INTRACELLULAR CALCIUM AND TRANSMEMBRANE CALCIUM FLUXES IN CHRONIC RENAL FAILURE PATIENTS

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Dedicated to my parents

ABSTRACT

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Intracellular calcium is a major determinant of a wide variety of cell functions and thus of organ function. In order to get a clear picture of the intracellular calcium status it is preferable to assess the content of the various intracellular calcium pools as well as the characteristics of the transmembrane calcium movements, i.e., the magnitude of the transmembrane Ca^{2+} flux upon stimulation and the rate of the subsequent return to baseline levels. The first aim of this study was to establish and evaluate the methods in the laboratory. The methods investigated include atomic absorption spectrometry, graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry for the determination of the total cell calcium content, fluorescence spectrophotometry for the determinations of intracellular free Ca^{2+} and transmembrane Ca^{2+} movements and transmission electron microscopy for the localisation of intracellular calcium. The methods eventually identified as feasible included fluorescence spectrophotometry for the determination of intracellular free Ca^{2+} and transmembrane Ca^{2+} movements and transmission electron microscopy for the localisation of intracellular calcium. The newly developed fluorescent calcium indicator, fura-PE3, was presently shown to be the most reliable fluorescent indicator for the intracellular free Ca^{2+} determinations. The best method for the calcium localisation by transmission electron microscopy was an adaptation of the antimonate precipitation technique. The following objectives were set in order to contribute to the knowledge in chronic renal failure; examination of the intracellular free Ca^{2+} content in the neutrophils of end stage renal failure patients on maintenance haemodialysis treatment, as the result of renal failure, dialysis treatment and medication combined; examination of the characteristics of the transmembrane Ca^{2+} movements; investigation of the intracellular calcium distribution in the neutrophils; exploration of a possible link between the alterations in intracellular calcium status and factors known to influence the calcium status, including the lipid composition of the membrane, the oxidative status as reflected by anti-oxidant vitamin levels, as well as the levels of parathyroid hormone, and ionised serum calcium.

This study involved 14 chronic renal failure patients on maintenance haemodialysis. An increase in intracellular free Ca^{2+}, the magnitude of the transmembrane Ca^{2+} flux upon fMLP stimulation and an increase in the rate of the subsequent decrease in intracellular free calcium were found. In separating the patients into those receiving rHuEPO and those not receiving rHuEPO, it was seen that the significance in the increase in intracellular free Ca^{2+} could be ascribed to the values obtained in those patients receiving rHuEPO – despite the fact that they were the only patients receiving calcium channel blockers. No overt indications of oxidative stress could be detected by anti-oxidant vitamin levels. Nevertheless, a decrease in the content of specific membrane fatty acids occurred, supporting the previous suggestions of the presence of a mild chronic inflammatory condition in the chronic renal failure patient on maintenance haemodialysis treatment. These results suggest that factors other than those associated with uraemia, such as rHuEPO administration, might result in an increase in intracellular free Ca^{2+} in cells of CRF/MHT patients. The magnitude of the rHuEPO–induced increase in intracellular free Ca^{2+} and the effects of the various calcium channel blockers need urgent further investigation as ineffective counteraction of the rHuEPO effect, as indicated by the relative ineffectivity of Norvasc, may have serious side-effects.

Keywords: Intracellular calcium, fluorescent calcium indicator, transmission electron microscopy, haemodialysis patients, recombinant human erythropoietin

ABSTRAK

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Graad:	**MSc Fisiologie**

Intrasellulêre kalsium speel 'n hoofrol in die regulering van verskeie selfunksies en dus van orgaanfunksie. Om 'n duidelike beeld te bekom van die intrasellulêre kalsium status is dit verkieslik nodig om die verskeie intrasellulêre kalsiumstore te ondersoek asook die eienskappe van die beweging van kalsium oor die membraan, insluitend die grootte van die kalsium fluks met stimulering van die sel en die tempo van die daaropvolgende verlaging na basaal vlakke. Die eerste doel van hierdie studie was die daarstelling en evaluering van die metodes in die laboratorium. Die volgende metodes was ondersoek, atoom absorpsie spektrometrie, grafiet-oond atoom absorbsie spektrometrie, induktief-gekoppelde plasma massa spektrometrie vir die bepaling van die totale kalsium inhoud in die sel, fluoressente spektrofotometrie vir die bepaling van intrasellulêre vry kalsium en kalsium flukse en transmissie elektron mikroskopie vir die lokalisering van intrasellulêre kalsium. Die metodes wat as voldoende geïdentifiseer was het die volgende ingesluit fluoressente spetrofotometrie vir die bepaling van intrasellulêre vry kalsium en kalsium flukse en transmissie elektron mikroskopie vir die lokalisering van intrasellulêre kalsium. Die onlangs ontwikkelde fluoressente kalsium indikator fura-PE3 was as die mees betroubare fluoressente indikator vir kalsium aangewys. Die mees betroubare metode vir kalsium lokalisering was 'n adaptasie van die antimoon-presipiterings tegniek. Die volgende was as mikpunte gestel vir die moontlike bydrae van nuwe inligting t.o.v. chroniese nierversaking; die ondersoek van die intrasellulêre vry Ca^{2+} inhoud in die neutrofiel van eindstadium nierversakings pasiënte wat hemodialise behandeling ontvang, soos bepaal deur die gesamentlike invloed van nierversaking, dialise en medikasie; die ondersoek van die eienskappe van die kalsium flukse; die ondersoek van die intrasellulêre kalsium distribusie in die neutrofiel; ondersoek na die moontlike verband tussen die verandering in die intrasellulêre kalsiumstatus en faktore wat die kalsiumstatus kan beïnvloed insluitend die volgende, die lipied komposisie van die membrane, die oksidatiewe skade soos gereflekteer deur die anti-oksidatiewe vitamien vlakke, asook paratiroïedhormoonvlakke en geïoniseerde serum kalsium.

Veertien chroniese nierversakingspasiënte wat hemodialise behandeling ontvang was ingesluit in die studie. Die volgende veranderings is aangetoon, 'n verhoging in die intrasellulêre vry kalsiumvlakke, 'n verhoging in die grootte van die intrasellulêre kalsium fluks met fMLP stimulering en 'n verhoogde tempo van die daaropvolgende verlaging in intrasellulêre vry kalsium. Met die verdeling van die pasiënte op grond van eritropoïetien behandeling of nie kan die waargenome verhoging in intrasellulêre vry kalsium toegeskryf word aan die pasiënte wat eritropoïetien ontvang, t.s.v. die feit dat hierdie pasiënte kalsium kanaal blokkers ontvang. Die vitamienvlakke dui nie op 'n verhoging in oksidatiewe stres nie, alhoewel 'n verlaging in spesifieke vetsure in die membrane aangetoon word. Hierdie verlaging in spesifieke membraan vetsure ondersteun vorige aanduidings van 'n chroniese lae-graadse inflammatoriese toestand teenwoordig in chroniese nierversakings pasiënte wat hemodialise behandeling ontvang. Die resultate impliseer dat faktore anders as die wat met uremie geassosieër word, soos rekombinante eritropoïetien toediening, moontlik 'n verhoging in intrasellulêre vry kalsium in selle van chroniese nierversakings pasiënte wat hemodialise behandeling ontvang mag veroorsaak. Die omvang van die rekombinante eritropoïetien geïnduseerde toename in intrasellulêre vry kalsium en die effekte van verskeie kalsium kanaal blokkers benodig dringende verdere ondersoek omrede die oneffektiewe blokkering van die rekombinante eritropoïetien effekte, soos tans aangedui vir Norvasc, mag lei tot ernstige newe-effekte.

Sleutelterme: Intrasellulêre kalsium, fluoressente kalsium indikator, transmissie elektron mikroskopie, hemodialise pasiënte, menslike rekombinante eritropoïetien

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Chapter 1

Theoretical background and aim of the study

The etiology and pathogenesis of renal diseases vary widely yet as chronic renal failure progresses, the manifestations in different patients are remarkably similar. These similarities make it possible to formulate common principles of pathophysiology that apply to the greater majority of patients, irrespective of the underlying pathogenic (infections, immunological, vascular or other) mechanism responsible for renal function destruction. (1) Intracellular calcium dyshomeostasis would appear to be one such common principle of the pathophysiology of chronic renal failure. This disturbance in intracellular calcium homeostasis involves many different cell types in the chronic renal failure patient. It is therefore possible that the abnormal intracellular calcium homeostasis, indicated in many different cell types in the chronic renal failure patient, may involve the body as a whole. Intracellular calcium homeostatic mechanisms are complex, and a large number of pathological factors are known to cause intracellular calcium dyshomeostasis. Many of these factors generally known to alter intracellular calcium status are indeed found in the chronic renal failure patient.

The present study comprises the investigation of intracellular calcium homeostasis and possible factors influencing intracellular calcium homeostasis in the chronic renal failure patient on maintenance haemodialysis treatment (MHT). The first introductory chapter deals with the theoretical background to the study and includes discussions on intracellular calcium signal transduction, intracellular calcium dyshomeostasis, possible manifestations of intracellular calcium

dyshomeostasis, status of intracellular calcium homeostasis in the chronic renal failure patient on maintenance haemodialysis treatment, and possible factors contributing to intracellular calcium dyshomeostasis in these patients. The aim of this study is presented in conclusion.

1)   Intracellular calcium signal transduction

Ionised calcium (Ca^{2+}) is the most common signal transduction factor in cells ranging from bacteria to specialised neurons. (2) Unlike many of the other signal transduction elements, Ca^{2+} is required throughout the life of a typical cell since Ca^{2+} regulates many diverse cellular processes ranging from gametogenesis, fertilisation, cell proliferation, and specialised control functions including muscle contraction, exocytosis, energy metabolism, chemotaxis, and synaptic plasticity during memory and learning. (3) The vast quantity of the Ca^{2+} sensitive effectors responsible for the regulation of these processes are located in the cytosol. (4) It is therefore the free ionised Ca^{2+} fraction in the cytosol that is the most critical with regard to the regulation of these intracellular events. Unlike any other signal transduction element, Ca^{2+} is not synthesised or subsequently degraded during the signal transduction process. In contrast Ca^{2+} is moved and/or released and subsequently sequestered for every signal transduction cycle. In order for Ca^{2+} to be available for signal transduction functions, the cell is endowed with at least two mechanisms to increase Ca^{2+} in the cytosol, firstly by releasing Ca^{2+} from the intracellular storage sites and secondly by moving Ca^{2+} from the extracellular medium into the cytosolic space where the effectors are functionally distributed. (5) The intracellular storage sites include Ca^{2+} bound to the plasma membrane (anionic sites consisting of either phospholipids or membrane proteins), Ca^{2+} sequestered within intracellular organelles such as the endoplasmic reticulum, and Ca^{2+} both free and bound within the cytosol. (6) The various mechanisms capable of generating a rise in cytosolic free Ca^{2+} are

all passive processes since the cytosolic free Ca^{2+} is lower than the Ca^{2+} concentration in any of the calcium pools surrounding the cytosolic space. It is therefore not necessary to expend any energy to move calcium down its concentration gradient into the cytosol upon agonist activation. (3, 7) In contrast with these passive processes capable of generating a rise in the cytosolic free Ca^{2+}, the processes necessary to reduce the surplus Ca^{2+} at the end of the signal transduction cycle require the expenditure of energy. These energy expensive processes responsible for rapidly decreasing cytosolic free Ca^{2+} include the Ca^{2+}-ATPase in the plasma membrane and the Ca^{2+}-ATPase in the endo/sarcoplasmic reticulum or calciosome. The activation of these calcium pumps result in the transfer of Ca^{2+} from the cytosol to the extracellular medium and the sequestration of Ca^{2+} into the intracellular calcium storage organelles. The subsequent decrease in cytosolic free Ca^{2+} results in termination of the calcium response. (2, 8)

2) Intracellular calcium dyshomeostasis

It is imperative that the cytosolic free Ca^{2+} levels be maintained within normal physiological limits, since unlike any other signal transduction element, calcium dyshomeostasis is an intracellular trigger for cellular dysfunction and damage. (9) The derangements in intracellular calcium homeostasis, resulting in cellular dysfunction and damage, include the intracellular redistribution of calcium pools during the important early stages of cellular damage, followed by the influx of Ca^{2+} from extracellular to intracellular spaces. (6) Many pathogenic factors, resulting in cellular dysfunction and damage, affect cellular calcium regulation because their initial effect impair one or more of the following; energy metabolism, plasma membrane integrity, calcium translocation systems or calcium signalling. (10) The ensuing intracellular calcium dyshomeostasis aggravates the initial impairment of various cellular functions, due to the inappropriate activation of

various calcium sensitive enzymes. These calcium sensitive enzymes include various enzymes regulating the intracellular calcium signalling process and a whole array of degradative enzymes: such as phospholipases, proteases and endonucleases. Intracellular targets for these degradative enzymes include the plasma membrane, the mitochondria, the endoplasmic reticulum and the cytoskeleton. The calcium activated degradative processes result in plasma membrane bleb formation, nuclear membrane bleb formation, deterioration of mitochondrial structure and function, as well as chromatin condensation. (11) Since an inappropriate elevation in intracellular free calcium results in the activation of so many degradative processes, the cell is furnished with several calcium translocation systems to carefully regulate intracellular calcium activity. The function of the Ca^{2+}-ATPase pump, as explained above, is to rapidly obviate the surplus calcium at the end of a transduction cycle. (8) In addition, these calcium pumps also play an important role in decreasing intracellular free Ca^{2+} under conditions of relative intracellular Ca^{2+} overload. These pumps are well suited for their roles since they display dynamic, low capacity enzyme kinetics. A rise in cytosolic free calcium results in immediate activation of these pumps and the rapid extrusion out of the cell, and sequestration into intracellular organelles of calcium. In the face of a prolonged elevation in intracellular free calcium, a second high capacity, slow to become activated, calcium extrusion pump plays a more important role namely the Na^+/Ca^{2+}-exchanger. This pump extrudes Ca^{2+} ions to the outside of the cell in exchange for Na^+ ions. The sodium concentration gradient – high sodium concentration on the outside of the cell and low sodium concentration on the inside – provides the energy needed for Na^+/Ca^{2+}-exchanger activities. This sodium gradient is maintained by the Na^+/K^+-ATPase. (7) During prolonged intracellular free calcium elevation the roles of the Na^+/Ca^{2+}-exchanger and in combination the buffering of calcium by the mitochondria become more important. (11)

In fact, the mitochondrium becomes the single most important membrane system in the cell to act as a long-term calcium buffer and to handle large amounts of calcium whenever the need arises. (11) All the pumps mentioned play a significant role in maintaining calcium homeostasis. Plasma membrane integrity, on the other hand, fulfills a dual function in the maintenance of intracellular calcium homeostasis. An intact plasma membrane, impermeable to calcium ions forms a barrier to unwanted calcium inflow into the cell. Plasma membrane phospholipid and fatty acid composition are furthermore important in supporting the structure and function of the calcium homeostasis pumps. (12) Impairment of any of the mechanisms mentioned may lead to intracellular calcium dyshomeostasis.

3)   Manifestations of intracellular calcium dyshomeostasis

A rise in intracellular free calcium can potentially activate a number of calcium sensitive enzymes that could culminate in cell dysfunction and damage. This cellular dysfunction and damage, resulting from the activation of calcium sensitive enzymes, could subsequently result in further aggravation of the initial rise in intracellular free calcium.

• As mentioned mitochondria become the single most important membrane system in the cell to act as long-term calcium buffers, but with the subsequent increase in mitochondrial calcium content functional and structural changes are triggered. The prolonged uptake of calcium by the mitochondria results in a reduction of cellular ATP content, since mitochondrial calcium transport takes precedence over oxidative phosphorylation. Calcium uptake occurs by an electrophoretic uniport that involves transport of calcium ions across the mitochondrial membrane without direct coupling to the movement of another ion. This net inward movement of positive charges, associated with due calcium flux, causes depolarisation of the mitochondrial membrane, which is immediately compensated for by an increase in the respiratory

activity followed by an increase in H^+ extrusion. Thus mitochondrial H^+ efflux coupled to electron transfer along the respiratory chain is the active energy-linked process driving the electrophoretic calcium uptake. This utilisation of the electrochemical gradient across the mitochondrial membrane for calcium uptake makes the electrochemical gradient unavailable for ATP production. (6) Furthermore, calcium uptake is followed by the activation of a mitochondrial associated phospholipase A_2. Phospholipase A_2 hydrolyses ester bonds of membrane phospholipids, specifically phosphatidylcholines containing the fatty acid arachidonic acid in the sn_2 position. Activation of this enzyme may therefore be responsible for the dramatic ultrastructural changes in the crystal membranes and mitochondrial swelling with a prolonged elevation in intracellular calcium. (9 - 11) The structural and functional changes of mitochondria upon prolonged elevation in intracellular calcium, most notably result in the further decrease of ATP production. Many of the enzymes responsible for decreasing intracellular free calcium are energy-dependent, therefore decreased ATP production could possibly result in decreased calcium extrusion. Therefore, mitochondrial dysfunction following the initial elevation in intracellular free calcium could result in the further progressive aggravation of intracellular calcium dyshomeostasis.

• An elevation in intracellular free calcium can possibly result in the activation of calpain, a neutral protease. Calpain activation results in degradation of cytoskeletal proteins such as actin and tubulin, and cytoskeletal and plasma membrane organisational proteins that are responsible for anchoring cytoskeletal elements to the plasma membrane. This results in alterations in the microfilament network and subsequently plasma membrane blebbing. Many of these organisational proteins restrict different membrane proteins to specific regions of the plasma membrane. Degradation of these organisational proteins

necessary for the restriction of membrane proteins results in the subsequent loss of cell polarisation and cell dysfunction. (9 - 11, 13)

• An inappropriate elevation in intracellular free calcium results in the unwanted production of oxygen radicals by phagocytic cells. Phagocytic cells such as neutrophils possess a H_2O_2/O_2^--generating NADPH oxidase. NADPH oxidase activation is triggered by the influx of calcium resulting in the production of oxygen radicals. (9, 14) Therefore, an inappropriate elevation in intracellular free calcium can result in the unwanted activation of the NADPH oxidase and the excessive production of oxygen radicals. In addition, oxidative stress, a condition in which the pro-oxidant: anti-oxidant ratio favours the former, is known to affect intracellular calcium homeostasis mechanisms. Disruption of many of the intracellular calcium homeostasis mechanisms due to an increased production of oxygen radicals, can potentially result in the elevation of basal intracellular free calcium. (15, 16) This disruption of intracellular calcium homeostasis mechanisms is the result of the reaction of oxygen radicals with cellular constituents. The superoxide anion is one of the oxygen radicals produced by the NADPH oxidase. The superoxide anion is not particularly reactive but is capable of diffusing over relatively large distances in the cell where, in the presence of iron or copper, a metal-catalysed Haber-Weiss reaction can occur with the formation of the highly reactive hydroxyl radical. (9) This hydroxyl free radical rapidly reacts with cellular constituents in its immediate vicinity and is the key radical species in causing cellular damage. With regard to intracellular calcium homeostasis the reaction with membrane lipids (to cause non-enzymatic lipid peroxidation), and the reaction with the sulphydryl groups contained in cellular proteins appear to be of particular importance. Lipid peroxidation can result in the loss of membrane integrity and a further influx of calcium into the cytosol due to the breakdown of the membrane barrier between the cytosol and the high

calcium containing pools. (9, 15, 17) Additionally, a change in membrane phospholipid and fatty acid composition as a result of lipid peroxidation may cause an alteration in the lipid microdomain of the Ca^{2+}-ATPase and the other enzymes responsible for lowering of intracellular free Ca^{2+}. The lipid microdomain surrounding an integral membrane protein determines the activity of the protein. This is mainly due to the support of the three-dimensional structure of the protein. Therefore, an optimal lipid environment is an important factor that can influence membrane protein function such that a change in the microdomain of the calcium pumps can result in the suppression of calcium extrusion. (12, 18-23) Furthermore, the calcium pumps responsible for decreasing calcium with the unwanted accumulation of calcium in the cytosol are highly sensitive to oxygen radicals. The unchecked production of oxygen radicals can possibly react with protein sulphydryl groups. The sulphydryl groups form part of the active center of the calcium pump, oxidation of these groups result in the suppression of calcium extrusion. (16, 24) Oxidative stress can also result in disruption of microfilament organisation. Thiol oxidation of actin molecules perturbs microfilament organisation. Microfilament organisation determines the size and distribution of the intracellular calcium stores, through the interaction with intracellular calcium store membranes. A change in the distribution of intracellular calcium stores can affect intracellular calcium signalling processes. During conditions of oxidative stress, the loss of both reduced pyridine nucleotides and protein thiols inhibits mitochondrial calcium sequestration resulting in the loss of this vital defence mechanism against elevated calcium while at the same time releasing calcium into the cytosol. (9) Lastly, one of the important properties of the calcium extrusion pump is its stimulation by calmodulin, which is due to a direct interaction of calmodulin with the pump. (25) Calmodulin binds four calcium ions, and this binding of calcium is accompanied by a substantial

conformational change whereupon the protein assumes an active form. The active form of calmodulin is now capable of interaction with the calcium pump. These high-affinity calcium-binding sites on the surface of calmodulin include methionine residues. Oxidation of these residues, due to the reaction of oxygen radicals with the sulphydryl group results in the alteration of calcium binding properties with the apparent loss of high-affinity calcium binding sites. The loss of the high-affinity calcium binding sites results in decreased activation of calmodulin and diminished capability of the up-regulation of calcium-pump activity. (26) This can result in decreased calcium-pump activity and the subsequent rise in intracellular free Ca^{2+}.

• An elevation in intracellular free calcium results in the activation of a cytosolic phospholipase A_2 (PLA$_2$). PLA$_2$ hydrolyses specific ester bonds of a specific membrane phospholipid; phosphatidylcholine with arachidonic acid in the sn_2 position. The activation of PLA$_2$ as a result of an elevation in intracellular free calcium upon agonist stimulation is a normal physiological response. It is when an unwanted elevation in intracellular free calcium occurs that PLA$_2$ activation can result in substantial membrane damage. PLA$_2$ activation consequently has been considered a candidate for causing cellular damage, since the loss of membrane integrity could result in an unwanted influx of calcium from the extracellular medium. (9, 27) Membrane integrity play an important role in the maintaining of cell viability, therefore an increase in membrane breakdown will result in an increase in membrane repair mechanisms. Acyltransferase is one of the enzymes responsible for membrane phospholipid and fatty acid resynthesis. For the adequate functioning of this enzyme ATP is required. One of the possible results of an elevation in intracellular free calcium is the reduced production of ATP. An unwanted elevation in intracellular free Ca^{2+} could therefore result in the perturbation of membrane repair due to the inadequate ATP production for acyltransferase activity.

- To ensure adequate signalling responses cells have evolved an ingenious mechanism of signalling based on presenting calcium as brief spikes organised as oscillating regenerative waves. (28) It is generally accepted today that calcium spiking is an important signalling mechanism and that various patterns of intracellular calcium oscillations are of physiological significance. (5) These kinds of responses are both spatially and temporally regulated and are obtained when cells are stimulated with concentrations of agonists representing the physiological situation. (5) This is in contrast with the calcium response obtained when cells are stimulated with high concentrations of agonist, i.e., a sustained elevation in intracellular free calcium and the subsequent return to baseline levels. Calcium appears to be the transduction element in these responses since receptors and enzymes displaying various degrees of sensitivity to the surrounding calcium concentration regulates the generation of these calcium patterns. Therefore, perturbation in intracellular free Ca^{2+} could result in the unwanted activation of these receptors and enzymes resulting in inappropriate calcium signalling responses. Following, is an account of these receptors and enzymes sensitive to calcium and the possible influence of an unwanted rise in intracellular free Ca^{2+} on calcium signalling processes. Agonist-receptor interaction stimulates these intracellular calcium oscillations. Initially, agonist-receptor interaction stimulates the activity of a phosphatidyl inositol specific phospholipase C (PI-PLC). Activation of this enzyme leads to the hydrolysis of phosphatidyl inositol 4,5-bisphosphate and formation of downstream effectors, water soluble 1,4,5-trisphosphate ($InsP_3$) and lipid soluble diacygliserol (DAG). The initial PI-PLC isoform activated as a result of receptor stimulation involves the activation of a specific PI-PLC isoform that is coupled to the receptor via a G-protein. This PI-PLC isoform appears to be largely insensitive to calcium. However, during the later phase of the response a second PI-PLC isoform, a calcium-dependent

isoform is activated. It is hypothesised that the activation of this calcium dependent PI-PLC isoform by an increase in intracellular free calcium, plays a role in the generation of calcium oscillations. An inappropriate elevation in intracellular free calcium, therefore can result in perturbation of these intracellular calcium oscillations due to the unwanted activation of the calcium-dependent PI-PLC. It is the primary action of InsP$_3$ to liberate calcium ions from intracellular organelles, mostly calciosomes (the equivalent of the calcium pool in the sarcoplasmic reticulum of skeletal muscle) into the cytosol. (29, 30) InsP$_3$ releases calcium from these stores by binding to the InsP$_3$-sensitive calcium channels in the membranes of these organelles. The InsP$_3$-activated channel is composed of four non-covalently bound identical subunits. The InsP$_3$ binding domain resides at the extreme N-terminal region, whilst the C-terminal portion contains six putative membrane-spanning domains that make up, or at least contain the calcium channel. The region between the InsP$_3$-binding domain and the putative calcium-channel constitutes the so-called 'coupling' domain, some 1400-1500 amino acids in length, which lies in the cytoplasm. This so-called 'coupling' domain constitutes the site for modulatory actions. (5) This domain contains a site for calcium binding. There are at least four different types of InsP$_3$ receptors derived from three or four distinct genes. Between these different types of InsP$_3$ receptors there exist a strong homology in the membrane-spanning domains but large divergencies are indicated in the cytoplasmic modulatory domains. It is hypothesised that since the InsP$_3$-receptor contains a site for calcium binding in the modulatory domain and in the light of the fact that there exists receptor subtypes containing large divergencies in these modulatory domains, that the functional distribution of these InsP$_3$-receptor subtypes play a significant role in the regulation of these spatially and temporally organised calcium patterns. Indeed there are many indications that the InsP$_3$-receptor plays the central role as

possible regulator of these complex intracellular calcium signalling patterns. There are indications that an inappropriate elevation in intracellular free calcium may affect these $InsP_3$-receptor functions. This can be the result of one of two factors. An elevation in intracellular free calcium results in cytoskeletal disorganisation followed by a change in the structure of the calcium stores. The structure of these calcium stores can be controlled by the integrity of the cytoskeleton since the internal membranes are associated with the elements of the cytoskeleton and it has been shown that cytoskeletal disruption leads to fragmentation of the $InsP_3$-sensitive calcium stores. The intracellular calcium signalling patterns are spatially organised and these spatially organised calcium patterns are possibly determined by the functional distribution of $InsP_3$-receptor subtypes. Therefore the structure of the $InsP_3$-sensitive calcium store is an important determinant for the generation of these spatially organised calcium signalling patterns. The following calcium signalling processes are determined by the distribution of $InsP_3$-receptor subtypes: Upon agonist activation the intracellular calcium signal is initiated at a discrete subcellular locus adjacent to a small region of the plasma membrane. This intracellular calcium wave initiation domain does not simply result from a polarised distribution of agonist receptors but indications are that there may be specialised subcellular regions for intracellular calcium release containing $InsP_3$-receptor subtypes that are more sensitive to $InsP_3$. (31) Various intracellular calcium responses do not involve the entire cell and are therefore localised within a specific region. To avoid the undesirable activation of calcium-dependent processes elsewhere in the cell, calcium signalling patterns are produced locally which are confined to the relevant region. The production of such local calcium signalling patterns are largely determined by the spatial arrangement of the $InsP_3$-sensitive stores. (31) In cells displaying chemotaxis the polarisation of the cell changes with every new movement. There are indications of a calcium gradient within these

cells that underlies the activities of calcium-dependent processes that favour the polarisation and directed locomotion of these cells. The gradient changes continually as the polarisation of the cell changes. The direction of the new calcium gradient within cells is established by the reorganisation of the $InsP_3$-sensitive stores. (31) An elevation in intracellular free calcium not only results in the disorganisation of the calcium stores and the subsequent altered distribution of the $InsP_3$-receptors, but the free calcium concentration can also modulate the degree of $InsP_3$–evoked channel opening. The free calcium concentration is an important factor in determining the degree of $InsP_3$-gated channel opening. These $InsP_3$–gated calcium channels display a bell-shaped calcium response curve. In the physiological calcium concentration range (0.1 – 1 μM) small changes in the calcium concentration can have dramatic effects on the probability of channel opening. When the intracellular free calcium concentration is increased from 0.1 to about 0.3 μM there is a marked increase in the open-state probability, but when the calcium concentration is further increased to 1 μM a marked fall in the degree of $InsP_3$–evoked channel opening is observed. Therefore an increase in intracellular free calcium concentration to above 0.3 μM can result in an inhibition of $InsP_3$–evoked calcium release. (30) $InsP_3$ liberation not only results in releasing of calcium from intracellular calcium stores as explained in the previous section, but also activates calcium entry pathways in the plasma membrane. It is most probably not $InsP_3$ per se that activates the calcium channels in the plasma membrane, but a product of the enzymatic modification of $InsP_3$ namely inositol-1,3,4,5 tetrakisphosphate ($InsP_4$). The plasma membrane $InsP_3$-receptor has a lower affinity for $InsP_3$ but a higher affinity for $InsP_4$ when compared to the well-known endoplasmic reticulum $InsP_3$-receptor, therefore providing for such a role for $InsP_4$. $InsP_4$ is formed as a result of the

phosphorylation of $InsP_3$ by $InsP_3$-kinase. These two processes, i.e., the release of calcium from the intracellular calcium storage organelles and the influx of calcium from the extracellular medium, are highly coordinated and possibly regulated by calcium. The $InsP_3$–kinase responsible for the formation of $InsP_4$ is calcium activated, therefore $InsP_3$ liberation results in the releasing of intracellular calcium and subsequently the activation of $InsP_3$-kinase. The subsequent formed $InsP_4$ triggers the influx of calcium from the extracellular medium. An unwanted increase in intracellular free Ca^{2+} could result in the perturbation of the interaction between these processes. (30) It is not only the calcium release and calcium influx pathways that are regulated by intracellular free calcium, but the pathways resulting in the termination of intracellular calcium signalling responses are also influenced by intracellular free calcium. These processes; calcium release, calcium influx and calcium efflux are highly coordinated. It was indicated that pulsatile calcium extrusion occurred synchronously with the cytosolic calcium spikes. The activity of the Ca^{2+}-ATPase mostly regulates the calcium extrusion and calcium sequestration at the end of the response. In the calcium range $0.1 - 0.3$ μM, where $InsP_3$-gated channel opening is increasing very steeply with a rise in intracellular free calcium, calcium extrusion is moderately activated. In the calcium range $0.3 - 1$ μM where $InsP_3$–gated calcium channel opening decreases with increasing free intracellular calcium, calcium extrusion rises sharply as intracellular free calcium increases. (30) Intracellular calcium per se plays an important role in the coordination of these processes. Three processes activated by the increase in intracellular free calcium results in the extrusion of calcium by the Ca^{2+}-ATPase. An increase in intracellular free calcium results in calmodulin activation. In the absence of calmodulin only a small increase in the calcium transport rate is indicated with the increase in intracellular free calcium. But in the presence of calmodulin, a small increase in intracellular free calcium

results in a dramatic increase in calcium pumping activity. The calcium-calmodulin-dependent activation of the Ca^{2+}-ATPase calcium pump increases the capacity or Vmax of the pump 15-20 fold and the efficiency two-fold, i.e., lowers the Km for calcium. The calcium-calmodulin complex binds to the Ca^{2+}-ATPase resulting in an increase in pump activity and the more rapid extrusion of calcium. The enzyme protein kinase C (PKC) also plays a significant role in the activation of the Ca^{2+}-ATPase. PKC is activated as a consequence of both the initial rise in the diacylglyserol (DAG) content of the plasma membrane and the transient rise in intracellular free calcium. Upon activation PKC moves from the cytosol to the plasma membrane. When PKC associates with the plasma membrane, it undergoes a conformational change to become a calcium-sensitive, plasma membrane associated protein kinase. The activity of this form of the kinase is regulated by the rate of calcium-cycling across the plasma membrane. (29) PKC activated by calcium leads to phosphorylation of the Ca^{2+}-ATPase pump, resulting in a 4 - 6 fold increase in pump activity. A third process possibly contributing to the coordination of calcium oscillations is the calcium-mediated activation of PLA_2. PLA_2 activation leads to the release of arachidonic acid. Arachidonic acid possibly performs a dual function in the coordination of intracellular calcium signalling patterns, i.e., arachidonic acid can inhibit $InsP_3$-evoked calcium mobilisation and can at the same time activate calcium extrusion. (31) In conclusion, many calcium sensitive receptors and enzymes are responsible for the generation of the complex calcium signalling patterns. In order to assure adequate signalling responses, these various processes are highly coordinated. Since the receptors and enzymes responsible for the generation of the calcium signalling patterns are calcium sensitive, calcium seems to be the modulator of its own signalling cycle. Therefore, perturbation in intracellular calcium could culminate in disturbed calcium signalling responses.

4) Intracellular calcium status in chronic renal failure

It was Massry who suggested that chronic renal failure is a state of calcium toxicity. (32) Soft tissue calcification is indeed a common finding in chronic renal failure (CRF), with an increase in calcium content known to occur in the cornea, skin, blood vessels, brain, peripheral nerves, heart, lungs, pancreas, liver, epididymal fat and testis of patients as well as animals with chronic renal failure. (33) Intracellular free calcium would appear to reflect this increased tissue content of calcium. An elevation in basal intracellular free calcium in various cell types in CRF is documented, indicating a potential association between this increase in the calcium content of tissues with an elevation in basal intracellular free calcium. A summary of published results of basal intracellular free calcium in humans and rats with chronic renal failure can be seen in Table 1. The cell types in which the levels have already been determined include brain synaptosomes, pancreatic islets, cardiac myocytes, thymocytes, B cells, T cells, neutrophils, adipocytes, and platelets. (33-37, 40-42, 44-46, 48, 50-51)

4.1) Pathological factors associated with CRF possibly contributing to the derangements in intracellular calcium homeostasis

There are a multitude of possible factors contributing to the elevation in basal intracellular free calcium in the CRF patient including secondary hyperparathyroidism, oxidative stress, deranged phospholipid and fatty acid metabolism and reduced ATP concentrations.

