CHAPTER 4

DISCUSSION

1) INTRODUCTION

Ferritin is the intracellular protein responsible for the sequestration, storage and release of iron. Ferritin can accumulate up to 4500 iron atoms as a ferrihydrite mineral in a protein shell and releases these iron atoms when there is an increase in the cell's need for bio-available iron (1). The ferritin protein shell consists of 24 protein subunits of two types, the H-subunit and the L-subunit. These ferritin subunits perform different functions in the mineralization process of iron (2). The ferritin protein shell can exist as various combinations of these two subunit types, giving rise to heteropolymers or isoferritins. Isoferritins are functionally distinct and it would appear that characteristic populations of isoferritins are found depending on the type of cell, the proliferation status of the cell and the presence of disease (3). The synthesis of ferritin is regulated both transcriptionally and translationally. Translation of ferritin subunit mRNA is increased or decreased, depending on the labile iron pool and is controlled by an iron-responsive element present in the 5'-untranslated region of the ferritin subunit mRNA (4). The transcription of the genes for the H-subunit and L-subunit of ferritin is controlled by hormones and cytokines, which can result in a change in the pool of translatable mRNA (5). The levels of intracellular ferritin are determined by the balance between synthesis and degradation. Degradation of ferritin in the cytosol results in complete release of iron, while degradation in secondary lysosomes results in the formation of haemosiderin and protection against iron toxicity (6). The majority of

ferritin is found in the cytosol. However, ferritin with slightly different properties can also be found in organelles such as nuclei and mitochondria (7, 8).

Most of the ferritin produced intracellularly is harnessed for the regulation of iron bio-availability, however some of the ferritin is secreted and internalized by other cells (9). In addition to the regulation of iron bio-availability ferritin may contribute to the control of myelopoiesis and immunological responses (10).

Plasma ferritin is increased as an acute phase protein during conditions of infection, inflammation and malignancies, but its expression is also up-regulated in the cytosol of various cells in conditions with uncontrolled cellular proliferation, in any condition marked by excessive production of toxic oxygen radicals, and by infectious and inflammatory processes (11). Under such conditions ferritin up-regulation is predominantly stimulated by increased reactive oxygen radical production and by cytokines (5, 12). The major function of ferritin in these conditions is to reduce the bio-availability of iron in order to stem uncontrolled cellular proliferation and excessive production of reactive oxygen radicals (13). Ferritin is, however, not indiscriminately up-regulated in these conditions as a marked shift towards a predominance in H-subunit rich ferritins would appear to occur (14, 15, 16, 17, 18, 19, 20, 21, 22).

In the present study ultrastructural immunolocalisation of the H-subunit and L-subunit of ferritin in different cells of the bone marrow was undertaken to quantitatively measure the expression of the subunits of ferritin at the single cell level. In this study the quantitative expression of the H-subunit and L-subunit of ferritin in bone marrow macrophages and cells of the erythron was ultrastructurally evaluated by post-embedding immunolocalisation with immunogold transmission electron microscopy in combination with the inflammatory status of patients with chronic immune stimulation.

2) EXPERIMENTAL GROUPS

Forty-eight patients attending the Department of Internal Medicine, Kalafong Hospital for treatment of chronic diseases, with a high prevalence of human immunodeficiency virus (HIV) infection were included in the study. Ten patients scheduled for hip replacement at the Department of Orthopaedics at the Pretoria Academic Hospital were included in the study as a group of patients with less severe immune stimulation. These patients were all diagnosed with osteoarthritis and were HIV-negative.

The diagnosis of the patients from Kalafong Hospital were diverse and included various types of infections (tuberculosis (TB), malaria, HIV), cancers (lung, breast), pancytopenias as a result of bone marrow suppression or peripheral destruction of blood cells, organ failures including renal failure, heart failure and liver failure, anaemias with different etiologies and various other pathologies that resulted in inflammatory reactions. This resulted in an extremely heterogenous group of patients. For the purpose of this study the immune status and iron status, respectively, were used to group these patients. The osteoarthritis patients were treated as a separate group.

3) AIM OF THE STUDY

The primary aim of the present study was to quantitatively measure the expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with chronic immune stimulation. A second aim was to investigate the possible role that the expression of the H-subunit and L-subunit of ferritin may have in the establishment and maintenance of an iron transfer block in patients with chronic immune stimulation.

The discussion will be presented in the following sections:

1) Subdivision of the patients according to their immune status.

2) Expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with a pro-inflammatory immune status.

3) Prevalence of the iron transfer block in patients with a pro-inflammatory immune status.

4) Expression of the H-subunit and L-subunit of ferritin in a group of Kalafong patients with iron transfer block.

5) Expression of the H-subunit and L-subunit of ferritin in the group of osteoarthritis patients.

