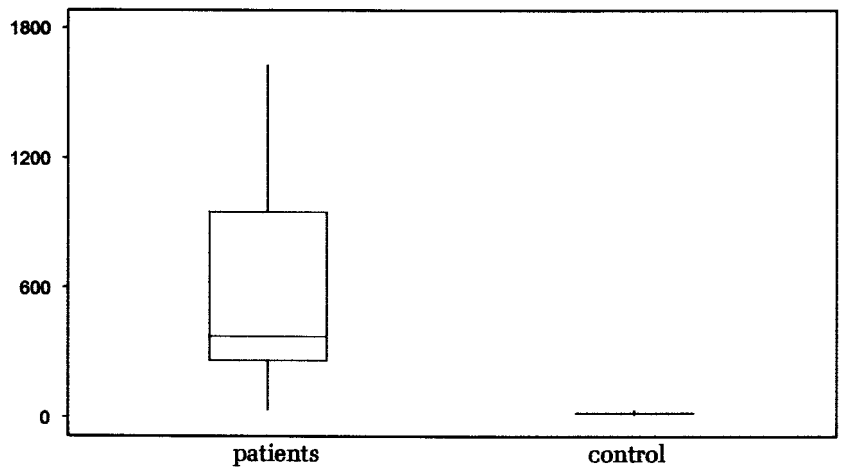

Figure 23. Box and Whisker plot – PTH (ng/l)

2. The vitamin C levels of the MHTtotal patients are not significantly different from the vitamin C levels of the control subjects. Nevertheless, it must be mentioned that the vitamin C levels of the MHTtotal patients display high variability. Refer to figure 24 – Scatter plot.

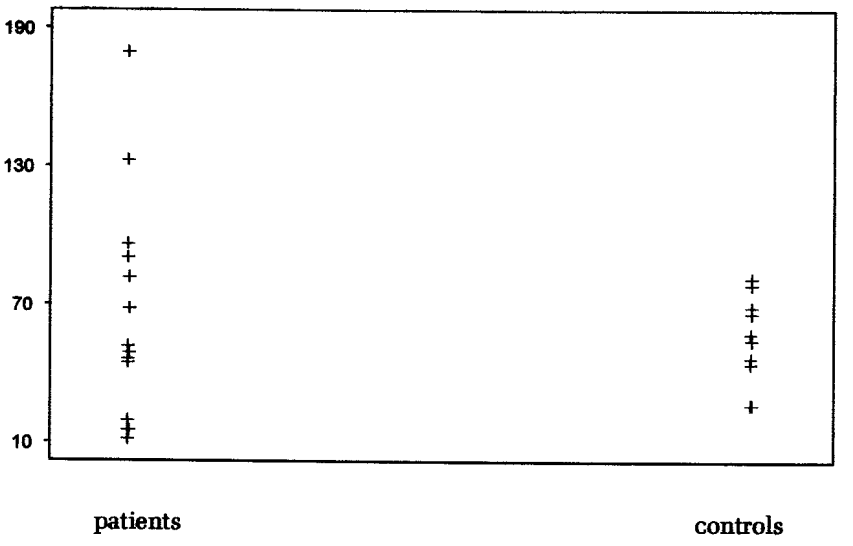

Figure 24. Scatter Plot- vitamin C (µmol/l)

3. The vitamin A levels of the MHTtotal patients are significantly higher than that for the control subjects, p-value < 0.001. Refer to figure 25 – Box and Whisker plot.

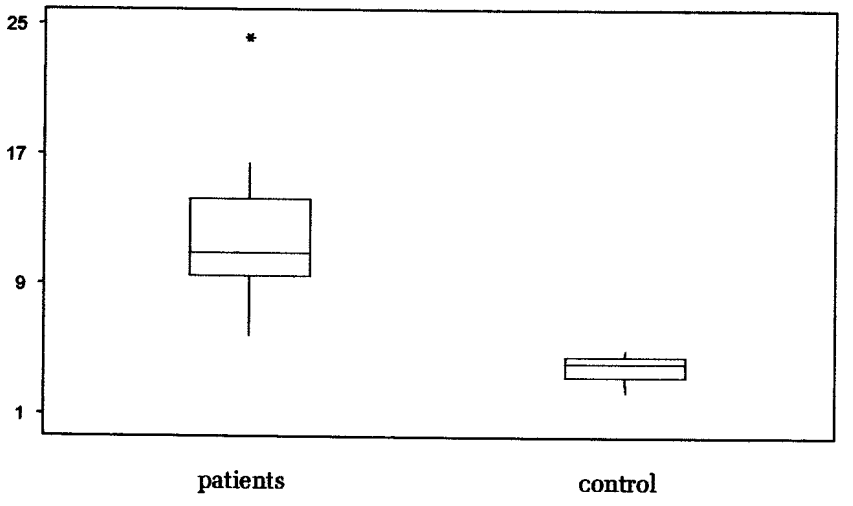

Figure 25. Box and Whisker Plot – vitamin A (µmol/l)

4. No significant differences for vitamin E levels are indicated between the MHTtotal patients and control subjects.

5. The serum ionised calcium is significantly lower in the MHTtotal patients compared to the control subjects, p-value 0.0404. Refer to figure 26 – Box and Whisker plot.

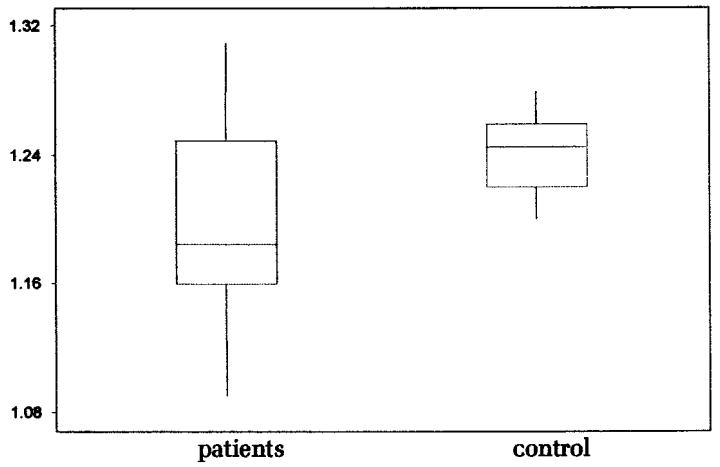

Figure 26. Box and Whisker Plot – ionised serum calcium (mmol/l)

The following significant differences are indicated between MHTnon-epo and MHTepo patients

1. No significant difference is indicated for PTH between the MHTnon-epo and MHTepo patients.

2. A significant difference for vitamin C is indicated between MHTnon-epo patients and MHTepo patients, p-value 0.0204. MHTnon-epo patients display higher vitamin C levels compared to MHTepo patients. Refer to figure 27 – Box and Whisker plot.

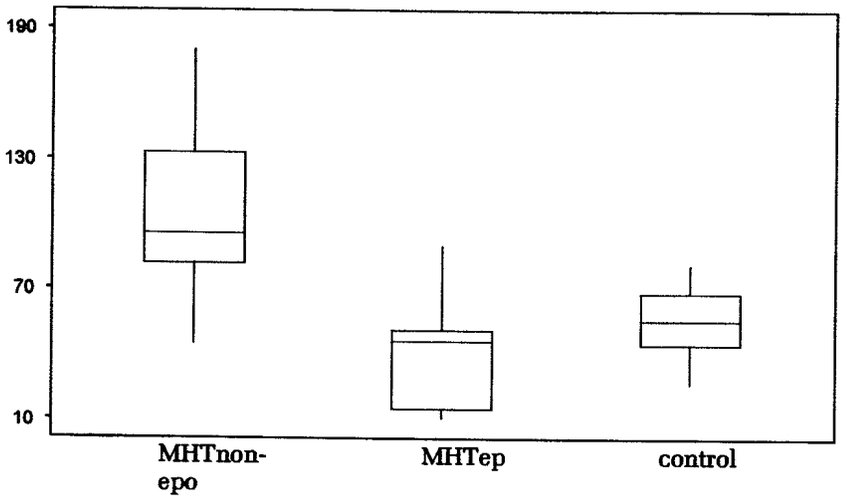

Figure 27. Box and Whisker plot – vitamin C (µmol/l)

3. No significant differences are indicated for vitamin A and E between the MHTnon-epo and MHTepo patients.

4. No significant difference is indicated for serum ionised calcium between the MHTnon-epo and MHTepo patients.

E. Correlations between variables

Table 13 contains the correlations between variables for the MHTtotal patients.

Table 13. Correlations between variables for MHTtotal patients

Variable	Variable	r-value	p-value
Intracellular free calcium	Transmembrane calcium flux	0.6304	0.0157
Intracellular free calcium	D5-Desaturase	-0.5662	0.0348
Intracellular free calcium	n-6 20:3	0.6351	0.0147
Intracellular free calcium	n3 20:3	0.5384	0.047
Transmembrane calcium flux	D5-Desaturase	-0.523	0.055
Transmembrane calcium flux	Decrease of intracellular free calcium - rate y	0.7063	0.0048
Transmembrane calcium flux	n-6 20:3	0.5265	0.0531
Decrease of intracellular free calcium - rate y	F16:1	0.6323	0.0152
Decrease of intracellular free calcium - rate y	F16:1	0.6189	0.0183
PTH	PUFA's	-0.5528	0.0403
PTH	n-3 fatty acids	-0.5887	0.0268
PTH	n-6 fatty acids	-0.5118	0.0614
PTH	PUFA's/SFA's	-0.5415	0.0455
PTH	PS	0.5232	0.0549
PTH	n-6 22:5	0.5197	0.0568
PTH	n-9 20:1	0.5082	0.0635

Table 13. (continue) Correlations between variables for MHTtotal patients

Variable	Variable	r-value	p-value
PTH	n-6 18:2	-0.6141	0.0195
PTH	n-3 22:5	-0.5698	0.0334
PTH	n-3 22:6	-0.5379	0.0473
PTH	F16:0	0.5861	0.0276
Vitamin A	n-6 fatty acids	0.6086	0.0209
Vitamin A	PUFA's	0.5965	0.0243
Vitamin A	PUFA's/SFA's	0.584	0.0339
Vitamin A	SFA's	-0.4748	0.0863
Vitamin A	n-6 18:2	0.5242	0.0543
Vitamin A	F16:0	-0.5643	0.0355
Vitamin C	TPL	0.6498	0.0119
Vitamin C	PS-P	-0.5357	0.0483
Vitamin C	PS	-0.5913	0.0259
Vitamin C	n-9 22:1	0.4983	0.0698
Vitamin C	n-6 20:4	0.5592	0.0376
Vitamin C	n-3 20:5	0.516	0.0589
Vitamin C	F22:0	-0.6871	0.0066
Vitamin C	F24:0	-0.6433	0.0131

F. Calcium distribution in the neutrophil

Figure 28 to 30 contains the electron microscopy photographs for the localisation of intracellular calcium in the neutrophil for patients and control subjects. Figure 28 a and b contain examples of the electron microscopy photographs for the control subjects. Intracellular calcium is localised in the following compartments, i.e., cytosol, nucleus and the space between the outer and inner nuclear membranes. Figures 29 and 30 contain examples of the electron microscopy photographs for the patients. Two strikingly different calcium localisation patterns were obtained. Figures 29 a and b contain examples of the electron microscopy photographs representative of the one pattern – intracellular calcium is localised in the nucleus and the cytosol. Figures 30 a and b contain examples of the electron microscopy photographs representative of the other pattern – the prominent localisation of intracellular calcium in the space between the inner and outer nuclear membranes.