4.1.1) Secondary hyperparathyroidism

It is hypothesised that an elevation in PTH levels in these patients could be the main cause for the increase in basal intracellular free calcium in the various cell types. (33, 44)

Table 1. Intracellular free Ca $^{2+}$ in various cell types in humans and rats with CRF

Cell type	Model	CRF [Ca^{2+}]i	Normal [Ca^{2+}]i	Fluorescent calcium indicator	Reference
Neutrophils	HD patients	73±3.6nM	42±0.09nM	Fura-2	34
Neutrophils	HD patients	92nM	60nM	Fura-2	35
Neutrophils	HD patients	60±1.2nM	46±1.2nM	Fura-2	36
Neutrophils	HD patients	79±5.1nM	52±3.4nM	Fura-2	37
Neutrophils	HD patients	68±1.1nM	57±1.1nM	Fura-2	38
	CRF rats	133±3.1nM	104±1.3nM		
Neutrophils	CRF rats	149±2.7nM	108±2.4nM	Fura-2	39
Platelets	CRF patients	117±33nM	47±14nM	Fura-2	40
Platelets	HD patients	137±8.86nM		Fura-2	41

Table 1. (continue) Intracellular free Ca $^{2+}$ in various cell types in humans and rats with CRF

Cell type	Model	CRF [Ca^{2+}]i	Normal [Ca^{2+}]i	Fluorescent calcium indicator	Reference
Platelets	CRF patients	138±16nM	83±7nM	Fura-2	42
Platelets	CRF patients	173±44nM	150±30nM	Indo-1	43
T-cells	HD patients	87nM	55nM	Fura-2	44
B-cells	HD patients	115±5.2nM	86±4.4nM	Fura-2	45
Brain synaptosomes	CRF rats	437±18nM	345±9nM	Fura-2	46
Brain synaptosomes	CRF rats	445±10nM	353±7.5nM	Fura-2	47
Islets of Langerhans	CRF rats	252±7.4nM	137±4.5nM	Fura-2	48
Heart cells	CRF rats	101±3.2nM	56±1.6nM	Fura-2	49

Indeed, this hypothesis finds much credit, since prevention of the elevation in PTH levels by parathyroidectomy or vitamin D therapy, or by the prevention of the action of PTH by calcium channel blocker administration, resulted in the normalisation of basal intracellular free calcium levels. (35, 37, 48, 51-53) It is generally accepted that patients with CRF have secondary hyperparathyroidism and elevated blood levels of PTH. (45) PTH tightly regulates plasma calcium and phosphate concentrations by modulating the activity of specific cells in the bone and kidney. (54, 55) In the normal functional kidney, PTH is known to increase calcium reabsorption and to stimulate phosphate excretion. PTH also activates the renal synthesis of the active vitamin D metabolite, which, in turn is able to stimulate or inhibit intestinal absorption of calcium and phosphate and is able to increase or decrease osteoclast mediated bone resorption depending on the concentration of vitamin D. (56) These actions of PTH are classical hormonal functions. It is with an abnormal elevation in PTH levels that PTH targets many other cell types. It has been shown that PTH can cause a sharp increase in the basal intracellular free calcium content of pancreatic islets cells, thymocytes (57), cardiac myocytes, hepatic cells, adipocytes, kidney cells, and osteoblasts. This effect of PTH is receptor-mediated. PTH activates the voltage-dependent calcium channels. This PTH-mediated calcium influx can be blunted or prevented by calcium channel blockers such as verapamil or nifedipine. The influence of PTH on non-conventional targets is supported by recent data which demonstrated that the mRNA for the PTH/PTHrP receptor is present in non-classical targets for PTH such as heart, brain, spleen, aorta, ileum, skeletal muscle, lung and testis. (33, 54) In addition to the experimental evidence for the central role of PTH in the elevation of intracellular free calcium in different cell types, indications suggest deranged cellular functions in association with an elevation in PTH. These include studies indicating deranged cellular functions in combination with

either an elevation in PTH or an elevation in PTH accompanied by an elevation in intracellular free calcium. Intracellular free calcium plays a paramount role in the regulation of a multitude of cellular functions. Intracellular calcium dyshomeostasis in CRF may thus be a major contributor to abnormal cellular function in uraemia.

4.1.2) Oxidative stress

Chronic renal failure patients receiving maintenance haemodialysis treatment (MHT) are continuously exposed to oxidative stress. Oxidative stress is a state of increased pro-oxidant levels and/or a deficit in the anti-oxidant capacity. In the MHT patient both an increase in pro-oxidant production and a decrease in anti-oxidant mechanisms are indicated. The increase in pro-oxidant production is mainly due to the relentless activation of polymorphonuclear leucocytes (PMNL) during haemodialysis. During extracorporeal circulation activation of PMNL's results in increased free oxygen radical production. (58) This activation of PMNL's is the result of the exposure of the blood constituents to the dialyser membrane. Various pathways are activated resulting in PMNL activation. These pathways include both complement-dependent and complement-independent PMNL activation. (59) Complement-dependent activation leads to the production and binding of C5a and C5a des Arg to specific PMNL receptors inducing aggregation, adherence to endothelial cells, releasing of oxygen radicals, degranulation, arachidonic acid metabolism, and enhanced phagocytotic capacity. (60) Complement-independent activation of PMNL's is the result of either direct stimulation of PMNL's due to interaction with the dialyser membrane or binding of monocyte-derived cytokines to their respective PMNL receptors. Cytokine exposure primes the respiratory burst to generate elevated levels of oxidants. (9, 61) There are indications that the activation of PMNL's during extracorporeal circulation involves an increase in intracellular free calcium. (59, 60, 62, 63) The process

responsible for the reduction of molecular oxygen to superoxide anion is catalysed by a multicomponent NADPH-oxidase and this process is activated and regulated by intracellular free calcium. It is therefore anticipated that an increase in intracellular free calcium will result in an increase in oxygen radical production. (64) Anti-oxidant mechanisms include various enzymes responsible for the reduction of the oxygen radicals to water. These anti-oxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase. Superoxide dismutase results in the dismutation of the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). In the following reactions the H_2O_2 formed is reduced to H_2O by either glutathione peroxidase or catalase. This subsequent reduction of H_2O_2 prevents the potential formation of hydroxyl radicals. If H_2O_2 accumulates due to insufficient glutathione peroxidase and catalase activity, hydroxyl radicals may be formed by the heavy metal catalysed Fenton reaction. During the glutathione peroxidase catalysed reduction of hydrogen peroxides, glutathione (GSH) serves as an electron donor, and the glutathione disulphide (GSSG) formed in the reaction is subsequently reduced back to GSH by glutathione reductase at the expense of nicotinamide adenine dinucleotide phosphate (NADPH). (9) NADPH is produced by normal activity of the hexose monophosphate shunt. (65) In MHT patients, a metabolic block of the pentose phosphate shunt has been described that impairs the reduction of oxidised glutathione due to the reduced NADPH formation. (65, 66) Most of the enzymes responsible for the reduction of oxygen radicals to H_2O contain certain trace elements at their active sites. Trace element levels are altered in MHT patients. The dialysis procedure further could appreciably alter the body's supply of trace elements by removing some, whereas contaminating trace elements in the dialysis fluid could be transferred to the patient. A reduction in the trace elements necessary for anti-oxidant enzyme activity can result in reduced anti-oxidant activity and therefore an increase in oxidative stress. The concentrations

of Zn and Se for instance are reduced in the MHT patient. These trace elements are necessary for normal activity of the anti-oxidant enzymes; superoxide dismutase and glutathione peroxidase. (67-69) Other trace elements catalyse the production of hydroxyl radicals from hydrogen peroxide, via the heavy metal catalysed Fenton reaction. An increase in these trace elements would therefore result in an elevation in oxidative stress. (68) Under conditions of oxidative stress, when the cell must cope with large amounts of H_2O_2, the role of glutathione oxidation exceeds the slower rate of GSSG reduction by glutathione reductase, and GSSG accumulates. To avoid the detrimental effects of increased intracellular levels of GSSG (e.g. formation of mixed disulphides with protein thiols) the cell actively secretes GSSG, which can lead to depletion of the intracellular glutathione pool. (9) In addition vitamins A, E and C are powerful anti-oxidants. Vitamin E and t-carotene are lipid-soluble anti-oxidants preventing radical mediated breakdown of poly-unsaturated fatty acids and vitamin C acts as a reductant in the cell, both regenerating oxidised vitamin E and preventing oxidation of cytosolic thiols. (9, 65, 66) In the CRF/MHT patient an increase in oxidative stress can result in the overconsumption of available anti-oxidant vitamins.

The increase in the oxidative stress in the CRF patient receiving maintenance haemodialysis due to both an increase in pro-oxidant production and a decrease in anti-oxidant activity, can potentially result in intracellular calcium dyshomeostasis. Unwanted oxygen radical production can result in the oxidation of membrane polyunsaturated fatty acids. Oxidation of membrane polyunsaturated fatty acids can subsequently result in an increase in the permeability for calcium ions causing an unwanted influx of calcium ions. (58, 65, 70) Furthermore, an unwanted oxidation of membrane polyunsaturated fatty acids can result in the derangement of membrane fatty acid composition possibly

due to an increase in the saturated fatty acid/unsaturated fatty acid ratio. The membrane fatty acid composition is an important factor regulating membrane-associated protein function. It is well known that various membrane-associated enzymes are highly sensitive to the membrane micro-environment. The Ca^{2+}-ATPase calcium pump responsible for lowering intracellular free calcium is one such membrane-associated enzyme that is very sensitive to membrane fatty acid composition. (71-73) An increase in oxidative stress will furthermore result in the oxidation of cellular proteins. Many of the membrane-associated proteins are rich in SH-groups which are targets for oxygen radical action. The Ca^{2+}-ATPase is a thiol-rich protein at the cytosol-membrane surface. (74) Therefore, an increase in the oxidative stress may result in oxidation of the SH-groups of the pump and suppression of the pump's activity. (75)

4.1.3) Inhibition of the calcium pump

A circulating inhibitor of the calcium pump is indicated in the plasma of CRF patients. This inhibitor is dialysable, smaller than 3000 molecular weight, heat-stable, and said to be protease resistant. (76, 77) It is hypothesised that this inhibitor of the calcium pump may be one of the factors contributing to the elevation in intracellular free calcium in various cells in the CRF patient.

4.1.4) Inhibition of the Na⁺-K⁺-ATPase

There are indications of an elevation in plasma levels of natriuretic factor in CRF. Natriuretic factor is a potent inhibitor of the Na^+-K^+-ATPase. Na^+-K^+-ATPase plays an important role in the maintenance of intracellular calcium homeostasis. Na^+-K^+-ATPase maintains the sodium gradient across cellular membranes, a high sodium concentration extracellularly and a low sodium concentration on the inside. This sodium gradient provides the energy necessary for the

extrusion of calcium via the Na^+-Ca^{2+}-exchanger. A decrease in the activity of the Na^+-K^+-ATPase may ultimately result in an elevation in intracellular free calcium. (78)

4.2) Results of an elevation in basal intracellular free calcium in chronic renal failure

4.2.1) Deranged membrane phospholipid metabolism

It is indicated that the content of phospholipids in brain synaptosomes and specifically phosphatidyl inositol (PI), phosphatidyl serine (PS), and phosphatidyl ethanolamine (PE) are significantly reduced in CRF. This reduction in the phospholipid content is attributed to the increase in basal intracellular free calcium caused by the chronic exposure to PTH. (79) Phospholipids are important components of the cell membrane and play an important role in maintaining membrane fluidity and membrane protein function. (80) Changes in phospholipid composition of cell membranes may therefore affect the activity of proteins and enzymes present in the membranes. (81, 82) The various enzymes responsible for lowering of intracellular free calcium, Ca^{2+}-ATPase, Na^+/Ca^{2+}-exchanger and the Na^+/K^+-ATPase are extremely sensitive to membrane phospholipid composition. (83, 84) Indeed, the changes in synaptosomal phospholipid composition are associated with changes in Na^+/K^+-ATPase activity. This change in the activity of the enzymes responsible for lowering of intracellular free calcium may thus possibly aggravate the initial increase in intracellular free calcium. Chronic exposure to PTH has been shown to inhibit the activity of many cellular enzymes. It is therefore possible that chronic exposure to excess PTH may also inhibit the activity of enzymes involved in the synthesis of phospholipids. (79, 85)

4.2.2) Deranged membrane fatty acid synthesis

It is indicated in the CRF patient that intracellular free calcium accumulation results in inhibition of various cellular enzymes. One of the enzymes possibly inhibited by an elevation in intracellular free calcium is carnitine palmitoyl transferase (CPT). (79) Adequate CPT activity is important in the maintaining of a favourable acyl CoA/free CoA ratio. CPT maintains a favourable acyl CoA/free CoA ratio by forming acylcarnitine from carnitine and acyl CoA. Membrane fatty acid turnover depends on both long chain acyl CoA synthetase and lysophospholipid acyl CoA transferase (LAT). The former generates acyl CoA from free fatty acids and CoA, while the latter reacylates lysophospholipids by using acyl CoA as substrate. When the rate of formation of acyl CoA becomes different from that of its utilisation for reacylation, acyl CoA and the acyl CoA/free CoA ratio become altered, leading to disturbances in membrane fatty acid turnover. In the CRF patient reduced activity of CPT is indicated. (85) Under such circumstances, the lack of buffering activity of CPT alters the acyl CoA/free CoA ratio, and eventually the availability of fatty acid moieties for reacylation are impeded. Additionally, the relatively high concentrations of free CoA may further act as an inhibitor of the enzyme LAT. This possible reduced activities of CPT and LAT may result in the inadequate repairing of membrane fatty acids damaged by free radicals and the loss of membrane integrity. (85)

4.2.3) Decreased ATP production

An elevation in basal intracellular free calcium inhibits mitochondrial oxidation, resulting in the decrease of basal and stimulated ATP production. (53, 78)

5) Aim of this study

Over the past decade a significant amount of work has been done on intracellular calcium in chronic renal failure. It should however be

remembered that the term intracellular calcium is used relatively non-specifically, as can be seen from the various publications on the subject where the expression *intracellular calcium* is used to denote anything from total cellular calcium to diffusible calcium content, depending on the methods available in specific laboratories. The complete picture of the intracellular calcium status, including a comparison between the intracellular content of the various calcium compartments, the characteristics of the transmembrane calcium movement, the possible causes which may contribute to the assumed intracellular calcium disturbances in CRF, as well as the contribution of such disturbances to the pathogenesis of chronic renal failure still need to be clarified. At this point in time it can, with a relative amount of certainty, be assumed that both the total intracellular calcium content and the intracellular free Ca^{2+} are increased in end stage chronic renal failure patients. This despite a small number of contradictory reports. Very little is, however, known about the movement across the cell membranes as well as about the intercompartmental shifts. The parathyroid hormone disturbance in chronic renal failure is at present seen as the major contributor to the deranged calcium status, but the evidence for such an assumption is, however tempting, still far from conclusive.

The aim of this study was to contribute to the knowledge on intracellular calcium in chronic renal failure by

a)   Examining the intraneutrophilic free cytosolic Ca^{2+} content in end stage chronic renal failure patients on maintenance haemodialysis treatment. The aim is not to evaluate the influence of renal failure *per se* on calcium metabolism, but rather to determine the status of intracellular calcium in the maintenance haemodialysis patient as it presents as a result of renal failure, dialysis treatment and medication. The rationale is that firstly the disturbed cellular functions and other

effects of the disturbed calcium content are functions of the existing calcium status and secondly that the application of stringent exclusion criteria would eliminate too many patients from the already small available group.

b) Examining the characteristics of the transmembrane movement of calcium, i.e., the calcium fluxes.

c) Investigating the intracellular distribution of calcium in the neutrophils of the patients by means of electron microscopy.

d) Exploring the possibility of a link between the alterations in intracellular calcium status and factors known to influence the calcium status. The factors investigated in this study include the lipid composition of the membrane, the oxidative status as reflected by anti-oxidant vitamin levels, the levels of parathyroid hormone, and ionised serum calcium.

In order to establish conditions inducive to accurate research, the techniques for calcium determinations, which did not previously exist in the current laboratory, had to be developed and evaluated. This constituted a rather large part of the project.

The rest of the chapters of this dissertation are presented in the following sequence

Chapter 2: Evaluation of the techniques for the determination of intracellular free calcium, transmembrane calcium fluxes and intracellular distribution of calcium

Chapter 3: Materials and methods

Chapter 4: Results

Chapter 5: Discussion and conclusions

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Chapter 2

Theoretical background and experimental evaluation of the techniques for the determination of intracellular free calcium, transmembrane calcium fluxes and intracellular distribution of calcium

1) Introduction

In the present study aspects of intracellular calcium regulation and signalling were investigated in the neutrophil, both in healthy subjects and in chronic renal failure patients on maintenance haemodialysis treatment (MHT). The various aspects of intracellular calcium regulation and signalling investigated included: basal intracellular free calcium, transmembrane calcium fluxes upon agonist stimulation, and the intracellular distribution of calcium between the various subcellular compartments. The techniques available to determine any one of these aspects are cumbersome and requires experimental evaluation as their success rate vary from cell type to cell type and small variations in the techniques would appear to have a major impact on the results. The following 4 paragraphs will refer to the techniques originally considered. Where after the techniques eventually employed will be discussed in more detail.

1.1) Basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation

The determination of basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation by using the fluorescent calcium indicators were evaluated. The determination of intracellular free calcium using the fluorescent calcium indicators,

introduced into the cytosol of the cell as the membrane permeant acetoxy methyl esters, is said to surpass all the other available techniques regarding avoidance of disruption of cell integrity and sensitivity. It is of the utmost importance that the technique employed should involve minimum disruption of cell integrity, since experimental manoeuvres that cause cell damage can subsequently result in disturbances in basal intracellular free calcium followed by the perturbation of transmembrane calcium fluxes. In this study initial values obtained with purely laboratory experimental work the results appeared to be quite satisfactory. However, results obtained from patients versus age and sex matched individuals forced rethinking and reevaluation of the technique. A large part of this chapter is therefore committed to the theoretical background and evaluation of this technique.

1.2) Total calcium content of the neutrophil

The neutrophil is endowed with powerful mechanisms to regulate basal intracellular free calcium in the cytosol. These mechanisms buffer abnormal elevations in basal intracellular free calcium and can include: sequestration of calcium into intracellular organelles and an increase in the pool of calcium bound to intracellular molecules. A short discussion of these mechanisms is provided in chapter 1. It is therefore possible that basal intracellular free calcium can be maintained within physiological limits despite elevations in the total calcium content of the neutrophil. Eager to include a method for the total calcium content, three techniques available for determining the total intracellular calcium content in a sample were tried and tested. The techniques included conventional flame atomic absorption spectrometry, graphite furnace atomic absorption spectrometry and inductively coupled plasma mass spectrometry. (1 - 3) The techniques were tested on a neutrophil sample size similar to that available from MHT samples. None of these methods

were successful in the determination of the total calcium content in the neutrophil. The major reason was most probably the small cell number available after removal of neutrophils from the isolated neutrophil pool for fluorescence and transmission electron microscopy (TEM) studies. This in combination with background calcium levels rendered the methods not suitable. Two alternatives remained, i.e., to increase the volume of blood collected at one sampling or to do the determination on separate occasions. In view of the anaemia of MHT patients, as well as the daily variability of intracellular calcium, neither alternative was feasible.

1.3) Localisation of intracellular calcium

Transmission electron microscopy was employed to investigate the distribution of intracellular calcium between the various subcellular compartments in the neutrophil. During the preparation of the neutrophils for transmission electron microscopy, in situ precipitation of calcium was performed, resulting in the formation of electron-dense particles containing calcium. The distribution of these electron-dense particles was investigated by means of the transmission electron microscope. The main concern about this technique was the specificity of the precipitation reaction for calcium, since the employed pyroantimonate anion can also precipitate other available cations. Adaptations and evaluations of the technique are presented in the second part of the chapter.

1.4) Spatial and temporal measurements of intracellular free calcium changes using video-rate confocal microscopy

It has become known that cellular calcium regulation and signalling are more complex than envisioned from calcium measurements made in cell populations. Intracellular calcium regulation and signalling involve more dynamic processes than these previous rigid concepts of a set

intracellular free calcium level at rest, and a transient rise and fall in intracellular free calcium upon agonist stimulation. These processes include temporally and spatially organised calcium oscillations and waves. These spatial and temporal patterns become obscured when measured in cell populations since cells, even from the same cell type, display heterogeneity in these intracellular calcium patterns. It is therefore necessary to investigate these spatially and temporally organised calcium oscillations and waves in a single cell. This can be achieved with the use of a video-rate confocal microscope. (4, 5) The use of the confocal microscope at the University of the Witwatersrand was tentatively envisaged and the possibility of using their facilities arranged. However, due to impracticalities such as sample size and cellular change with travel time as well as the fact that the standardisation of such a technique would fall outside the scope of the Masters degree's specifications, investigation of the temporal and spatial calcium patterns were not performed. After the evaluation and consideration of these various techniques the following techniques were considered practical although further standardisations and evaluations were necessary, i.e., determination of intracellular free Ca^{2+} by fluorescence spectrophotometry, determination of the transmembrane Ca^{2+} flux by fluorescence spectrophotometry and the localisation of intracellular calcium by transmission electron microscopy.

2) The measurement of basal intracellular free calcium and transmembrane calcium fluxes upon agonist stimulation by the employment of the fluorescent calcium indicators

2.1) Theoretical background

2.1.1) Biological fluorescence measurements

Fluorescence is an electromagnetic radiation phenomenon. A molecule, capable of fluorescing is excited by exposure to electromagnetic radiation of the appropriate wavelength - which usually coincides with absorption maxima - resulting in the excitation of electrons to a higher energy level. These excited electrons, with a half-life in the order of 10^{-8} seconds, revert to ground state energy levels giving rise to detectable emitted fluorescence. (6) Spatial and temporal fluorescence measurements have widely been used in studies to investigate cellular structure and function. Since fluorescence markers fluoresce more brightly than cellular constituents, they can be used to 1) indicate the presence of a specific molecule, 2) report the change in the structure of a specific molecule or 3) measure the fluctuations in the concentrations of ions. (4)

1) Spatial studies involve the use of probes, furnished with a fluorophore, which specifically bind the molecule of interest. For example:

- immunofluorescence labeling
- fluorescent analog chemistry
- vital staining of organelles and
- assessment of cell morphology (4)

2) To indicate conformational changes in a protein, probes are directed to specific protein sites. These probes report the change in conformation taking place at that specific protein site. (6)

3) Fluorescing molecules that can directly, specifically and reversibly bind an ion, and undergo a measurable change in fluorescence spectral properties upon this binding, allow the continuous monitoring of the fluctuation in the ion's concentration with various manipulations. (4, 6)

2.1.2) The fluorescent calcium indicators

In order for a molecule to resolve and report intracellular free calcium changes it should be able to directly, specifically and reversibly bind the free calcium ions. Upon this binding the molecule should undergo a measurable change, and this measurable parameter should change at a faster rate than the changes in the free calcium ion concentrations take place. A change in emitted fluorescence upon excitation takes place at an appropriate rate to be able to resolve intracellular free calcium changes. Such fluorescent indicators need to work at suitable wavelengths, fluoresce brightly, resist bleaching, sense their intended stimulus, reject interfering influences, localise in the correct cellular or tissue compartment, and perturb cell function as little as possible. It was with this in mind that R Tsien designed and synthesised the well-known fluorescent calcium indicators. (7)

i)   The characteristics of the fluorescent calcium indicators

- **The structure of the fluorescent calcium indicators**

The structure of the fluorescent calcium indicators has been modelled on the widely used calcium-chelator EGTA (ethylene glycol bis (beta-aminoethyl ether)-N, N, N', N',-tetraacetic acid). (4, 6, 7) EGTA encapsulates calcium in a ring-like cage via the 8 oxygen atoms donated from the 4 carboxylate groups. This calcium chelation ring is retained in the structure of the fluorescent calcium indicators, but the two ethylene groups of EGTA are substituted for a moiety containing a stilbene-type backbone with two aromatic rings connected via a two carbon alkyl chain. This combination affords a maximised quantum efficiency and photochemical stability for the fluorophore. A fifth carboxylate group at the end of the chromophore tail is not

involved in chelation but contributes to the indicator's hydrophilic characteristics. (8)

- **The specificity of the fluorescent calcium indicators for calcium**

The fluorescent calcium indicators show a selectivity for calcium over magnesium of 5 to 6 orders of magnitude, therefore the fluorescent calcium indicators are capable of buffering calcium in the physiological range of $10^{-8} - 10^{-6}$ M in the presence of physiological magnesium concentrations of $10^{-4} - 10^{-3}$ M. (6) This aversion for magnesium in the presence of calcium ions is the result of the smaller size of the magnesium ion, such that no more than half of the liganding groups can contact the ion simultaneously to form a stable complex. Also monovalent cations do not form detectable specific complexes, probably because their charge is inadequate to organise the binding pocket in the face of the electrostatic repulsion of the negative carboxylates.

- **Negligible interference of hydrogen ion binding with calcium binding at physiological pH**

Calcium binding to EGTA is very sensitive at physiological pH, this is the result of the binding of hydrogen ions to the lone pair electrons of nitrogen at pH 7. Hydrogen binding to these electrons, results in a decrease in the availability of the negative charges at the carboxylate anion site for calcium chelation. The fluorescent calcium indicators are insensitive to hydrogen ion binding at a physiological pH, due to the incorporation of the aromatic rings in the structure of the fluorescent calcium indicators. The incorporation of the aromatic rings results in diversion of the nitrogen lone pair electrons due to the

overlapping p-orbitals and a resulting decrease in the pKa of the amine nitrogens to 6.5 or below. (4, 6, 9)

- **The fluorescence properties of the fluorescent calcium indicators**

The fluorescence properties of the fluorescent calcium indicators are determined by the electronic configuration of the aromatic rings. The electronic configuration of these aromatic rings can be manipulated by the binding of calcium to the four carboxylate groups. The amino substituents connected to the aromatic rings contain pi-electrons that can delocalise across the aromatic rings via overlapping p-orbitals. (10) Calcium binding, involving the 4 carboxylate anions in an octa-coordinate ligand complex, results in the constraint on the conformation of the molecule and the subsequent diversion of the nitrogen lone pair electrons away from the aromatic rings. (4, 9) Thus the nitrogen lone pair electrons no longer delocalises across the aromatic rings and as a result of this change in the electronic configuration of the indicator large fluorescence spectral shifts take place. (4, 6, 9)

ii) **The introduction of the fluorescent calcium indicators into the cytosol of the cell**

- **Problems associated with the introduction of the fluorescent calcium indicator into the cytosol of the cell**

The fluorescent calcium indicator is a highly charged, hydrophilic molecule that cannot cross the cell membrane, therefore procedures such as various membrane permeabilisation techniques or liposome fusion must be employed to introduce the fluorescent calcium indicator into the cytosol of the cell. These procedures are all problematic. Any disruption of membrane

integrity would lead to an uncontrollable rise in basal intracellular free calcium levels and the loss of soluble cytoplasmic constituents. Introduction of the fluorescent calcium indicators into the cytosol via liposome fusion results in the incorporation of foreign lipid into the cell membrane and the releasing of only trace quantities of the trapped indicator into the cytoplasm. (5, 8, 11)

- **Synthesis of a membrane-permeable form of the fluorescent calcium indicator**

To reduce these problems an uncharged, lipophilic derivative of the fluorescent calcium indicator was synthesised that could easily be introduced into the cytosol of cells by direct crossing of the membrane. This lipophilic derivative is synthesised by the formation of ester bonds to the 5 carboxylate ion groups of the calcium indicator, resulting in the concealment of the groups responsible for the molecule's hydrophilic characteristics. (6, 8, 12) Once inside the cell, esterases hydrolyse these ester bonds randomly, resulting in the formation of membrane impermeable calcium indicator molecules. Theoretically the calcium indicator is now trapped inside the cytosol of the cell. (5, 6, 8, 12) A variety of ester derivatives of the fluorescent calcium indicators have been found to cross the cell membrane, but the acetoxy methyl ester form is the most readily activated by cellular esterases. The simplest esters, methyl or ethyl are not hydrolysed. Anhydrides, imidazolides or activated aryl esters are rejected because the end products would probably include indicator molecules bound to intracellular nucleophiles. Trimethylsilyl esters are too rapidly hydrolysed extracellularly and t-butyldimethylsilyl esters are potently haemolytic. (11)

- **Loading efficiency of different cell types**

The acetoxy methyl (AM) ester form of the fluorescent calcium indicator is the most readily activated in most cell types but loading is never more than 50% efficient. More importantly, the loading efficiency varies between different cell types. This results from the variability in esterase activity in different cell types. In some cell types it is impossible to introduce the fluorescent calcium indicator into the cytosol as the acetoxy methyl ester derivative since these cells lack the esterases necessary to cleave the ester bonds. (6) A trial and error approach is thus necessary to determine a specific cell type's ability to accumulate the acetoxy methyl ester derivative of the indicator.

iii) **The fluorescent calcium indicators as intracellular calcium binding molecules**

When introduced into the cytosol of a cell, the fluorescent calcium indicators bind the available free calcium ions. This binding of the free calcium ions results in the buffering and subsequent lowering of cytosolic free calcium. In response to the lowering of the intracellular free calcium concentration, the pump/leak balance of the cell membrane for calcium is altered and steady state intracellular free calcium concentrations are restored. (5, 13) The restoration of intracellular free calcium concentrations to basal levels, will only result if the amount of fluorescent calcium indicator introduced into the cytosol is not extreme. In any case calcium buffering by the indicators tends not to have much of an effect on basal intracellular free calcium levels or elevated basal intracellular free calcium levels. If restoration to basal levels do not occur, this buffering of the free calcium ions can result in a reduction in the magnitude and

extent of calcium fluxes upon agonist stimulation, and the prolonged reversal of calcium levels to a new steady state. (5)

iv) **The fluorescent calcium indicator-calcium chelation complex**

Upon cleavage of all the ester bonds the highly charged penta-anion form of the indicator are generated. It is the penta-anion form of the indicator that is capable of chelating calcium via the octa-coordinate binding site of the molecule. These formed chelation complexes are dynamic, reversible molecules that follow the rules of chemical equilibria. In reversible equilibrium systems the amount of bound chelate-calcium complex will depend on the total concentration of target ion – Ca^{2+}, and the affinity of the chelator for the ion. The following formula is derived from the chemical equilibrium equation:

$$[\text{Chelate-Ca}^{2+} \text{ complex}] = [\text{Ca}^{2+}] \,.\, \text{Kd} \,.\, [\text{Chelator}]$$

v) **A change in the fluorescence spectral properties of the indicator caused by the binding of calcium**

In order for a fluorescent indicator to report temporal changes in the concentration of calcium ions, the indicator must undergo significant changes in its fluorescence spectra caused by any change in the concentration of calcium ions. (6)

These spectral changes can be either one of the following:

- a change in fluorescence intensity
- a shift in the excitation wavelength maximum
- or a shift in the emission wavelength maximum

Thus two types of indicators are available, those that do not change wavelength maxima – wavelength stable calcium indicators, and those that change their wavelength maxima upon calcium binding – wavelength shifting calcium indicators.

Wavelength shifting calcium indicators allow for the treatment of fluorescence data as the ratio of fluorescence intensities at two wavelengths, rather than the absolute intensity at a single wavelength. It is this property that makes the application of wavelength shifting calcium indicators less problematic, since fluorescence intensities are dependent on the illumination intensity, emission collection efficiency and indicator concentration. To account for these factors absolute fluorescence intensity values should be corrected, (7) but treating fluorescence data as the ratio of fluorescence intensities at two wavelengths, result in the cancelling of these interfering factors, since these interfering factors have a similar effect on fluorescence intensities at both wavelengths. (4 - 8)

vi)   **The ratio methodology for measuring intracellular free calcium concentrations using the fluorescent calcium indicators**

The ratio methodology for intracellular free calcium measurements using the fluorescent calcium indicators is best explained on the basis of the following equilibrium equation. Fura-PE3 was the fluorescent calcium indicator of choice for the present study for reasons as will be explained in subsequent sections of this chapter. Therefore fura-PE3 is specified in the following equations. Binding of calcium to the indicator molecule can be related to the following equilibrium equation:

$$[Ca^{2+}] \; + \; [FURA\text{-}PE3] \; \Leftrightarrow \; [CaFURA\text{-}PE3]$$
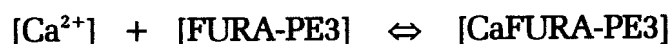
Following from the above equilibrium equation -

- an increase in $[Ca^{2+}]$ will result in an increase in the [CaFURA-PE3] complex and at the same time a decrease in the free [FURA-PE3] indicator

-   a decrease in [Ca^{2+}] will result in a decrease in the [CaFURA-PE3] complex and at the same time an increase in the free [FURA-PE3] indicator

It is clear that an increase in the [CaFURA-PE3] complex is accompanied by a decrease in the free [FURA-PE3] indicator molecules and visa versa. The CaFURA-PE3 complex and the free FURA-PE3 indicator differ in their fluorescence properties. Firstly, both the CaFURA-PE3 complex and the free FURA-PE3 indicator molecules are highly fluorescent, but in all cases the CaFURA-PE3 complex has a quantum efficiency between 1.3 and 2.1 fold higher than the free FURA-PE3 indicator, therefore the CaFURA-PE3 complex is more fluorescent than the free FURA-PE3 indicator. (7) Secondly, the CaFURA-PE3 complex and the free FURA-PE3 indicator differ in their excitation maxima wavelengths. For optimal separation of fluorescence due to the two forms of the indicator, the excitation maxima for the CaFURA-PE3 complex are set at 340nm and the excitation maxima for the free FURA-PE3 indicator are set at 380nm. At every measured time point the fluorescence intensities are obtained at both the wavelengths. At a wavelength of 340nm the fluorescence obtained is proportional to the [CaFURA-PE3] complex and at a wavelength of 380nm the fluorescence obtained is proportional to the free [FURA-PE3] indicator molecules. Following from the explanation above, these fluorescence intensity values obtained at the two different excitation wavelengths change in opposite directions. An increase in the [CaFURA-PE3] complex results in an increase of the fluorescence emission intensity obtained at the 340nm wavelength. At the same time a decrease in the free [FURA-PE3] molecules occurs and thus results in a decrease of the fluorescence emission

intensity obtained at the 380nm wavelength. These two values are represented as a ratio. The fluorescence emission value obtained at the 340nm wavelength was arbitrarily chosen as the numerator and the fluorescence emission value obtained at the 380nm wavelength as the denominator: 340nm/380nm = ratio. Such that the ratio value changes in the same direction as the intracellular free calcium concentration. (7) Thus an increase in $[Ca^{2+}]$ resulting in an increase in the [CaFURA-PE3] complex and a decrease in the free [FURA-PE3] indicator leads to an increase in the ratio value and visa versa. (6)

vii) **Calculation of intracellular free calcium concentrations**

Under conditions of constant illumination and in a fixed chemical and physical environment, the intensity of fluorescence from a molecular species is proportional to its concentration. Thus, fluorescence intensity can be used as a quantitative tool. (6) As explained above, at any ratio value a mixture of the CaFURA-PE3 complex and the free FURA-PE3 indicator exist. The ratio value is obtained by measuring the emitted fluorescence at the two excitation wavelengths: 340nm and 380nm. The total fluorescence intensities at these two wavelengths will be given by the following:

$$F_{340nm} = S_{FURA\text{-}PE3(340nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(340nm)} \cdot C_{CaFURA\text{-}PE3}$$

$$F_{380nm} = S_{FURA\text{-}PE3(380nm)} \cdot C_{FURA\text{-}PE3} + S_{CaFURA\text{-}PE3(380nm)} \cdot C_{CaFURA\text{-}PE3}$$

Where

F = fluorescence intensities

S = proportionality coefficients (the product of excitation intensity, ln10, extinction coefficient, path length, quantum efficiency, and the instrumental efficiency of collecting emitted photons)

C = concentration

However, $C_{FURA-PE3}$ and $C_{CaFURA-PE3}$ are related to $[Ca^{2+}]$ by the above mentioned equation for 1:1 complexation:

$C_{CaFURA-PE3} = C_{FURA-PE3} [Ca^{2+}] / Kd$

Where

Kd = dissociation constant

The fluorescence ratio R is obtained by calculating:

$F_{340nm} / F_{380nm} = S_{FURA-PE3(340nm)} \cdot C_{FURA-PE3} + S_{CaFURA-PE3(340nm)} \cdot C_{CaFURA-PE3} / S_{FURA-PE3(380nm)} \cdot C_{FURA-PE3} + S_{CaFURA-PE3(380nm)} \cdot C_{CaFURA-PE3}$

Thus: $Ratio = (S_{FURA-PE3(340nm)} + S_{CaFURA-PE3(340nm)} \cdot [Ca^{2+}] / Kd) / (S_{FURA-PE3(380nm)} + S_{CaFURA-PE3(380nm)} \cdot [Ca^{2+}] / Kd)$

Solving for $[Ca^{2+}]$ yields the following calibration equation:

$[Ca^{2+}] = Kd [R - (S_{FURA-PE3(340nm)} / S_{FURA-PE3(380nm)})] / [(S_{CaFURA-PE3(340nm)} / S_{CaFURA-PE3(380nm)})-R] [S_{FURA-PE3(380nm)} / S_{CaFURA-PE3(380nm)}]$

In practice the S factors are obtained from the fluorescence intensities of calibration solutions containing known concentrations of the free and the calcium-saturated indicator.
Note that $(S_{FURA-PE3(340nm)} / S_{FURA-PE3(380nm)})$ is simply the limiting value that R can have at zero $[Ca^{2+}]$ and so may be considered Rmin, while $(S_{CaFURA-PE3(340nm)} / S_{CaFURA-PE3(380nm)})$ is the analogous limiting value that R can have at saturating $[Ca^{2+}]$ giving Rmax.