4) SUBDIVISION OF THE PATIENTS ACCORDING TO THEIR IMMUNE STATUS

In order to investigate the expression of the H-subunit and L-subunit of ferritin it was necessary to subdivide the patients in groups according to their immune status. The immune status is determined to a major extent by cytokines and it has been shown that certain cytokines can influence the expression of the H-subunits and L-subunits to different extents. Patients were subdivided according to their immune status based firstly on C-reactive protein levels and secondly based on neopterin levels. In order to validate the subdivisions in terms of C-reactive protein and neopterin and to investigate more specifically the cytokines that may contribute to changes in the expression of the H-subunit and L-subunit of ferritin the cytokine profiles of these patients were determined.

4.1) C-reactive protein as an indicator of immune stimulation

C-reactive protein is an acute phase protein and rises sharply with the onset of inflammation reaching peak concentrations within 24-48 hours. Inflammatory processes accompanying tissue injury, infection, malignancy, autoimmune diseases and cardiovascular diseases can all result in an increase in C-reactive protein levels (23). Not

only does C-reactive protein correlate with the progression of the inflammatory process but C-reactive protein also reflects the resolution of inflammation. C-reactive protein is synthesised and secreted by hepatocytes upon stimulation by acute phase protein-inducing cytokines such as interleukin-6 (Il-6) (24). C-reactive protein is a pattern recognition molecule and binds to specific molecular configurations that are typically exposed during cell death or found on the surfaces of pathogens (25). By binding to the molecules exposed with cell death C-reactive protein increases the clearance of apoptotic cells. C-reactive protein binds to the stimulatory receptors, FcgammaRI and FcgammaRII, increasing phagocytosis and the release of inflammatory cytokines. In addition, C-reactive protein binds to the inhibitory receptor FcgammaRIIb, blocking activating signals (26). C-reactive protein is therefore clearly not only a by-stander molecule during inflammation but actively takes part in pro-inflammatory, as well as anti-inflammatory, processes. C-reactive protein levels are seen by some as the most accurate reflection of the inflammatory state.

In the present study, in order to subdivide the Kalafong patients into groups, C-reactive protein was determined as an overall indicator of the inflammatory status. A value of 10 mg/l and less was taken as normal C-reactive protein levels, whereas a value of more than 10 mg/l as elevated C-reactive protein levels. For the group of Kalafong patients (n = 19) with normal C-reactive protein the mean and SD was 2.8 ± 2.8 mg/l, whereas for the group of Kalafong patients (n = 29) with elevated C-reactive protein the mean and SD was 82.2 ± 76.2 mg/l. The mean and SD for C-reactive protein for the osteoarthritis group was 3.4 ± 5.1 mg/l (n = 10). The C-reactive protein was significantly higher (p-value < 0.0001) in the group of Kalafong patients with elevated C-reactive protein than in the group of osteoarthritis patients. There was no significant difference between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

This subdivision of the Kalafong patients clearly showed a group of patients with increased C-reactive protein and a group with normal C-reactive protein. The group of osteoarthritis patients showed C-reactive protein levels similar to that of the group of Kalafong patients with normal C-reactive protein levels.

An increase in C-reactive protein is seen as an indicator of the involvement of the classically activated macrophage (pro-inflammatory macrophage) in the orchestration of the immune reponse. In the group of Kalafong patients with elevated C-reactive protein, the macrophage takes on a pro-inflammatory role, which is characteristic of a T-helper cell type-1 immune response.

Not all Kalafong patients with elevated C-reactive protein necessarily had elevated neopterin and *vice versa*. However, the group of Kalafong patients with elevated C-reactive protein showed a significantly higher level for neopterin (p-value = 0.0002), (29.5 ± 24.8 ng/ml; n = 29) than the group of Kalafong patients with normal C-reactive protein (7.7 ± 11.7 ng/ml; n = 19). Compared to the osteoarthritis patients (2.2 ± 0.52 ng/ml; n = 10), both the group of Kalafong patients with elevated C-reactive protein (p-value < 0.0001) and the group of Kalafong patients with normal C-reactive protein (p-value = 0.017) showed significantly higher neopterin levels.

4.2) Neopterin as an indicator of immune stimulation

The group of Kalafong patients were subsequently subdivided according to another immunological indicator namely neopterin. Neopterin is synthesised by macrophages upon stimulation with the T-helper cell type-1 secretory product, interferon-γ (INF-γ). Consequently, neopterin has thus been used as a measure of both macrophage activity and cell-mediated immunity. In the pro-inflammatory macrophage neopterin is produced from guanosine triphosphate (GTP). Activation of GTP cyclohydrolase I

results in an accumulation of 7,8-dihydroneopterin triphosphate, followed by the conversion of 7,8-dihydroneopterin triphosphate to neopterin and 7,8-dihydroneopterin by phosphatases (27). The rate of rise of neopterin is slower than that for C-reactive protein, peaking at 9-12 days after contraction of viral infection. Although IFN-γ is the major cytokine responsible for neopterin production (28), IFN-γ mediated neopterin production can be superinduced by lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) (27). Various other cytokines such as interleukin-2 (Il-2) which are able to induce IFN-γ release from T-helper cell type-1 cells can also provoke neopterin release (27). Furthermore, increasing the number of monocytes/macrophages by administration of granulocyte macrophage-colony stimulating factor (GM-CSF) also results in increased neopterin production (27). However, it has also been shown that IFN-γ is not essential for neopterin production. For instance, patients suffering from Mendelian susceptibility to mycobacterial disease (MSMD), characterised by impaired or absent IFN-γ production or function, show neopterin production when challenged by mycobacterium (29).