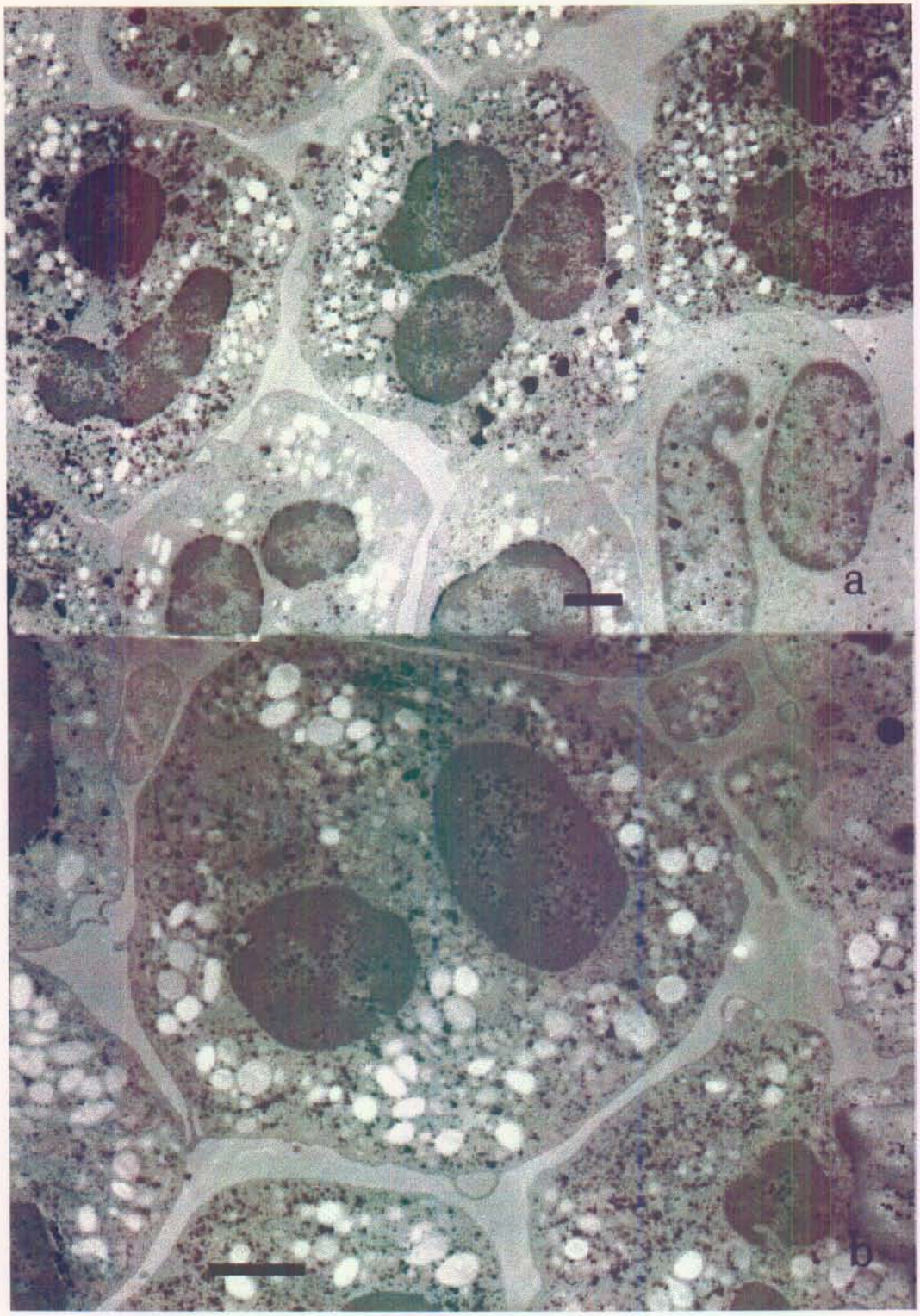

Figure 28 a and b. Electron microscopy photographs indicating the localisation of intracellular calcium in the cytosol, in the nucleus and in the space between the inner and outer nuclear membranes in the neutrophils of the control subjects. Scale bar 1 μm.

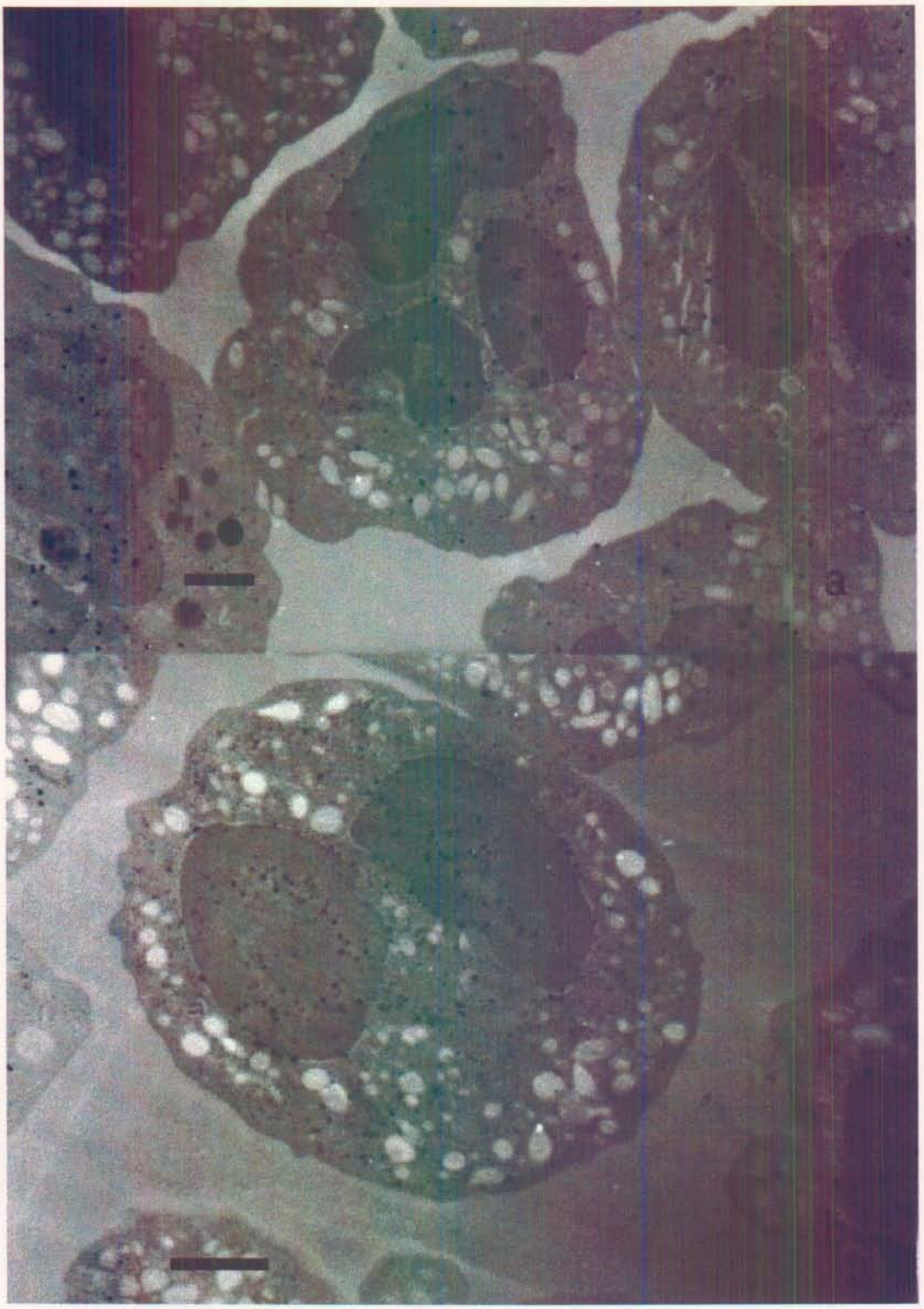

Figure 29 a and b. Electron microscopy photographs indicating the localisation of intracellular calcium in both the nucleus and cytosol in the neutrophils of some of the patients. Scale bar 1 μm.