Thus the following Grynkiewicz equation is obtained:

$[Ca^{2+}] = Kd . (R - Rmin / Rmax - R) (S_{FURA-PE3(380nm)} / S_{CaFURA-PE3(380nm)})$

Assumptions:

1) The indicator forms a simple 1:1 complex with calcium
2) The indicator behaves in cells as it does in calibration media
3) The indicator is sufficiently dilute for fluorescence intensity to be linearly proportional to the concentrations of the fluorescent species (6, 7, 12)

- Calibration of the obtained fluorescence values

Ultimately, the obtained fluorescence values should be converted to intracellular free calcium concentrations. As explained above, this can be achieved by solving the Grynkiewicz equation. To be able to solve for intracellular free calcium concentrations, an end of the experiment calibration should be performed. Performing the calibration procedure, results in determination of fluorescence ratio values of the indicator in solutions with known calcium concentrations. In this case: at maximum calcium concentration – 1 mM, and at minimum calcium concentration – after EGTA addition. Both these values are accounted for in the Grynkiewicz equation.

- Autofluorescence

Autofluorescence is not accounted for when using the ratio method, therefore autofluorescence should be subtracted from the fluorescence intensities obtained at both the wavelengths before calculating the ratios. The generated cellular autofluorescence can mostly be attributed to the pyridine nucleotides. (6 - 8)

viii) Processes interfering with the accurate measurement of intracellular free calcium concentrations using the fluorescent calcium indicators

a)   Incomplete hydrolysis of the acetoxy methyl ester bonds

Determination of intracellular free calcium concentrations by the employment of the fluorescent calcium indicators assumes the complete conversion of the AM ester precursor of the indicator to the calcium sensitive fluorescent indicator. According to the Grynkiewicz equation it is from the obtained fluorescence intensities that intracellular free calcium concentrations are determined, therefore any contribution to these fluorescence intensity values from molecular species not directly involved in calcium binding would lead to the inaccurate determination of intracellular free calcium concentrations. Such calcium-insensitive, fluorescent molecular species include unhydrolysed AM ester indicator molecules and/or incompletely hydrolysed indicator molecules. In different cell types the contribution of the different fluorescent molecular species to the total fluorescence has been described. The completely hydrolysed calcium indicator molecule is said to contribute only 11.8% to the total fluorescence in neuroblastoma cells, 7.6% in human pulmonary artery endothelial (HPAE) cells and 66.5% in hepatocytes. Of the total fluorescence the more than 30 potential incompletely hydrolysed molecules resulting from the random cleavage of the five ester bonds appear to contribute 5.1% in neuroblastoma cells, 5.5% in HPAE cells, and 23.7% in hepatocytes. (8) In a separate study involving neutrophils, it was shown that intermediates of the de-esterification process also contribute to the total fluorescence. Nevertheless, washing of the cells in a medium containing BSA, after the completion of the loading process, results in the removal of unhydrolysed and incompletely hydrolysed acetoxy methyl indicator molecules. (6, 14)

b)   Unwanted binding of the fluorescent calcium indicators to cellular constituents

The acetoxy methyl ester derivatives of the calcium indicator are highly lipophilic and therefore can easily bind to cell membranes and escape enzymatic hydrolysis. Even the fully de-esterified indicator or injected free acid form can interact with cellular constituents, be it cell membranes or cellular proteins, resulting in the alteration of their fluorescent properties. As a deterrent of hydrophobic binding to membranes of the indicators via the long chromophore tail, the terminal carboxylate was incorporated into the structure of the fluorescent calcium indicator. (7)

c)   Differences in the uptake and cellular processing of the indicators in various cell types

In the following table a comparison is presented of possible differences in the uptake and processing in three different cell types. These factors which include differences in the rate of hydrolysis of the acetoxy methyl ester bonds and differences in the rate of extrusion of the indicator should be taken into consideration when the fluorescent calcium indicators are employed in the measurement of intracellular free calcium concentrations. (8)

	Murine NIE-115 neuroblastoma cells	Human pulmonary artery endothelial cells	Rat hepatocytes
Indicator precursor concentration	10 micromolar	10 micromolar	10 micromolar
Rate of indicator formation	9.7 pmol/min/10^6 cells	2.6 nmol/min/10^6 cells	77.9 pmol/min/ 10^6 cells
Concentration of the indicator after 60 minutes	137 micromolar	141 micromolar	Peak concentration after 15 min 82 microM
[Indicator] after washing with medium containing 2% BSA	40 micromolar	Almost completely lost	
[Indicator precursor] after wasing with medium containg 2% BSA	90 micromolar	58 micromolar	
Half-time loss after washing	34 minutes	74 minutes	

d)   Sequestration of the indicator into intracellular organelles

Fluorescent calcium indicator molecules sequestered in intracellular organelles are insensitive to the changes in the free calcium concentration in the cytosol. (12) More importantly these sequestered indicator molecules report the higher calcium concentration present in intracellular organelles. Therefore a prerequisite for employing the fluorescent calcium indicators as probes of cytosolic free calcium concentrations, is the homogenous distribution of the indicator throughout the cytosol without any sequestration of the indicator into intracellular

organelles. (15) Independent studies involving different cell types indicated three possible mechanisms resulting in the sequestration of the fluorescent calcium indicators into intracellular organelles:

- **Crossing of intracellular organelle membranes by the lipophilic acetoxy methyl ester derivative**

Not all cell types contain sufficient cytosolic esterase activity to rapidly cleave the acetoxy methyl ester bonds and produce the membrane impermeable penta-anion form of the indicator. This can result in the movement of the acetoxy methyl ester derivative across the membranes of intracellular organelles. Once inside the intracellular organelle the acetoxy methyl ester bonds can be hydrolised, since many intracellular organelles such as the secretory granules contain esterase activity. The hydrolysis of the ester bonds results in the subsequent entrapment of the membrane impermeable indicator in the organelle. (12, 16)

- **Endocytosis as the means of sequestering the fluorescent calcium indicator into intracellular organelles**

Human skin fibroblasts (HSF) and 3T3 fibroblasts loaded with the membrane permeable acetoxy methyl ester (AME) derivative displayed fluorescent spots concentrated in the perinuclear region occupied by the membrane bound acidic vesicles, lysosomes. It is suggested that the means of indicator accumulation is by endocytosis followed by the subsequent hydrolysis of the acetoxy methyl ester derivative and entrapment of the indicator, since these cells – HSF and 3T3 fibroblasts – with high endocytic activity show greater accumulation of fluorescent spots in the perinuclear region than cells with less endocytic activity. (15)

- **Sequestration of the fluorescent calcium indicator into intracellular organelles by an anion transporter**

There are indications that fluorescent calcium indicator sequestration in mouse peritoneal macrophages, J774 cells, PC12 cells, and N2A cells are mediated by an organic anion transport system, since this sequestration of the fluorescent calcium indicators can be blocked by the anion transport inhibitors, probenecid and sulfinpyrazone. In a study involving the J774 macrophage cell line, the indicator was introduced into the cells either as the acetoxy methyl ester or penta-anion forms of the indicator. In both cases, sequestration of the indicator into membrane-bound cytoplasmic vacuoles were detected 90 minutes after the onset of the loading procedures, and in both these cases sequestration could be blocked by the organic anion transport inhibitor, probenecid. (17) The concentrations of these agents necessary to effect near-maximal inhibition of the unwanted transport of the indicator in J774 cells were 5mM probenecid and 2.5 mM sulfinpyrazone. Incubation of macrophages in medium containing probenecid for as long as 3h apparently has no untoward effects on cell viability, although resting intracellular free calcium could be about 20% higher than in macrophages incubated without probenecid. Importantly, the transient increase in calcium induced by agonists such as platelet activating factor or by phagocytosis of IgG-coated erythrocytes is not affected. Other cells, i.e., PC12 rat pheochromocytoma cells and N2A mouse neuroblastoma cells incubated in probenecid, indicated a reduced rise in intracellular free calcium of 50% triggered by depolarisation of the plasma membrane and by the neuropeptide bradykinin. (17, 18)

e) Leakage of the penta-anion form of the fluorescent calcium indicator into the extracellular medium

The penta-anion form of the calcium indicators can actively be extruded from cells by an anion transporter. In the spectrofluorometer cuvette, leakage of the penta-anion form of the calcium indicator out of the cell into the extracellular medium would result in binding of the calcium indicator with extracellular calcium ions and the measurement of inaccurate cytosolic calcium concentrations. This process can be inhibited by the anion transport inhibitors, probenecid or sulfinpyrazone or by the lowering of the temperature from 37°C to 30-34°C. Extrusion of the calcium indicator can also be circumvented by the attachment of the indicator to a macromolecule such as dextran. (4, 12, 15, 17)

f) Photobleaching of the fluorescent calcium indicators

A decline in fluorescence intensities due to photobleaching is typical of fluorescent molecules and the fluorescent calcium indicators are not immune to this photobleaching effect. It was shown that exposure of the fluorescent calcium indicators to the excitation light, whether in cells or in calibration solutions, resulted in photobleaching. It was initially assumed that bleaching was functionally equivalent to decreasing the concentration of indicator, and that the ratio value would not be affected since the fluorescence intensities comprising the ratio are influenced to the same extent. (19) This assumption is incorrect, since in certain circumstances, photobleaching results in the formation of a calcium indicator intermediate that is still fluorescent but not sensitive to calcium over the same range as the completely hydrolysed fluorescent calcium indicator. The formation of such fluorescent calcium indicator intermediates

with altered calcium binding affinities violates the assumption on which the ratio method of calibration is based, that is, that the only fluorescent species present are the calcium-bound and the free anion forms.

g) Different fluorescence properties of the calcium indicator in the cytosolic milieu as compared to standard calibration solutions

There are indications of spectral differences between the indicator present in the cytosol and the indicator present in standard calcium concentration solutions. This may be the result of the actual binding of the indicator to cellular constituents or effects of the alterations in ionic strength, hydrogen ion concentration, viscosity and lipid and protein buffering of calcium intracellularly, therefore the in situ Kd may vary greatly from the in vitro Kd. If this is the case, the Kd of the fluorescent calcium indicator should be determined in situ. To accomplish this the intracellular free calcium concentration should be clamped to known values and the fluorescence ratio values measured subsequently. In short, the procedure involves the attainment of extracellular medium calcium, set to a known calcium concentration via the right combination of EGTA/calcium solutions, which subsequently must be equilibrated with the intracellular calcium concentration. The strategy for equilibration involves the utilisation of a divalent cation ionophore to elevate inherent cellular calcium leakage to such an extent that calcium pump activity is overcome. Two ionophores, ionomycin and 4-bromo-A23187, display high selectivity for calcium over other divalent cations. Ideally, after equilibration, extracellular and intracellular calcium concentrations are identical. (6, 7, 20 - 23)

ix) Available fluorescent calcium indicators / advantages and disadvantages

Quin-2

Advantages: 1) Hydrolysis of quin-2/AM seems easier, reaches higher cytosolic concentrations of the indicator and is less often complicated by compartmentalisation into membranous compartments than is observed with acetoxy methyl esters of higher molecular weight and lesser H_2O solubility

2) Low Kd for calcium – well suited for resolving resting calcium concentrations

Disadvantages: 1) Does not show a usable wavelength shift, therefore calibration of fluorescence intensities calls for the standardisation of absolute fluorescence intensities which is directly dependent on indicator concentration, specimen thickness, and instrumental factors (24)

2) Poor discrimination of calcium over magnesium, Kd for magnesium: 1mM

3) Low Kd for calcium, limiting the maximum measurable calcium concentration to about 1 µmolar, since the indicator approaches saturation and looses resolution

4) Severe photobleaching

5) Interference by heavy metal ions and pH

6) Responds to other divalent ions besides calcium

7) Relatively low quantum yield for the calcium-quin-2 complex, therefore require cellular concentrations of the indicator in the millimolar range and thus intracellular calcium buffering can be

substantial, resulting in the buffering of intracellular calcium transients

8) Unsuitable for single cell studies (13)

Fluo-3   **Advantages**: 1) Excitation at visible wavelengths rather than near UV wavelengths, this permits the use of these indicators in cell types that routinely exhibit significant levels of background fluorescence

2) Very large enhancement in fluorescence intensity about 40-fold upon binding calcium

3) Significantly weaker calcium affinity, Kd 400nM, permitting measurement of intracellular free calcium concentrations of up to 5-10 μmolar

Disadvantages: 1) Negligible wavelength shifts in either excitation or emission spectra, so that fluo-3 like quin-2 is limited to intensity changes without wavelength pairs to ratio

2) Difficult calibration: treatment of the cells with ionophore, heavy metals, and/or detergent at the end of every experiment (4)

Fura-2   The most widely used of the fluorescent calcium indicators and is the indicator of choice for imaging studies

Advantages: 1) 30-fold brighter fluorescence, thus smaller concentrations of the indicator is necessary to be introduced into the cells, which results in a smaller calcium-buffering effect (4, 6 - 8, 17), fura-2 – micromolar, quin-2 – millimolar

2) Lower concentration of indicator, as low as 1µM – less acidity and toxicity due to either indicator itself or acetic acid and formaldehyde by-products

3) Large spectral wavelength shifts upon calcium binding (7)

4) Lower affinities for calcium, can measure intracellular free calcium levels up to several micromolar (6)

5) Improved selectivity for calcium over magnesium, manganese, zinc and iron (6, 7, 17)

6) One to one stoichiometry upon formation of the complex with calcium (5)

7) Negligible binding to membranes (13)

8) Very much more resistant to photodestruction than quin-2

9) Extra heterocyclic bridge to reinforce the ethylenic bond of the stilbene and to reduce hydrophobicity

Disadvantages: 1) Propensity to enter subcellular organelles such as the sarcoplasmic reticulum, nucleus and secretory vesicles

2) Possibility that some of the fluorescent product or starting material remains in the cellular membranes (25)

Indo-1   **Advantages**: 1) Has a rigidised stilbene fluorophore with reduced hydrophobic characteristics, like fura-2, due to the extra heterocyclic bridge to reinforce the ethylenic bond of the stilbene

2) Has the unique property that its emission, not just its excitation spectrum shifts to shorter wavelengths when the molecule binds calcium

3) Less compartmentalisation than with fura-2

Disadvantages: 1) The blue and violet wavelengths of indo-1 emission overlap cellular autofluorescence from pyridine nucleotides more severely than the green of fura-2

2) Indo-1 bleaches several fold faster than fura-2 (4)

2.2) Experimental evaluation of the technique used in the present study

It was deemed necessary to evaluate various aspects of the technique employed because:

1. initial studies indicated unreliable values for both basal intracellular free calcium and the magnitude of the transmembrane calcium flux in both the healthy subjects group and the patients group

2. there are many processes interfering with the accurate measurement of intracellular free calcium as discussed in the previous section

3. there are studies involving MHT patients, which indicate inconsistencies in the values published results for basal intracellular free calcium in the neutrophil.

2.2.1) Materials and methods

a) Isolation of the neutrophils

The intracellular free calcium determinations were performed in the neutrophil.   The neutrophils were isolated from ACD-anticoagulated (acid citrate dextrose) whole blood according to a modification of the method of Böyum. (26, 27) In short, ACD-anticoagulated whole blood was loaded onto a layer of hypaque-ficoll. The various blood cells were

separated into different layers according to densities. The polymorphonuclear leucocytes formed a layer on top of the red cell mass in the bottom of the tube. The contaminating red cells were lysed with an ammonium chloride solution. The polymorphonuclear leucocyte layer contained the neutrophils, eosinophils and basophils. Since eosinophils contribute only 0.5-6% and basophils only 0-1% of the total amount of circulating white blood cells (28) no further separation of the neutrophils were performed from the other polymorphonuclear leucocytes. Finally the neutrophils were washed and suspended in Hanks balanced salt solution.

b) Loading of the fluorescent calcium indicators

The suspension of neutrophils was loaded with the calcium indicators. In short the neutrophil suspension was incubated with the acetoxy methyl ester form of the indicators. During this incubation period the acetoxy methyl ester form of the indicator traverses the cell membrane where after the acetoxy methyl ester bonds are hydrolysed. Upon the hydrolysis of these bonds trapped penta-anion indicator molecules are generated. The neutrophil suspension was washed and suspended in Hanks balanced salt solution.

c) Fluorescence intensity determinations

The fluorescence intensity determinations were performed on the Perkin Elmer LS-50B Luminescence spectrometer containing a Xenon lamp excitation source.

2.2.2) Initial results according to the method

In the initial study intracellular free calcium and transmembrane calcium fluxes were performed in the neutrophils of the MHT patient versus race, sex and age matched healthy controls. Results: intracellular free calcium and transmembrane calcium fluxes in nM.

Combination	Patient		Race, age and sex matched control	
	$[Ca^{2+}]_i$	Ca^{2+} flux	$[Ca^{2+}]_i$	Ca^{2+} flux
1	89 nmolar	406 nmolar	49 nmolar	461 nmolar
2	62 nmolar	408 nmolar	87 nmolar	246 nmolar
3	60 nmolar	322 nmolar	86 nmolar	310 nmolar
4	90 nmolar	437 nmolar	91 nmolar	490 nmolar
5	110 nmolar	578 nmolar	89 nmolar	379 nmolar
6	86 nmolar	435 nmolar	86 nmolar	272 nmolar
7	154 nmolar	403 nmolar	169 nmolar	466 nmolar
8	56 nmolar	300 nmolar	89 nmolar	379 nmolar

According to these results, no consistencies were indicated for intracellular free calcium values in the control group. In combination 7 both the patient's and control subject's intracellular free calcium were unexpectedly high. Since these two determinations were performed on the same day this indicated a possible experimental flaw.

2.2.3) In search of the most suitable fluorescent calcium indicator for intracellular free calcium measurements in the neutrophil

In the present study intracellular free calcium and transmembrane calcium fluxes were determined in the neutrophil in suspension. There are many fluorescent calcium indicators available for the determination of intracellular free calcium of cells in suspension. The most common of these fluorescent calcium indicators for cuvette-based measurements is fura-2, although the use of fura-2 can be problematic in some cell types. The major problem associated with the employment of fura-2 for the measurement of intracellular free calcium is fura-2's propensity to leak out of the cytosol into the extracellular medium. In this study fura-2 was initially used according to many published intracellular free calcium studies where fura-2 was employed. A new fluorescent calcium indicator became available, namely fura-PE3 which is claimed to be less

problematic than fura-2 because of fura-PE3's resistance to leakage. Both these indicators were evaluated for their ability to measure intracellular free calcium in the neutrophil.

2.2.4) The following evaluations were performed

i)   Evaluation of loading procedures

                    With BSA

                    Without BSA

ii)   Evaluation of loading procedures

                    With agitation

                    Without agitation

iii) Determination of acetoxy methyl ester derivative concentrations in the loading medium in order to attain sufficient quantities of the indicator in the cytosol for calcium measurements

                    Fura-2

                    Fura-PE3

iv)   Comparison of determined intracellular free calcium concentrations in neutrophil populations with different quantities of the fluorescent calcium indicator in the cytosol

v)   Determination of the extent of leakage via the nickel quench technique for the indicators fura-2 and fura-PE3

vi)   Determination of the extent of the inaccurately determined intracellular free calcium concentration as a result of leakage of the indicator into the extracellular medium

vii) Characterisation of the leakage process

viii) Investigation of the possible interference from heavy metals in the cytosol with the determination of intracellular free calcium

ix) Evaluation of the calibration procedures

x) Evaluation of the reproducibility of the technique for the determination of intracellular free calcium

xi) Evaluation of the sensitivity of the technique for the determination of a change in intracellular free calcium

2.2.5) Experimental evaluations

i) Evaluation of the loading procedures – with and without BSA

Bovine serum albumin (BSA) acts as a dispersing agent and assures the availability of the indicators for crossing of the membrane. An example of fura-2 loading with and without BSA is presented.

- Loading medium without BSA - Refer to figure 1, red and blue graphs
- Loading medium containing 0.25% BSA – Refer to figure 1, purple and green graphs
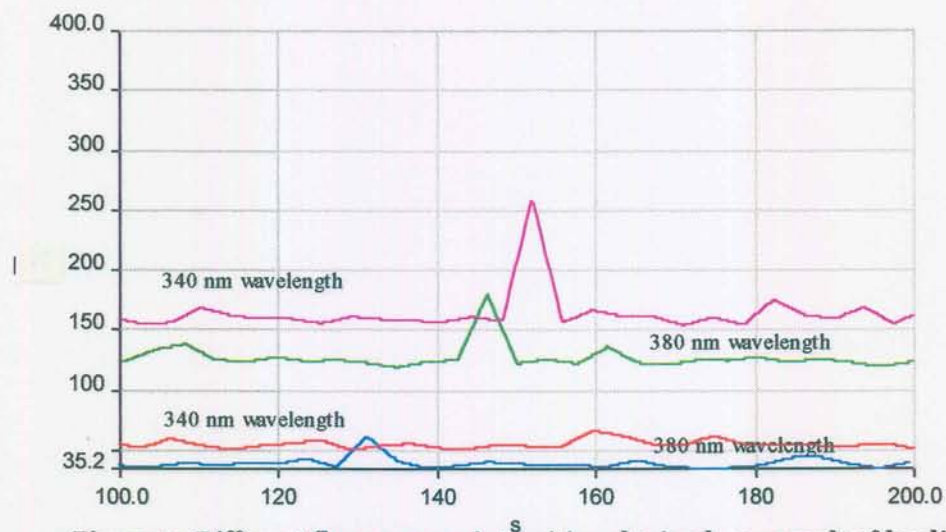
Figure 1. Different fluorescence intensities obtained as a result of loading with and without BSA

The fluorescence intensities obtained after loading in a medium without BSA were found to be equal to background fluorescence intensities resulting from cellular constituents which indicated no accumulation of the indicator in the cytosol, refer to figure 1; red and blue graphs. An increase in the fluorescence intensities well above background fluorescence was obtained when loaded in a medium containing 0.25% BSA, refer to figure 1; purple and green graphs.

ii)   **Evaluation of the loading procedures - with and without agitation**

To facilitate the ester derivative of the indicators in crossing of the cell membrane the neutrophil suspension was continuously agitated, a shaking water bath was employed. An example of fura-2 loading with and without agitation is presented.

-      Neutrophil suspension continuously agitated during the loading period – Refer to figure 2, purple and green graphs

-      Neutrophil suspension kept still for the duration of the loading period - Refer to figure 2, red and blue graphs
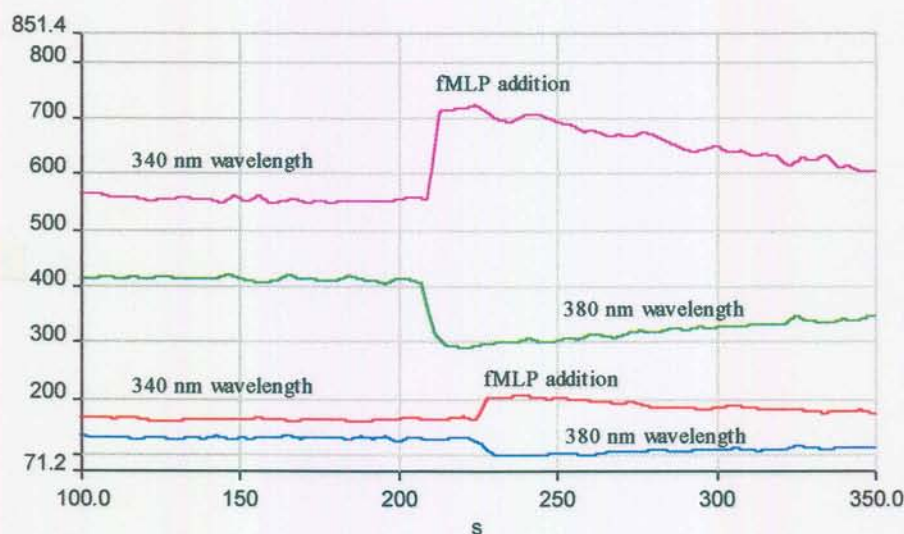
Figure 2. Different fluorescence intensities obtained as a result of continuous agitation or no agitation during the loading period

The fluorescence intensities obtained were higher when the neutrophil suspension was continuously agitated during the loading period, refer to figure 2; green and purple graphs. The red and blue graphs represent the fluorescence intensities obtained when the indicator was loaded without agitation.

iii)   The concentration of the acetoxy methyl ester form of the indicator in the loading medium and the incubation time and temperature for loading of fura-2/AM and fura-PE3/AM

The fluorescent calcium indicators were introduced into the cytosol of the neutrophil as the membrane permeant acetoxy methyl ester derivatives of the indicators. Different cell types vary in their ability to accumulate the fluorescent calcium indicators due to different cytosolic esterase activities, therefore the loading protocols were determined on a trial and error basis. The following protocols were sufficient for loading of the calcium indicators fura-2 and fura-PE3.

<u>Fura-2</u>

-     Concentration of fura-2/AM in the loading medium 5μM and % DMSO 0.05 %

-     Incubation: 37°C for 10 minutes in a shaking water bath and for a further 20 minutes at room temperature

-     Refer to figure 3
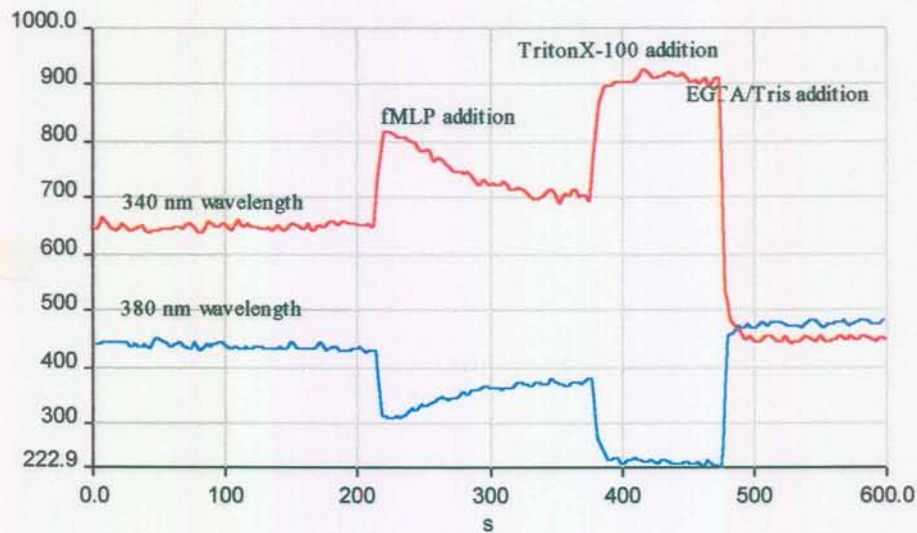
Figure 3. Determination of intracellular free calcium by employing fura-2

The lowest fluorescence intensity obtained from either excitation at the 340 nm or the 380 nm wavelength was well above 100 to be able to subtract background fluorescence. Refer to figure 3.

The concentration of the loaded neutrophils in the cuvette was chosen such that the fluorescence intensity increase upon Triton X-100 addition did not fall outside the limits for instrument detection. Refer to figure 4. Upon Triton X-100 addition the fluorescence intensities obtained for the 340 nm wavelength was outside the range for instrument detection.
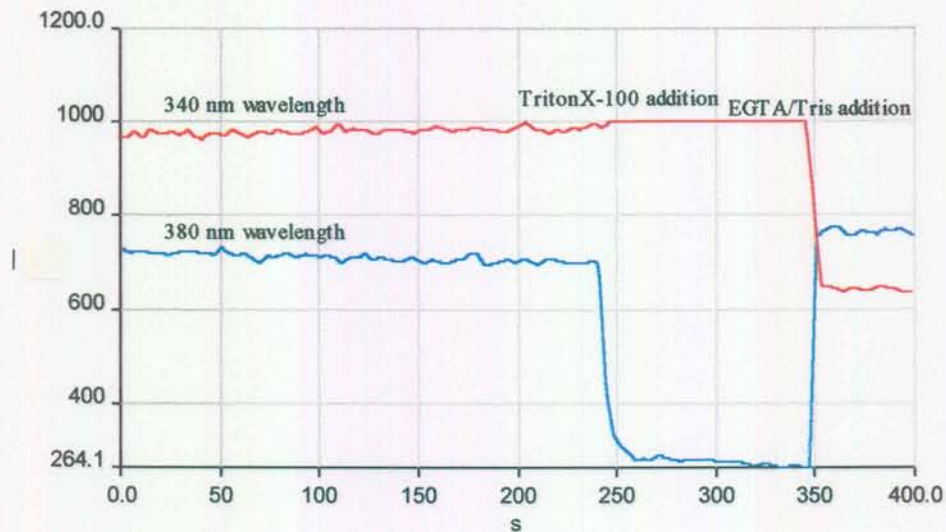
Figure 4. Too high fluorescence intensities obtained for instrument detection

According to the above results the working concentration of neutrophils in the cuvette was determined: 2 to 9 x 10^5 neutrophils/ml for fura-2.

Fura-PE3

- Concentration of fura-PE3/AM in the loading medium 2µM and the % DMSO 0.025%
- Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature
- Refer to figure 5
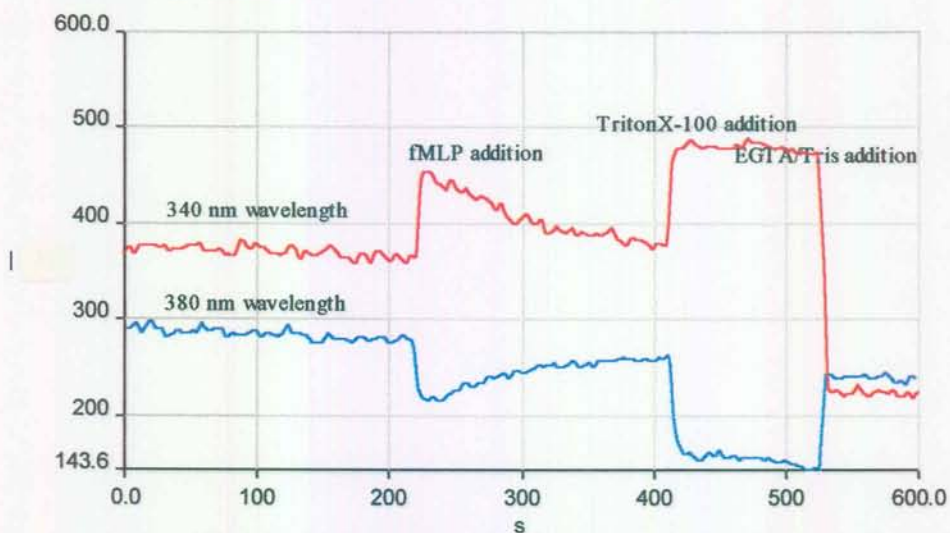
Figure 5. Determination of intracellular free calcium by employing fura-PE3

As for fura-2 evaluations were performed (not shown) in order to determine the working concentration of neutrophils in the cuvette: $9x10^5$ to $1x10^6$ neutrophils/ml for fura-PE3. This concentration range of neutrophils ensured that the maximum obtained fluorescence intensities did not fall outside instrument detection limits and that the minimum fluorescence intensities obtained were high enough to be able to subtract background fluorescence.

iv)   Comparison of determined intracellular free calcium concentrations in neutrophil populations with different quantities of the fluorescent calcium indicator in the cytosol

- **The obtainment of different cytosolic indicator quantities**

1)   Concentration of fura-PE3/AM in the loading medium 2μM

-    Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature

-    Concentration of neutrophils in the cuvette $9x10^5$/ml

-    Refer to figure 6, red and blue graphs

2)   Concentration of fura-PE3/AM in the loading medium 6μM

-    Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature

-    Concentration of neutrophils in the cuvette $9x10^5$/ml

-    Refer to figure 6, purple and green graphs
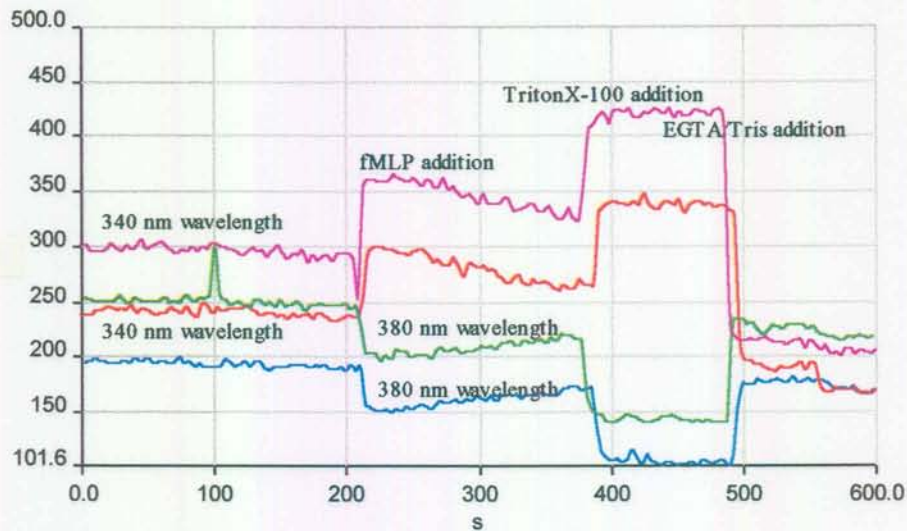
Figure 6. Different fluorescence calcium indicator quantities obtained as a result of different acetoxy methyl ester derivative concentrations in the loading medium

Different fluorescence intensities were obtained for these two protocols indicating different quantities of the indicator in the cytosol. Loading with the higher concentration of fura-PE3/AM resulted in the obtainment of higher quantities of the indicator in the cytosol (refer to figure 6; purple and green graphs) and loading with the lower concentration of fura-PE3/AM in lower quantities of the indicator in the cytosol (refer to figure 6; red and blue graphs).