Neopterin production does not only indicate macrophage activation, but neopterin levels are also shown to correlate with the degree of macrophage activation. Therefore, neopterin levels in plasma can be employed to monitor the magnitude of cellular immune activation/involvement during an immune response (27). When cell-mediated immunity dominates, high neopterin levels will be present and when humoral immunity takes the lead, neopterin levels will be low (27). *In vivo* increased neopterin production was demonstrated in patients suffering from infections with viruses and intracellular bacterial or protozoal pathogens, in patients with rheumatoid arthritis, systemic lupus erythromatosus and in patients with acute cellular graft rejection or graft-versus-host disease (28).

In the present study neopterin was determined in order to indicate the involvement of cell-mediated immunity orchestrated by the classically activated macrophage (pro-inflammatory macrophage). In this study a value of less than 3.4 ng/ml was taken as normal neopterin and a value equal to and more than 3.4 ng/ml as elevated neopterin. This resulted in a group of 13 Kalafong patients with normal neopterin (2.2 ± 0.47 ng/ml) and a group of 35 Kalafong patients with elevated neopterin (27.9 ± 23.5 ng/ml). For the osteoarthritis patients neopterin was 2.2 ± 0.52 ng/ml (n = 10). The neopterin values were significantly higher (p-value < 0.0001) in the group of Kalafong patients with elevated neopterin than in the group of osteoarthritis patients. There was no significant difference for neopterin between the osteoarthritis patients and the group of Kalafong patients with normal neopterin.

As for the C-reactive protein subdivision, the subdivision of the Kalafong patients based on neopterin clearly showed a group of patients with increased neopterin and a group with normal neopterin. The group of osteoarthritis patients showed neopterin levels similar to that of the group of Kalafong patients with normal neopterin.

The group of Kalafong patients with elevated neopterin showed a significantly higher level for C-reactive protein (p-value = 0.001), (64.7 ± 77.2 mg/l; n = 35) than the group of Kalafong patients with normal neopterin (13.1 ± 24.4 mg/l; n = 13). Compared to the osteoarthritis patients (3.4 ± 5.1 mg/l; n = 10), only the group of Kalafong patients with elevated neopterin showed a significantly increased C-reactive protein (p-value < 0.0001).

C-reactive protein and neopterin correlated positively for the Kalafong patients (r = 0.48, p-value = 0.0006). However, not all Kalafong patients with elevated C-reactive protein necessarily had elevated neopterin and *vice versa*. Specific disease types show characteristic elevations in C-reactive protein and/or neopterin (25, 28, 30). Therefore,

depending on the type and stage of immune/inflammatory process and resolution thereoff, C-reactive protein and neopterin might be elevated to different extents and may be cleared at different time-points. Consequently, it was considered feasible to continue the investigation based on both the subdivision according to C-reactive protein and the subdivision according to neopterin. For the rest of the discussion reference will often be made to groups with elevated or normal C-reactive protein and groups with elevated or normal neopterin with the understanding that the reference is made to subdivisions according to C-reactive protein and neopterin and that there are overlapping in patients between the groups.

5) THE CYTOKINE RESPONSE OF PATIENTS WITH ELEVATED C-REACTIVE PROTEIN AND PATIENTS WITH ELEVATED NEOPTERIN

In order to validate the subdivision according to C-reactive protein and neopterin and to investigate more specifically the cytokines that may contribute to changes in the expression of the H-subunit and L-subunit of ferritin, cytokine profiles of these patients were determined. The cytokines investigated included interferon-γ (INF-γ), tumor necrosis factor-α (TNF-α), interleukin-1β (Il-1β), interleukin-6 (Il-6), interleukin-12 (Il-12), interleukin-2 (Il-2), interleukin-8 (Il-8), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-4 (Il-4), interleukin-5 (Il-5), transforming growth factor-β (TGF-β) and interleukin-10 (Il-10).

5.1) Background

Macrophage activation has many phenotypes during an immune response. These macrophage phenotypes can be classified into three major types of activated macrophages, each with distinguishable, but also some overlapping functions. These different macrophage phenotypes are sequentially encountered during an immune

response. During the first phase of an immune response the macrophage (type-1 activated macrophage – classically activated macrophage) takes on a pro-inflammatory role. The function of the type-1 activated macrophage is as an effector cell in T-helper cell type-1 cellular immune responses. The type-2 activated macrophage is anti-inflammatory and preferentially induces T-helper cell type-2 humoral immune responses to antigen. The third type of activated macrophage, the alternatively activated macrophage appears to be involved in immunosuppression and tissue repair in the last phase of the immune response (31, 32, 33). The magnitude and duration of each of these phases of an immune response, orchestrated by one of the three types of activated macrophages, depend on the complexity of the signalling pattern encountered by the macrophage. Signal complexity depends on the type, combination and quantity of stimuli (such as cytokines) and the temporal sequence in which the stimuli (such as cytokines) are presented to the macrophage (34).