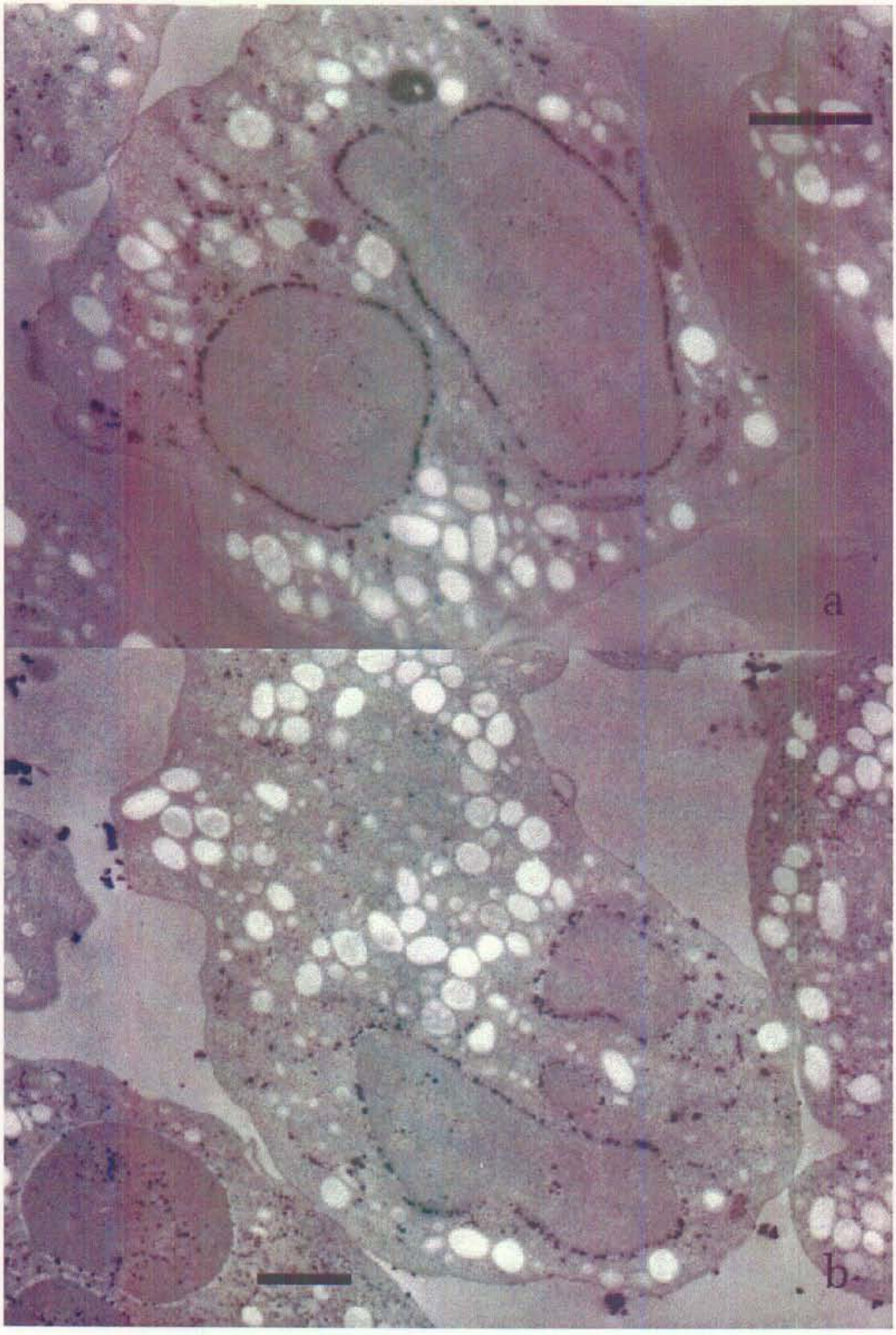

Figure 30 a and b. Electron microscopy photographs indicating the prominent localisation of intracellular calcium in the space between the inner and outer nuclear membranes in the neutrophils of some of the patients. Scale bar 1 μm.

References

1) Girelli D, Azzini M, Oliviero O, Guarini P, Trevisan MT, Lupo A, et al. Red Blood Cells and Platelet Membrane Fatty Acids in Non-Dialyzed and Dialyzed Uremics. Clinica Chimica Acta 1992; 211: 155-166.

2) Christensen JH, Aaroe J, Knudsen N, Dideriksen K, Kornerup HJ, Dyerberg J, et al. Heart Rate Variability and n-3 Fatty Acids in Patients with Chronic Renal Failure – a Pilot Study. Clinical Nephrology 1998; 49(2): 102-106.

3) Ramsay B, Cream JJ, Curtis JR, Manku MS, Stewart JCM. Erythrocyte Membrane Docosahexanoic Acid in Haemodialysis Patients on Epoetin. The Lancet 1992 May 16; 339: 1232-1233.

Chapter 5

Discussion and Conclusions

Intracellular calcium regulates many cellular functions and intracellular calcium dyshomeostasis culminates in cellular dysfunction and ultimately cell death. Thus, intracellular calcium activation brings about both good and bad responses. It is for both these two responses of intracellular calcium activation that studies are conducted to determine the mechanisms involved in intracellular calcium regulation. Intracellular calcium is contained in various intracellular calcium storage pools. These intracellular calcium storage pools include a pool consisting of calcium bound to the cytosolic side of the plasma membrane and to macromolecules in the cytosol, a pool consisting of free calcium ions in the cytosol, and a pool consisting of calcium sequestered into various intracellular organelles. (1) It is the free calcium ions in the cytosol that regulate the activities of the different calcium-sensitive proteins and it is in turn these calcium-sensitive proteins that determine the intracellular calcium responses. (2) These proteins differ in their calcium response curves such that at differing intracellular free calcium concentrations specific proteins are activated, or specific proteins are activated to different degrees. (3) It is therefore of the utmost importance that this pool of intracellular free Ca^{2+} ions be precisely regulated. To assure the precise regulation of this cytosolic free Ca^{2+} ion pool the cell is endowed with powerful mechanisms to decrease intracellular free Ca^{2+} when an unwanted elevation occurs. One of these mechanisms involves the sequestration of intracellular free Ca^{2+} ions into the mitochondrium. (4) Mitochondria can buffer an

elevation in intracellular free Ca^{2+} ions although not without any deleterious effects to the mitochondrium itself.

Probably the most important function of the intracellular organelles responsible for sequestering calcium is the ability to release calcium into the cytosol upon agonist stimulation. (2) The intracellular organelles capable of releasing Ca^{2+} upon agonist stimulation are $InsP_3$-sensitive. (5) $InsP_3$ is generated as an intracellular messenger upon agonist binding to the receptor. The $InsP_3$-sensitive intracellular calcium storage organelles comprise the endoplasmic reticulum in excitable cells and the calciosome in non-excitable cells. (6) The space between the inner and outer nuclear membranes forms yet another intracellular calcium storage site. It is the inner nuclear membrane that contains the $InsP_3$-receptor and therefore stimulation of these receptors result in the releasing of calcium into the nucleus. (7)

The membranes of the endoplasmic reticulum form an interconnected boundary that includes the outer membrane of the nuclear envelope, thus forming an enclosed luminal space for the movement of Ca^{2+} ions between different calcium storage sites. (8) It is proposed that the distribution and connectivity of these calcium storage organelles (endoplasmic reticulum and nuclear envelope) underlie functioning, such as the regulation of localised calcium signals and sustained calcium gradients. (8) The interconnectivity between the calcium storage organelles is dynamically regulated, resulting in an increase or decrease in the quantity of releasable calcium. (9) It is also indicated that hormones and growth factors can modulate the components responsible for the integrity of the luminal communication between calcium storage organelles. (9)

It is also in the neutrophil that intracellular free calcium regulates many diverse cellular responses. These cellular responses, both good and bad, include chemotaxis, adhesion, extravasation, migration, phagocytosis, phagosome-granule fusion, and production of oxygen radicals and apoptosis. (10 - 13) In the neutrophil the intracellular calcium storage organelle is the calciosome. These intracellular calcium storage organelles are distributed over the entire cytoplasm. (6, 14) But as with many other cell types, redistribution of the intracellular calcium stores are indicated during the performing of a specific cell function such as adhesion, chemotaxis, and phagocytosis. (12, 15, 16) Therefore, cellular events that induce distributional changes of the intracellular calcium stores can significantly interfere with different types of calcium signals. (8)

To get a clear picture of the calcium status in a cell it is preferable to assess the various intracellular calcium pools, as well as the characteristics of transmembrane calcium movements. To perform this type of study where many aspects of intracellular calcium are investigated simultaneously on neutrophils isolated from the same blood collection, a whole team of workers will have to be involved to prevent a time delay which would compromise the functional integrity of the neutrophils. In the present study the aim was to investigate more than one aspect of the intracellular calcium status simultaneously in the neutrophils of CRF patients receiving maintenance haemodialysis treatment. The first objective was to establish the methods in the laboratory and to evaluate the techniques, since none of the techniques were available in the Department of Physiology, UP. This became a rather time consuming aspect of the study, partly due to the dependency on other laboratories for the use of equipment such as the inductively coupled plasma mass spectrometer, graphite furnace atomic absorption spectrometer and transmission electron microscope. The developmental

aspects of the study are presented in Chapter 2. Initially, five techniques were investigated, i.e., atomic absorption spectrometry, graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry for determining the total calcium content in the neutrophil, fluorescence spectrophotometry for the determination of intracellular free calcium and transmembrane calcium movements, and transmission electron microscopy for intracellular calcium localisation in the neutrophil, (refer to chapter 2). The three techniques eventually identified as feasible to perform within the confines and prerequisite of the study, include the determination of free cytosolic calcium by fluorescence spectrophotometry, the transmembrane calcium movements by fluorescence spectrophotometry and the localisation of intracellular calcium within the neutrophil by transmission electron microscopy. The major factors which determined the final choice of assessments were the volume of blood which could be collected at one time from the already anaemic patients, the ability of one person to complete the blood collection, the required neutrophil isolations and the calcium determinations without a confounding time delay which could result in the alteration of neutrophil properties. Some limitation was imposed on extending the study towards CRF patients on other forms of renal replacement treatments. However, it was not considered a stumbling block with regard to completion of the dissertation as this particular work is seen as the first in a series of studies on the calcium status in CRF. The evaluation studies are presented in chapter 2. The experimental work showed fura-PE3 to be the most reliable fluorescent calcium indicator. Fura-PE3 was thus employed in all the subsequent neutrophil intracellular free Ca^{2+} determinations. Initial experimental work involved the determination of one patient and one control per day. Evaluations, however, showed that intracellular free calcium determinations in the neutrophils of only one subject could be performed on one occasion, since the time delay after loading of the

indicator resulted in the leaking of the indicator into the extracellular medium. The method developed for calcium localisation gave satisfactory results, but in retrospect, can further be refined. The final experimental procedures are described in the evaluation chapter, i.e., Chapter 2.