- **The obtainment of similar intracellular free calcium concentrations**

Calibration of the previously obtained fluorescence intensities followed by the calculation of intracellular free calcium concentrations gave similar results. Refer to figure 7.
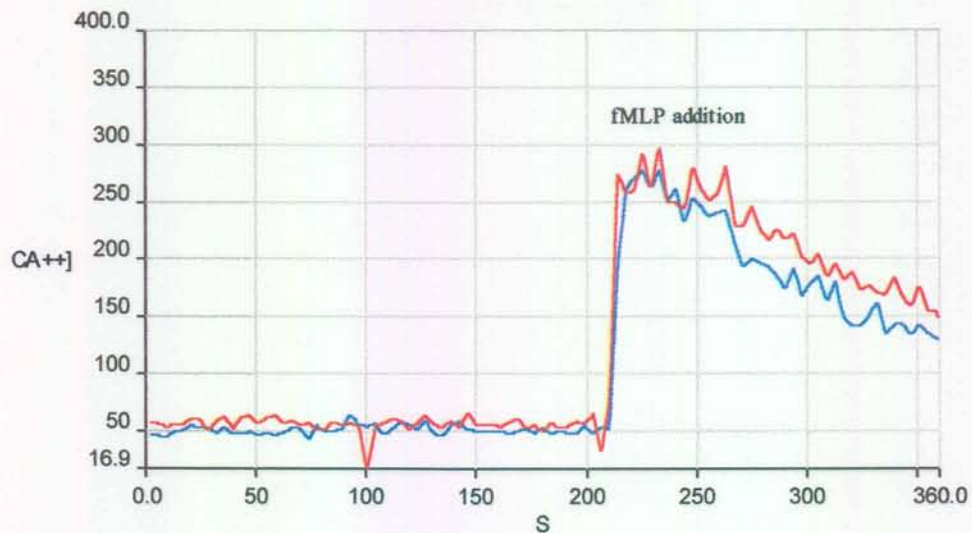
Figure 7. Similar intracellular free calcium concentrations obtained

The red graph (figure 7) represents the intracellular free Ca^{2+} concentration obtained from the purple and green graphs (figure 6). The blue graph (figure 7) represents the intracellular free Ca^{2+} concentration obtained from the red and blue graphs (figure 6). The red and blue graphs (figure 7) are identical. Therefore, different indicator quantities result in the determination of identical intracellular free Ca^{2+} concentrations.

v) **Determination of the extent of leakage via the nickel quench technique for the indicators fura-2 and fura-PE3**

Due to the initial results and the many published studies indicating leakage of the indicators in different cell types, it was deemed necessary to determine the leakage rate for both the indicators in the neutrophil. Determinations of the rate of leakage across the cell membrane into the extracellular medium were performed via the nickel quench technique. Nickel quenched the fluorescence output of the available indicator molecules in the extracellular medium, therefore a decrease in the measured ratio values upon nickel chloride addition were indicative of

the quantity of fluorescent calcium indicators present in the extracellular medium due to leakage from the cytosol. The extent of leakage of the two indicators were determined and subsequently compared. The protocol for loading of fura-2 was changed to the protocol for loading of fura-PE3 in order that all the conditions were the same for the two indicators. Loading of fura-2 according to the protocol for fura-PE3 still resulted in sufficient quantities of the indicator in the cytosol.

<u>Fura-2</u>

- Incubation: 37°C for 30 minutes in a shaking water bath and for a further 30 minutes at room temperature

- Washing and resuspension - 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes

- Working medium: the same as the washing medium, incubate on ice for a further 2 minutes and for another 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

- Refer to figure 8, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.26 to 1.21 was obtained
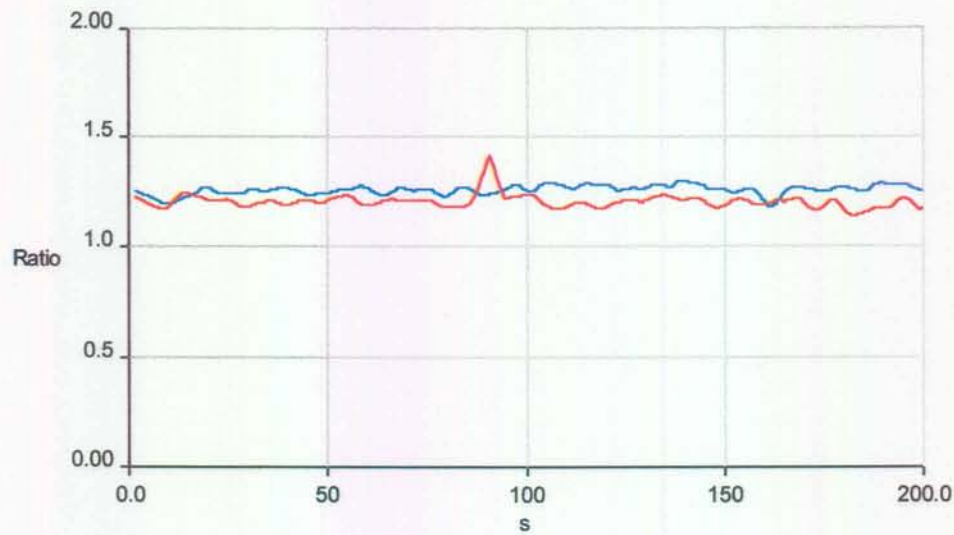
Figure 8. Decrease in the ratio value upon nickel quenching of fura-2 molecules in the extracellular medium

- A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements
- Refer to figure 9, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.36 to 1.26 was obtained
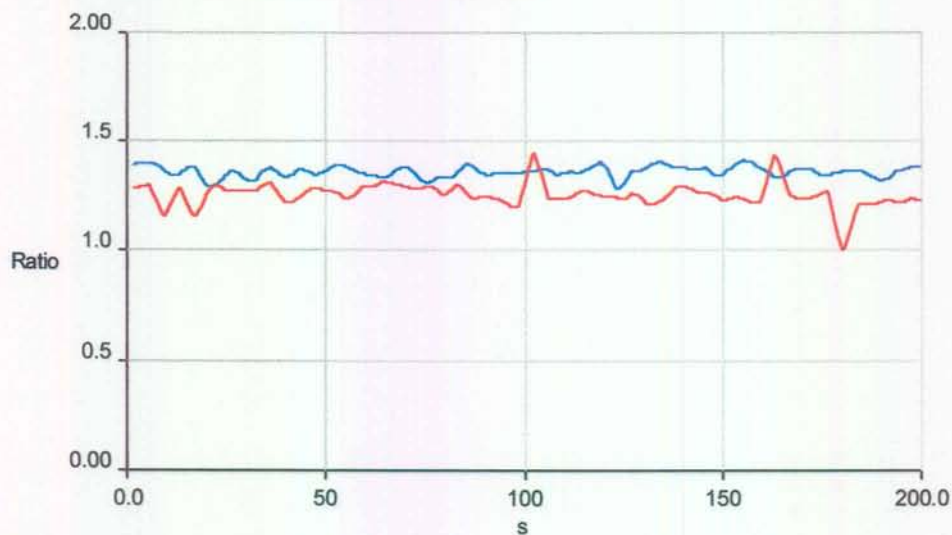
Figure 9. Decrease in the ratio value upon nickel quenching of fura-2 molecules in the extracellular medium

Fura-PE3

-   Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature

-   Washing and resuspension – 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes

-   Working medium: the same as the washing medium, incubate on ice for 2 minutes and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

-   Refer to figure 10, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.19 to 1.17 was obtained
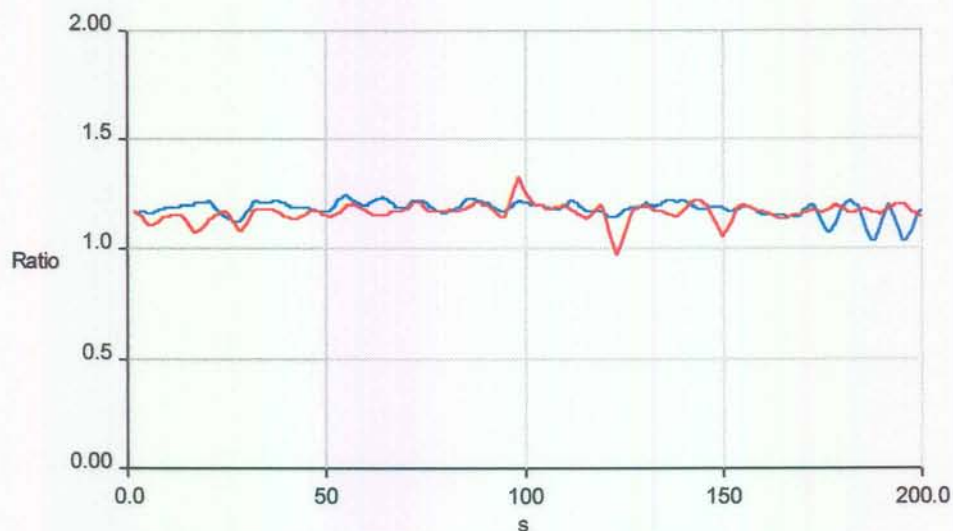
Figure 10. Decrease in the ratio value upon nickel quenching of fura-PE3 molecules in the extracellular medium

-   A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

-   Refer to figure 11, blue graph and after nickel quenching – red graph. A decrease in the ratio value upon nickel chloride addition from 1.26 to 1.17 was obtained
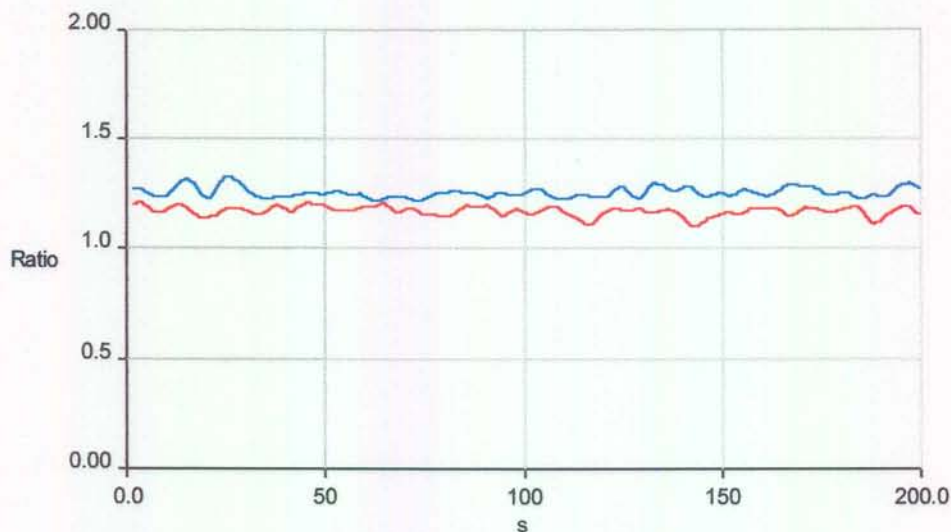
Figure 11. Decrease in the ratio value upon nickel quenching of fura-PE3 molecules in the extracellular medium

In summary: Ratio values before and after nickel chloride addition

	Before	After	Difference
Fura-2: 2 min on ice	1.26	1.21	0.05
Fura-PE3: 2min on ice	1.19	1.17	0.02
Fura-2: 50 min on ice	1.36	1.26	0.1
Fura-PE3: 50 min on ice	1.26	1.17	0.09

Both the indicators leak from the cytosol into the extracellular medium. Nevertheless, fura-PE3 to a lesser extent than fura-2 and more so during the 2 minute incubation period than during the 50 minute incubation period.

vi)   **Determination of the extent of the inaccurately measured intracellular free calcium concentration as a result of the leakage of the indicator into the extracellular medium**

Calibration of the measured fluorescence intensities cannot be performed in the presence of nickel chloride, therefore a separate set of experiments was performed to determine the extent of the inaccurately determined intracellular free calcium concentration represented by the indicator extruded into the extracellular medium. Fura-PE3 was the indicator of choice according to the previously obtained results due to less leakage from the cytosol compared to fura-2, and was therefore used in the subsequent investigations.

-   Incubation: 37°C for 30 minutes in a shaking water bath and a further 30 minutes at room temperature

-   Washing and resuspension – 2 x Hanks medium containing BSA at 1200 r.p.m. 12°C for 10 minutes

-   Working medium: the same as the washing medium, incubate on ice for 2 minutes and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

-   Determine the intracellular free calcium concentration at the exact time point coinciding with the nickel chloride addition as for the previous set of experiments

-   Refer to figure 12, blue graph

-   A separate aliquot of the same lot of cells was incubated on ice for a further 50 minutes after washing away of the loading medium, and for a further 2 minutes at 37°C in the cuvette before commencing with the fluorescence intensity measurements

-   Determine the intracellular free calcium concentration at the exact time point coinciding with nickel chloride addition as for the previous set of experiments

- Refer to figure 12, red graph
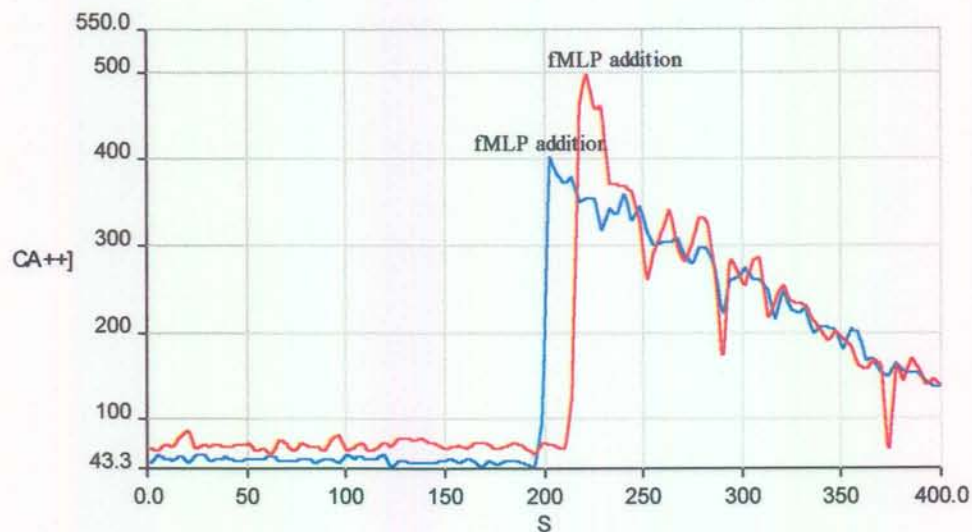
Figure 12. The extent of the inaccurately determined intracellular free calcium concentrations as a result of fluorescent calcium indicator leakage

In figure 12 the red and blue graphs represent the intracellular free Ca^{2+} concentrations determined for either after the 2 minute incubation period (blue graph) or after the 50 minute incubation period (red graph). The difference in the intracellular free Ca^{2+} concentration between these two graphs can be seen, this represent the inaccurately determined intracellular free Ca^{2+} as a result of leakage of the indicator during the 50 minute incubation period.

vii)   Characterisation of the leakage process

• **The leakage process is temperature dependent**

A series of fluorescence intensity measurements was obtained at different temperature settings for a single cell suspension aliquot. The ratio values were calculated and the results plotted. Refer to figure 13 – the six ratio value graphs were obtained with a time interval of 20 minutes between measurements. The bottom two graphs – the red and blue graphs were obtained at 25°C. The following green graph was

obtained at 30°C. The remaining three graphs were obtained at 37°C with the time interval still being 20 minutes. An increase in the leakage rate occurred with an increase in temperature.
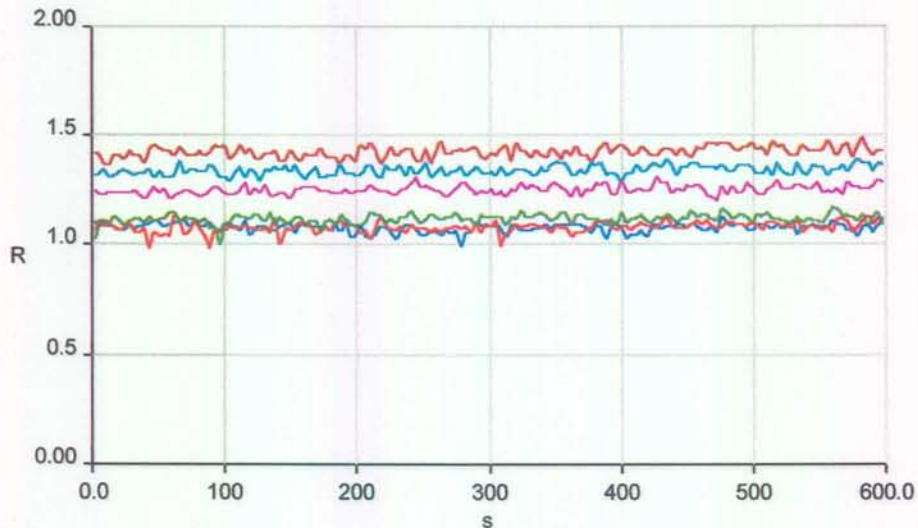
Figure 13. An increase in the rate of indicator leakage due to a rise in temperature

- **The leakage process is independent of magnetic bar stirring in the cuvette**

A series of fluorescence intensity measurements was obtained at different temperature settings and the ratio values calculated. Refer to figure 14 – nine ratio value graphs were obtained with a time interval of 20 minutes between measurements. The bottom four graphs were obtained from the first cell suspension aliquot – the red and blue graphs at 30°C and the green and pink graphs at 37°C. The following three graphs were obtained from a second cell suspension aliquot kept on the water bath and not continuously stirred. The top two graphs were obtained from a third cell suspension aliquot kept on the water bath and not continuously stirred. An increase in the leakage rate not different from the first cell suspension aliquot in the cuvette occurred for both the

cell suspension aliquots kept on the water bath. Therefore, the leakage process is not accelerated as a result of agitation of the neutrophils.
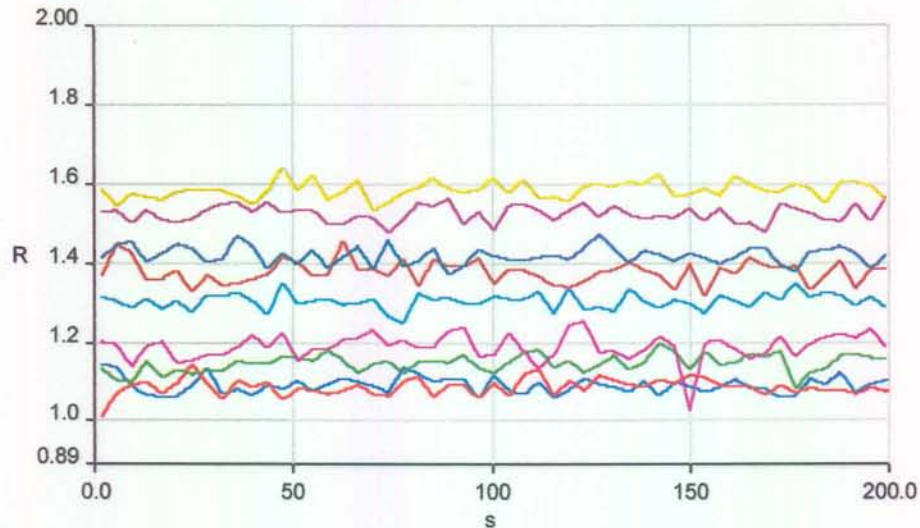
Figure 14. Comparison of the rates of the leakage process for the fluorescent calcium indicator as a result of magnetic bar stirring

• **Determination of the possible indicator leakage occurring during a 1000s period**

Fluorescence intensity measurements were performed for 1000 seconds. During this period no significant amount of leakage occurred. Refer to figure 15. The duration of the subsequent fluorescence determinations in the neutrophils for both patients and healthy subjects were less – 650 seconds in total. Therefore leakage of the indicator could not have interfered with the fluorescence intensity measurements for the duration of the experiment.
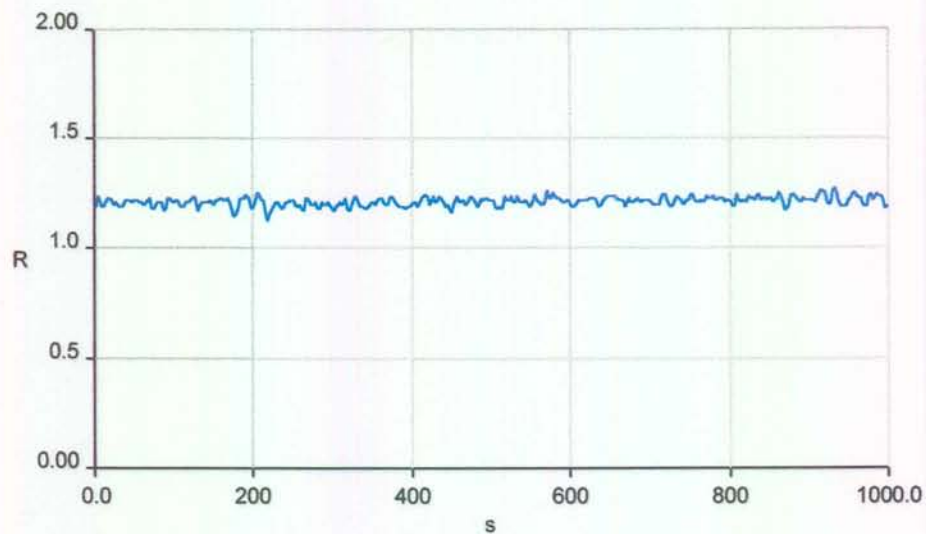
Figure 15. **Constant ratio values during a similar time period as for intracellular free calcium determinations in the patients and control subjects**

viii) Investigation of the possible interference from heavy metals present in the cytosol with the determination of intracellular free calcium

Possible heavy metal ion quenching of the calcium indicator was investigated in the neutrophils of both patients and healthy subjects. This was achieved by the addition of TPEN (N, N, N', N', - tetrakis(2-PYRIDYLMETHYL)ETHYLENEDIAMINE) at a concentration of 20µM during the measurement of the fluorescence intensities. A decrease in the ratio value would indicate heavy metal ion quenching of the indicator. No heavy metal ion quenching was detected.

ix) Evaluation of the calibration procedure

Intracellular free calcium concentrations were determined by solving the Grynkiewicz equation – refer to section on the theoretical background of the technique. This equation contains two calibration points – the ratio value obtained at maximum calcium and the ratio value obtained at minimum calcium. At the end of the experiment the calibration procedure is performed. The fluorescence ratio value for maximum

calcium is determined by the addition of TritonX-100 to the cell suspension. The addition of TritonX-100 results in the breakdown of the cell membranes and the subsequent releasing of the indicator into the medium containing a calcium concentration of 1mM. Fluorescence intensity determinations are continuously performed. After the addition of TritonX-100 the fluorescence ratio values at maximum calcium concentration are determined for a period of 100 seconds. At the completion of this period EGTA/Tris is added. EGTA chelates the available calcium ions resulting in attainment of fluorescence ratio values at minimum calcium concentration. Refer to figure 16.
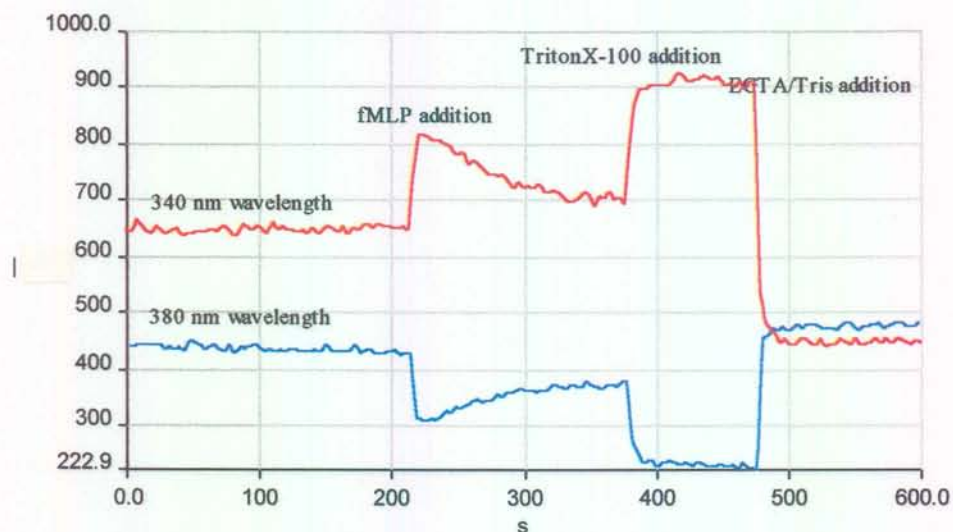
Figure 16. **An end of the experiment calibration procedure, the calcium indicator is released into the extracellular medium where the calcium concentration is subsequently manipulated**

For this end of the experiment calibration procedure the indicator is released into the extracellular medium where the calcium concentration is manipulated. There are indications that the fluorescence properties of the indicators are influenced by the characteristics of the medium, i.e., viscosity and ion concentrations. The cytosol differs from the extracellular medium in this regard therefore the release of the indicator into the extracellular medium might result in a change in fluorescence

ratio values independent of a change in the calcium concentration. If this is the case the calibration of the fluorescence ratio values should be performed whilst the indicator is still in the cytosol. This possibility was investigated. Intracellular free calcium concentrations should be manipulated in order to calibrate the fluorescence ratio values in situ. This was achieved by the addition of calcium ionophores to the neutrophil suspension.

- **Manipulation of intracellular free calcium concentrations in order to calibrate the fluorescence ratio values in situ – a suitable calcium ionophore**

The calcium ionophore A23187

Addition of the ionophore A23187 at a concentration of 40 μM resulted in the obtainment of fluorescence intensity values above the limits for instrument detection and obscuring of the calcium indicator's fluorescence signals, since the calcium ionophore A23187 is highly fluorescent at an excitation wavelength of 340nm and 380nm. Refer to figure 17.
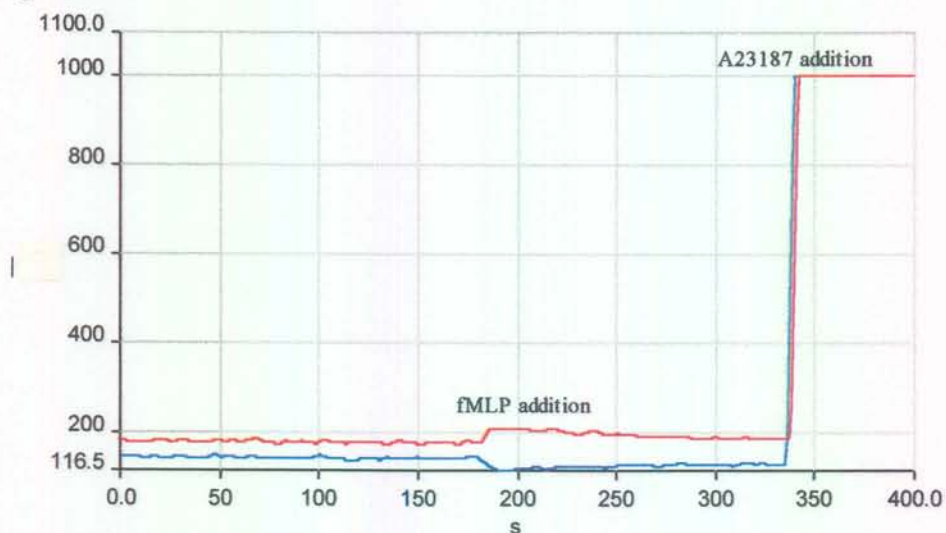
Figure 17. The addition of the calcium ionophore A23187 in order to equilibrate the intracellular free calcium with extracellular medium calcium

At a concentration of 2 µM the ionophore's fluorescence intensities were significantly less but still large enough to obscure the indicator's fluorescence signals. Refer to figure 18.

Figure 18. The addition of the calcium ionophore A23187 in order to equilibrate intracellular free calcium with extracellular medium calcium

At a lower ionophore concentration the ability to rapidly equilibrate extracellular calcium with cytosol calcium was abolished.

The calcium ionophore: ionomycin

The addition of ionomycin to a final concentration of 12.5 µM resulted in the immediate elevation of the fluorescence ratio value and thus an increase in the cytosolic free calcium concentration. Refer to figure 19.
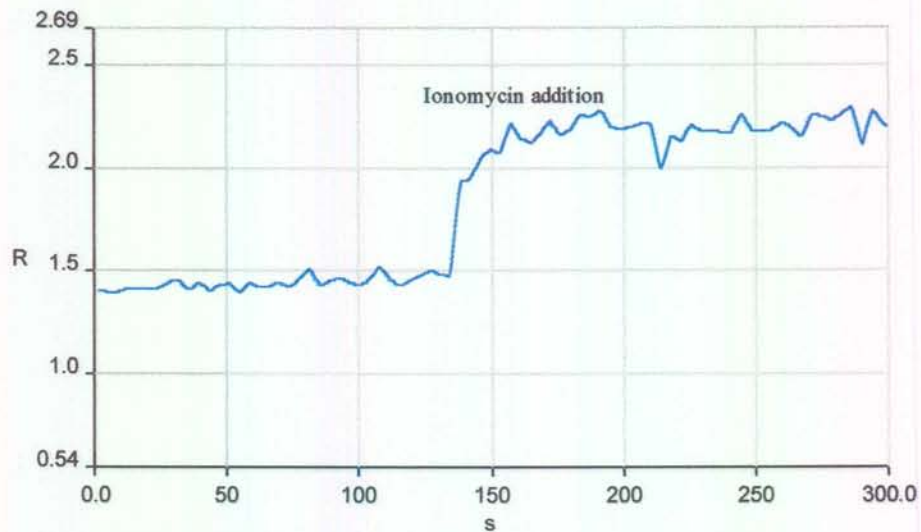
Figure 19. The addition of ionomycin in order to equilibrate intracellular calcium with extracellular medium calcium

Most importantly was the attainment of true end-point values for maximum intracellular free calcium. The evidence that ionophore-induced alterations reflect the true end points rests upon comparison to results obtained after cell lysis. To validate if maximum cytosolic free calcium is reached, it is suggested that subsequent to the addition of ionomycin TritonX-100 should be added. Any increase in the ratio value upon this TritonX-100 addition would indicate that the complete equilibration of medium calcium with cytosolic calcium did not take place. Upon the subsequent addition of TritonX-100 a small increase in the fluorescence ratio values were obtained, possibly indicating that the equilibration of cytosolic free calcium with medium calcium is incomplete. Refer to figure 20.
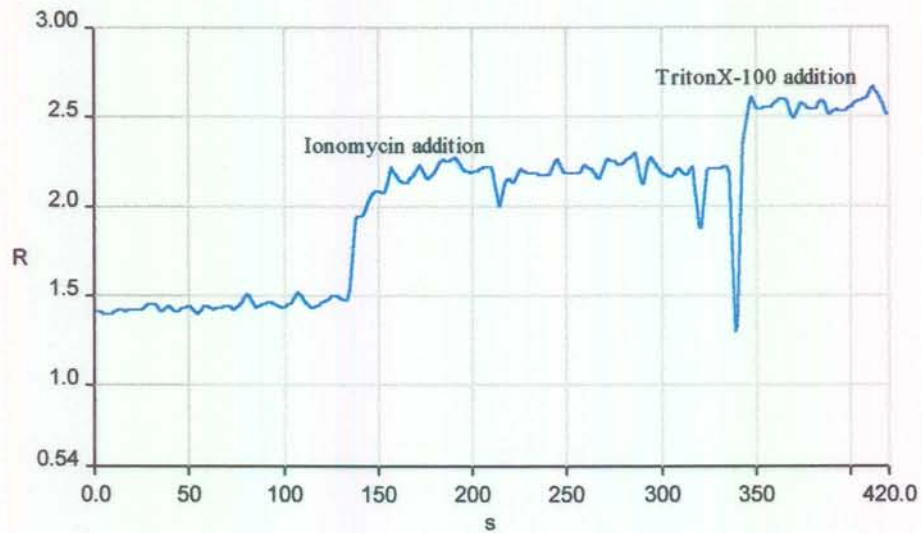
Figure 20. An increase in the ratio value upon the addition of TritonX-100 after the addition of ionomycin

However, investigation of the fluorescence intensities comprising the ratio values indicated that upon TritonX-100 addition a small, insignificant increase occurred in the 340nm wavelength graph compared to a larger decrease in the 380nm wavelength graph. Refer to figure 21.
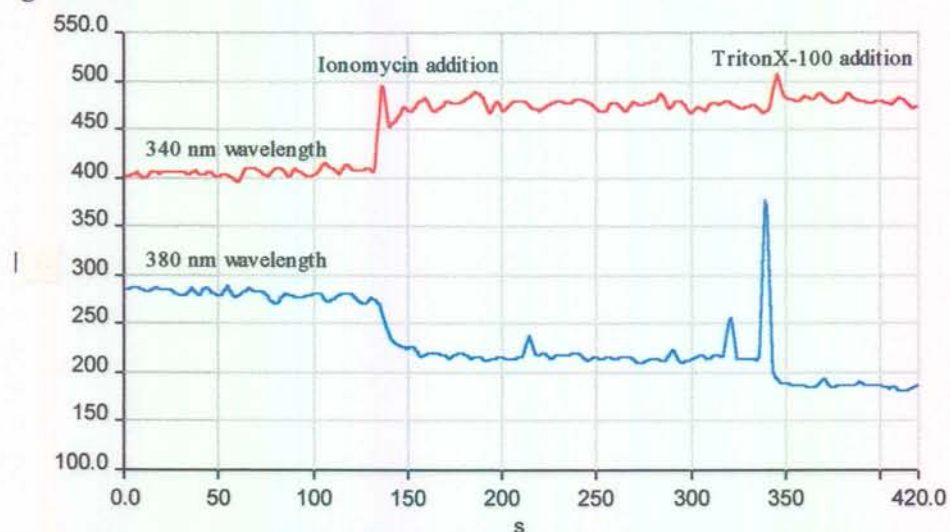
Figure 21. An insignificant increase in the 340 nm wavelenth intensity and a larger decrease in the 380 nm wavelength intensity incompatible with a rise in intracellular free calcium

This is incompatible with an increase in intracellular free Ca^{2+}, since with a true increase in intracellular free Ca^{2+} the increase in fluorescence intensity values for the 340 nm wavelength should always be greater than the magnitude of the decrease in the fluorescence intensity for the 380 nm wavelength. This subsequent increase in the ratio value upon TritonX-100 addition therefore was not due to the incomplete equilibration of extracellular medium calcium with cytosolic calcium. This increase in the ratio value could rather be the result of the change in the fluorescence characteristics of the fluorescent calcium indicator due to the release of the indicator into a medium different in constitution than the cytosol.