The activating signals for the classically activated macrophage (type-1 activated macrophage) include INF-γ, TNF-α and Il-2 with the secretory products TNF-α, Il-12, Il-1β, Il-6 and Il-8 (31, 34, 35). IFN-γ is the cytokine of major importance in the classical activation of the macrophage. IFN-γ has been shown to augment the activation of macrophages in response to bacterial products, such as LPS, pro-inflammatory cytokines including TNF-α, Il-1β and Il-12, or activated T-lymphocytes through the CD40 ligand (36). When the macrophage is activated by one of these stimuli without IFN-γ being present, the absolute amount of cytokines and toxic nitrogen radicals released are many-fold lower than for macrophages that have been exposed to activating stimuli with IFN-γ (36). Il-6 tends to be more of a secondary mediator, fundamental for the acute phase response in the liver and has a regulatory function in the immune response (35). The chemokine, Il-8, is responsible for the recruitment of leukocytes such as the neutrophil to the inflammatory site (37).

The activating signals for the type-2 activated macrophage include IgG complexes and Toll-like receptor (TLR) ligation with the secretory products Il-10, TNF-α and Il-6. The activating signals for the alternatively activated macrophage include Il-4, TGF-β and glucocorticoids with the secretory products Il-1 receptor antagonist (Il-1RA) and Il-10 (31). The T-helper cell type-2 cytokines Il-4, Il-10 and transforming growth factor-β (TGF-β) induces type-2 macrophage phenotypes. Type-2 macrophages are not simply deactivated macrophages but are macrophages that display anti-inflammatory properties together with the ability to bring about a more mature type-2 immune response characterised by up-regulated humoral activities. Therefore, none of these T-helper cell type-2 cytokines can be categorised as purely macrophage deactivating since they also need to exert macrophage-activating effects in order for the macrophage to orchestrate a type-2 immune response (38). T-helper cell type-2 activated macrophages show enhanced capacity for antigen presentation and enhanced phagocytosis of debris and particles, but not to pathogens. Furthermore, the T-helper cell type-2 activated macrophage generates anti-inflammatory cytokines, suppresses the synthesis of pro-inflammatory cytokines and is resistant to re-activation (36). Both Il-10 and TGF-β are shown to reduce the potential for inflammation in the local microenvironment, down-regulate ongoing inflammation and polarize adaptive immunity towards anti-inflammatory and/or humoral responses (39). TGF-β, Il-4 and Il-10 deactivate macrophages in numerous ways including indirectly by suppression of T-cell proliferation and/or directly by decreasing IFN-γ production. Furthermore, they can each induce antagonists of cytokines released by macrophages such as Il-2 receptor antagonist. Il-4 preferentially stimulates type-2 T-helper cells in an autocrine function. These type-2 T-helper cells produce Il-10 a potent inhibitor of monocyte/macrophage-dependent IFN-γ production by type-1 T-helper cells, cytotoxic T-cells and natural killer cells. TGF-β and Il-4 also suppress IFN-γ production by T-lymphocytes, human blood

mononuclear cells, or natural killer cells (38). Not only does these type-2 cytokines bring about alternatively activated macrophages during an immune response, but can also cause naïve macrophages to become resistant to type-1 cytokine exposure and the attainment of a pro-inflammatory status. When naïve macrophages are exposed to T-helper cell type-2 lymphokines such as Il-4 or TGF-β, cytokines Il-10 and Il-13 and glucocorticoids, the macrophage does not attain the classically activated (pro-inflammatory) phenotype, even when such macrophages are challenged with pro-inflammatory cytokines (36). These T-helper cell type-2 cytokines can all suppress nitric oxide (NO) production by various mechanisms including inducible nitric oxide synthase (iNOS) down-regulation and a decrease in iNOS substrate availability (40).

TGF-β is a potent anti-inflammatory cytokine, which plays a central role in the resolution of inflammation and tissue repair. It has been shown that TGF-β production can be stimulated early in the inflammatory response (41). TGF-β is the most potent inhibitor of the iNOS pathway and generation of NO. TGF-β has been shown to inhibit the synthesis of the iNOS protein and to destabilize iNOS mRNA (40). Furthermore, TGF-β suppresses the production of pro-inflammatory cytokines by macrophages including Il-8, GM-CSF and TNF-α (41, 42). TGF-β appears to be further involved in the reparative phase of inflammation due to its ability to stimulate formation of matrix components by fibroblasts. This enhanced production of matrix proteins coupled with the unique ability of TGF-β to both inhibit matrix-degrading enzymes and increase the secretion of protease inhibitors results in a pronounced pro-fibrotic influence of TGF-β (43).

5.2) Cytokine profiles of the Kalafong and osteoarthritis patients

The cytokines are presented in groups in the following order. Firstly, cytokines involved in the pro-inflammatory immune state, followed by the cytokines mostly involved in an alternative T-helper cell type-2 driven immune reaction (predominantly anti-inflammatory) and lastly, the cytokines most probably involved in the resolution of the immune response and tissue repair.