The results on the analysis of intracellular free Ca^{2+} showed a significant elevation in the neutrophils of the total group of patients receiving maintenance haemodialysis treatment (MHTtot), (x 53.071; SD 12.761) compared to the group of control subjects, (x 43.2; SD 6.4601, p-value 0.0242). Refer to figure 2, page 159. In separating the patients into those receiving recombinant human erythropoietin (rHuEPO) (MHTepo), (x 58; SD 13.162), and those not receiving rHuEPO (MHTnon-epo), (x 44.2; SD 5.4498), the intracellular free Ca^{2+} was significantly elevated for the MHTepo patients compared to the control subjects, (p-value 0.0016) but not for the MHTnon-epo patients. Refer to table 6, page 158. In comparing the intracellular free Ca^{2+} levels between MHTepo and MHTnon-epo, intracellular free Ca^{2+} was significantly higher in the MHTepo patients, (p-value 0.0164). Refer to figure 6, page 162. This despite the fact that the MHTepo patients were receiving the calcium channel blocker, Norvasc. The fact that the intracellular free Ca^{2+} was significantly higher in the patients receiving both rHuEPO and calcium channel blockers, suggest a) either a dramatic effect of rHuEPO on intracellular free Ca^{2+} or b) a decrease in the effectivity of the calcium channel blocker. The efficacy of rHuEPO treatment in the correction of anaemia of uraemic patients on maintenance haemodialysis is well established. (17) This is due to rHuEPO binding to the EPO-receptor on erythroid precursor cells, which occur in the adult bone marrow. Binding of rHuEPO to the EPO-receptor induces differentiation and proliferation of these precursor cells. It is suggested that rHuEPO binding to the EPO-receptor causes elevation in intracellular free Ca^{2+}

levels through voltage-independent Ca^{2+} channels in the erythroid precursors. (17) This rHuEPO-induced increase in intracellular free Ca^{2+} in human erythroblasts is dose-dependent, dependent on extracellular Ca^{2+} and blocked by high doses of nifedipine. (18) However, the bone marrow is not the only EPO target tissue and as a result, rHuEPO administration has been associated with complications that are independent of its haemotologic effects, most notably an increased incidence of hypertension. (19 - 21) There are also indications of rHuEPO influencing various other cell types and that stimulation of such cell types possibly involves the influx of Ca^{2+}. Such cells include neurons (22, 23), vascular smooth muscle cells (19), vascular endothelial cells (24), and platelets (19, 21, 25) The assumption that the rHuEPO be the cause of the high intracellular free Ca^{2+} in the neutrophils of the MHTepo group is further hinted at by various other findings such as a) the detection of EpoR-mRNA in a stem-cell subclone dependent on granulocyte colony stimulating factor for growth, (26) b) various other studies which demonstrated modulation of calcium-dependent neutrophil functions such as phagocytosis and oxygen radical production by rHuEPO administration in maintenance haemodialysis patients. (17, 27) The increase in intracellular free Ca^{2+} indicated by this study might very well be an underlying factor to the other rHuEPO-induced effects.

As previously mentioned in this study, the increase in intracellular free Ca^{2+} levels were found in the MHTepo patients despite calcium channel blocker administration. Certain data suggest that neutrophils may not have L-type voltage–dependent calcium channels that are blocked by calcium channel blockers, nevertheless some investigators previously reported inhibition of $^{45}Ca^{2+}$ influx in neutrophils by calcium channel blockers. In addition, many studies involving neutrophils in CRF patients indicate the prevention of a rise in intracellular free Ca^{2+} with

the administering of the calcium channel blockers – nitrendipine and verapamil. (28 - 31) The calcium channel blocker, amlodipine was administered to the patients involved in the present study. No published data could be found on the prevention of an increase in intracellular free Ca^{2+} in neutrophils by amlodipine. Nevertheless, one study administered amlodipine to diabetic rats to prevent a rise in intracellular free Ca^{2+} in the neutrophil. (32) Therefore, it is predicted that amlodipine should prevent the possible pathological rise in intracellular free Ca^{2+} in the neutrophil in these patients. At first glance, the fact that the intracellular free Ca^{2+} was higher in those patients receiving calcium channel blockers, seemed difficult to explain. However, when other factors are considered a possible explanation may be found. Results from previously published work showed that different types of fatty acids may interfere with the action of agonists and antagonists of the Ca^{2+}-channels by specifically binding to the Ca^{2+}-channel at or near the binding site for the agonist or antagonist. (33) This interaction between the calcium channel and the specific fatty acid might interfere with the pharmacological action of calcium channel blockers. It seems logical to assume that this be one of the causes for the increase in intracellular free Ca^{2+} in the neutrophils of MHTepo patients despite calcium channel blocker administration. This assumption gains credibility by the positive correlation seen in this study between intracellular free Ca^{2+} levels and two of the poly-unsaturated fatty acids, i.e., n-6 20:3, (r-value 0.6351, p-value 0.0147) and n-3 20:3 (r-value 0.5384, p-value 0.047). Refer to table 13, page 199. It should be mentioned that red blood cell membranes were analysed, since the volume of blood necessary to obtain enough neutrophils for membrane lipid analysis would have resulted in the drawing of too large a volume of blood in these anaemic patients. It has previously been shown, in various published studies, that the lipid composition in the membranes of red blood cells is representative of that of various other cell types,

therefore red blood cells are considered a reasonable model to study the lipids of plasma membranes in general. (34 - 37) It would seem, from other studies, that the fatty acids eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) may inhibit the effect of agonists and antagonists on Ca^{2+}-channel current. This may possibly be a result of an interaction between the fatty acids and the calcium channel protein due to the fatty acid's intrinsic structural conformational properties related to the extent of unsaturation. (33) These types of fatty acids and their interactions with membranes, chiefly govern the physicochemical properties and function of membranes. It may be explained by an alteration to a specific protein-lipid or lipid-lipid interaction. The protein in this case being the calcium channel.

The calcium studies were performed in a single blind fashion, i.e., the fact whether patients received rHuEPO or not and whether patients received calcium channel blockers or not were not known or taken into consideration. All available patients, as was previously mentioned, who gave permission and who did not have a recent change in treatment or medication regimen were included. The negative off-spin of this non-selectivity was the eventual unfortunate unequal distribution of males vs females, as well as an unequal race distribution between epo/non-epo groups. The MHTnon-epo patients included whites only while all but one of the MHTepo group were black. The MHTnon-epo group included only one male and the MHTepo group only one female. Two factors should therefore be considered when comparing the MHTepo patients to the MHTnon-epo patients. Firstly, race and therefore nutrition may be a contributor to the difference and secondly that gender could be a confounding factor. In taking albumin as an indicator of nutritional status there would not appear to have been a significant difference in the nutritional status between the groups. A further indication that the difference in intracellular free Ca^{2+} is in fact valid and ascribable to the

effect of rHuEPO is the fact that except for vitamin C no other significant differences could be found between the two groups – even for membrane phospholipid and fatty acid composition.

In discussing the calcium fluxes to and from the cytosol, two aspects were considered. Firstly, the magnitude of the inward movement of calcium upon fMLP stimulation and secondly the subsequent rate of intracellular free Ca^{2+} decrease. In this study the transmembrane Ca^{2+} flux upon fMLP stimulation of the neutrophils was significantly elevated for the total group of patients (MHTtot), (x 380.29; SD 126.68) compared to that of the control subjects, (x 201.2; SD 20.335, p-value 0.0002). Refer to figure 3, page 160. In separating the patients into those receiving rHuEPO (MHTepo), (x 424.56; SD 117.21), and those not receiving rHuEPO (MHTnon-epo), (x 300.6; SD 111.03), the transmembrane Ca^{2+} flux upon fMLP stimulation was significantly elevated compared to the control subjects for the MHTepo patients, (p-value < 0.001) but no significant difference was found for the MHTnon-epo group compared to the control subjects. Refer to table 6, page 158. No significant difference was indicated between MHTepo and MHTnon-epo patients. In order to clarify the presentation of the efflux of calcium in the present study, a couple of explanatory points are necessary. The return to intracellular free Ca^{2+} baseline levels after the elevation upon fMLP stimulation is the result of both the sequestration of Ca^{2+} into the calciosome and the pumping of Ca^{2+} to the extracellular medium. (2) Therefore, the rate of the decrease in intracellular free Ca^{2+} to baseline levels is determined by various pumps responsible for the lowering of intracellular free Ca^{2+}. However, the Ca^{2+}-ATPase in the calciosome membrane and the Ca^{2+}-ATPase in the plasma membrane is mostly responsible for the decrease in intracellular free Ca^{2+} in order to terminate the neutrophil's response upon fMLP stimulation, (see chapter 1). In the present study the rate of the decrease in intracellular free Ca^{2+}

was characterised in two ways. Firstly, the rate of the decrease in intracellular free Ca^{2+} was represented as the slope of a line fitted to the real-time calcium measurements, rate y. Secondly, the rate of the decrease of intracellular free Ca^{2+} was represented as the slope of a line fitted to the ln values of the real-time calcium measurements, rate lny. By calculating the ln of the real-time calcium measurements it was aimed to do away with the initial stimulation of the calcium pump by the increase in intracellular free Ca^{2+} upon agonist stimulation. As an increase in intracellular free Ca^{2+} stimulates the Ca^{2+}-ATPase (see chapter 1), the rate lny probably reflects functioning of the Ca^{2+}-ATPase as determined by membrane status. In comparing the values for rate lny between the total group of patients (MHTtot), (x -0.0052; SD 0.001686), to the control subjects, (x -0.0038; SD 0.001488), no significant difference was indicated, (p-value 0.0952). Refer to table 5, page 157. In separating the patients into those receiving rHuEPO (MHTepo), (x -0.00508; SD 0.001536), and those not receiving rHuEPO (MHTnon-epo), (x -0.00541; SD 0.002106) no significant difference was seen between any of the groups. Refer to table 6, page 158. In comparing the values for rate y, a significant increase was seen in the rate of intracellular free Ca^{2+} return to baseline levels for the MHTtot patients, (x -1.0533; SD 0.5566) to that of the control subjects, (x –0.476; SD 0.1811, p-value 0.0017). Refer to table 5, page 157. In separating the patients into those receiving rHuEPO (MHTepo), (x –1.1036; SD 0.4544), and those not receiving rHuEPO (MHTnon-epo), (x –0.963; SD 0.7602), a significant difference was indicated for the MHTepo patients, (p-value 0.0011) but not for the MHTnon-epo patients compared to the control subjects. Refer to table 6, page 158. No significant difference was found between the MHTepo and MHTnon-epo patients. These results show a significant increase in the magnitude of the transmembrane calcium flux upon fMLP stimulation in the group of MHTtot patients compared to the control subjects. These results also show a significant increase in the

rate of intracellular free Ca^{2+} decrease for the MHTtot patients compared to the control subjects. The differences in the MHTtot patient's group relative to the control group is however due to the values obtained for those patients receiving rHuEPO.