In order to clamp the cytosolic free calcium concentration to a minimum EGTA/Tris was added. Refer to figure 22.
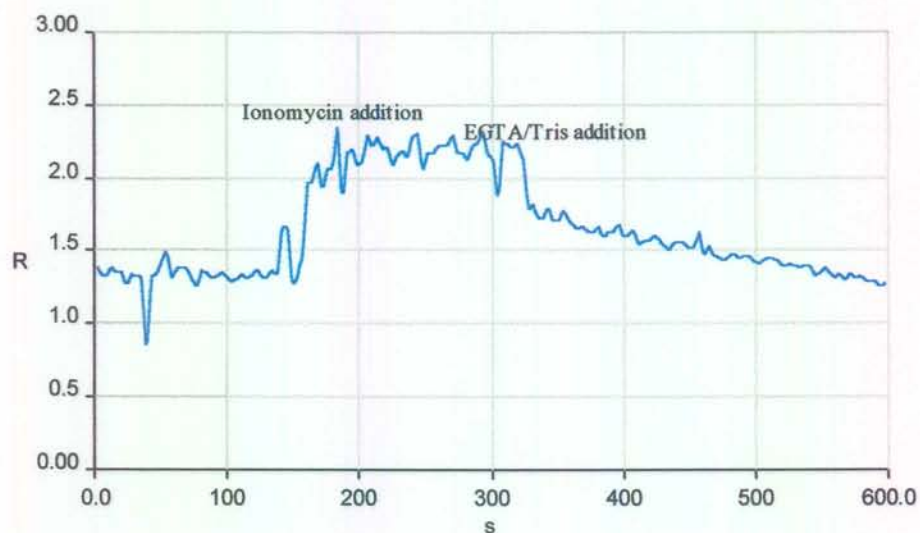
Figure 22. Addition of a EGTA/Tris solution in order to attain minimum calcium concentrations

Equilibration of the cytosolic calcium with the medium calcium containing minimum calcium was a slow process, and the attainment of minimum calcium concentrations in the cytosol uncertain.

It seems that the fluorescence characteristics of the fluorescent calcium indicator do change as a result of the releasing of the indicator into the extracellular medium but the result of this change on the ratio values are negligible. Furthermore, according to these results, the attainment of the minimum fluorescence intensity values was uncertain. Therefore the end of the experiment calibration of the obtained fluorescence intensities were performed following cell lysis.

x) **Evaluation of the reproducibility of the technique for the determination of intracellular free calcium and transmembrane calcium fluxes when performed over a time period**

At this stage it became necessary to determine whether the technique gave reproducible results when the intracellular free calcium and transmembrane calcium fluxes are determined on several occasions for the same subject. To evaluate the reproducibility and accuracy of the technique, basal intracellular free calcium and transmembrane calcium fluxes were determined for the same subject on different days. The attained intracellular free calcium and transmembrane calcium fluxes indicate the reproducibility of the technique and also the tight regulation of intracellular free calcium. Refer to figure 23.
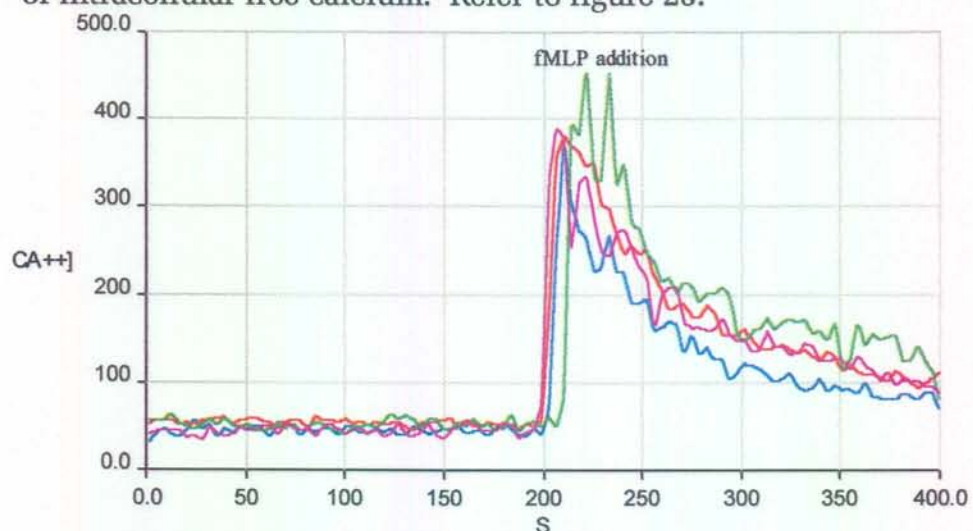
Figure 23. The attainment of reproducible results in the same subject on different occasions

The basal intracellular free Ca^{2+} concentrations were: 45 nM, 54 nM, 52 nM, 45 nM and the magnitude of the transmembrane Ca^{2+} flux upon fMLP stimulation were: 379 nM, 381 nM, 464 nM, 390 nM.

In order to determine the range of basal intracellular free calcium and transmembrane calcium fluxes in different subjects, measurements were performed for 6 subjects on 6 different days:

Refer to figure 24, white female 25y, white female 24y, black female 27y, black female 40y, white male 61y and white female 65y. The attained basal intracellular free calcium and transmembrane fluxes indicate the reproducibility of the technique and also the range of basal intracellular free calcium and transmembrane calcium fluxes in different subjects.
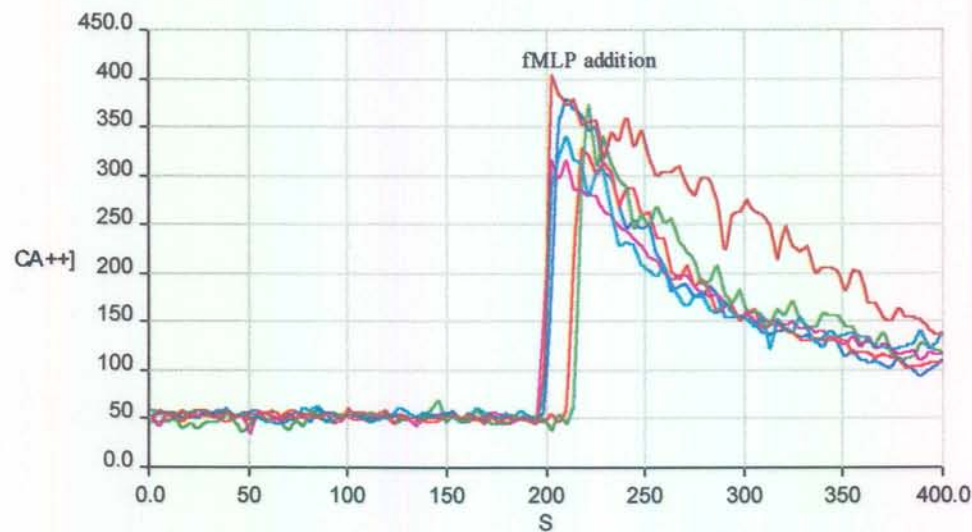
Figure 24. The attainment of reproducible results for different subjects on different occasions

The basal intracellular free Ca^{2+} concentrations were: 50 nM, 52 nM, 50 nM, 52 nM, 57 nM, 53 nM and the magnitude of transmembrane Ca^{2+} fluxes were: 328 nM, 464 nM, 380 nM, 318 nM, 341 nM, 405 nM.

xi)   Evaluation of the sensitivity of the technique for the determination of a change in intracellular free calcium

The following measurements were performed to test whether the technique would indeed be sensitive enough to determine variations and whether this would follow changes in the degree of sickness such as in sepsis. Dyshomeostasis of the immune system in septic patients is the result of the dysfunction of many of the cells involved in host immunity, including the neutrophils. There are indications that one of the contributing factors to neutrophil dysfunction is intracellular calcium dysregulation. (29, 30) Intracellular free calcium at rest and upon fMLP stimulation were determined in this group of patients as an indication of the potential of the optimised experimental procedure for detecting a change in intracellular free calcium, both at rest and upon fMLP stimulation.

- **Determination of neutrophil intracellular free calcium in patients**

-       Patient 1: Refer to figure 25, blue graph and upon partial recovery, red graph. Basal intracellular free calcium and the magnitude of the transmembrane calcium flux are decreased with an improvement in the patient's condition.
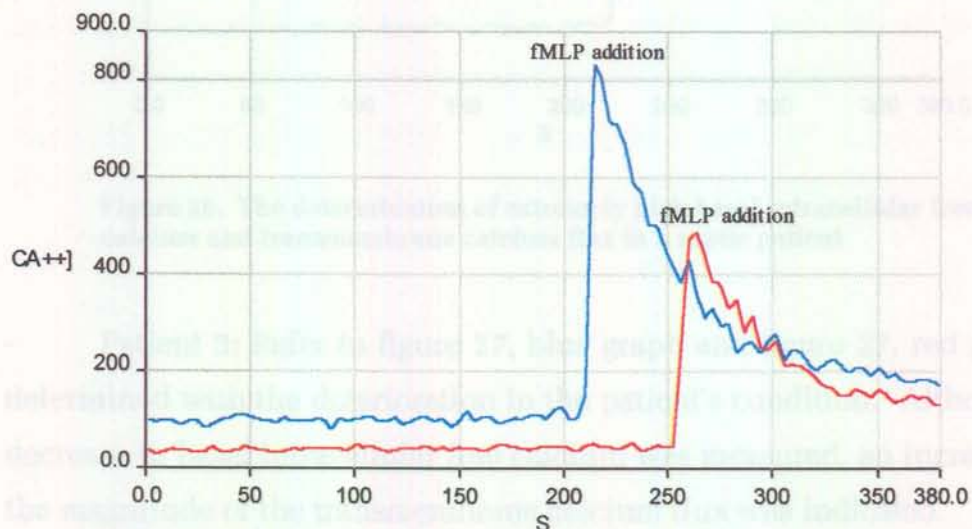
Figure 25. A decrease in intracellular free calcium and transmembrane calcium flux upon partial recovery of a septic patient

-       Patient 2: Refer to figure 26, an elevation in basal intracellular free calcium and the transmembrane calcium flux was determined. The patient's condition deteriorated and the patient died, preventing a follow-up determination. The magnitude of the patient's neutrophil intracellular free calcium and transmembrane calcium flux were extremely high. Refer to figure 24 for the normal range of basal intracellular free Ca^{2+} and transmembrane Ca^{2+} fluxes.
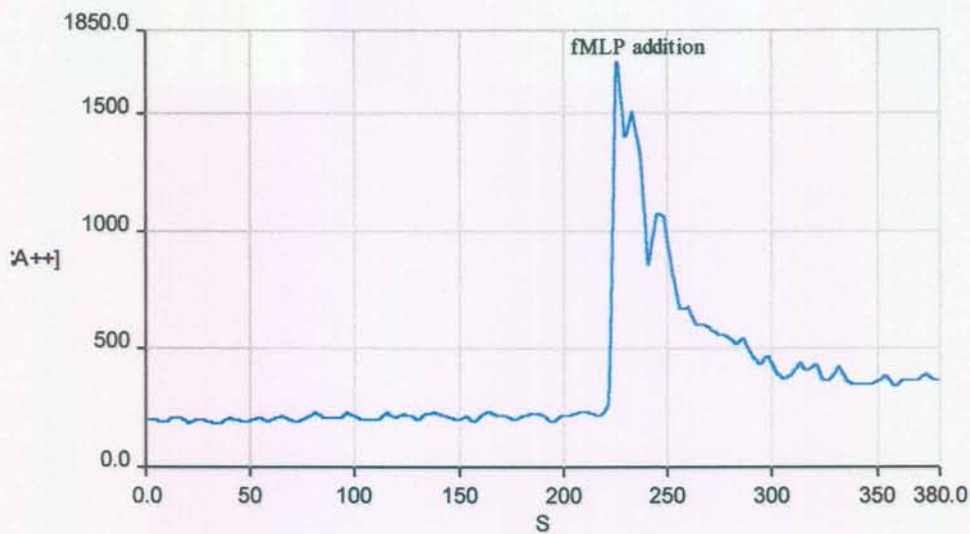
Figure 26. The determination of extremely high basal intracellular free calcium and transmembrane calcium flux in a septic patient

-       Patient 3: Refer to figure 27, blue graph and figure 27, red graph, determined with the deterioration in the patient's condition. Although a decrease in basal intracellular free calcium was measured, an increase in the magnitude of the transmembrane calcium flux was indicated.
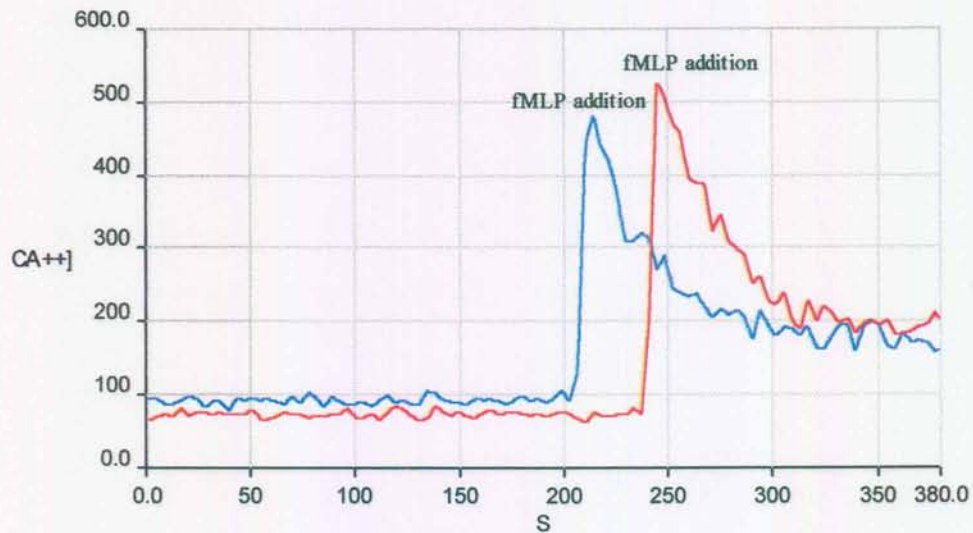
Figure 27. A decrease in basal intracellular free calcium and an increase in the transmembrane calcium flux of a septic patient with the deterioration of the patient's condition

- Patient 4: Refer to figure 28. The blue graph represents the first determination of basal intracellular free calcium and transmembrane calcium flux, whereas the red graph the second determination. A lowering in basal intracellular free calcium and the transmembrane calcium flux accompanied the improvement in the patient's condition.
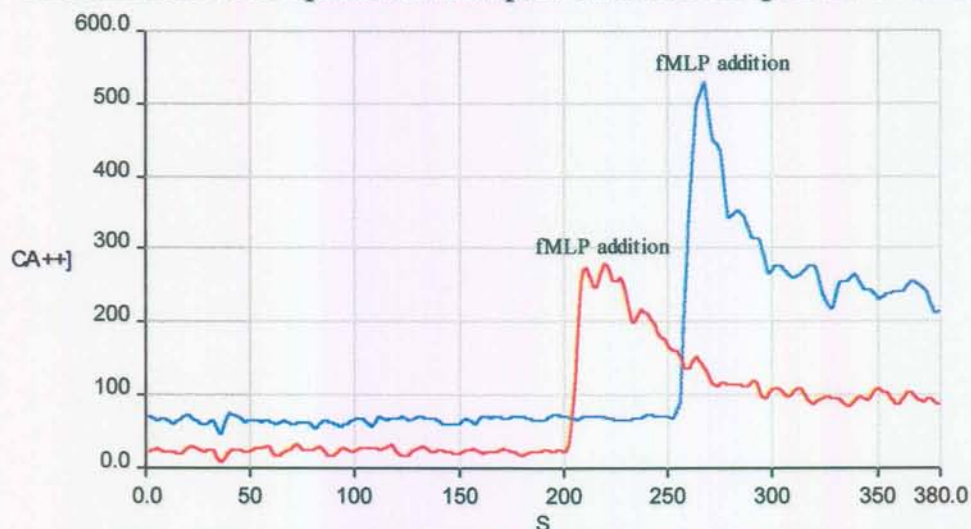
Figure 28. A decrease in basal intracellular free calcium and a decrease in the transmembrane calcium flux in a septic patient, the patient's condition improved

2.2.6) Discussion

There are many available fluorescent calcium indicators for cuvette-based measurements of intracellular free calcium. The most commonly used of these fluorescent calcium indicators is fura-2. Fura-2 is also the fluorescent calcium indicator of choice in many studies investigating intracellular free calcium in the neutrophil. The employment of this calcium indicator gave good results in many of these studies, but there are indications that leakage and compartmentalisation of the indicator into intracellular organelles might interfere with intracellular free calcium determinations in some cell types. In some cell types indicator extrusion can be so severe, that in these cell types it become difficult to load enough indicator to be sufficient for fluorescence determinations for the duration of the experiment. More commonly, cells initially load evenly, but soon the indicator leaks out of the cytosol while the remaining indicator molecules are associated with the intracellular organelles. This leakage and compartmentalisation of the indicator complicate many aspects of calcium studies, including the determination of true resting intracellular free calcium levels in cuvette-based measurements, since in the presence of external calcium a constant leakage of the indicator could be misinterpreted as a slow rise in intracellular free calcium. Indicator extrusion and compartmentalisation are not limited to cells loaded with the acetoxy methyl ester form of the indicator, since cells microinjected with the penta-anion form of the indicator display similar problems. These problems can be partly remedied with organic anion transport inhibitors such as probenecid or with an indicator linked to dextran, however the use of probenecid adds unknown variables to the experiment, and indicator-dextran conjugates must be micro-injected. (17, 18) It would therefore be desirable to develop indicators that do not leak out of cells or are compartmentalised in intracellular organelles, yet retain the ability to load as an acetoxy methyl ester. It was with this in mind that

the experimentalists developed a fluorescent calcium indicator that resisted leakage from the cytosol and compartmentalisation into intracellular organelles. The new indicator fura-PE3, an analog of fura-2 is designed to resist leakage and compartmentalisation by virtue of an added positive charge. The incorporation of an additional positive charge produced a molecule with zwitterionic characteristics, ensuring a much slower extrusion from the cytosol and compartmentalisation into intracellular organelles. It was shown by the patent holders that fura-PE3 can be loaded in much the same way as fura-2, as an acetoxy methyl ester derivative, but gives more uniform loading. In addition, cells loaded with fura-PE3 is said to remain uniformly loaded for hours, whereas fura-2 quickly accumulates in organelles and leaks out of the cell. Despite its benefits, fura-PE3 is still not the ideal cytosolic calcium indicator. Leakage and compartmentalisation are dramatically reduced but not eliminated. (31, 32) In search of the most suitable fluorescent calcium indicator for intracellular free calcium studies in the neutrophil both fura-2 and fura-PE3 were tried and tested. Both these indicators are well suited to resolve basal intracellular free calcium levels with a Kd for fura-2 of 224nM and a Kd for fura-PE3 of 250nM. Kd values closest to the concentration level to be measured, assures the largest change in fluorescence intensities as a result of a change in intracellular free calcium and thus good resolving capabilities.

Various aspects of the protocol for the determination of intracellular free calcium in the neutrophil were evaluated, since as discussed in the first section of this chapter the determination of intracellular free calcium by employing the florescent calcium indicators is not problem-free. Firstly, sufficient quantities of the fluorescent calcium indicator are to be obtained in the cytosol of the neutrophil to determine intracellular free calcium. The following precautions assisted in the attainment of

sufficient quantities of the fluorescent calcium indicators, both fura-2 and fura-PE3 in the cytosol.

- The stock solutions which consisted of the acetoxy methyl ester derivative of the indicator dissolved in DMSO was premixed in the appropriate buffer containing bovine serum albumin (BSA). Bovine serum albumin acted as a dispersing agent and assured the availability of the indicator for crossing of the membrane. Omission of BSA resulted in inadequate accumulation of the indicator in the cytosol of the neutrophil.

- The agitation of the neutrophil suspension during the loading period facilitated the acetoxy methyl ester derivative in crossing of the membrane and ensured the obtainment of sufficient quantities of the indicator in the cytosol.

Other factors also determined the efficiency of the loading procedure. These factors included the temperature at which loading of the indicator was performed, the concentration of the ester derivative of the indicator in the incubation medium and the duration of the loading procedure. These three factors were altered on a trial and error basis and the following protocols for loading of the indicator established.

- The protocol for loading of fura-2/AM is formulated according to a combination from published methods and suggestions. Attempts to optimise the loading procedure for the fura-2 indicator indicated sufficient quantities of the indicator in the cytosol with a concentration of the acetoxy methyl ester derivative of the indicator in the loading medium of 2 – 5 μM. For the loading of the fura-2 indicator a concentration of the AM ester of 5 μM was employed.

- There are no published studies on intracellular free calcium investigations in the neutrophil employing fura-PE3, but it is suggested that the concentration of the fura-PE3/AM ester in the incubation medium should be lower (1 - 2 μM) and the incubation

time longer than that for the fura-2/AM ester. This, due to the fact that the fura-PE3/AM ester has a greater tendency to crystallise or precipitate than seen with the fura-2/AM ester. For the loading of the fura-PE3 indicator a concentration of the AM ester of 2 µM was employed and found to be sufficient. The working concentration of neutrophils in the cuvette was 2 to 9 x 10^5 / ml for fura-2 and 9 x 10^5 to 1 x 10^6 for fura-PE3. This difference was the result of the obtainment of different quantities of the indicators in the cytosol due to the different loading protocols and as a result of differences in the rate of cleavage of the ester bonds for these two indicators. These working concentrations of neutrophils assured fluorescence intensities above background fluorescence values and below the limits for instrument detection.

The wavelength shifting fluorescent calcium indicators like fura-2 and fura-PE3 can be employed in ratio mode, that is at every measured time-point two fluorescence intensity values at two different wavelengths are obtained and subsequently the ratio consisting of these values is calculated for every time-point. The advantage of this feature is that factors influencing fluorescence intensity values such as indicator concentration cancel when the ratio is calculated, since indicator concentration influence the fluorescence intensity values to the same extent. To evaluate this feature two aliquots of the same neutrophil suspension were loaded with different concentrations of the fura-PE3/AM ester, resulting in the subsequent attainment of unequal quantities of fura-PE3 in the cytosol. Calibration of the obtained fluorescence intensities followed by the calculation of intracellular free calcium concentrations gave similar results. Refer to figure 7.

Indicator extrusion complicated intracellular free calcium determinations during the initial study, since leakage of the indicator into the extracellular medium resulted in an incorrectly measured higher

basal intracellular free calcium concentration. Therefore the extent of leakage for both the fluorescent calcium indicators, fura-2 and fura-PE3 were determined in the neutrophil. A negligible decrease in the ratio values occurred for both fura-2 and fura-PE3 upon nickel chloride addition after the cell suspension was kept on ice for a very short period of only two minutes – 0.05 for fura-2 and 0.02 for fura-PE3. Nevertheless, the decrease in the ratio value for fura-2 is larger than that for fura-PE3. This according to other published results in different cell types indicating that fura-PE3 is better retained in the cytosol of the specific cell types. It must be mentioned that a small amount of the indicator is not removed with repeated washing of the cell suspension after incubation in the loading medium. (6) This fraction of unremovable indicator molecules might represent indicator molecules bound to the cell membrane, therefore quenching of the fluorescence of these indicator molecules upon nickel chloride addition might have been the cause of the measured decrease in the ratio values and not as a result of the extrusion of the indicators from the cytosol. For both types of indicators a much larger decrease in the ratio values occurred upon nickel chloride addition after the 50 minute incubation period, although again a small difference existed between the two indicators – 0.1 for fura-2 and 0.09 for fura-PE3. This in contrast to other published results in different cell types indicating that fura-2 is extruded from the different cell types to a much larger extent than fura-PE3. According to these results leakage of both the fluorescent calcium indicators are mentionable during a 50 minute incubation period. But for both the indicators the leakage of the indicator during a 2 minute incubation period are negligible, more so for fura-PE3. Since leakage of the fluorescent calcium indicator into the extracellular medium results in the obtainment of higher basal intracellular free calcium, the incorrectly measured higher basal intracellular free calcium concentration as a result of the extrusion of the indicator to the extracellular medium

containing a millimolar calcium concentration was determined. An example of one of 20 determinations is presented - refer to figure 12. The blue graph was obtained after the first incubation period – 2 minutes on ice and a further 2 minutes at 37°C in the cuvette. The red graph was obtained after the second incubation period – 50 minutes on ice and a further 2 minutes at 37°C in the cuvette. The mean intracellular free calcium concentration calculated for the blue graph equals 53 nM and for the red graph 70 nM. Thus the calculated difference in the ratio values obtained before and after nickel chloride addition after an incubation period of 50 minutes which is 0.09 represents an inaccurately determined elevation of intracellular free calcium equal to: 70 – 53 nM = 17 nM. A decrease in the ratio value after the first short incubation of 0.02 would more or less represent a negligible elevation in the intracellular free calcium concentration of approximately 2 nM.

There are indications that heavy metals present in the cytosol in various cell types may interfere with intracellular free calcium determinations performed by using the fluorescent calcium indicators. Heavy metals for instance Fe^{2+} and Zn^{2+} quench the fluorescence quantum yield and would therefore result in the lowering of the determined intracellular free calcium levels. (6) It is well known that chronic renal failure patients on maintenance haemodialysis, display an increase in heavy metal ion levels in the cytosol of the red cell. (33) In both the patient's group and the healthy subject's group the possible interference of heavy metal quenching was therefore investigated. This was achieved by the addition of TPEN (N, N, N', N', - tetrakis(2-PYRIDYLMETHYL)ETHYLENEDIAMINE). TPEN is a lipid-soluble heavy metal chelator that can permeate cellular membranes and bind heavy metals in the cytosol with an extraordinary high affinity while rejecting calcium and magnesium. (34) No heavy metal ion quenching of the

calcium indicators could be detected at a final concentration of TPEN of 20μM. This could be due to fura-PE3's low affinity for heavy metals, since there is no ring nitrogen that would promote heavy metal binding.

Intracellular free calcium concentrations were determined by solving the Grynkiewicz equation – refer to section on the theoretical background of the technique. This equation contains two calibration points – the ratio value obtained at maximum calcium and the ratio value obtained at minimum calcium. At the end of the experiment the calibration procedure was performed. For this end of the experiment calibration procedure the indicator was released into the extracellular medium where the calcium concentration was manipulated. There are indications that the fluorescence properties of the indicators are influenced by the characteristics of the medium, if this is the situation calibration of the fluorescence values should be performed in situ. This was investigated. Intracellular free calcium concentrations should be manipulated in order to calibrate the fluorescence ratio values in situ. This was achieved by the addition of calcium ionophores to the neutrophil suspension. The addition of an ionophore specific for calcium would result in the equilibration of medium calcium with the intracellular free calcium. It is therefore possible to clamp intracellular free calcium levels to known concentrations and subsequently determine the fluorescence ratio values in situ. The ionophorous antibiotics are lipid-soluble molecules, capable of forming complexes with alkali metal cations followed by the transporting of the cations across a variety of membranes. (35) Physical studies indicate that the complexation-decomplexation kinetics and diffusion rates of ionophores and their complexes across lipid barriers are so favourable that their transport turnover numbers across biological membranes attain values of thousands per second, exceeding the turnover numbers of most macromolecular enzymes. (36) Initially the calcium ionophore A23187

- a carboxylic acid antibiotic with a high specificity for the divalent cations calcium and magnesium, (35) was employed for the in situ calibration procedure, but this ionophore is highly fluorescent at both the wavelengths 340 nm and 380 nm. Therefore, the non-fluorescent calcium ionophore ionomycin was subsequently employed. Ionomycin is a diacidic polyether antibiotic having high affinity for calcium ions. (37) Similar to A23187, ionomycin forms lipid-soluble complexes with calcium and traverses cellular membranes. The addition of ionomycin resulted in rapid equilibration of medium calcium with intracellular free calcium resulting in maximum cytosolic calcium. Most importantly was the attainment of true end-point values for maximum intracellular free calcium. To validate if maximum cytosolic calcium is reached, it is suggested that subsequent to the addition of ionomycin TritonX-100 should be added and any increase in the ratio value upon this TritonX-100 addition would indicate that the complete equilibration of medium calcium with cytosolic calcium did not take place. This evaluation procedure was employed, after the addition of ionomycin, TritonX-100 was added. A small increase in the ratio value did take place and according to the above-mentioned suggestion indicated incomplete equilibration of medium calcium with cytosolic calcium. However, investigation of the fluorescence intensities comprising the ratio values indicated that upon TritonX-100 addition a small, insignificant increase occurred in the 340nm wavelength graph compared to a larger decrease in the 380nm wavelength graph. Refer to figure 21. This is incompatible with an increase in intracellular free calcium, since with a true increase in intracellular free calcium the increase in fluorescence intensity values for the 340nm wavelength should always be greater than the decrease in fluorescence intensities for the 380nm wavelength. This subsequent increase in the ratio value upon TritonX-100 addition therefore was not due to the incomplete equilibration of extracellular medium calcium with cytosolic calcium, but seems to rather be the result of the change in

the fluorescence characteristics of the calcium indicator due to the releasing of the indicator into a medium different in constitution than the cytosol. In order to clamp cytosolic free calcium to a minimum EGTA/Tris was added, the attainment of minimum calcium in the cytosol is a slow process and when and if minimum calcium could be attained in the cytosol uncertain. According to these results, the releasing of the fluorescent calcium indicator into the extracellular medium resulted in a small change in the indicator's fluorescence characteristics, but since the attainment of minimum calcium concentrations in the cytosol was uncertain the calibration of fluorescence intensities were performed following cell lysis. (7)

Finally the reproducibility of the optimised protocol was evaluated, the range of basal intracellular free calcium levels determined, and the potential for the determination of a difference in basal intracellular free calcium levels and transmembrane calcium fluxes investigated. Reproducible results were obtained, refer to figures 24 and 25 and indications are that the optimised protocol is adequately sensitive to determine a change in intracellular free calcium and transmembrane calcium fluxes, refer to figures 25 to 28.

2.2.7) Conclusions

The optimised protocol is presented.

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.

10) If the pellet is still contaminated with red blood cells, the red blood cell lysis step can be repeated.

11) Discard the supernate and gently dissolve the pellet in 0.25% BSA supplemented Hanks (8°C).

12) Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.

13) Discard the supernate and dissolve the pellet in 2 ml 0.25% BSA supplemented Hanks (neutrophil cell suspension).

14) 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 number of neutrophils in all 5 cell chambers = N

Number of neutrophils in one cell chamber = N/5

Volume of one cell chamber = 0.1 mm³

Concentration of neutrophils in 1 mm³ after dilution with Turk's blood solution = N/5 . 10

Concentration of neutrophils in 1 mm³ 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.

Loading of the neutrophils with the fura-PE3/acetoxy methyl ester

1) Incubate the neutrophil suspension at 37°C for 10 minutes – stabilisation and equilibration of ion gradients.

2) Dissolve 2 µl of the fura-PE3/AM stock solution in 2 ml 0.25% BSA/Hanks.

3) Add 0.5 ml of this mixture to 1.5 ml of the neutrophil suspension. The final fura-PE3/AM concentration is 2 µM and the final % DMSO is 0.025%.

4) Incubate for 30 minutes at 37°C in a gently shaking water bath 50 r.p.m. - protect from light – and for a further 30 minutes at room temperature.

5) Centrifuge at 1200 r.p.m. (12°C) for 10 minutes.

6) Wash the pellet twice with 0.25% BSA/Hanks (8°C), centrifuge at 1200 r.p.m. (12°C) for 10 minutes.

7) Discard the supernate and dissolve the pellet in 2 ml 0.25% BSA/Hanks.

8) Incubate on ice for 2 minutes prior to the commencement of the fluorescence measurements.

Measurement of the fluorescence intensities

1) Add 900 µl of the neutrophil suspension and 1100 µl 0.25% BSA/Hanks to the cuvette.

2) Place the cuvette in the spectrofluorometer cuvette chamber and incubate for 2 minutes with the magnetic bar stirrer switched on.

3) OK to start.

fMLP stimulation of the neutrophils

1) Mix 10 µl of the fMLP stock with 190 µl 0.25% BSA/Hanks.

2) Add 20 µl of the previously prepared solution to the 2 ml neutrophil suspension in the cuvette. Final fMLP concentration 1 µM and the further addition of 0.05% DMSO.

Calibration of the fluorescence ratio data

1) To obtain the fluorescence ratio value at maximum calcium – Rmax. Add 100 µl of a 1% TritonX-100 solution to the 2ml neutrophil suspension in the cuvette. TritonX-100 lyses the cells, resulting in the releasing of the fura-PE3 molecules into the extracellular medium. Since the extracellular medium contains a

calcium concentration of 1 mM, attainment of the Rmax value follows.

2) To obtain the fluorescence value at minimum calcium – Rmin. Add 100 µl of a 0.5 M EGTA/3 M Tris solution pH 8.7 to the 2 ml neutrophil suspension in the cuvette. EGTA chelates the available calcium ions and the attainment of Rmin follows.

Calculation of intracellular free calcium concentrations

The Grynkiewicz equation is solved, refer to page 52.

3)   Intracellular calcium localisation explored in the neutrophil via transmission electron microscopy

Localisation of intracellular calcium is considered a major problem to most workers in the field (personal communication Head of electron microscopy, UP).   Localisation of calcium in neutrophils therefore present a severe challenge for the uninitiated as further technical pitfalls such as isolation of neutrophils from blood and proper cell packing should be performed without disturbance of the intracellular composition of the cell.   It was therefore considered essential to start the procedure by an attempt to prepare transmission electron microscopy (TEM) preparations after neutrophil isolation, before starting on the Ca^{2+} precipitation technique.   Since no local expertise could be identified on the preparation of neutrophil TEM samples, a thorough study of the available literature was conducted.   After completion of the literature study several protocols were designed and evaluated in order to find the most suitable for the TEM preparation of neutrophils.   The most critical step that determines the success of the preparation procedure is the adequate fixation of the cells.   Broken plasma membranes, washed out figures, coagulated-appearing cytoplasm, and swollen or shrunken cells are produced at the time of fixation.   (38)   To preserve such cellular detail glutaraldehyde has gained superiority over osmium tetroxide as a primary fixative.   It seems likely that this superiority of glutaraldehyde over osmium tetroxide as a primary fixative, particularly for non-membranous components of the cytoplasm is partly due to the faster rate of reaction of glutaraldehyde with cellular components resulting in fixation before any noticeable extraction of cellular components by the buffer.   As mentioned osmium tetroxide is not the primary fixative of choice but post-fixation with osmium tetroxide can result in emphasising of membrane structures since osmium tetroxide reacts with those lipids that are not fixed by glutaraldehyde and osmium tetroxide

also acts as a stain. (39) It is conceivable that the ideal composition of the primary fixative should match the physiological environment of the cell with respect to pH, osmolarity and ionic constitution. An unsuitable fixative composition during primary fixation, before the cellular components have become adequately preserved, may result in extraction of cellular materials and/or deposition of fixative compounds. The properties required for the vehicle of the fixative include

• Ability to maintain a constant pH during fixation. Nevertheless, there is little evidence that the pH is at all critical within the range 6.5 – 8.0.

• Suitable osmolarity (when mixed with the fixing agent) so that cells and organelles neither swell nor shrink. Many prefer a somewhat hyperosmotic solution.