5.2.1) Cytokine levels in patients with elevated C-reactive protein, patients with normal C-reactive protein and osteoarthritis patients

Specific cytokines determine the production of C-reactive protein and others are raised concomitantly, but independently. Various cytokines both pro-inflammatory and anti-inflammatory were determined in the patients. As shown below, significant differences for the pro-inflammatory cytokines were found between the group of Kalafong patients with elevated C-reactive protein levels and the group of Kalafong patients with normal C-reactive protein levels, while the osteoarthritis group's pro-inflammatory cytokines largely corresponded with that of the group of Kalafong patients with normal C-reactive protein.

Pro-inflammatory cytokines

INF-γ was

- significantly higher (p-value = 0.0008) in the group of Kalafong patients with elevated C-reactive protein (25.6 ± 73.8 pg/ml; n = 28) compared to the group of Kalafong patients with normal C-reactive protein (1 ± 2.6 pg/ml; n = 19),
- significantly higher (p-value = 0.0008) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (0.1 ± 0 pg/ml; n = 10) and

- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

Similar to INF-γ, TNF-α was also

- significantly higher (p-value = 0.007) in the group of Kalafong patients with elevated C-reactive protein (4.1 ± 2.9 pg/ml; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (2.5 ± 0.97 pg/ml; n = 19),
- significantly higher (p-value = 0.014) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (2.5 ± 0.96 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

Furthermore, the pro-inflammatory secretory product Il-1β was

- significantly higher (p-value = 0.013) in the group of Kalafong patients with elevated C-reactive protein (6.5 ± 10.3 pg/ml; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (1.3 ± 2.3 pg/ml; n = 19),
- significantly higher (p-value = 0.029) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (1.6 ± 3.3 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

Another of the pro-inflammatory secretory products, Il-6, was

- significantly higher (p-value < 0.0001) in the group of Kalafong patients with elevated C-reactive protein (807.6 ± 2384.6 pg/ml; n = 29) compared to the

group of Kalafong patients with normal C-reactive protein (7.2 ± 12.6 pg/ml; n = 19),

- significantly higher (p-value < 0.0001) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (5 ± 2.1 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

The only pro-inflammatory secretory product not higher in the group of Kalafong patients with elevated C-reactive protein compared to the group of Kalafong patients with normal C-reactive protein was Il-12. However, the osteoarthritis patients differed from both the groups of Kalafong patients. Il-12 was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (4.6 ± 4 pg/ml; n = 29) and the group of Kalafong patients with normal C-reactive protein (4.7 ± 3.8 pg/ml; n = 19) and
- significantly higher in both the group of Kalafong patients with elevated C-reactive protein (p-value = 0.0001) and the group of Kalafong patients with normal C-reactive protein (p-value = 0.001) compared to the osteoarthritis patients (1.1 ± 1.3 pg/ml; n = 10).

The above may perhaps be explained by the reduced concentration of CD4 cells present in the group of Kalafong patients with elevated C-reactive protein, since Il-12 is produced by T-helper cell type-1 lymphocytes. The CD4 count was marginally lower (p-value = 0.072) in the group of Kalafong patients with elevated C-reactive protein (293 ± 397.7 x 10^6/l; n = 20) compared to the group of Kalafong patients with normal C-reactive protein (449.3 ± 664.9 x 10^6/l; n = 10). However, the CD4 counts were not

determined in all patients that were HIV-negative. If the CD4 counts of these patients were included the group of Kalafong patients with normal C-reactive protein are very likely to have had a higher mean CD4 count. Normal ranges for CD4 counts are 500-2010 x 10^6/l.

In addition, Il-2 was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (10.7 ± 6.3 pg/ml; n = 29) and the group of Kalafong patients with normal C-reactive protein (9.9 ± 8.2 pg/ml; n = 19),
- marginaly higher (p-value = 0.051) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (6.7 ± 4.9 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

The finding of no significant difference between the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein for Il-2 is also possibly accounted for by the low CD4 counts in the group of Kalafong patients with elevated C-reactive protein.

The chemokine Il-8 was

- significantly higher (p-value < 0.0001) in the group of Kalafong patients with elevated C-reactive protein (334.6 ± 845.2 pg/ml; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (20.1 ± 19.9 pg/ml; n = 19),

- significantly higher (p-value = 0.0009) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (18.9 ± 6.4 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal C-reactive protein and the osteoarthritis patients.

In the present study GM-CSF was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (6.1 ± 9.4 pg/ml; n = 28), the group of Kalafong patients with normal C-reactive protein (23.4 ± 55.5 pg/ml; n = 19) and the osteoarthritis patients (3.6 ± 6.2 pg/ml; n = 10).