The transmission electron microscopy calcium distribution studies were performed in an attempt to identify any possible changes in intracellular calcium storage pools in CRF, i.e., either an increase in calcium content or the redistribution of calcium between the various calcium storage organelles. As mentioned, cellular events that induce distributional changes of the intracellular calcium stores can significantly interfere with different types of Ca^{2+} signals. In the present study, in a group of the MHTtot patients, pronounced calcium-pyroantimonate precipitate formation was seen in the nuclear envelope calcium stores compared to the rest of the calcium stores in the neutrophil, (refer to figure 30, page 204). In this group of patients the vitamin A levels were elevated (12, 15.1, 16.4 μmol/l) compared to the group of patients not displaying the pronounced calcium-pyroantimonate precipitate formation in the nuclear envelope calcium stores (5.8, 6.8, 7.3, 9.5, 10.2 μmol/l), (refer to figure 29, page 203). This may imply modulation of neutrophil intracellular calcium signalling by vitamin A. (38, 39) Additional conclusions from the TEM results would be unfounded without refinement of the technique and the concomitant assessment of the total calcium pool.

The increase in intracellular free Ca^{2+} for the total group of patients seen in this study supports the findings of previous workers. However, this increase in intracellular free calcium can be contributed only to the values obtained in those patients receiving rHuEPO. The increased intracellular free Ca^{2+} in the MHTepo group supports the possibility of an increase in intracellular free Ca^{2+} in the neutrophil caused by

rHuEPO. (28, 40 – 55) Several of these investigators indicated a significant decrease in the magnitude of the transmembrane calcium flux upon agonist stimulation in the neutrophils and other cell types of CRF patients. (40, 42, 46, 49, 52, 56) These findings resulted in the proposition that a reduced intracellular free Ca^{2+} flux upon stimulation might underlie the impaired cellular responses characteristic of these patients. Examples of such abnormal cellular activities ascribed to subnormal transmembrane Ca^{2+} fluxes include:

- Impaired phagocytosis upon ligation of FcgRIII receptors with 3G8 monoclonal antibody in neutrophils is possibly the result of a smaller rise in intracellular free calcium. (40)
- Decreased oxygen consumption during the respiratory burst upon fMLP stimulation is possibly the result of a reduced intracellular free Ca^{2+} response in PMNL of both humans and rats with CRF. (42)
- Reduced intracellular Ca^{2+} response was associated with the decreased aggregation of platelets. (56)
- Glucose-induced calcium signal is reduced in pancreatic islets and this possibly results in impaired insulin secretion. (46, 52)
- Reduced calcium signal in response to anti-CD3 antibody, and these cellular derangements may interfere with the proper response of T cells to mitogens. (49)

In contrast two recent studies found elevations in the calcium signal upon agonist stimulation in cells from CRF patients including:

- In PMNL's, an elevation in the Ca^{2+} signal upon fMLP stimulation. (57)
- An elevation in the Ca^{2+} signal upon ionophore-stimulation in the platelets of chronic renal failure patients. (44)

The results of the present study is in accordance with the results of the latter two studies, i.e., an elevated Ca^{2+} response upon fMLP stimulation. It is therefore suggested that the intracellular calcium response upon fMLP stimulation in the neutrophils of MHT patients is not impaired, and that the subnormal cellular activities seen in MHT patients are not the direct result of an impaired calcium flux upon stimulation. A decrease in the magnitude of the intracellular Ca^{2+} flux upon stimulation is not expected but rather an increase in the Ca^{2+} flux when basal intracellular free Ca^{2+} levels are elevated. Following is a possible explanation for such an increase in the magnitude of the rise in intracellular free Ca^{2+}. A rise in cytosolic free Ca^{2+} is an important intracellular messenger in neutrophils. It is thought that the increase consists of two components, a release of Ca^{2+} from intracellular stores and an influx of Ca^{2+} across the plasma membrane. (11, 57) The initial event resulting in the rise of intracellular free Ca^{2+} comprises the binding of the agonist, in this case fMLP, to a specific receptor. fMLP occupancy of the receptor will result in the activation of a phosphatidylinositol 4,5-bisphosphate specific phospholipase C (PLC). Phosphatidylinositol 4,5-bisphosphate cleavage by PLC will then generate IP_3 and DAG, whereupon IP_3 will bind to IP_3-receptors on the membranes of intracellular calcium stores. The IP_3-receptor molecules contain calcium channels and therefore IP_3 binding to the receptor will result in the releasing of calcium from the calcium-containing organelles. IP_3-receptor molecules display bell-shaped calcium sensitivity. (57) This characteristic of the IP_3-receptor calcium channel results in an increase in the open-probability of the calcium channel in the intracellular Ca^{2+} concentration range from basal levels up to 300 nM, and a decrease in the open-probability of the calcium channel above 300 nM. In addition, the calcium channels situated in the plasma membrane governing the movement of calcium from the extracellular medium into the cytosol also display an increase in the open probability in the Ca^{2+} concentration

range from basal levels up to 300 nM. Therefore, a pathological rise in the resting levels of intracellular Ca^{2+} up to 300 nM (as found in the MHTepo patients) is likely to result in an increase in the magnitude of the transmembrane Ca^{2+} flux upon agonist stimulation. Indeed, a positive correlation between basal intracellular free Ca^{2+} and the magnitude of the Ca^{2+} flux was indicated in the present study, (r-value 0.6304, p-value 0.0157). Refer to table 13, page 199. In addition, an increase in the rate of intracellular free calcium decrease was seen in the MHTtot patients, this may possibly have been the result of the higher intracellular free calcium attained upon fMLP stimulation, since a positive correlation is indicated between the magnitude of the transmembrane calcium flux and rate of intracellular free calcium decrease, (r-value 0.7063, p-value 0.0048). Refer to table 13, page 199. This could be expected since an increase in intracellular free calcium upon agonist stimulation is known to stimulate the activity of the calcium pumps. (57)

In the third part of this study a number of factors which may influence intracellular free Ca^{2+} were investigated. A multitude of factors may theoretically contribute to the elevation in basal intracellular free calcium in the CRF patient, including secondary hyperparathyroidism, increased oxidative stress or reduced anti-oxidant mechanisms, deranged phospholipid and fatty acid metabolism and reduced ATP concentrations. Figure 1 schematically presents the various factors that may influence intracellular free Ca^{2+}. Suffice to say that not all implicated factors can without doubt be assumed to be major effectors and not all of them are specific correlates of the CRF patient on MHT. In the present study a number of factors were measured as a pilot study for finding a relationship with the anticipated changes in intracellular free Ca^{2+}. They include PTH and the oxidative status as indicated by the anti-oxidative vitamins, phospholipid and fatty acid composition of the