Alcohol dehydration is generally preferred to acetone dehydration since acetone extracts membrane lipids resulting in inadequate preservation of the membranes. However, most methods for calcium localisation seem to employ acetone. In the neutrophil, as in most other cell types, intracellular calcium is functionally distributed between the various subcellular compartments. These compartments include: binding of calcium to the cytosolic side of the plasma membrane either to phospholipids or plasma membrane associated proteins, binding of calcium to macromolecules including proteins free in the cytosol and sequestration of calcium in various membranous organelles. (40) In order to study the distribution of intracellular calcium ions between various subcellular compartments, the transmission electron microscopy preparation procedure employed should preserve and present the *in vivo* distribution pattern of intracellular calcium. Depletion and translocation of any unbound calcium ions may occur during tissue fixation and in some instances the process of fixation may release calcium ions that were previously bound to organic macromolecules. To circumvent this problem two methods have been described to localise

and precipitate calcium ions in situ. In order to bind and retain calcium in these various subcellular compartments, either inorganic phosphates or oxalate anions can be employed. The methods are based on a) stabilisation and/or trapping of calcium during the primary fixation step in glutaraldehyde by the phosphate or oxalate anions, (theoretically phosphate and oxalate anions target different calcium compartments) b) the subsequent wash-out of all non-trapped cations such as sodium, potassium and magnesium and c) the conversion of the complexed or trapped calcium into an electron-dense and insoluble calcium pyroantimonate salt during post-fixation. This distribution of electron-dense particles can subsequently be explored by transmission electron microscopy. (41) Omission of pyroantimonate from the post-fixative solution results in the complete absence of deposits in both procedures. (42) The precipitation of the calcium ions as part of a pyroantimonate complex is said to assure only negligible loss of calcium ions since the Ca-Sb precipitate displays very low solubility and translocation of precipitates is unlikely because of their crystalline nature. The basis of this reaction is that under the appropriate fixation conditions potassium-antimonate infiltrates the neutrophil, followed by the replacement of potassium ions by calcium ions to form an insoluble electron dense calcium-pyroantimonate precipitate. (42 - 44) The employment of phosphate in combination with pyroantimonate is said to result in the visualisation of acidic phospholipid-bound calcium by formation of a stable complex between inorganic phosphate, calcium and acidic phospholipids. The acidic phospholipids are retained on the cytoplasmic side of the plasma membrane, therefore this phosphate-based procedure principally localises calcium on the cytoplasmic side of the plasma membrane. The subsequent addition of antimonate results in the conversion of the preformed complex into an insoluble calcium-pyroantimonate precipitate so that during the subsequent preparatory steps dissolution or displacement of the complex is prevented. (42, 45)

The oxalate anion is relatively specific for calcium in the presence of physiologic concentrations of other biological cations and therefore can be employed to retain calcium during primary fixation. With the addition of potassium-pyroantimonate during post-fixation the electron-dense precipitate is formed. (40)

Free cellular calcium is not likely to be responsible for a significant fraction of the precipitates subsequently formed as a result of antimonate binding. The low concentration of free calcium in most cells being at or below 10^{-8} M is at or below the threshold for antimonate precipitation, especially when phosphate buffer is employed and most is expected to be lost with other soluble cations during aqueous processing steps. The remaining calcium making up the vast bulk of the cell's total calcium exists in subcellular compartments with differing calcium affinities and degrees of exchangeability. Some of this calcium is unable to react with antimonate. One such form of calcium that probably does not react with antimonate is the insoluble amorphous tricalcium phosphate $Ca_3(PO_4)_2$ found in mitochondria. This has led to the suggestion that the antimonate reaction product represents only more loosely bound exchangeable calcium populations. (41)

It is known that antimonate has a high affinity for various intracellular cations, and that changes in reaction parameters strongly influence the retention and precipitation of physiological cations relative to each other. Nevertheless, the careful choice of reaction conditions can make the phosphate-antimonate technique highly selective for calcium in comparison to other cations that are capable of precipitation. (41) Following are a few important findings from investigators concerning in situ calcium precipitation by the antimonate anion.

• Theoretically, glutaraldehyde fixation preserves more of the cytoplasmic proteins than the other available fixatives, therefore a

greater amount of calcium ions should be retained since many cytoplasmic proteins bind calcium.

• The importance of employing glutaraldehyde as the primary fixative followed by osmium tetroxide post-fixation was discussed in the previous section. In many intracellular calcium localisation studies, cells fixed directly in the potassium-pyroantimonate/osmium reagent usually are poorly preserved when compared to those exposed to glutaraldehyde prior to the potassium-pyroantimonate/osmium reagent. (46) In addition to the morphological preservation superior to that seen with osmium-antimonate or antimonate alone, glutaraldehyde does not affect the antimonate-calcium precipitation thresholds. (41)

• Employing the preferred above-mentioned regime; primary fixation with glutaraldehyde followed by osmium-antimonate post-fixation only results in the precipitation of calcium during the osmium tetroxide post-fixation step and therefore can result in the translocation of calcium during the prior preparatory steps. Nevertheless, there are indications of almost complete retention of calcium during a 0.1 M phosphate-buffered pH 7.2-8.0 glutaraldehyde fixation, although antimonate was used only during osmium post-fixation. This suggests that in the absence of antimonate, phosphate-buffered glutaraldehyde effectively retains calcium in situ until it can be precipitated and made visible by osmium-antimonate. Fixation with unbuffered glutaraldehyde totally eliminated the subsequent formation of antimonate deposits, thus buffer components and not glutaraldehyde appear to be responsible for retaining calcium ions during primary fixation. (41)

• To accomplish maximisation of the precipitation and retention of calcium versus other cations, thus circumventing the non-specific nature of the antimonate precipitation technique the phosphate anion can aid in the precipitation of mobile calcium ions and therefore can enhance the specific localisation of calcium. This possibly by decreasing the sensitivity of antimonate for sodium and potassium ions. It is indicated

that phosphate inhibits the reaction with sodium, potassium and calcium both *in vitro* and in situ but apparently does so differentially, severely restricting precipitation of monovalent ions while only slightly reducing formation of calcium-antimonate precipitates. The employment of a phosphate buffer therefore results in the relative specificity for precipitation of the cell's divalent cations. Thus, while total numbers of intracellular deposits may be lower due to reduction in potassium and sodium precipitation, the precipitates present predominantly contains calcium.

• If antimonate-reactive cations are mainly those bound loosely enough to cellular components to allow antimonate to displace the component we might expect the bound half of total magnesium to precipitate, however in most instances it appears that nearly all magnesium is mobilised and lost during fixation. (41, 47)

• Even if a significant amount of these interfering cations are retained they form relatively soluble sodium, potassium and magnesium antimonate salts which can be removed by rinsing the neutrophil pellet in a non-antimonate containing solution prior to dehydration. (42)

3.1) Experimental evaluation of different neutrophil preparation procedures for TEM suitable for intracellular calcium localisation

In search of a method

a) which would produce acceptable neutrophil micrographs

b) which could be used on Hanks isolated neutrophils (the medium in which the neutrophils were suspended after isolation for the fluorescence techniques)

c) which could be used as basis for the subsequent intracellular calcium localisation.

3.1.1) Methods

- **Isolation of the neutrophils**

The neutrophils were isolated from ACD-anticoagulated (acid citrate dextrose) whole blood according to a modification of the method of Böyum. (26, 27) In short, ACD-anticoagulated whole blood was loaded onto a layer of hypaque-ficoll. The various blood cells were separated into different layers according to densities upon centrifugation. The polymorphonuclear leucocytes formed a layer on top of the red cell mass in the bottom of the tube. The supernate was discarded. The contaminating red cells were lysed with a 0.25 mM EDTA containing ammonium chloride solution. The polymorphonuclear leucocyte layer contained the neutrophils, eosinophils and basophils. Since eosinophils contribute only 0.5-6% and basophils only 0-1% of the total amount of circulating white blood cells (28) no further separation of the neutrophils were performed from the other polymorphonuclear leucocytes. Finally the neutrophils were washed and suspended in Hanks balanced salt solution.

- **Formation of the neutrophil pellet suitable for preparation for TEM**

A volume of 1 ml of the neutrophil cell suspension was pipetted into an eppendorff. A neutrophil pellet was subsequently formed by centrifugation at 2000 r.p.m. for 5 minutes. The supernatant was discarded.

- **TEM preparation procedure**

Four different protocols were designed and tested – (refer to figure 29)

In an effort to determine the most successful neutrophil preparation procedure for transmission electron microscopy suitable for intracellular calcium localisation, primary fixation by 2.5% glutaraldehyde followed by 1% osmium tetroxide post-fixation was employed in all the variations

Neutrophil EM Adaptation 1a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in 0.1 M PBS 1 hr (room temp)
Rinse 3x 0.1 M PBS
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x 0.1 M PBS

Dehydration: 15 min/concentration
1st alcohol=50% ethanol
2nd alcohol=50% ethanol + 1.5% uranyl acetate
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 1b

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in 0.1 M PBS 1 hr (room temp)
Rinse 3x 0.1 M PBS
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x 0.1 M PBS

Dehydration: 15 min/concentration
1st alcohol=50% ethanol + 1.5% uranyl acetate
2nd alcohol=50% ethanol
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 2a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in Sabatini's solution (0.1 M PBS
with 6.8% sucrose) 1 hr (room temp)
Rinse 3x Sabatini's solution
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x Sabatini's solution

Dehydration: 15 min/concentration
1st alcohol=50% ethanol
2nd alcohol=50% ethanol + 1.5% uranyl acetate
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Neutrophil EM Adaptation 3a

Neutrophil isolation - Hanks
Centrifuge 15 min

Fix 2.5% GA in Hanks 1 hr (room temp)
Rinse 3x Hanks
Post fix 1% OsO_4 1 hr (room temp)
Rinse 3x Hanks

Dehydration: 15 min/concentration
1st alcohol=50% ethanol + 1.5% uranyl acetate
2nd alcohol=50% ethanol
3rd alcohol=70% ethanol
4th alcohol=95% ethanol
5th alcohol=100% ethanol
6th alcohol=100% ethanol

Embed in quetol
Section
Stain: lead citrate and uranyl acetate

Figure 29. Protocols for the neutrophil transmission electron microscopy preparation procedures

of the preparation procedure. Within this framework different adaptations were made concerning the vehicle for the primary fixative. A volume of 1 ml of the various fixatives was pipetted into the eppendorff containing the neutrophil pellet. Primary fixation of the neutrophil pellet was carried out for 1 hour, whereafter the neutrophil pellet was washed 3 times in the appropriate buffer. The supernate was discarded and a volume of 1 ml of the post-fixative was pipetted into the eppendorff containing the neutrophil pellet. Post-fixation of the neutrophil pellet was carried out for 1 hour, whereafter the neutrophil pellet was washed 3 times in the appropriate buffer. The supernate was discarded and the dehydration procedure started. Dehydration was performed in a range of ethanol solutions. The range included; 50%, 70%, 95% and 100% ethanol solutions. Dehydration in the various ethanol solutions was carried out for 10 minutes. After the final dehydration step the 100% ethanol solution was changed with fresh 100% ethanol solution. The dehydrated neutrophil pellets were left in this 100% ethanol and transported to the Electron Microscopy Unit, UP. The rest of the preparation procedure was conducted at the EM unit. Following dehydration the neutrophil pellet was embedded in a Quetol epoxy resin. (48) An equal volume of Quetol was added to the 100% ethanol solution in the eppendorff containing the neutrophil pellet. The neutrophil pellet was left in this 50:50 mixture of 100% ethanol and Quetol for a period of 1 hour. The supernate was discarded and replaced with a solution of Quetol only and left overnight. The following morning the supernate was discarded and replaced with fresh Quetol. The eppendorff containing the neutrophil pellet in Quetol was placed in an oven at 60°C for 6 hours. The thermal energy allowed the Quetol to polymerise and form a hard resin suitable for sectioning. The resin blocks containing the neutrophils were sectioned, stained and viewed. The sections were stained with both uranyl acetate and lead citrate. Uranyl acetate staining enhances the general contrast of the sections

especially structures that contain nucleic acids and ribosomes. (38) Whereas, lead citrate staining emphasises the cell membrane and glycogen particles most effectively. (38)

3.1.2) Results

Examples of the micrographs obtained can be seen in Figure 30.

1)   General

•   All 4 methods resulted in preservation of the ultrastructural features adequate to allow continuation of the establishment of the calcium localisation method.

2)   Characteristic features of the neutrophil as visualised with the transmission electron microscope

The neutrophil displayed various characteristics, which distinguished the neutrophil from other blood cells.

•   The nucleus is lobulated, and these segmented lobules are held together by thin filaments. These filaments are made up of dense chromatin and they are enclosed, like the rest of the nucleus, by the different membranes of the nuclear envelope. As a general rule the more segmented the nucleus, the more mature the neutrophil. The chromatin is very dense and made up of very dark masses of heterochromatin separated by smaller bands of paler euchromatin. These extremely condensed patches of heterochromatin are often discontinuous at the nuclear membrane at points that correspond to the nuclear pores. No trace of a nucleolus remains, which seems to indicate that protein synthesis has ceased. (49)

•   Ribosomes are scant and little or no endoplasmic reticulum is to be found.
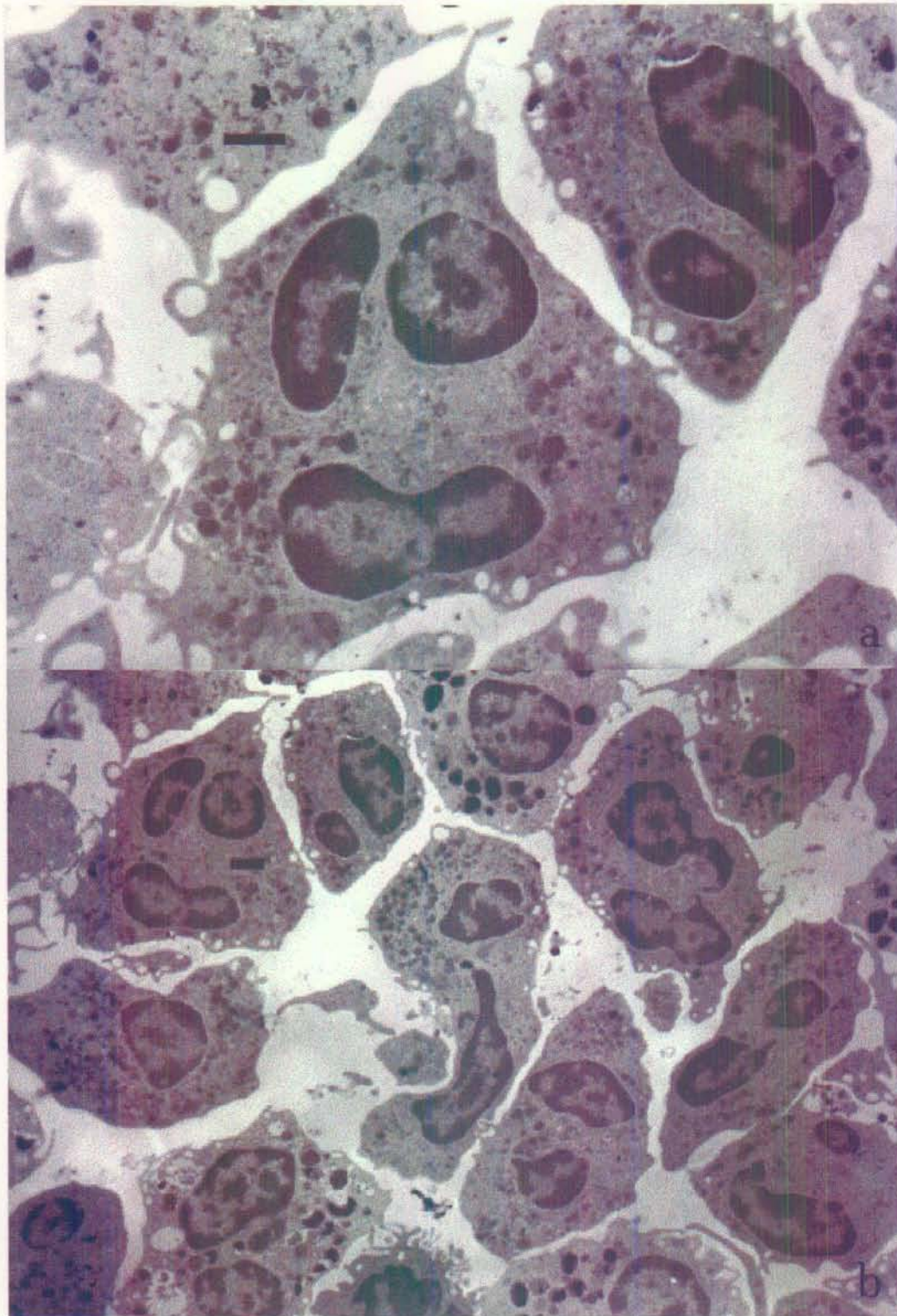
Figure 30 a and b. Electron microscopy photographs indicating suitable TEM neutrophil preparations for intracellular calcium localisation studies. Scale bar 1 µm.

- The Golgi body is inactive and forms a small sphere in the neutrophil center.

- The neutrophil center contains the two centrioles from which many microtubules emanate.

- Mitochondria are few, small and often elongated.

- A large number of glycogen particles, often quite large in size are present which serve as the major energy source for active anaerobic metabolism during phagocytosis.

- Distinctive cytoplasmic granules are present in the cytoplasm of the neutrophil. These granules include: primary granules or azurophilic granules which are large and round, secondary granules or neutrophilic granules which are elongated with an internal crystal structure, and tertiary granules which are small and often dumbbell shaped. The number of granules were variable, even in the strictly normal state, one may see neutrophils with few or no granules and others with numbers of granules which appeared to be distinctly more than in a average cell. (49)

- Fatty vacuoles are round and grayish in color. (39, 49, 50)

3.1.3) Discussion

There are contradictory indications in the literature concerning the employment of glutaraldehyde as primary fixative. On the one hand investigators claim that the effect of differences in the composition of the vehicle are more apparent in aldehyde fixatives. On the other hand since the reaction rate of glutaraldehyde with the cellular constituents is so fast the choice of the composition of the vehicle are not so important. Employment of glutaraldehyde as a primary fixative in the above mentioned vehicles with different compositions concerning osmotically active constituents, resulted in the adequate preservation of ultrastructural detail in all situations demonstrating that the choice of

the composition of the vehicle within limits during glutaraldehyde fixation is not so critical. (39, 49, 50) The morphology of granules are said to be influenced by a variety of technical factors, it is not uncommon for the water-soluble contents of the granules to be lost during primary fixation. The degree of granule content extraction can be influenced by factors including the nature and concentration of the fixative, the duration of the fixation, section thickness, and the method of staining of the sections. Considerable variation was seen in granule content extraction between these various preparation procedures.

3.2) Experimental evaluation of the calcium precipitation reaction: phosphate-pyroantimonate and oxalate-pyroantimonate reactions

3.2.1) Methods

- **Neutrophil isolation**

The neutrophils were isolated as previously described. The only difference being the omitting of the 0.25 mM EDTA from the ammonium chloride solution in some of the procedures. This in order to determine if the addition of EDTA during the isolation procedure could affect the calcium distribution patterns.

- **Formation of the neutrophil pellet suitable for preparation for TEM**

The neutrophil pellet was prepared for TEM as previously described.

- **TEM preparation procedure for the localisation of intracellular calcium**

Six adapted protocols were designed and tested for the localisation of intracellular calcium – (refer to figure 31).

Calcium localisation Adaptation 1a

Neutrophil isolation
+ EDTA

Fix 2.5% GA in 0.1 M NaPBS 1 hr (room temp)
Rinse 3x 0.1 M NaPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M NaPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 1b

Neutrophil isolation
- EDTA

Fix 2.5% GA in 0.1 M NaPBS 1 hr (room temp)
Rinse 3x 0.1 M NaPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M NaPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 2a

Neutrophil isolation
+ EDTA

Fix 2.5% GA in 0.1 M KPBS 1 hr (room temp)
Rinse 3x 0.1 M KPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M KPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

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Section
Stain

Calcium localisation Adaptation 2b

Neutrophil isolation
- EDTA

Fix 2.5% GA in 0.1 M KPBS 1 hr (room temp)
Rinse 3x 0.1 M KPBS
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x 0.1 M KPBS

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Calcium localisation Adaptation 3a

Neutrophil isolation
+ EDTA

Fix 2.5% GA, 90 mM potassium oxalate 1 hr (room temp)
Rinse 3x 90 mM potassium oxalate, 7.5% sucrose
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x alkalinised H_2O pH 10

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Calcium localisation Adaptation 3b

Neutrophil isolation
- EDTA

Fix 2.5% GA, 90 mM potassium oxalate 1 hr (room temp)
Rinse 3x 90 mM potassium oxalate, 7.5% sucrose
Post fix 1% OsO_4, 2% $KSb(OH)_6$ 1hr (room temp)
Rinse 3x alkalinised H_2O pH 10

Dehydration: 10 min/concentration
1st alcohol=50% ethanol
2nd alcohol=70% ethanol
3rd alcohol=95% ethanol
4th alcohol=100% ethanol
5th alcohol=100% ethanol

Embed in quetol
Section
Stain

Figure 31. Protocols for the intracellular calcium localisation procedures

The first part of the evaluation procedure was conducted in order to obtain a suitable preparation procedure for neutrophils for TEM. The most suitable preparation procedure is now employed for the localisation of intracellular calcium via TEM. As discussed all 4 the tested preparation procedures gave satisfactory neutrophil micrographs. Nevertheless, the preparation procedure containing phosphate buffered saline (Adaptation 1a or 1b) was the method of choice. The reason being that phosphate ions influence the pyroantimonate reaction; possibly making the reaction more specific for calcium. The optimal reaction conditions for the pyroantimonate reaction was discussed previously.

- **Control reactions**

In order to determine the composition of the formed pyroantimonate precipitates; whether the precipitates contain calcium or another cation, the sections were left in the presence of 0.2 M EDTA pH 8.4 at 60°C. Dissolution of the precipitates would indicate calcium as the major cation contained in the precipitates.

3.2.2) Results

Examples of the obtained micrographs for the intracellular calcium localisation in the neutrophil can be seen in Figure 32.

1) General

There are indications that the use of EGTA or EDTA prior to or during fixation results in the loss or diminution of deposits that had been shown by X-ray analysis to contain calcium. A 0.25 mM EDTA ammonium chloride solution is employed during neutrophil isolation therefore the effect of the use of EDTA on the magnitude of calcium precipitate formation was investigated. Refer to the various protocols, however no difference was found in the magnitude of precipitate formation in the absence or presence of EDTA.
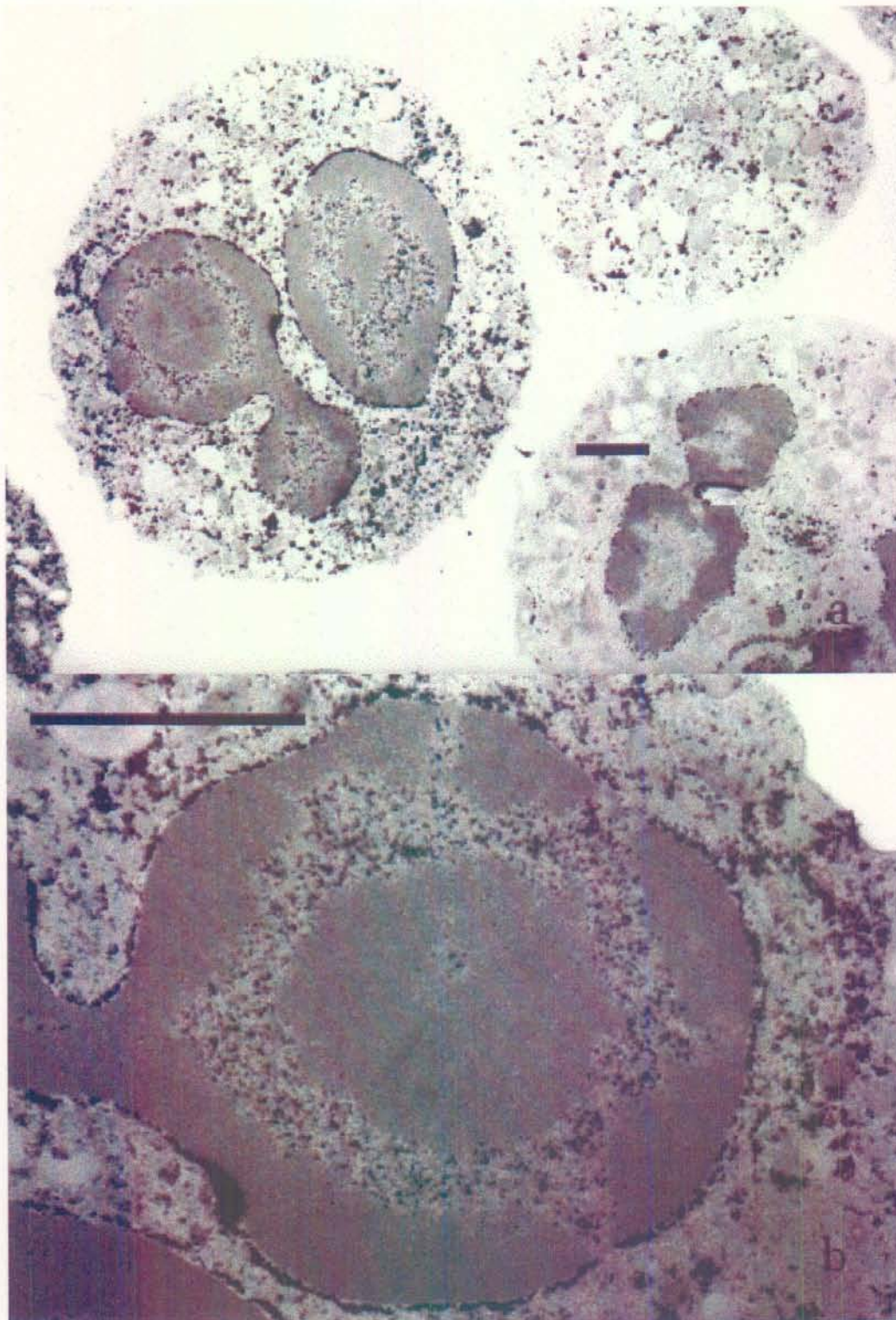
Figure 32 a and b. Electron microscopy photographs indicating calcium-pyroantimonate precipitate formation in neutrophils. Scale bar 1 μm.

2) Calcium distribution patterns obtained with phosphate-pyroantimonate and oxalate-pyroantimonate calcium precipitation reactions

Three different antimonate precipitation protocols were investigated. Refer to figure 31. In adaptation 1 and 2 the phosphate anion was employed to retain calcium, whereas in adaptation 3 the oxalate anion was employed to retain calcium. Only a few precipitates were noticed with the employment of the oxalate anion, indicating unsatisfactory reaction conditions during primary fixation. With the employment of the phosphate-pyroantimonate procedure various calcium precipitation patterns were obtained, and a significant difference was noticed between the employment of either the sodium or potassium phosphate salts. Employment of the sodium phosphate salt resulted in fewer calcium precipitates compared to the employment of the potassium phosphate salt.

3) Control reactions

During a control reaction, a 0.2 M EDTA solution pH 8.4 was employed to demonstrate the presence of calcium in the antimonate precipitation products. Sections were left in the presence of this EDTA solution for 1 hour at 60°C. This resulted in the complete disintegration of the precipitates and the formation of voids, indicating that mostly calcium was present in the antimonate-precipitates. Examples of the micrographs obtained after the removal of the precipitates can be seen in Figure 33.

3.2.3) Discussion

Various calcium distribution patterns were obtained for both the phosphate pyroantimonate procedures (potassium and sodium) except that fewer precipitates were detected for the sodium phosphate salt. The difference in the magnitude of precipitate formation between

Figure 33 a and b. Electron microscopy photographs indicating the complete disintegration of the calcium-pyroantimonate precipitates and the subsequent formation of voids upon EDTA addition in neutrophils. Scale bar 1 μm.

employment of either the sodium phosphate salt or the potassium phosphate salt might be the result of displacement of calcium by sodium since antimonate has a high affinity for sodium. Various calcium distribution patterns were obtained. Refer to figure 32. These very distinctive calcium precipitation patterns included: 1) pronounced precipitate formation around the nucleus of the neutrophil coinciding with the localisation of the calcium store in the nuclear envelope, 2) precipitate formation in the nucleus as part of the less dense euchromatin, this precipitate pattern projects outward to the nuclear envelope coinciding with the nuclear pores, and 3) throughout the cytoplasm scattered precipitates were noticed. (43, 51) One would expect calcium also to be associated with sites of high DNA content, heterochromatin staining would be attributable to calcium bound to nucleic acids as well as to reactive amino acids on histones. Nevertheless, the present results appear to indicate that the condensed chromatin in the nucleus is largely free of calcium ions. The presence of chelated calcium ions cannot be ruled out since they will not yield insoluble pyroantimonate salts. (45, 46) From these results it was apparent that different neutrophils, even adjacent to one another display variations in the magnitude of calcium precipitate formation. This might be a manifestation of the heterogeneity of calcium homeostasis in different neutrophils and also various neutrophils might be captured at different time-points during various calcium regulated cellular processes.

The chelators EGTA and EDTA are frequently used to demonstrate the presence of calcium in antimonate precipitation products. Monovalent cation-antimonate salts will not be removed by chelators, magnesium-antimonate will remain essentially unaffected by EDTA and will be only slightly diminished by EGTA, while calcium-antimonate deposits will almost totally be abolished by either chelator. (42, 47) With the

employment of a 0.2 M EDTA solution pH 8.4 to demonstrate the presence of calcium in the antimonate precipitation products, complete disintegration of the precipitates resulted.

3.3)   Conclusions
The optimised protocol is presented.

Neutrophil isolation

Calcium localisation for TEM
1)   Pipet a volume of 1 ml of the neutrophil suspension into an eppendorff.
2)   Centrifuge at 2000 r.p.m. for 5 minutes.
3)   Discard the supernatant.
4)   Pipet 1 ml of the 2.5% glutaraldehyde, 0.1 M KPBS, room temperature into the eppendorff containing the neutrophil pellet.
5)   Incubate 1 hour at room temperature - primary fixation.
6)   Discard the supernatant.
7)   Wash 3x with 0.1 M KPBS.
8)   Discard the supernatant.
9)   Pipet 1 ml of the 1% OsO_4, 2% $KSb(OH)_6$, room temperature.
10)   Incubate 1 hour at room temperature - post-fixation.
11)   Wash 3x with 0.1 M KPBS.
12)   Discard the supernatant.
13)   Add 1 ml of a 50% ethanol solution.
14)   Incubate for 10 minutes, room temperature.
15)   Discard the supernatant.
16)   Add 1 ml of a 70% ethanol solution.
17)   Incubate for 10 minutes, room temperature.
18)   Discard the supernatant.

19) Add 1 ml of a 95% ethanol solution.

20) Incubate for 10 minutes, room temperature.

21) Discard the supernatant.

22) Add 1 ml of 100% ethanol.

23) Incubate for 10 minutes, room temperature.

24) Discard the supernatant.

25) Add 1 ml of 100% ethanol.

26) Add 1 ml of a Quetol epoxy resin.

27) Incubate 1 hour at room temperature.

28) Discard the supernatant.

29) Add 1 ml of a Quetol epoxy resin.

30) Incubate overnight at room temperature.

31) Discard the supernatant.

32) Add 1 ml of a Quetol epoxy resin.

33) Incubate 6 hours at 60°C.

34) Section.

35) Stain uranyl acetate and lead citrate.

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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$_2$O (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 $0.22\mu m$ 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.