GM-CSF is a hemopoietic cytokine involved in differentiation and activation of cells in the myeloid compartment (44). GM-CSF was originally defined by its ability to generate *in vitro* granulocyte and macrophage colonies from bone marrow precursor cells. It now appears more likely that its major role lies in its ability to govern the properties of the more mature myeloid cells of the granulocyte and macrophage lineages, particularly during host defence and inflammatory reactions (45). As it was previously shown that GM-CSF could contribute to inflammation through recruitment, increased survival and/or priming of monocytes/macrophages for activation (45), GM-CSF was determined in this study. However, no differences were seen between any of the groups.

T-helper cell type-2 cytokines

The T-helper cell type-2 (predominantly anti-inflammatory) cytokines are involved during the later phase of an immune response. These cytokines were evaluated for the groups of patients. As shown below, and in contrast to the findings on pro-inflammatory cytokines, the level of only Il-10 was marginally higher in the group of

Kalafong patients with elevated C-reactive protein compared to the group of Kalafong patients with normal C-reactive protein.

Il-4 was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (2.5 ± 1.8 pg/ml; n = 29), the group of Kalafong patients with normal C-reactive protein (2.4 ± 2.2 pg/ml; n = 19) and the osteoarthritis patients (2.5 ± 2.2 pg/ml; n = 10).

Il-5 was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (2.8 ± 1.5 pg/ml; n = 29), the group of Kalafong patients with normal C-reactive protein (4.1 ± 5 pg/ml; n = 19) and the osteoarthritis patients (2.4 ± 1.6 pg/ml; n = 10).

TGF-β was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (12.4 ± 7.1 ng/ml; n = 28) and the group of Kalafong patients with normal C-reactive protein (10.5 ± 7.1 ng/ml; n = 19),
- significantly lower (p-value = 0.003) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (18.3 ± 4.1 ng/ml; n = 10) and
- significantly lower (p-value = 0.0007) in the group of Kalafong patients with normal C-reactive protein compared to the osteoarthritis patients.

Il-10 was

- marginally higher (p-value = 0.059) in the group of Kalafong patients with elevated C-reactive protein (28.1 ± 61.6 pg/ml; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (5.6 ± 3.3 pg/ml; n = 19),
- significantly higher (p-value = 0.035) in the group of Kalafong patients with elevated C-reactive protein compared to the osteoarthritis patients (2.7 ± 1.5 pg/ml; n = 10) and
- significantly higher (p-value = 0.004) in the group of Kalafong patients with normal C-reactive protein compared to the osteoarthritis patients.

In the present study there were no significant differences for the T-helper cell type-2 cytokines Il-4 and Il-5 between the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein. However, the T-helper cell type-2 cytokine, Il-10, was increased in the group of Kalafong patients with elevated C-reactive protein compared to the group of Kalafong patients with normal C-reactive protein. In addition, TGF-β was not significantly different between the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein.

In summary on the cytokine profiles of the C-reactive protein subdivision

The above cytokine results confirm the presence of a pro-inflammatory immune state in the group of Kalafong patients with elevated C-reactive protein levels. This was shown by the increase in various pro-inflammatory cytokines including INF-γ, TNF-α, Il-1β, Il-6 and Il-8. Nevertheless, Il-2 and Il-12 were not significantly different between the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein. These cytokines are both produced by T-helper

type-1 cells (CD4 cells). A lower CD4 count than that of normal was shown in the group of Kalafong patients with elevated C-reactive protein. A decrease in CD4 cells would result in the production of less Il-2 and Il-12. With a pro-inflammatory immune state it would be expected that the T-helper cell type-2 cytokines involved in predominantly anti-inflammatory processes will not be increased. The T-helper cell type-2 cytokines, Il-4, Il-5 and TGF-β, were not significantly different between the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein. However, the T-helper cell type-2 cytokine, Il-10, was marginally higher in the group of Kalafong patients with elevated C-reactive protein compared to the group of Kalafong patients with normal C-reactive protein.

5.2.2) Cytokine levels in patients with elevated neopterin, patients with normal neopterin and osteoarthritis patients

C-reactive protein and neopterin correlated positively for the Kalafong patients ($r = 0.48$, p-value = 0.0006). However, as previously mentioned, not all Kalafong patients with elevated C-reactive protein necessarily had elevated neopterin and *vice versa*. In the following paragraphs cytokines for the neopterin subdivision are evaluated. As shown below, significant differences for the pro-inflammatory cytokines were found between the group of Kalafong patients with elevated neopterin levels and the group of Kalafong patients with normal neopterin levels, while the osteoarthritis group's pro-inflammatory cytokines largely corresponded with that of the group of Kalafong patients with normal neopterin.