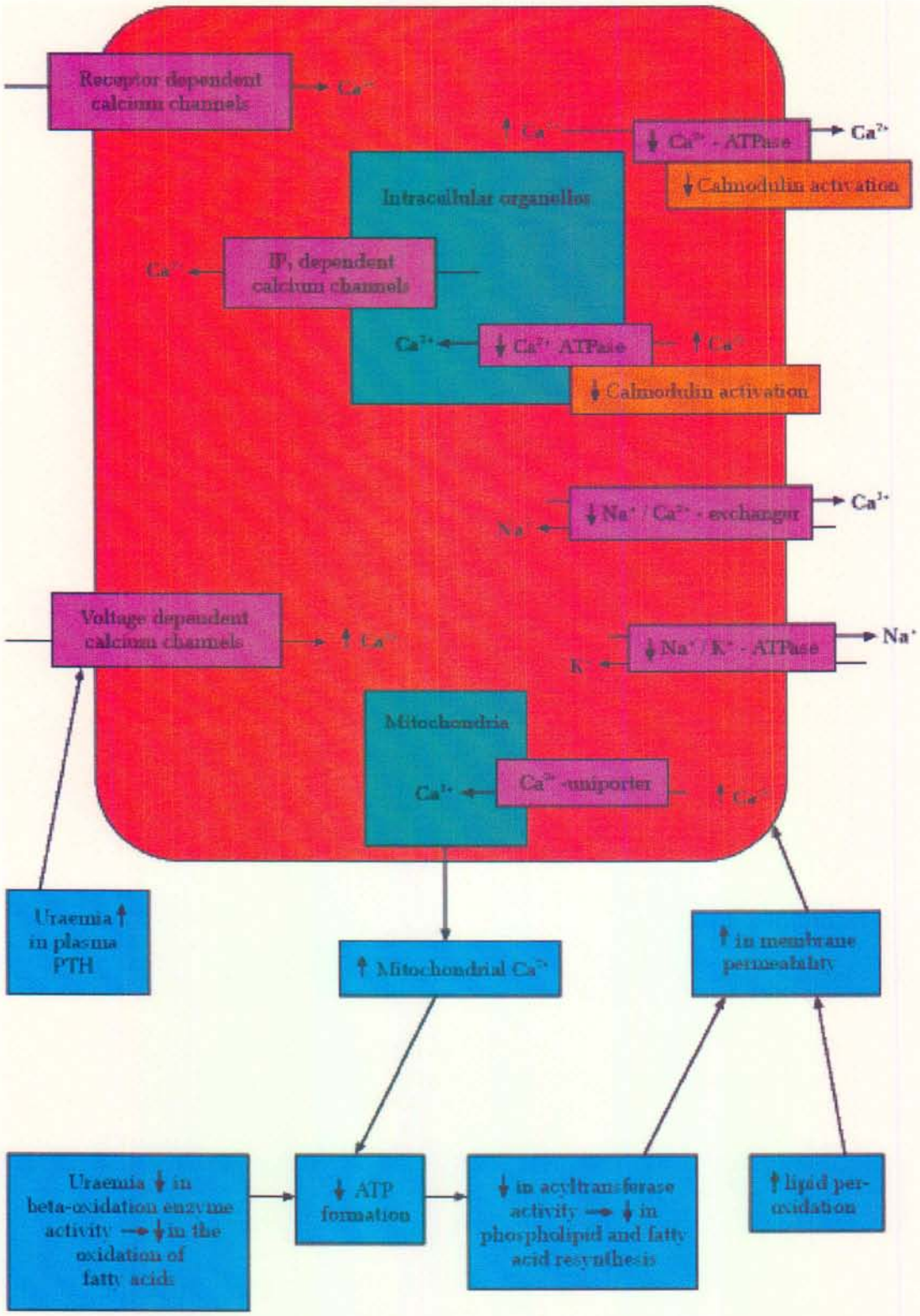

Figure 1. Schematic presentation of possible factors responsible for the perturbation in intracellular free Ca^{2+} in the chronic renal failure patient.

membranes, as well as the free serum calcium. Phospholipid and fatty acid composition of the membranes were determined both for their direct effect on calcium transfer and as indicators of possible oxidative damage.

The PTH levels in the MHTtot patients, (x 591.37; SD 477.16), were significantly elevated (p-value < 0.001) compared to the control subjects, (x 24.98; SD 6.4965). Refer to figure 23, page 195. CRF is associated with an elevation in PTH levels and Massry even hypothesised that PTH might be one of the uraemic toxins. (59) The classical target organs for parathyroid hormone (PTH) are the bone and kidney. However, there are indications that PTH may also affect the function of a number of non-classical organs and tissues besides the bone and kidney, including the brain, heart, smooth muscles, lungs, erythrocytes, lymphocytes, neutrophils, pancreas, adrenal glands, and testes, and there is evidence to believe that intracellular free Ca^{2+} may be increased in these non-classical organs and tissues by PTH. (52, 60) In CRF patients this increase in intracellular free calcium levels is said to initially be caused by an increased influx of calcium secondary to the activation of the L-type calcium channels by PTH, (59) but that this increase in the influx of calcium is not the only contributor to the pathological rise in basal intracellular free Ca^{2+} levels. It is proposed that an increased influx of calcium alone would not result in the pathological rise in basal intracellular free Ca^{2+} levels, since cells are endowed with powerful mechanisms to alleviate the unwanted influx of Ca^{2+}. It is only when a reduced calcium efflux in combination with an increased influx exists that a possible pathological rise in basal intracellular free Ca^{2+} levels are noted. This was indeed reported in CRF patients with secondary hyperparathyroidism. In various published studies reduced Ca^{2+}-ATPase and Na^{+}-K^{+}-ATPase activities were found with elevated PTH levels. It was also hypothesised that the reduced

calcium efflux is an indirect result of the initial rise in intracellular free calcium caused by PTH. This was said to be the result of the sustained PTH-mediated calcium entry causing an inhibition of mitochondrial oxidation and subsequently reduction in ATP production. Presumably, the decrease in ATP contributes to the observed impairment of the activities of the enzymes responsible directly - Ca^{2+}-ATPase or indirectly – Na^{+}-K^{+}-ATPase for calcium extrusion out of cells. (59, 60) In addition, the activity of the Na^{+}/Ca^{2+}-exchanger is reported to be impaired with elevated PTH levels. The exact mechanism responsible for the impairment of the Na^{+}/Ca^{2+}-exchanger has not been elucidated, but it is proposed that a change in the membrane phospholipid composition may be a factor. (59)

Neutrophils have previously been reported to be a target for PTH action in CRF. Various studies have shown an association between an elevation in neutrophil intracellular free calcium with elevations in PTH levels. (31, 40 - 42) In the present study PTH is significantly elevated in the maintenance haemodialysis patients, (p-value < 0.001). Although an increase in intracellular free Ca^{2+} was indicated for the MHTtot patients no correlation was found between PTH levels and intracellular free Ca^{2+}. Resistance to the action of PTH has however been reported in CRF patients due to the down-regulation of the PTH receptor. (61 - 63) In addition, an elevation in the rate of the decrease of intracellular free Ca^{2+} to baseline levels was indicated in the present study in contrast to the decrease in the activities of the various pumps responsible for the decrease in intracellular free Ca^{2+} as reported in various previous studies. This is perhaps more in line with those studies showing down-regulation of the PTH-receptor. It is therefore possible that PTH had no effect on intracellular free Ca^{2+} in the neutrophils of the MHT patients involved in the present study.

As oxidative damage to membrane lipids and membrane proteins may result in an increase in intracellular free Ca^{2+}, the anti-oxidant vitamin status in the MHT patients was determined. None of the patients received vitamin A and E supplements. Figure 2 schematically presents the oxidant-anti-oxidant disequilibrium that could present in renal failure patients receiving MHT. In MHT patients an increased production of oxygen radicals may occur, due to complement-dependent and complement-independent neutrophil activation during haemodialysis. (64) In addition, a decrease may develop in the anti-oxidant mechanisms, including decreased superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity. The decrease in SOD activity is said to result from a decrease in transition metals necessary for enzyme activity, whereas, a decrease in GPX activity may be the result of decreased pentose phosphate shunt function. (65, 66) The two major oxidation-induced effects that could lead to an intracellular free Ca^{2+} increase, are lipid peroxidation and protein oxidation. Poly-unsaturated fatty acids (PUFA's) represent the substrate damaged by oxidation such that excessive lipid peroxidation results in an increased saturated fatty acid (SFA) to poly-unsaturated fatty acid (PUFA) ratio and possibly a reduction in the activity of the enzymes responsible for lowering intracellular free Ca^{2+}. (67) The anti-oxidant enzymes are not the only mechanisms available to the body to combat an increase in oxidative stress. Vitamin A, E and C offer additional protection from oxidative damage to cell constituents. (68) It is said that vitamin E is the most important anti-oxidising system and in CRF patients a significant reduction in membrane damage is indicated with vitamin E administration. (69)

In the present study vitamin A, E and C levels were determined. The vitamin C levels for the MHTtot patients, (x 64.386; SD 48.675), were not different from the vitamin C levels of the control subjects, (x 55.35; SD

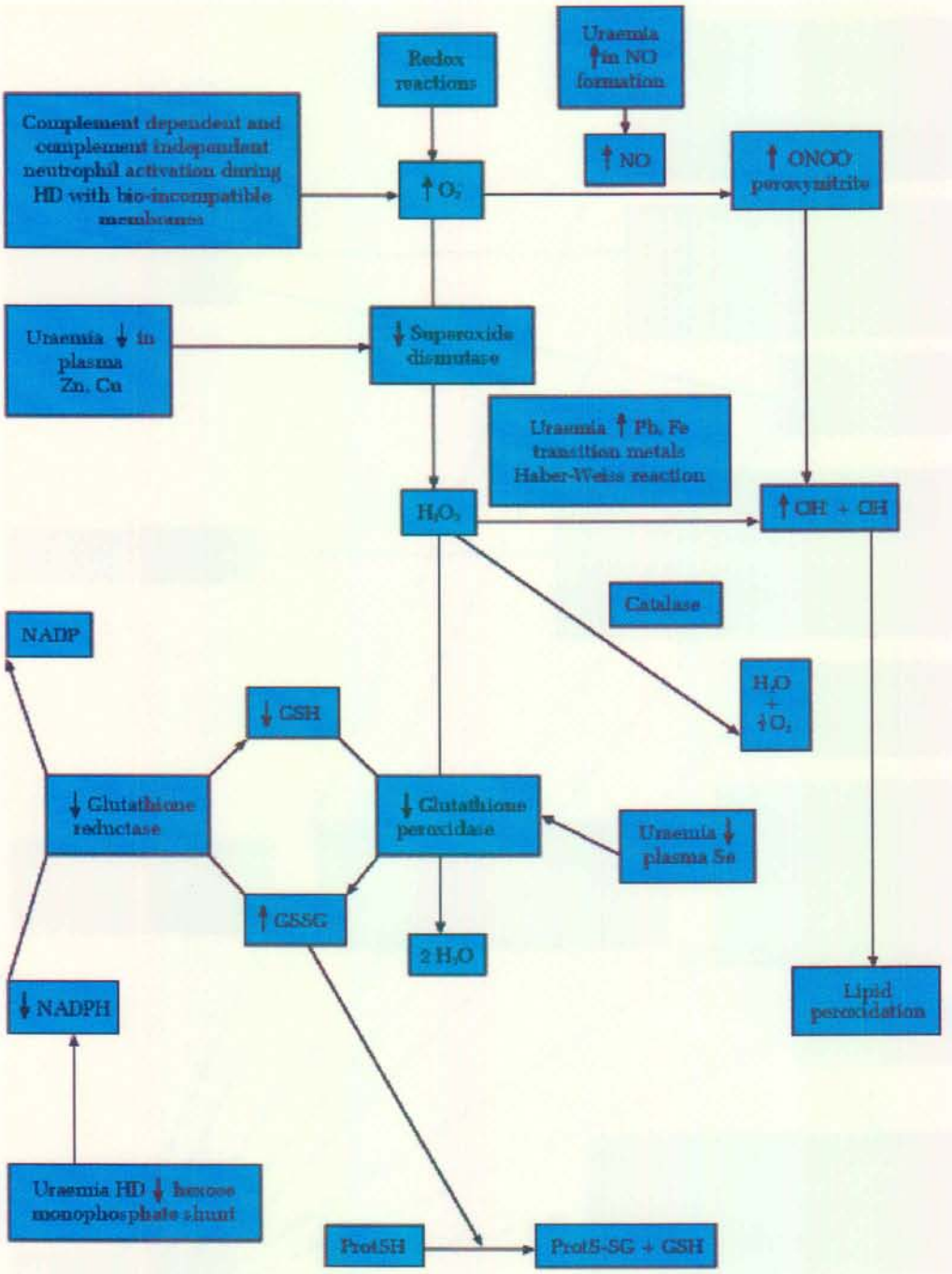

Figure 2. Schematic presentation of the possible oxidant-anti-oxidant disequilibrium in chronic renal failure patients receiving maintenance haemodialysis treatment. An increased production of oxygen radicals may occur, due to complement-dependent and complement-independent neutrophil activation during haemodialysis. In addition, a decrease may develop in the anti-oxidant mechanisms, including decreased superoxide dismutase and glutathione peroxidase activity. This resulting increase in oxidative stress may cause lipid peroxidation and protein oxidation.