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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
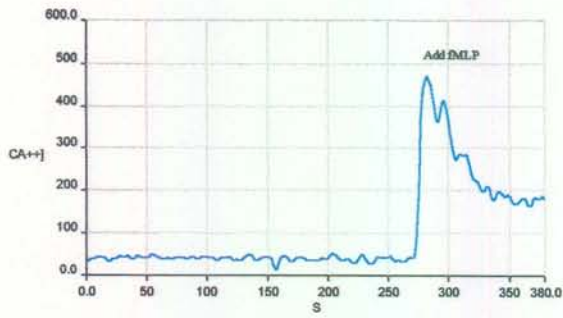
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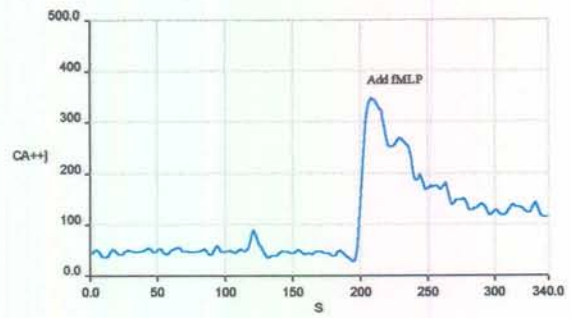
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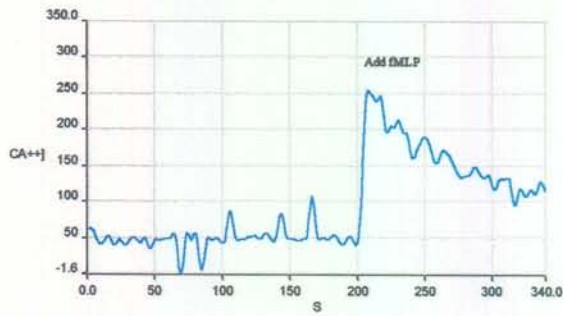
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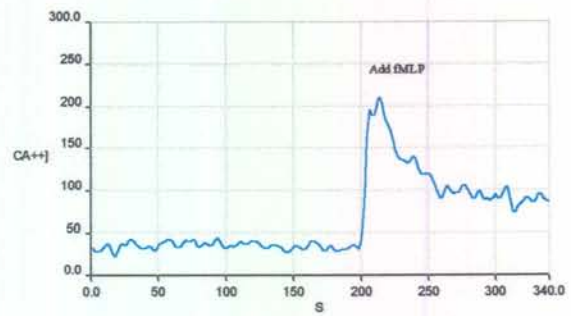
Figure 1d-patient 4
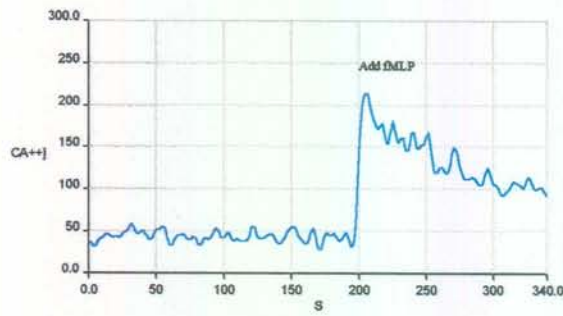
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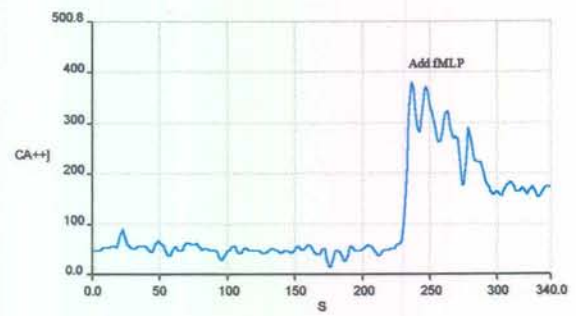
Figure 1f-patient 6
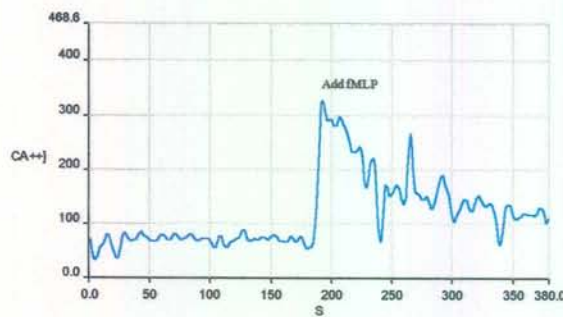
Figure 1g-patient 7
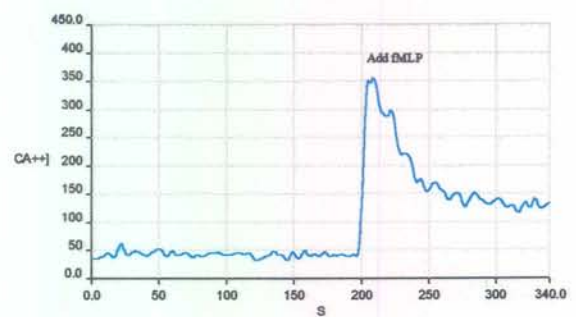
Figure 1h-patient 8

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)

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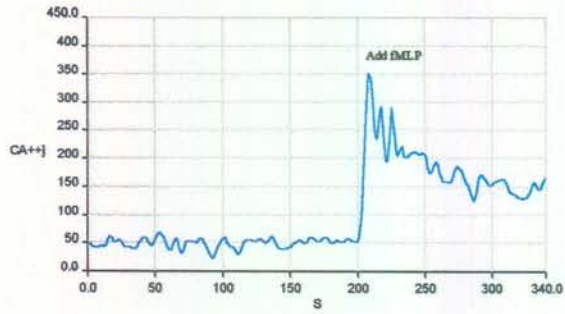
Figure 1i-patient 9
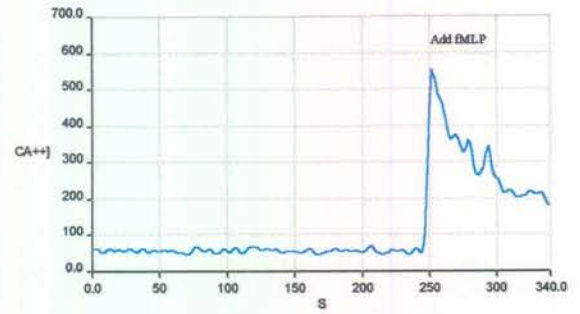
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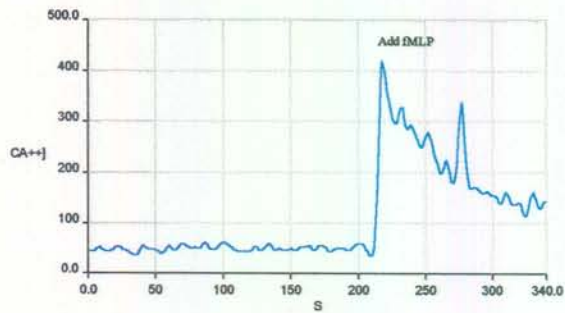
Figure 1k-patient 11
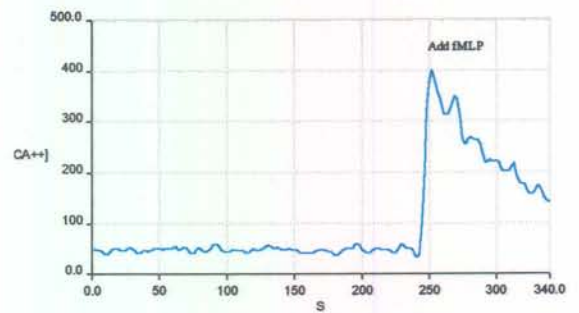
Figure 1l-patient 12
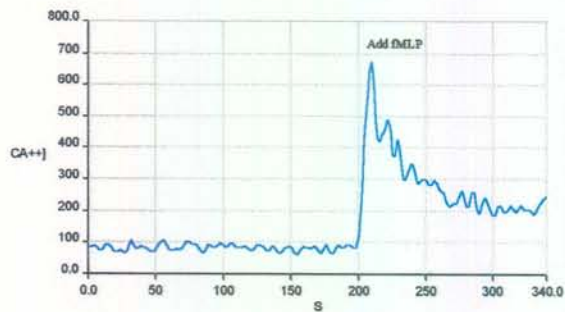
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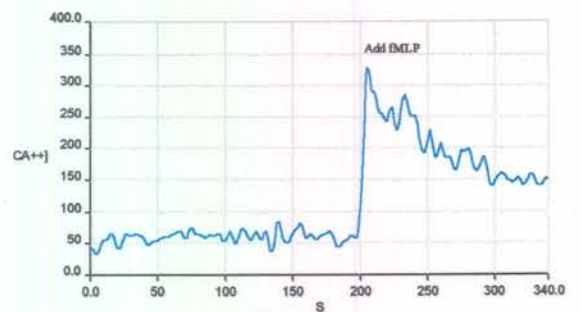
Figure 1n-patient 14

154
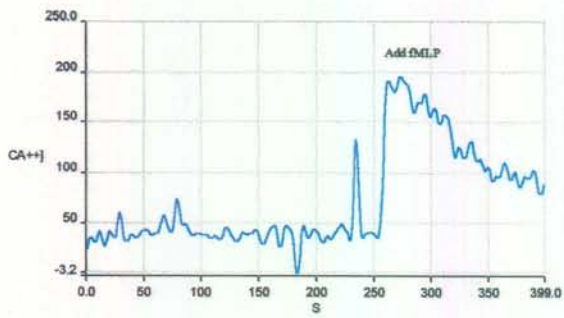
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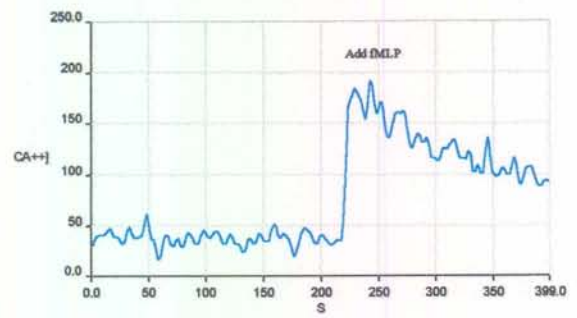
Figure 1p-normal 2
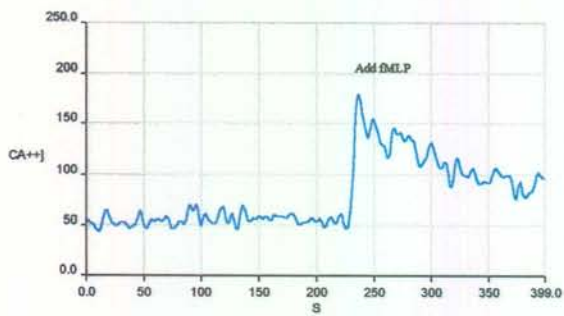
Figure 1q-normal 3
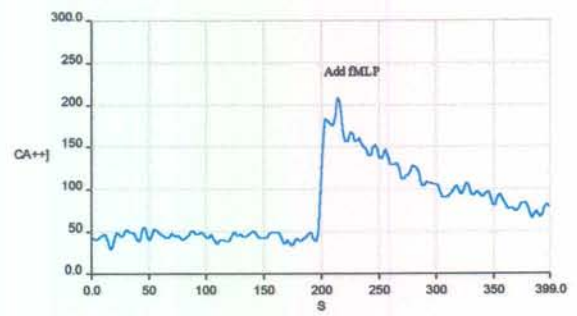
Figure 1r-normal 4
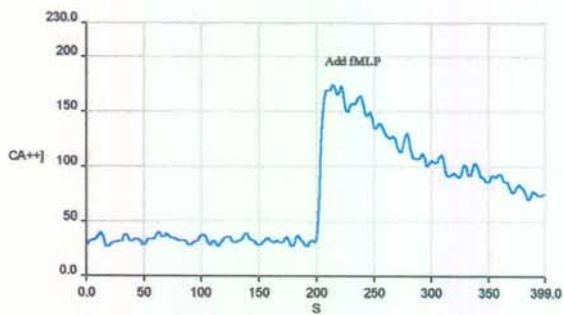
Figure 1s-normal 5
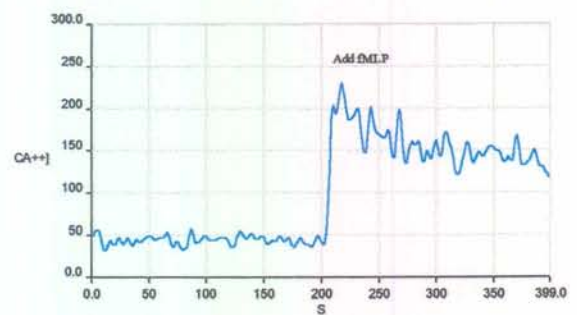
Figure 1t-normal 6
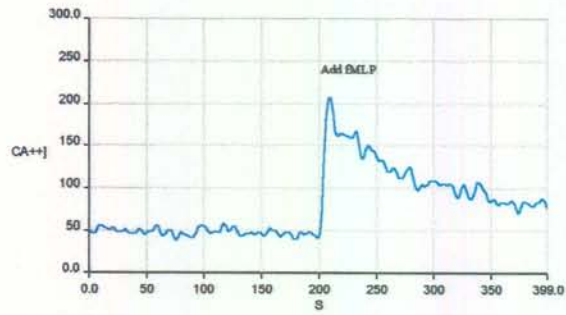
Figure 1u-normal 7
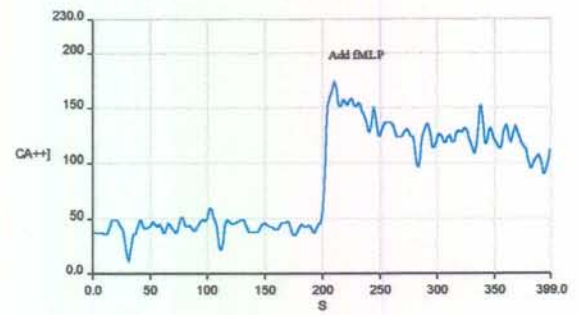
Figure 1v-normal 8
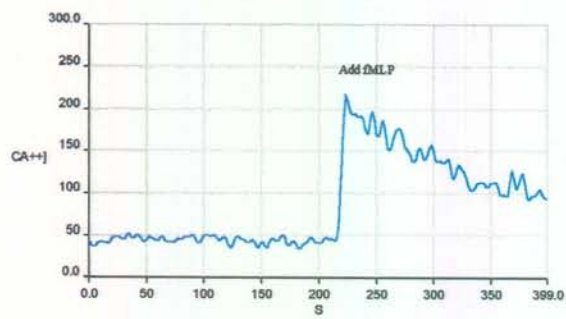
Figure 1w-normal 9

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	* 0.0242
	mean	53.071	43.2	
	SD	12.761	6.4601	
Transmembrane calcium flux	n	14	10	* 0.0002
	mean	380.29	201.2	
	SD	126.68	20.335	
Decrease of iCa rate lny	n	14	10	# 0.0952
	mean	-0.0052	-0.0038	
	SD	0.001686	0.001488	
Decrease of iCa rate y	n	14	10	* 0.0017
	mean	-1.0533	-0.476	
	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		e=ne
	mean	44.2	58	43.2	* 0.0016	ne=c
	SD	5.4498	13.162	6.4601		e>c
Transmembrane Ca flux	n	5	9	10		e=ne
	mean	300.6	424.56	201.2	* < 0.001	ne=c
	SD	111.03	117.21	20.335		e>c
Decrease of iCa rate lny	n	5	9	10		
	mean	-0.00541	-0.005077	-0.003799	0.2248	
	SD	0.002106	0.001536	0.001488		
Decrease of iCa rate y	n	5	9	10		c=ne
	mean	-0.963	-1.1036	-0.476	* 0.0011	ne=e
	SD	0.7602	0.4544	0.1811		e> c

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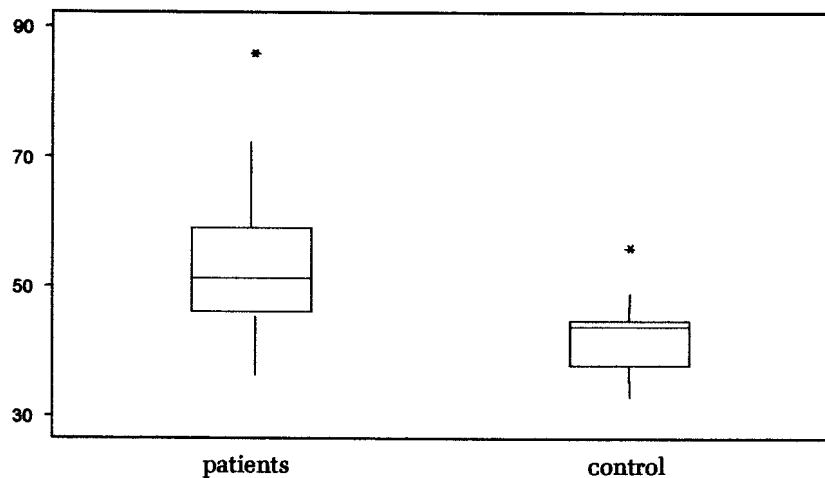
Figure 2. Box and Whisker Plot – intracellular free calcium (nM)

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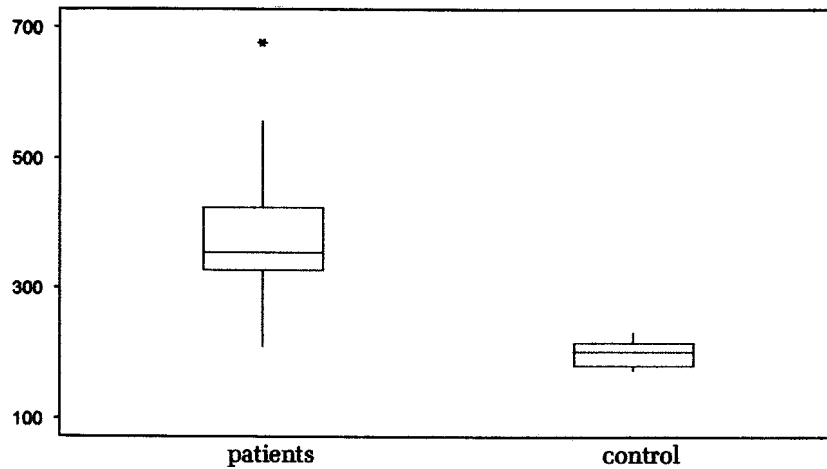
**Figure 3. Box and Whisker Plot –
transmembrane calcium flux (nM)**

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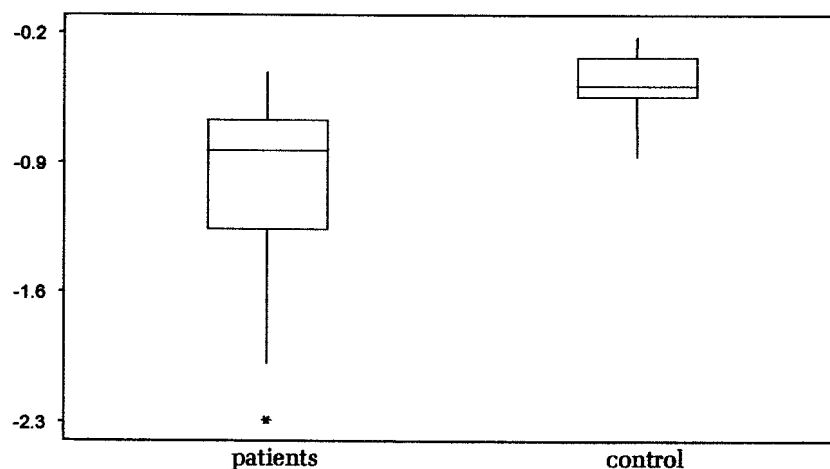
**Figure 4. Box and Whisker Plot – rate
of intracellular free calcium decrease
(nM/s)**

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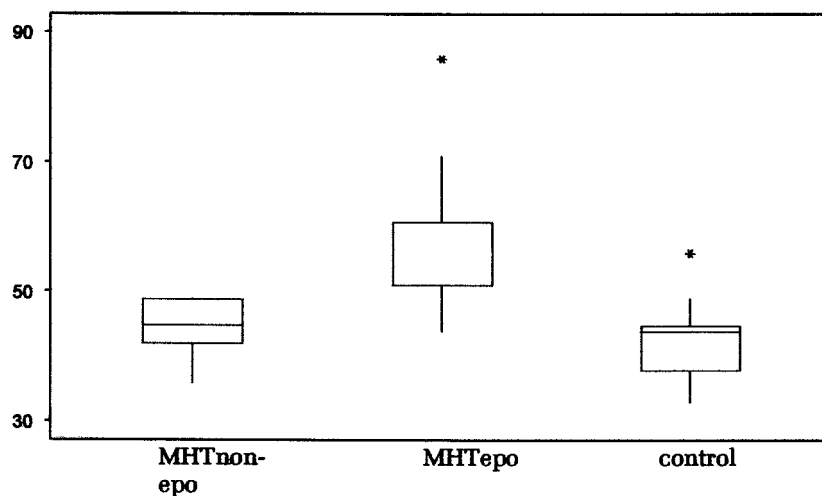
Figure 5. Box and Whisker plot – intracellular free calcium (nM)

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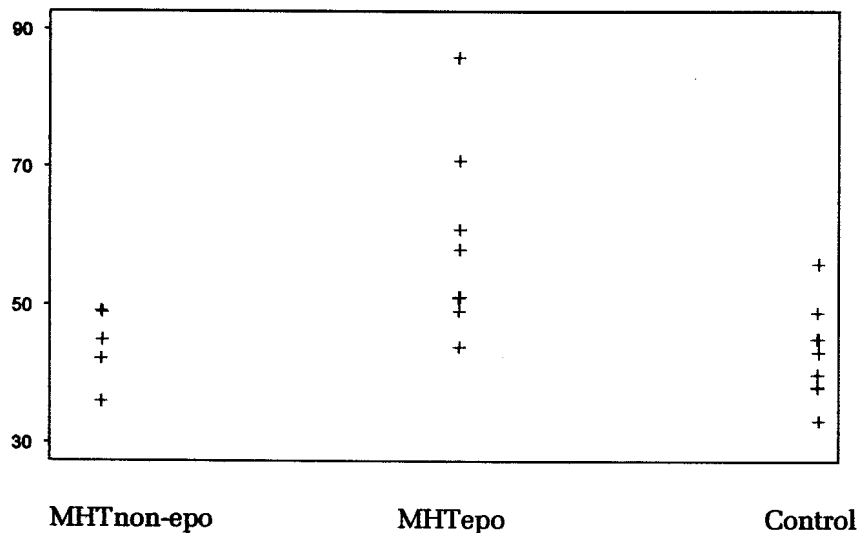
Figure 6. Scatter plot – intracellular free calcium (nM)

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	
	mean	2437.5	2344	2343.5	0.8249
	SD	255.08	147.94	203.39	
SM	n	5	9	10	
	mean	435.14	473.43	394.38	0.1554
	SD	103.98	89.619	60.91	
PC	n	5	9	10	
	mean	638.5	633.33	617	0.9739
	SD	128.73	72.64	34.335	
PEA	n	5	9	10	
	mean	487.16	510.84	451.4	0.1825
	SD	81.805	59.406	66.675	
PI	n	5	9	10	
	mean	144.35	147.66	118.4	0.4907
	SD	50.02	72.761	21.108	
PS	n	5	9	10	
	mean	225.55	230.15	208.15	0.4576
	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		

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

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

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	
	mean	3.74	3.9444	3.699	0.9301
	SD	0.562	0.6462	0.3949	
Fn-6 22:5	n	5	9	10	
	mean	0.466	0.7878	0.748	0.1823
	SD	0.407	0.464	0.2104	
Fn-3 22:5	n	5	9	10	
	mean	1.482	1.3633	1.564	0.4547
	SD	0.3192	0.2642	0.2228	
Fn-3 22:6	n	5	9	10	
	mean	4.05	3.5733	4.26	0.3087
	SD	0.5542	0.9103	0.6533	
F24:0	n	5	9	10	
	mean	2.962	3.8122	3.41	0.0509
	SD	0.6785	0.4481	0.5129	
Fn-9 24:1	n	5	9	10	
	mean	2.806	2.9122	2.807	0.8142
	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						
TPL-SUM	n	5	9	10		e=ne
	mean	1930.7	1995.4	1789.3	* 0.0182	ne=c
	SD	218.05	140.39	150.71		e>c
SM-P	n	5	9	10		
	mean	22.4	23.778	22	0.4522	
	SD	3.7815	3.8006	2		
PC-P	n	5	9	10		
	mean	33.2	31.556	34.7	0.1866	
	SD	6.0581	3.678	2.4967		
PEA-P	n	5	9	10		
	mean	25.2	25.444	25.2	0.9687	
	SD	1.7889	1.8782	2.6583		
PI-P	n	5	9	10		
	mean	7.38	7.3556	6.57	0.6047	
	SD	2.0729	3.3683	0.636		
PS-P	n	5	9	10		
	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 mean SD	5 44.236 2.6046	9 46.832 4.9676	10 43.577 1.6851	0.6468	
MUFA's	n mean SD	5 17.326 0.8688	9 16.619 0.8803	10 15.494 1.1897	* 0.0096	ne=e e=c ne>c
PUFA's	n mean SD	5 38.446 2.7467	9 36.54 4.6756	10 40.933 2.351	# 0.0948	
PUFA's/SFA's	n mean SD	5 0.8744 0.1136	9 0.7971 0.1805	10 0.9423 0.0884	0.1778	
n-3	n mean SD	5 5.962 0.6874	9 5.3344 1.1663	10 6.263 0.8952	0.2004	
n-6	n mean SD	5 32.484 2.194	9 31.206 3.7057	10 34.67 2.0548	# 0.0981	

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	0.5977
	mean	5.4811	6.0465	5.6261	
	SD	0.4452	1.1815	0.7728	
D5-Desaturase	n	5	9	10	0.5506
	mean	12.981	11.394	10.378	
	SD	4.7673	2.4121	2.6152	
D6-Desaturase	n	5	9	10	# 0.095
	mean	0.002531	0.005367	0.002765	
	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.
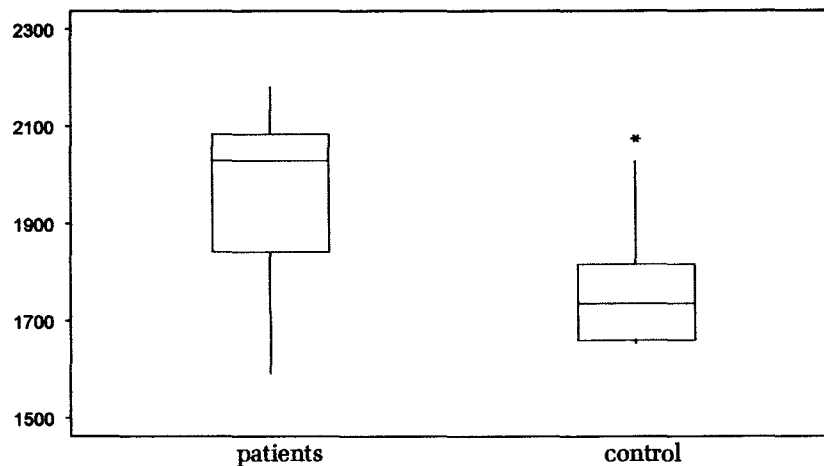
Figure 7. Box and Whisker plot – total phospholipids (µg/ml packed cells)

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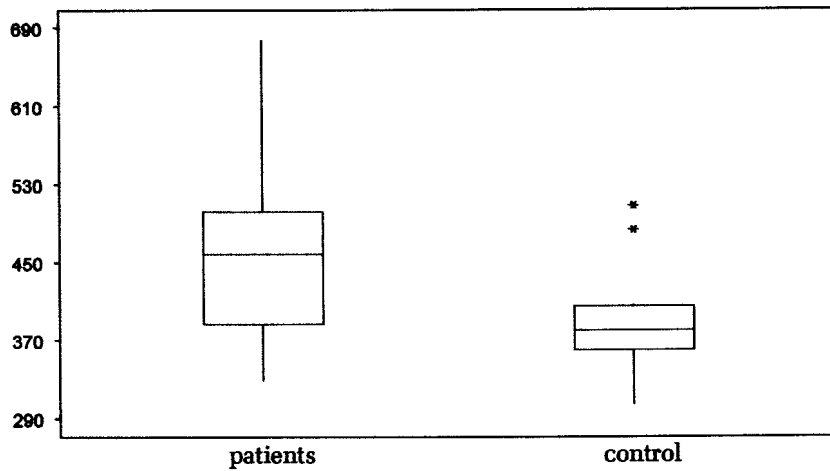
Figure 8. Box and Whisker plot – SM (µg/ml packed cells)

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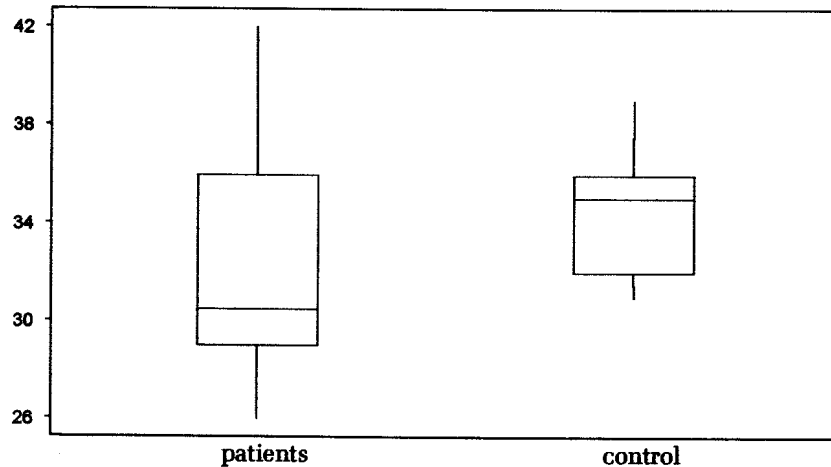
Figure 10. Box and Whisker Plot – PC (%)

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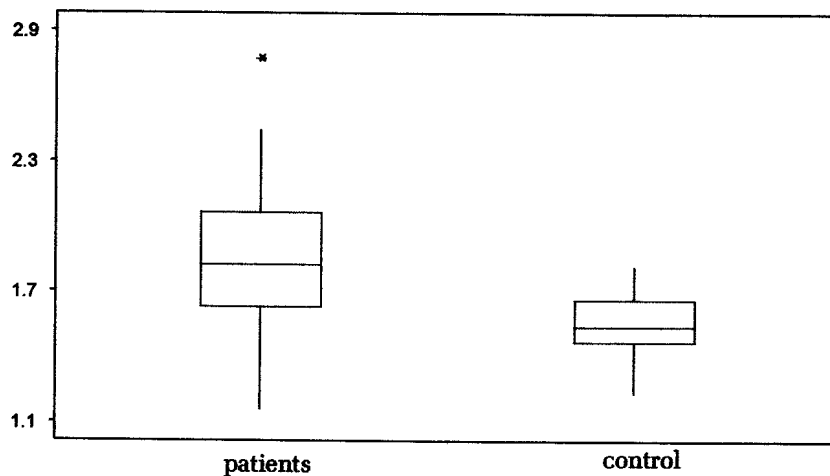
Figure 11. Box and Whisker Plot – 22:0 (weight %)

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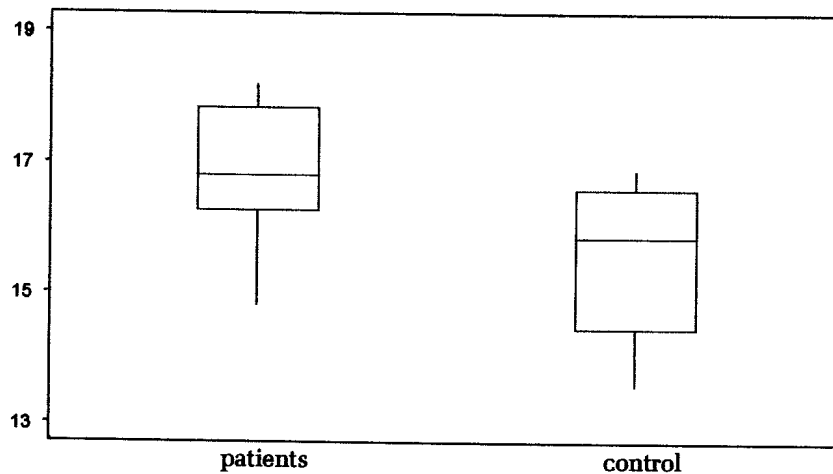
Figure 12. Box and Whisker Plot - MUFA's (weight %)

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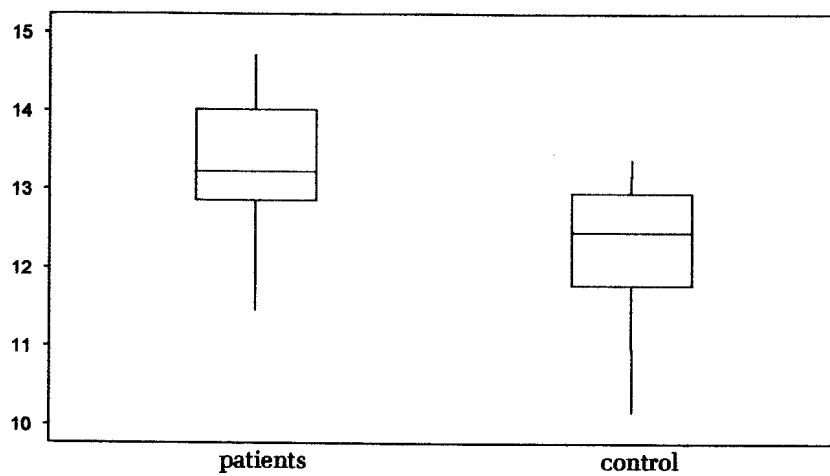
**Figure 13. Box and Whisker Plot – 18:1 (weight %)**

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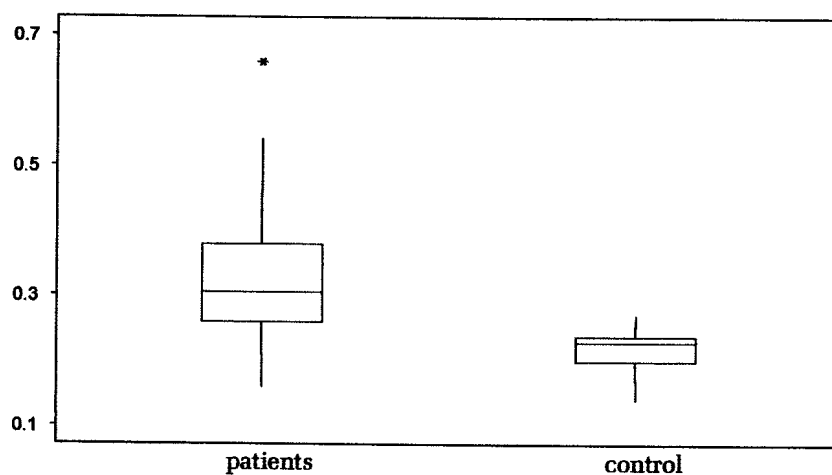
Figure 14. Box and Whisker plot – n-9 20:1 (weight %)

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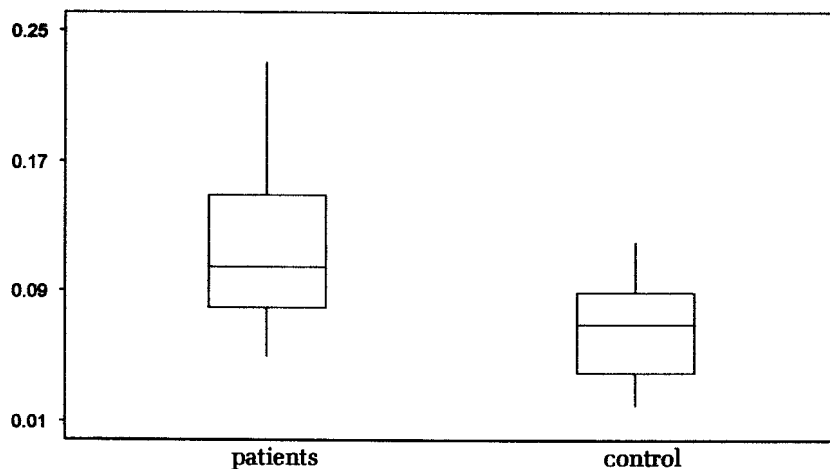
Figure 15. Box and Whisker Plot – n-9 22:1 (weight %)

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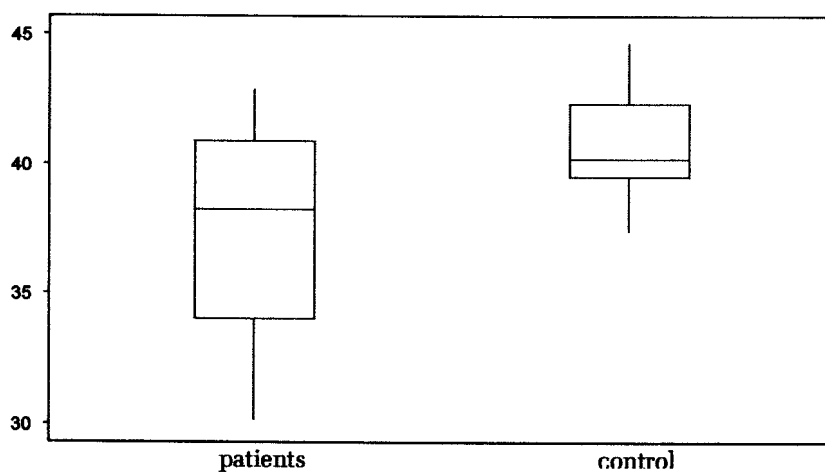
Figure 16. Box and Whisker Plot – PUFA's (weight %)

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
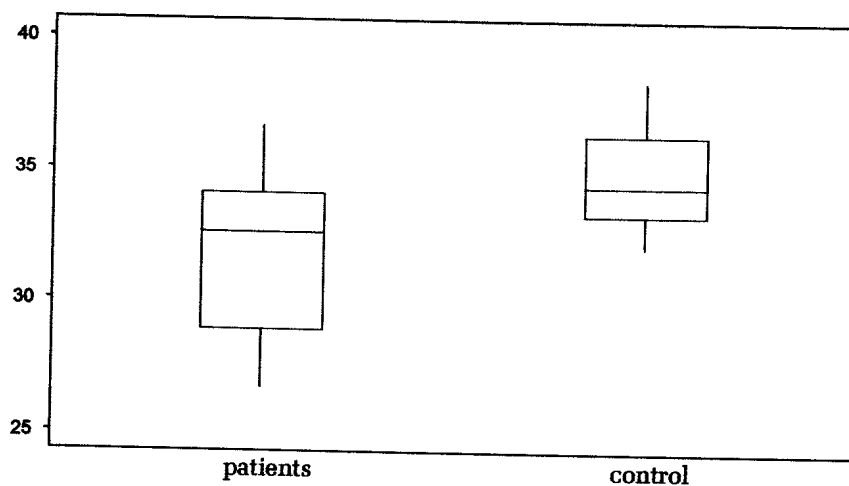

Figure 17. Box and Whisker plot – n-6 fatty acids (weight %)

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.
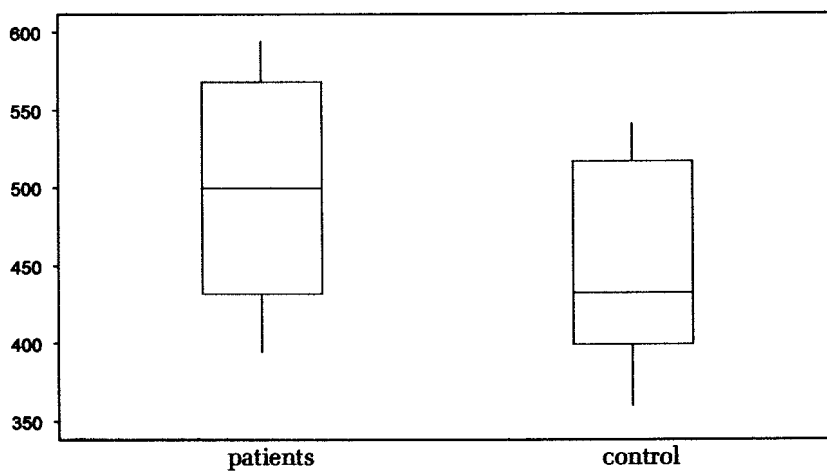