Pro-inflammatory cytokines

The major pro-inflammatory cytokine INF-γ was

- marginally higher (p-value = 0.056) in the group of Kalafong patients with elevated neopterin (21 ± 67.4 pg/ml; n = 34) compared to the group of Kalafong patients with normal neopterin (1.6 ± 3.3 pg/ml; n = 13),
- significantly higher (p-value = 0.003) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (0.1 ± 0 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

The pro-inflammatory secreted cytokine TNF-α was

- significantly higher (p-value = 0.048) in the group of Kalafong patients with elevated neopterin (3.8 ± 2.8 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (2.7 ± 0.89 pg/ml; n = 13),
- significantly higher (p-value = 0.035) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (2.5 ± 0.96 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

Furthermore, Il-1β was

- marginally higher (p-value = 0.075) in the group of Kalafong patients with elevated neopterin (5.4 ± 9.7 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (2 ± 2.8 pg/ml; n = 13),

- marginally higher (p-value = 0.057) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (1.6 ± 3.3 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

Il-6 was

- significantly higher (p-value = 0.0003) in the group of Kalafong patients with elevated neopterin (661.1 ± 2187.6 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (32.3 ± 93.6 pg/ml; n = 13),
- significantly higher (p-value = 0.0005) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (5 ± 2.1 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

Similar to what was found for the C-reactive protein subdivision, Il-12 was

- not significantly different between the group of Kalafong patients with elevated neopterin (4.5 ± 4.2 pg/ml; n = 35) and the group of Kalafong patients with normal neopterin (5.1 ± 3.1 pg/ml; n = 13) and
- significantly higher (p-value = 0.0001) in the group of Kalafong patients with elevated neopterin and in the group of Kalafong patients with normal neopterin (p-value = 0.0005) compared to the osteoarthritis patients (1.1 ± 1.3 pg/ml; n = 10).

In addition, similar to the C-reactive protein subdivision, Il-2 was

- not significantly different between the group of Kalafong patients with elevated neopterin (10.7 ± 7.1 pg/ml; n = 35) and the group of Kalafong patients with normal neopterin (9.3 ± 7.1 pg/ml; n = 13),
- significantly higher (p-value = 0.049) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (6.7 ± 4.9 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

Il-8 was

- significantly higher (p-value = 0.0001) in the group of Kalafong patients with elevated neopterin (282.4 ± 775.9 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (15.4 ± 8.3 pg/ml; n = 13),
- significantly higher (p-value = 0.007) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (18.9 ± 6.4 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

GM-CSF was

- not significantly different between the group of Kalafong patients with elevated neopterin (10.2 ± 15.6 pg/ml; n = 34) and the group of Kalafong patients with normal neopterin (20.8 ± 65.9 pg/ml; n = 13),

- marginally higher (p-value = 0.054) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (3.6 ± 6.2 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

T-helper cell type-2 cytokines

As shown below, and in contrast to the findings on the T-helper cell type-2 cytokines for the C-reactive protein subdivision, the level of Il-5 was significantly higher in the group of Kalafong patients with elevated neopterin compared to the group of Kalafong patients with normal neopterin.

Il-4 was

- not significantly different between the group of Kalafong patients with elevated neopterin (2.6 ± 1.8 pg/ml; n = 35), the group of Kalafong patients with normal neopterin (2.1 ± 2.3 pg/ml; n = 13) and the osteoarthritis patients (2.5 ± 2.2 pg/ml; n = 10).

However, different from the C-reactive protein subdivision, Il-5 was

- significantly higher (p-value = 0.011) in the group of Kalafong patients with elevated neopterin (3.9 ± 3.7 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (1.9 ± 1.4 pg/ml; n = 13),
- marginally higher (p-value = 0.072) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (2.4 ± 1.6 pg/ml; n = 10) and

- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

Il-10 was

- significantly higher (p-value = 0.032) in the group of Kalafong patients with elevated neopterin (24.9 ± 56.3 pg/ml; n = 35) compared to the group of Kalafong patients with normal neopterin (3.6 ± 2.1 pg/ml; n = 13),
- significantly higher (p-value = 0.026) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (2.7 ± 1.5 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

TGF-β was

- marginally lower (p-value = 0.082) in the group of Kalafong patients with elevated neopterin (10.2 ± 5.8 ng/ml; n = 34) compared to the group of Kalafong patients with normal neopterin (15.2 ± 8.9 ng/ml; n = 13),
- significantly lower (p-value = 0.0001) in the group of Kalafong patients with elevated neopterin compared to the osteoarthritis patients (18.3 ± 4.1 ng/ml; n = 10) and
- not significantly different between the group of Kalafong patients with normal neopterin and the osteoarthritis patients.

In summary on the cytokine profiles of the neopterin subdivision

The pro-inflammatory cytokines for the group of Kalafong patients with elevated neopterin was increased similar to the group of Kalafong patients with elevated C-

reactive protein. However, significant differences, not shown in the subdivision according to C-reactive protein, were shown for T-helper cell type-2 cytokines between the group of Kalafong patients with elevated neopterin and the group of Kalafong patients with normal neopterin. The increase in these cytokines may have included a more mature immune response in the group of Kalafong patients with elevated neopterin. The pro-inflammatory cytokines for which significant increases were shown in the group of Kalafong patients with elevated neopterin compared to the group of Kalafong patients with normal neopterin included INF-γ, TNF-α, Il-1β, Il-6 and Il-8. Furthermore, similar to the C-reactive protein subdivision, Il-2 and Il-12 were not significantly different between the group of Kalafong patients with elevated neopterin and the group of Kalafong patients with normal neopterin. This may also have been as a result of the decreased CD4 count in the group of Kalafong patients with elevated neopterin. Il-5, a T-helper cell type-2 cytokine, was significantly increased in the group of Kalafong patients with elevated neopterin. Furthermore, Il-10 with predominantly anti-inflammatory properties was significantly increased in the group of Kalafong patients with elevated neopterin. For TGF-β, a decrease was shown for the group of Kalafong patients with elevated neopterin compared to the group of Kalafong patients with normal neopterin.