19.344, p-value 1). Refer to figure 24, page 196. Nevertheless, positive correlations were found for vitamin C and 2 poly-unsaturated fatty acids (PUFA's). Vitamin C and n-6 20:4 (r-value 0.5592, p-value 0.0376), Vitamin C and n-3 20:5 (r-value 0.516, p-value 0.0589). Refer to table 13, page 199. There were also negative correlations indicated for vitamin C and two saturated fatty acids (SFA's). Vitamin C and 22:0 (r-value –0.6871, p-value 0.0066), Vitamin C and 24:0 (r-value –0.6433, p-value 0.0131). These correlations may be indicative of possible protection against unsaturated fatty acid oxidation and at the same time causing the expected decrease in membrane SFA content. However, this suggested possibility can at this stage only be seen as speculation.

No significant differences for vitamin E levels were found between any of the groups. In renal units where factors like impurities in the dialysis water supply lead to oxidative damage to cells, vitamin E levels were generally reported to be drastically decreased. (70) The vitamin E levels did not give any indication of oxidative stress.

The vitamin A levels of the MHTtot patients, (x 11.8; SD 4.7099), were significantly higher than the vitamin A levels of the control subjects, (x 3.95; SD 0.9229, p-value < 0.001). Refer to figure 25, page 196. The patients included in the present study did not receive vitamin A supplements. These results are in accordance with a previous study. (71) It is suggested that vitamin A might present an uraemic toxin capable of producing symptoms similar to vitamin A intoxication. It has further been shown that the catabolism of retinol binding protein is reduced in chronic renal failure and that vitamin A supplementation may aggravate the intoxication. Nevertheless, these high levels of vitamin A possibly offer some protection against PUFA oxidation. The following positive correlations were seen: vitamin A and total PUFA's, (r-value 0.5965, p-value 0.0243), vitamin A and n-6 fatty acids but not n-3

fatty acids, (r-value 0.6086, p-value 0.0209), vitamin A and PUFA's/SFA's, (r-value 0.584, p-value 0.0339), vitamin A and n-6 18:2, (r-value 0.5242, p-value 0.0543). The following negative correlation was seen; vitamin A and 16:0 (r-value -0.5643, p-value 0.0355). Refer to table 13, page 199. These correlations may indicate a possible protection against poly-unsaturated fatty acid oxidation and at the same time the concomitant decrease in membrane SFA content.

In the present study the red blood cell membrane phospholipid and fatty acid composition were determined in order to investigate two aspects. Firstly, to determine if a change in membrane phospholipid and fatty acid composition exists in this group of MHT patients and if so whether it correlates with alterations in intracellular free calcium. A change in the membrane phospholipid and fatty acid composition may, as previously mentioned, result in perturbation of membrane integrity and an alteration in calcium influx. Furthermore, a change in the membrane phospholipid and fatty acid composition may result in decreased activity of the calcium pumps responsible for calcium efflux and changes in intracellular free calcium. This because the calcium pumps responsible for the efflux of calcium are situated in the membranes and an optimal lipid environment assures appropriate calcium pump functioning. (72) Refer to figure 3. The second reason for the investigation of membrane phospholipid and fatty acid composition was for the investigation of the possible peroxidation of unsaturated fatty acids as a result of an increased oxidative stress.

With regard to the first aspect, no correlations were seen between an increase in basal intracellular free calcium and any of the membrane phospholipids or fatty acids. Although a positive correlation was approached between the magnitude of the rise in intracellular free calcium upon fMLP stimulation and the n-6 20:3 content, (r-value

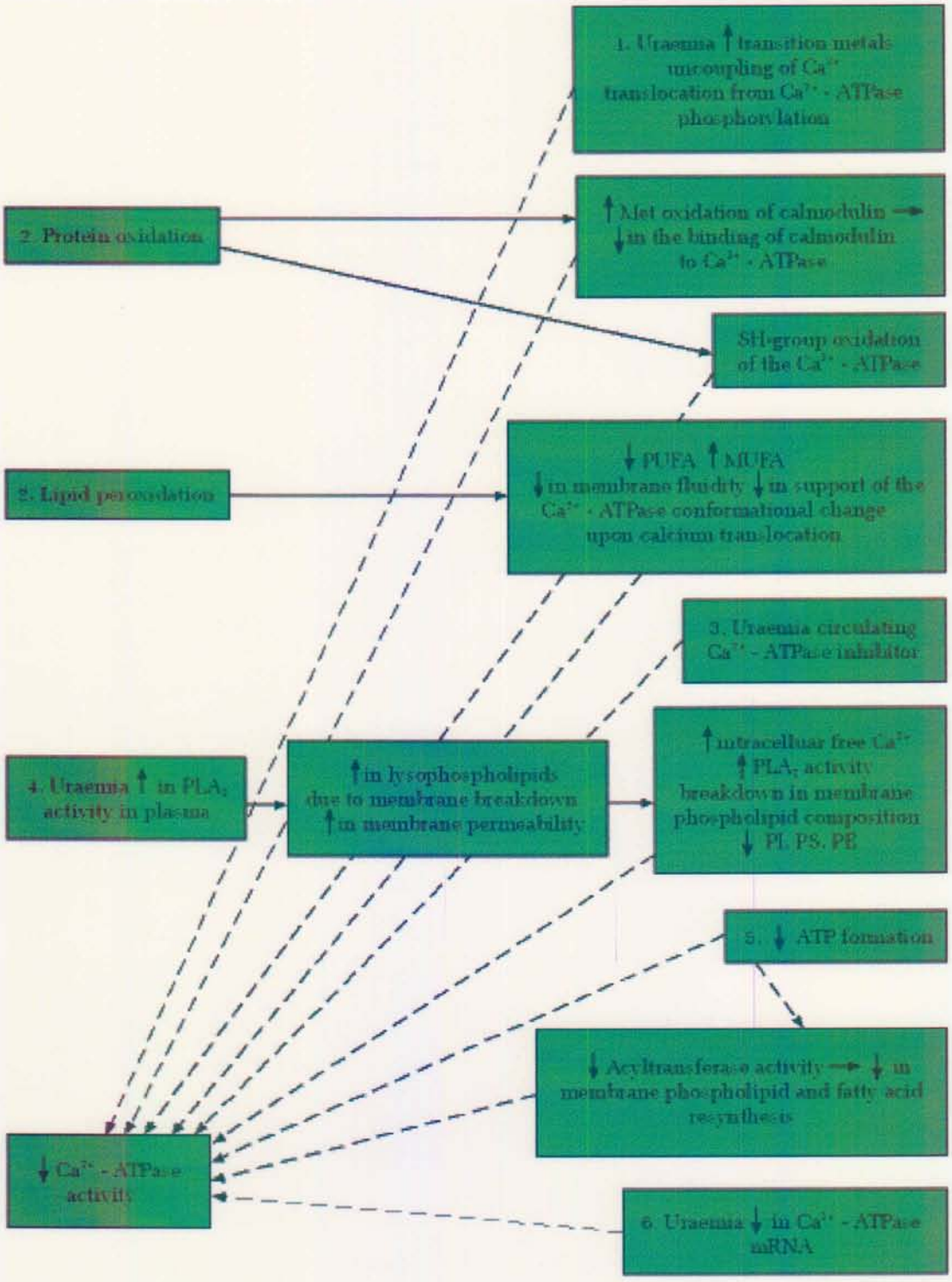

Figure 3. Schematic presentation of factors associated with chronic renal failure possibly causing a decrease in Ca^{2+} - ATPase activity.

0.5265, p-value 0.0531). Refer to table 13, page 199. The magnitude of the transmembrane calcium flux in MHTtot patients was significantly higher than that for the control subjects, however the n-6 20:3 content was significantly lower. The content of n-6 20:3 of the MHTtot patients, (x 1.3221; SD 0.3736) compared to the control subjects, (x 1.624; SD 0.4414, p-value 0.0465). Refer to figure 19, page 189. Therefore, the n-6 20:3 content could not have been be a major contributor to the increased transmembrane calcium flux, but could perhaps have modulated the activity of the calcium channel upon fMLP stimulation. The effects of an agonist or an antagonist on the activity of the calcium channel is influenced by specific fatty acids. (33) As for the influence of the phospholipid and fatty acid composition of the membranes on the activity of the calcium pumps responsible for the lowering of intracellular free calcium, correlations were indicated for the following, decrease of intracellular free calcium – rate y and 16:1, (r-value 0.6323, p-value 0.0152), and decrease of intracellular free calcium – rate lny and 16:1, (r-value 0.6189, p-value 0.0183). It is of interest that such a strong correlation existed between these factors. The results of this present study are in line with those of previous workers which showed that not only does calcium stimulate the activity of the calcium pumps but that specific membrane fatty acids might modulate calcium pump activity. (72)

The second reason for the measurement of membrane phospholipid and fatty acid composition was for the investigation of the possible peroxidation of unsaturated fatty acids as it has previously been reported to interfere with intracellular calcium status. Refer to chapter 1. In the previous section the findings on the anti-oxidant vitamin levels were discussed. No decrease in any of the vitamin levels in the MHT patients were indicated. If judged only by the levels of the anti-oxidant vitamins no undue disequilibrium in the oxidation-reduction status exists in the

investigated group of patients. The correlations between vitamin A, vitamin C and various fatty aids were an interesting observation. It is possible that these correlations may reflect a degree of anti-oxidant protection against unsaturated fatty acid oxidation and the concomitant decrease in membrane SFA content. Despite this implicated protection against oxidation by the high anti-oxidant vitamin levels, the content of fatty acids of this study point towards a degree of lipid peroxidation. A significant reduction in the contents of the following fatty acids were found; PUFA's, PUFA's/SFA's, n-6 fatty acids and n-3 20:5, and a non-significant reduction in the contents of the following fatty acids were found; n-6 18:2 and n-6 20:4. Refer to table 8, page 169.