Figure 19. Box and Whisker plot – n-6 20:3 (weight %)

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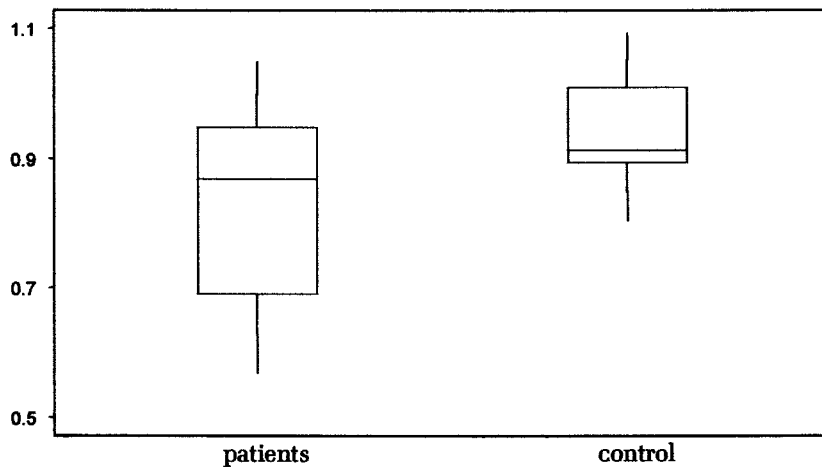
Figure 22. Box and Whisker plot – PUFA's/SFA's

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	* < 0.001
	mean	591.37	24.98	
	SD	477.16	6.4965	
Vitamin C	n	14	10	1
	mean	64.386	55.35	
	SD	48.675	19.344	
Vitamin A	n	14	10	* < 0.001
	mean	11.8	3.95	
	SD	4.7099	0.9229	
Vitamin E	n	14	10	0.4823
	mean	37.521	32.07	
	SD	13.554	6.7962	
Serum free Ca	n	14	10	* 0.0404
	mean	1.1943	1.239	
	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		e=ne
	mean	398.58	698.48	24.98	* < 0.001	e+ne>c
	SD	528.99	440.38	6.4965		
Vitamin C	n	5	9	10		ne=c
	mean	107.1	40.656	55.35	* 0.0204	e=c
	SD	51.434	27.449	19.344		ne>e
Vitamin A	n	5	9	10		e=ne
	mean	10.52	12.511	3.95	* < 0.001	ne+e>c
	SD	2.6892	5.5533	0.9229		
Vitamin E	n	5	9	10		
	mean	44.4	33.7	32.07	0.5078	
	SD	21.613	4.3549	6.7962		
Serum free Ca	n	5	9	10		
	mean	1.188	1.1978	1.239	0.1096	
	SD	0.0901	0.0452	0.0281		

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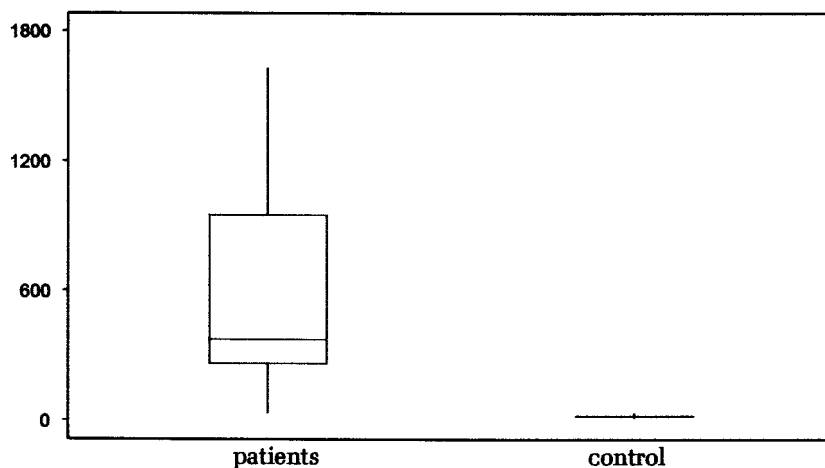
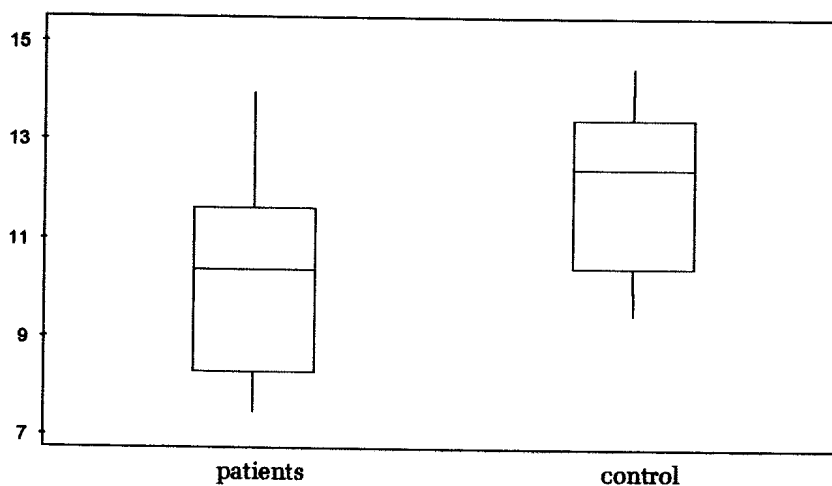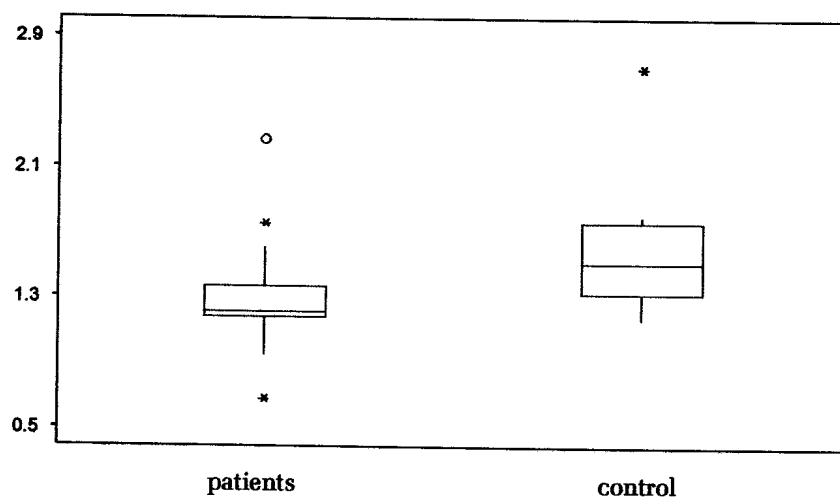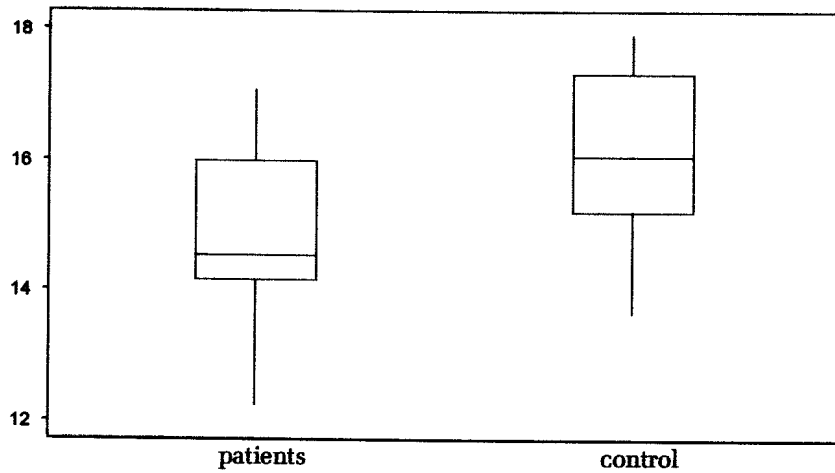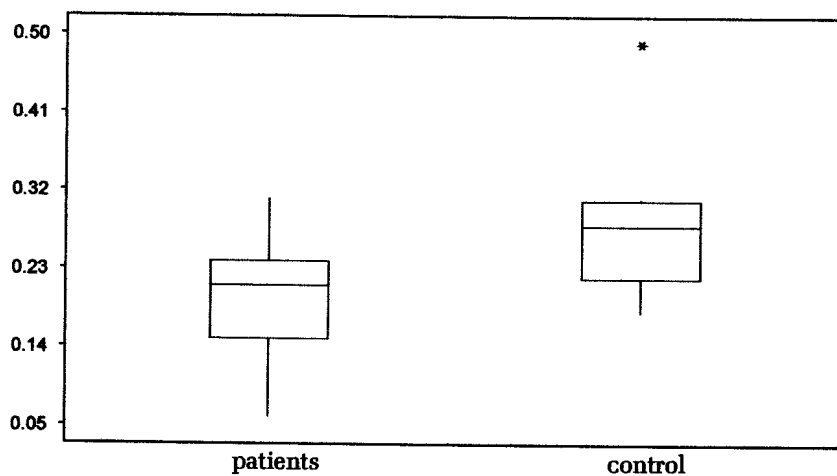**Figure 23. Box and Whisker plot – PTH (ng/l)**

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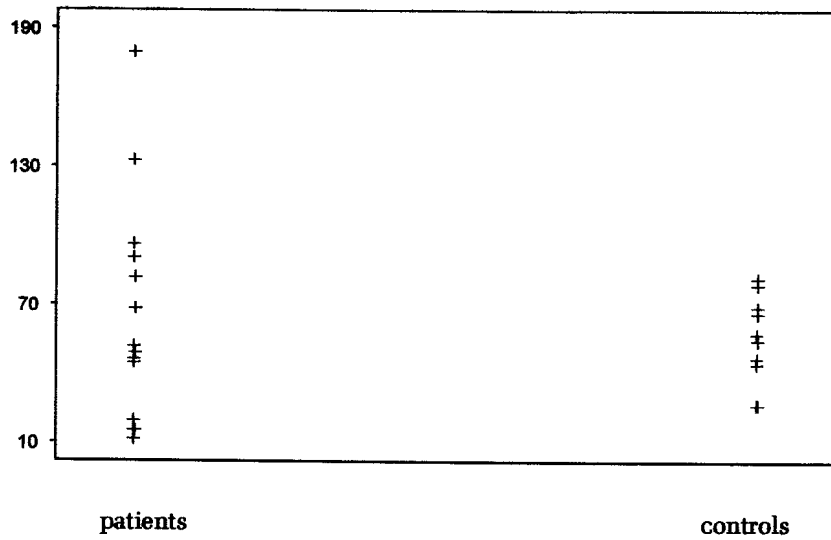
Figure 24. Scatter Plot- vitamin C (µmol/l)

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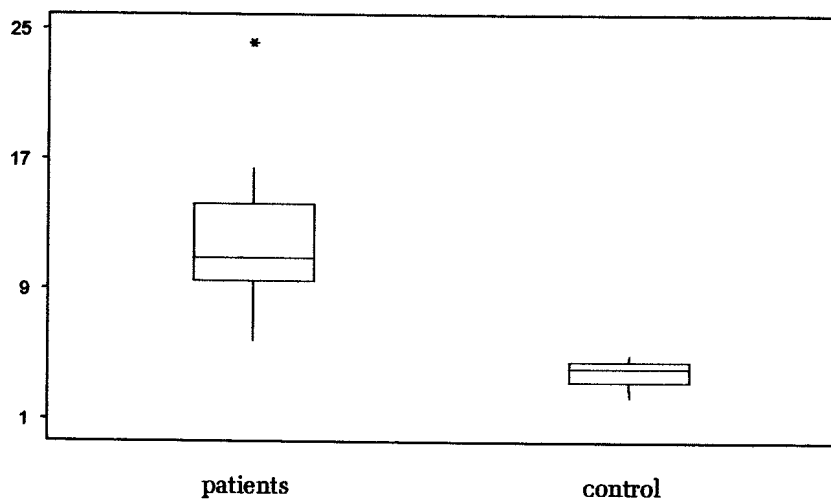
Figure 25. Box and Whisker Plot – vitamin A (µmol/l)

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.
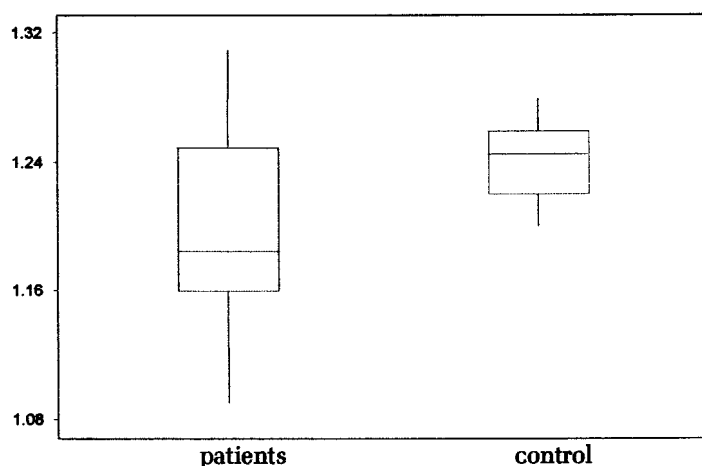

Figure 26. Box and Whisker Plot – ionised serum calcium (mmol/l)

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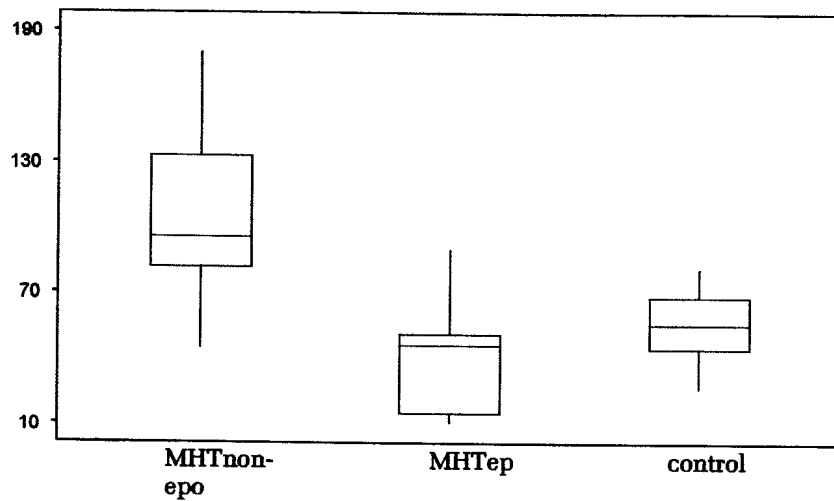
Figure 27. Box and Whisker plot – vitamin C (μmol/l)

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.
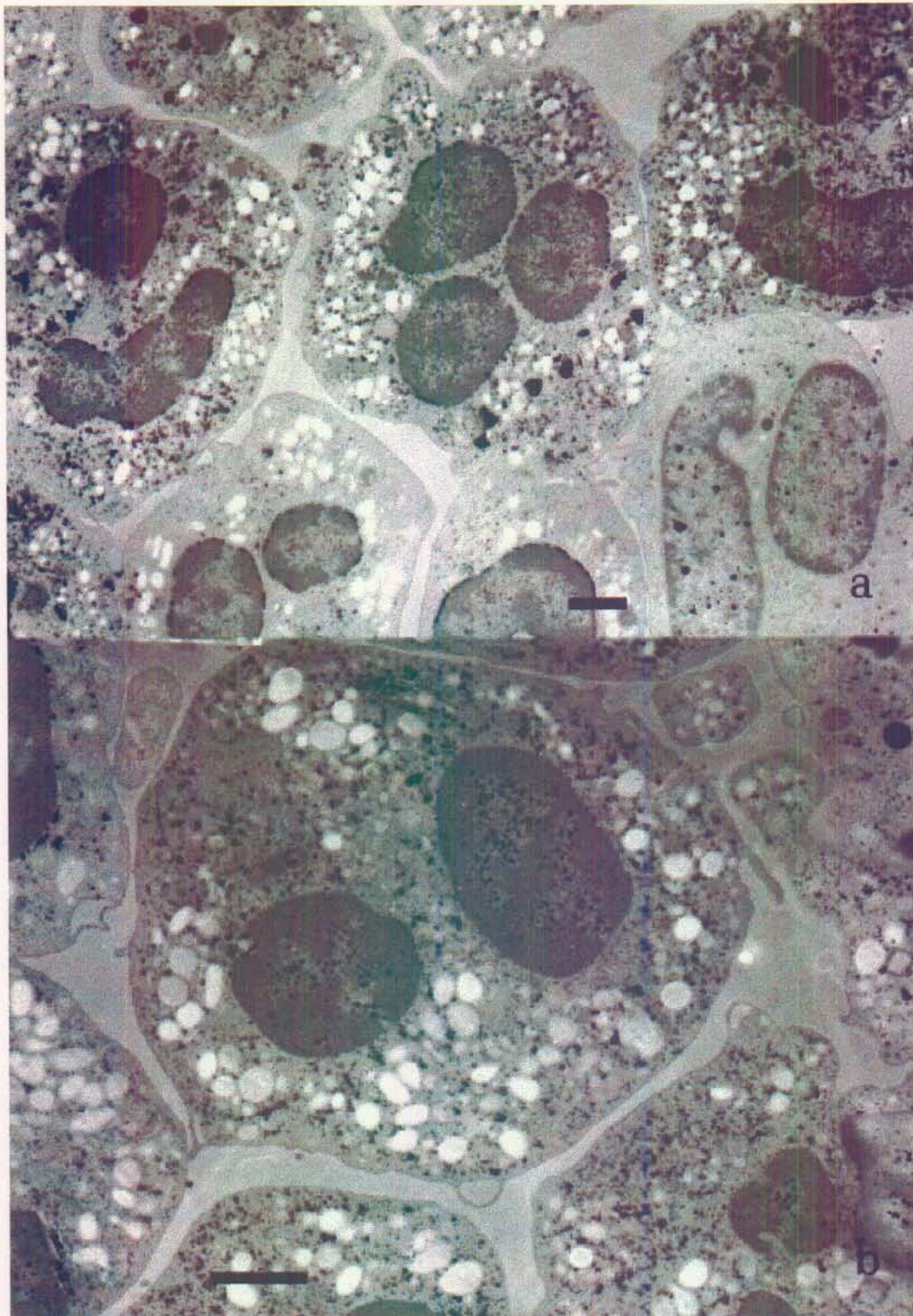
Figure 28 a and b. Electron microscopy photographs indicating the localisation of intracellular calcium in the cytosol, in the nucleus and in the space between the inner and outer nuclear membranes in the neutrophils of the control subjects. Scale bar 1 μm.
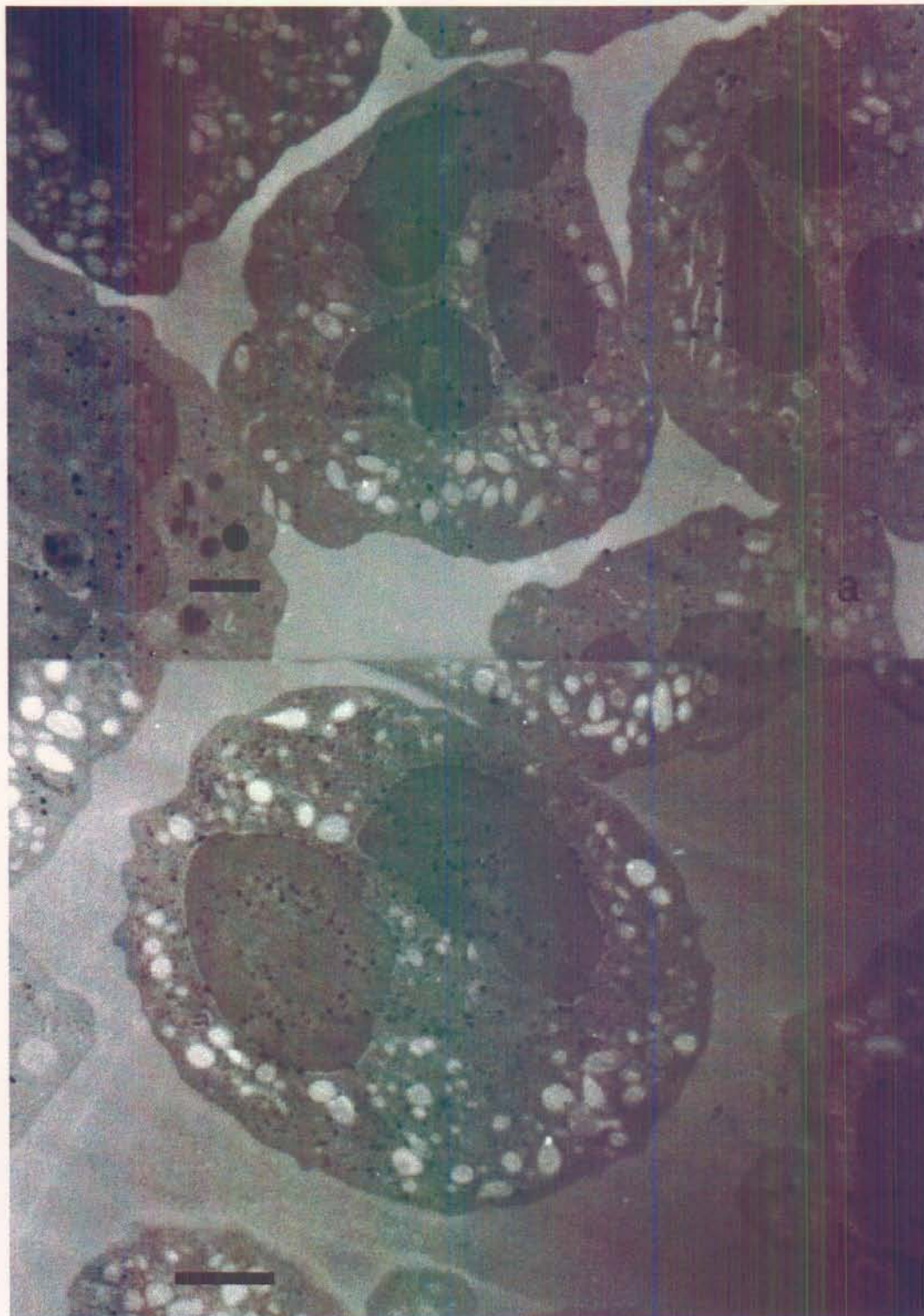
Figure 29 a and b. Electron microscopy photographs indicating the localisation of intracellular calcium in both the nucleus and cytosol in the neutrophils of some of the patients. Scale bar 1 μm.
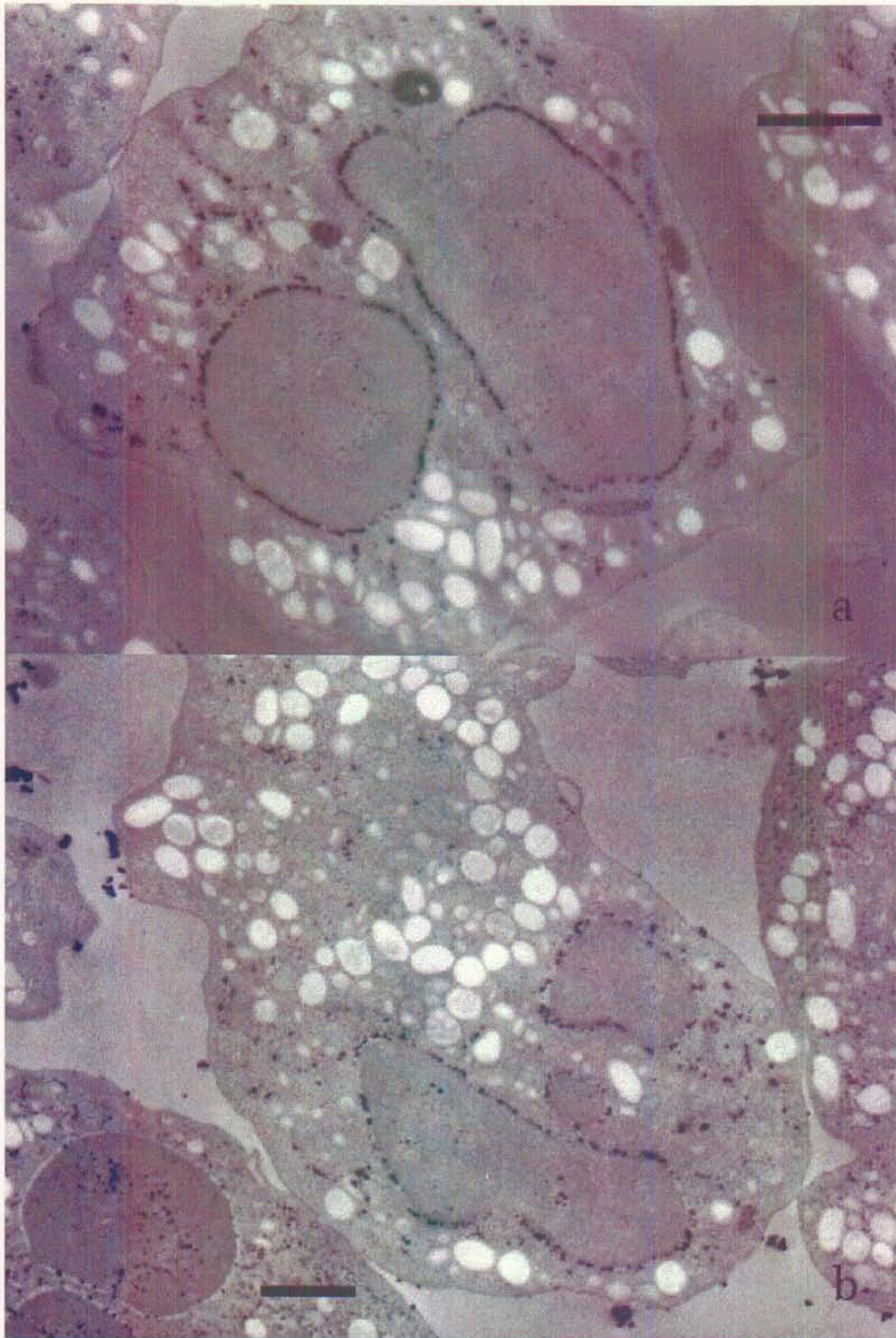
Figure 30 a and b. Electron microscopy photographs indicating the prominent localisation of intracellular calcium in the space between the inner and outer nuclear membranes in the neutrophils of some of the patients. Scale bar 1 μm.

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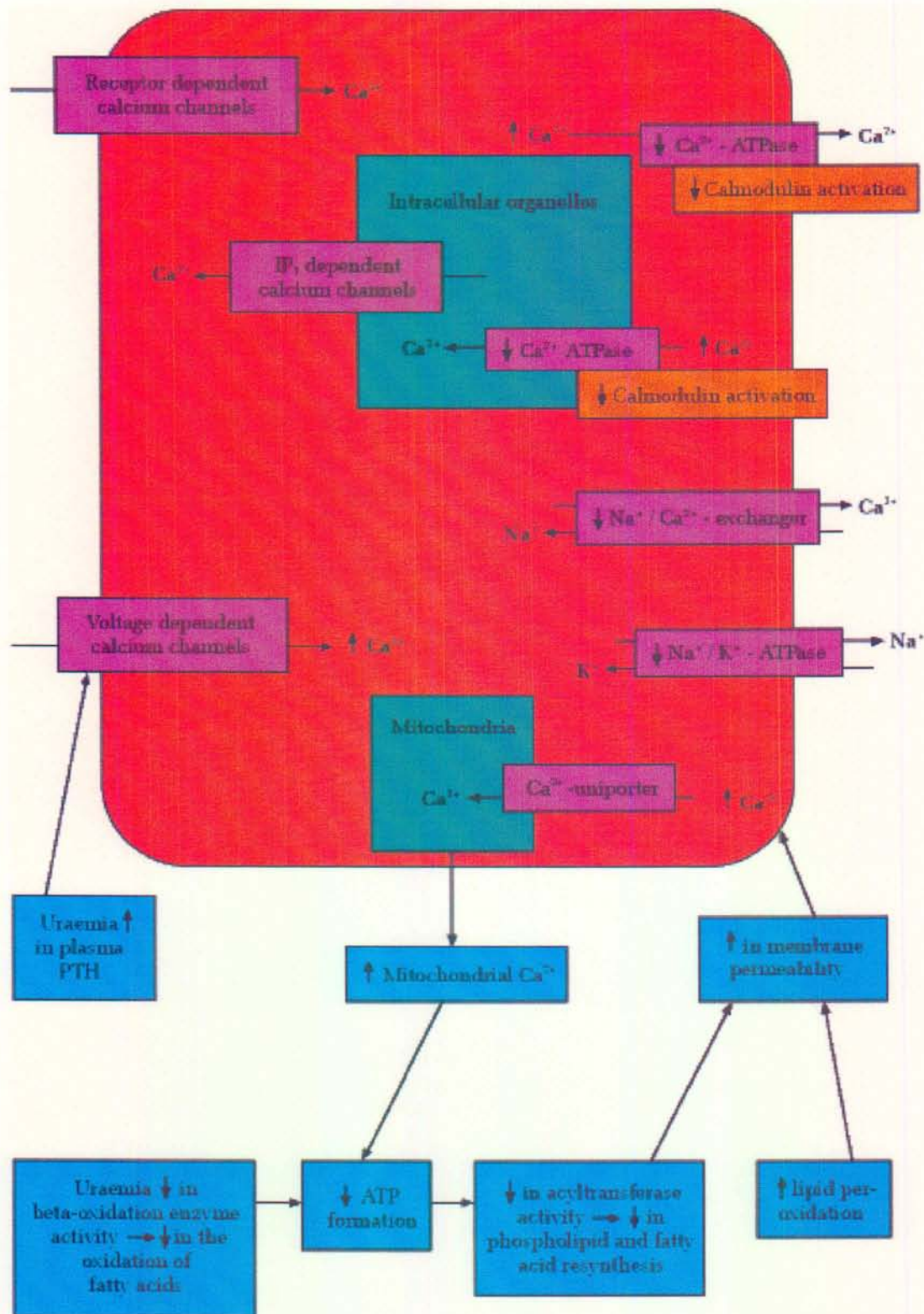
Figure 1. Schematic presentation of possible factors responsible for the perturbation in intracellular free Ca^{2+} in the chronic renal failure patient.

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

Complement dependent and complement independent neutrophil activation during HD with bio-incompatible membranes

Redox reactions

Uraemia ↑ in NO formation

↑ O_2

↑ NO

↑ ONOO peroxynitrite

Uraemia ↓ in plasma Zn, Cu

↓ Superoxide dismutase

Uraemia ↑ Pb, Fe transition metals Haber-Weiss reaction

H_2O_2

↑ OH + OH

Catalase

H_2O + ½O_2

NADP

↓ GSH

↓ Glutathione reductase

↓ Glutathione peroxidase

Uraemia ↓ plasma Se

↑ GSSG

2 H_2O

↓ NADPH

Lipid peroxidation

Uraemia HD ↓ hexose monophosphate shunt

ProtSH

ProtS-SG + GSH

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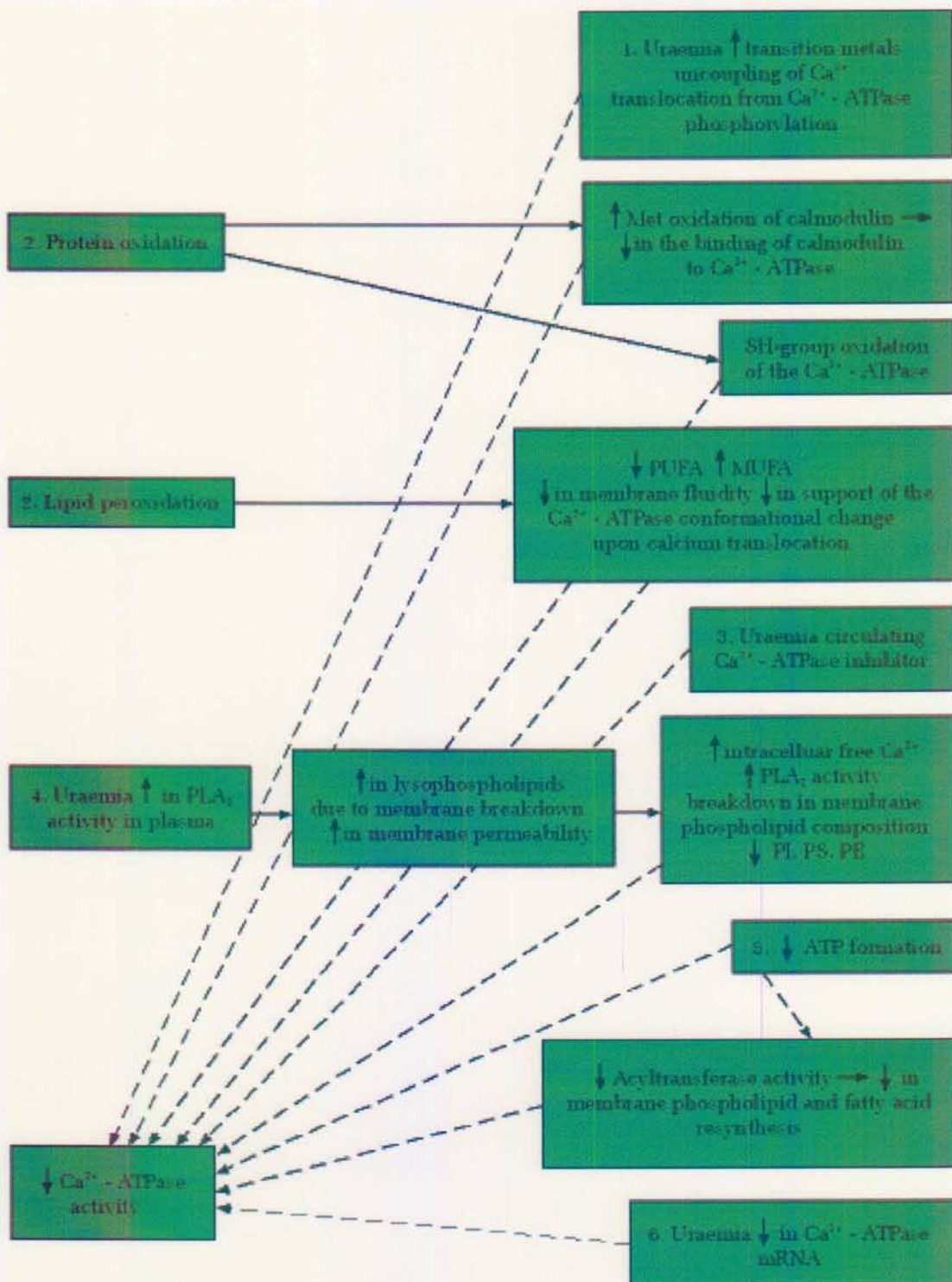
Figure 3. Schematic presentation of factors associated with chronic renal failure possibly causing a decrease in Ca^{2+} - ATPase activity.

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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