It was only with the final analysis of the phospholipid and fatty acid results that a third aspect of the phospholipid and fatty acid composition of the red blood cell membrane was noticed, i.e., the fact that the composition reflects that of a chronic inflammatory condition. Indications from both the determined fatty acid and phospholipid contents show that a chronic inflammatory condition might have been present in the MHT patients. One of the major functions of the PUFA's is as precursors for the families of eicosanoids. The 20-carbon series PUFA's give rise to eicosanoids; prostaglandins, thromboxanes and leukotrienes, which function as hormones in many sites in the body. Each family of the PUFA's is metabolised to a separate family of eicosanoids with different hormonal effects. (73) An activation of the eicosanoid system during haemodialysis has been proposed by previously published results. (74, 75) This activation of the eicosanoid system is said to be the result of the blood-membrane interaction during extracorporeal circulation, and include the activation of neutrophils, monocytes and platelets. (76, 77) In previously published studies an elevation in different eicosanoids including prostaglandins, leukotrienes and thromboxanes or a decrease in the fatty acid precursors were

indicated. The eicosanoids for which an increase was previously reported in MHT patients include 5-lipoxygenase products leukotriene C_4 and leukotriene B_4, and cyclo-oxygenase products thromboxane B_2 and prostaglandin E_2. (76, 78) Fatty acid profiles hinting at the activation of the eicosanoid system during haemodialysis have been described in various published studies. These fatty acid disturbances in CRF patients receiving MHT involve more or less the same fatty acids in different studies. Nevertheless, contradictions exist. It has even been reported that as a result of the constant eicosanoid production in HD patients, a fatty acid profile characteristic of essential fatty acid deficiency may develop in these patients. (36, 73, 74, 79 - 81) The occurrence of essential fatty acid deficiency in CRF (MHT) patients is highly possible taking into consideration the clinical symptoms characteristic of these patients. Clinical symptoms exhibited by MHT patients such as dry and scaly skin, abnormal perspiration, susceptibility to infection, delayed wound healing, increased hemolysis and hormonal aberrations are characteristic of essential fatty acid deficiency. (74, 75, 79) Furthermore, it is also indicated that the production of the various eicosanoids is inadequate when the need arise due to the decreased precursor content. Such as the decreased production of thromboxane B2 in response to thrombin induced platelet aggregation during spontaneous clotting and defective immune functions because of the deficiency of eicosanoids required for immune functions. (78) A decrease in linoleic acid, linolenic acid, arachidonic acid, and eicosapentonoic acid, together with an increase in oleic acid and eicosatrienoic acid are said to be characteristic of essential fatty acid deficiency indicated in MHT patients. All three of these metabolic products are precursors for eicosanoid formation. Dihomo-gamma linolenic acid for the "1-series" eicosanoids, arachidonic acid for the "2-series" eicosanoids and eicosapentanoic acid for the "3-series" eicosanoids. In the present study a decrease in the content of the

essential fatty acid linoleic acid and three of the essential fatty acid metabolic products; dihomo-gamma linolenic acid, arachidonic acid and eicosapentanoic acid were shown. The n-6 18:2 (linoleic acid - LA) content was however non-significantly reduced in the MHTtot patients, (x 10.393; SD 2.2057), compared to the control subjects, (x 12.111; SD 1.6661, p-value 0.0841). Refer to figure 18, page 189. The n-6 20:3 (dihomo-gamma linolenic acid – DGLA) content was significantly reduced in the MHTtot patients, (x 1.3221; SD 0.3736), compared to the control subjects, (x 1.624; SD 0.4414, p-value 0.0465). Refer to figure 19, page 189. The n-6 20:4 (arachidonic acid – AA) content was again non-significantly reduced in the MHTtot patients, (x 14.808; SD 1.404), compared to the control subjects, (x 15.923; SD 1.4697, p-value 0.1073). Refer to figure 20, page 190. For only one of the n-3 series fatty acids a significantly decreased content in the MHTtot patients was indicated. The n-3 20:5 (eicosapentanoic acid – EPA) content was significantly decreased in the MHTtot patients, (x 0.2021; SD 0.0707), compared to the control subjects (x 0.282; SD 0.883, p-value 0.0326). Refer to figure 21, page 190. Furthermore, in the present study 18:1 (oleic acid) content was significantly elevated for the MHTtot patients, (x 13.341; SD 0.8647), compared to the control subjects, (x 12.228; SD 0.9999, p-value 0092). Refer to figure 13, page 186. An elevation in the eicosatrienoic acid is also characteristic of essential fatty acid deficiency, but the content of eicosatrienoic acid was not determined in the present study. Eicosatrienoic acid is a n-9 fatty acid, containing a double bond on the n-9 position. In the present study an elevation in the content of two other n-9 fatty acids were found. The n-9 20:1 content was significantly elevated in the MHTtot patients, (x 0.3386; SD 0.1316), compared to the control subjects, (x 0.222; SD 0.0365, p-value 0.0034). Refer to figure 14, page 186. The content of n-9 22:1 was significantly elevated in the MHTtot patients, (x 0.064; SD 0.0334, p-value 0.0139) compared to the control subjects, (x 0.064; SD 0.0334, p-value 0.0139). Refer to figure 15,

page 187. The non-significance of the decrease in linoleic acid and arachidonic acid contents might very well be the result of the small subjects groups, since all other results point towards essential fatty acid deficiency possibly caused by the stimulated eicosanoid production.

As for the change in the fatty acid content in the present study a change in the phospholipid content also possibly indicate an increased eicosanoid production. The fatty acid precursors of the "1-series", "2-series", and "3-series" eicosanoids are metabolic products, due to carbon chain desaturation and elongation of two essential fatty acids, linoleic acid and alpha-linolenic acid. (73) The rate-limiting step in eicosanoid formation is the liberation of the fatty acid precursors from membrane phopholipids by phospholipase A_2. (82) Phospholipase A_2 (PLA_2) is usually activated only in response to particular stimuli, in the present situation by blood membrane interactions. (83) Indeed, an increased activity of PLA_2 has been observed before in the plasma of patients with uraemia. (84) Various studies have previously shown changes in the membrane phospholipid content as for the fatty acid content for these patients. However, substantial controversies exist. (85 - 87) In the present study an elevation in the sphingomyelin (SM) content for the MHTtot patients was found, although not significant, (x 459.76; SD 92.907), compared to the control subjects, (x 394.38; SD 60.91, p-value 0.0841). Refer to figure 8, page 183. Also a decrease in the phosphatidylcholine (PC) content was indicated for the MHTtot patients, although again not significant, (x 32.143; SD 4.504), compared to the control subjects, (x 34.7; SD 2.4967; p-value 0.0841). Refer to figure 10, page 184. There are indications of an elevated choline uptake in CRF patients. (88, 89) This increased capacity for choline uptake possibly results in the increased PC and SM synthesis. (88) Therefore, the increased SM content is possibly caused by an altered SM synthesis. As for the unexpected decrease in the PC content if an increased choline

uptake exists in these patients, an increase in PLA_2 (phosphatidylcholine 2-acylhydrolase) activity might result in the breakdown of PC. (90) This suggestion of a possible increase in eicosanoid production by previous workers and hinted at by the present results requires further studies to unequivocally prove this hypothesis in these patients.

Conclusions

The majority of studies describe above normal intracellular free Ca^{2+} levels and below normal transmembrane Ca^{2+} fluxes in CRF/MHT patients. The secondary hyperparathyroidism of CRF is suggested to be the major cause of this free Ca^{2+} disturbance, but oxidative damage could also be implicated. In the present study an increase in intracellular free Ca^{2+} and an increase in transmembrane Ca^{2+} fluxes were shown in the neutrophils of these patients. The increase in intracellular free Ca^{2+} was due solely to the effect of erythropoietin – a factor previously shown to increase intracellular free Ca^{2+}. Patients not receiving erythropoietin had normal intracellular free Ca^{2+} levels. The increase in intracellular free Ca^{2+} was not prevented by norvasc administration possibly due to a change in membrane fatty acid composition. No overt oxidative damage was present as indicated by the anti-oxidant vitamin levels, but a decrease in the content of specific membrane fatty acids occurred. The specific fatty acids for which the decrease in membrane content was shown involved the fatty acid precursors necessary for eicosanoid synthesis, possibly indicating a mild chronic inflammatory condition.

Suggestions for future research

In order to indicate rHuEPO unequivocally as the factor for causing the increase in intracellular free Ca^{2+} in the neutrophil of CRF/MHT patients and to investigate the action of norvasc, intracellular free Ca^{2+} should be determined before and after rHuEPO administration and before and after calcium channel blocker administration. Although the fatty acid precursors of the eicosanoids are shown to be decreased in the membranes of these patients and the fact that various published studies indicate an increase in eicosanoid synthesis in these patients, the plasma levels of the eicosanoids will be of interest in the patient group involved in the present study. No correlation is presently shown between the elevated PTH levels and the increase in intracellular free Ca^{2+}, but PTH receptor down-regulation has been shown to occur in previously published studies and therefore PTH might have a confounding effect on intracellular free Ca^{2+} earlier in the course of kidney failure. It is suggested that intracellular free Ca^{2+} and perhaps PTH-receptor kinetics should be determined from the initial stages of kidney failure as secondary hyperparathyroidism develops. The unfortunate ethnic bias in the patients leads to the situation where four of the nine patients on rHuEPO have hypertensive nephropathy as cause of the renal failure. Whether the reported link between the essential hypertension of blacks (see footnote) has any bearing on the results can not be ascertained from the results of the present study and should be further investigated.

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