5.2.3) Osteoarthritis patients

Osteoarthritis is a disease of articular joints with the progression characterised by the destruction of articular cartilage, sclerosis of underlying bone and osteophyte formation (46). It has been suggested that osteoarthritis is induced by mechanical stress manifested by cartilage destruction with minimal involvement of the immune response as compared to that of rheumatoid arthritis (47). Although osteoarthritis is considered to be a largely non-inflammatory disease, there is compelling evidence that subclinical inflammation is a common event, even in the absence of acute inflammatory flares (48). It has previously

been shown that the secretion of tumor necrosis factor-α (TNF-α) and interleukin-6 (Il-6) by stimulated blood cells of osteoarthritis patients was increased, similar to that of rheumatoid arthritis patients. However, in contrast to the rheumatoid arthritis patients no increased production was shown for interleukin-1β (Il-1β) (49).

Controversial opinions exist about cytokine involvement in osteoarthritis. This is mostly due to differences in the levels found for these substances in the general circulation. Nevertheless, they do play a significant role at the tissue level. Osteoarthritis is characterised by the involvement of both catabolic and anabolic cytokines in the progression of the disease (46). The catabolic cytokines, Il-1β and TNF-α, have been shown to be involved in primary cartilage damage, whereas the anabolic cytokine, TGF-β, is implicated in promotion of the repair and integrity of cartilage with osteoarthritis (46). Both these catabolic and anabolic cytokines play an important part in the maintenance of articular cartilage homeostasis and it is suggested that the balance between the signalling pathways of the catabolic cytokines, Il-1β and TNF-α, and the anabolic cytokine, TGF-β, is disrupted with osteoarthritis (50). The catabolic effects of Il-1β and TNF-α are exerted by enzymatic destruction through activation of metalloproteinases of cartilage matrix. Furthermore, inadequate synthesis of inhibitors of the actions for Il-1β and TNF-α can contribute to cartilage destruction with osteoarthritis (51). An increase in the expression of the anabolic cytokine, TGF-β, has been reported early in osteoarthritis with an increase in extracellular matrix production (52). This anabolic cytokine, TGF-β, causes the synthesis of proteoglycans and other cartilage matrix components in osteoarthritis patients (46) and is said to counteract the effects of the catabolic cytokines in the later stages of disease progression. Although TGF-β results in an anabolic state it is believed not to compensate for the overall catabolic insult to cartilage with disease progression (52).

In summary on the cytokine profiles of the osteoarthritis patients

The group of osteoarthritis patients showed C-reactive protein and neopterin levels similar to the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin, respectively. It is therefore suggested that the osteoarthritis patients did not have any pronounced pro-inflammatory immune stimulation. Furthermore, the cytokine profile of the osteoarthritis patients was, with one major exception, similar to the groups of Kalafong patients with normal C-reactive protein/normal neopterin. The exception was a significantly higher TGF-β level in the osteoarthritis patients.

In the present study no significantly higher levels were shown for the catabolic cytokines, Il-1β and TNF-α, in the osteoarthritis patients compared to the group of Kalafong patients with normal C-reactive protein and to the group of Kalafong patients with normal neopterin, respectively. In addition, no significantly higher levels for various other catabolic (pro-inflammatory) cytokines including INF-γ, Il-6, Il-8, Il-12, Il-2 and GM-CSF were shown in the osteoarthritis patients compared to the group of Kalafong patients with normal C-reactive protein and to the group of Kalafong patients with normal neopterin, respectively. Furthermore, no significantly higher levels for any of the following T-helper cell type-2 cyokines – Il-4, Il-5 or Il-10 – were shown in the osteoarthritis patients compared to either the group of Kalafong patients with normal C-reactive protein or to the group of Kalafong patients with normal neopterin. It should perhaps be stressed that circulating levels of cytokines do not necessarily reflect levels of cytokine activity at the tissue level (46). However, a significantly higher level was shown for TGF-β in the osteoarthritis patients compared to both the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with elevated C-reactive protein, respectively. In addition, a significantly higher level was shown for TGF-β in the osteoarthritis patients compared to the group of Kalafong patients with

elevated neopterin. However, the group of Kalafong patients with normal neopterin showed TGF-β levels similar to that of the osteoarthritis patients.

TGF-β, a cytokine with pleiotropic functions, does not only play a paramount role as an anti-inflammatory cytokine during an immune response but also stimulates the production of extracellular matrix proteins in joints with osteoarthritis. In the present study TGF-β was significantly higher in the group of osteoarthritis patients without any significantly higher levels for any of the pro-inflammatory or T-helper cell type-2 cytokines compared to the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin, respectively. It is therefore suggested that TGF-β was increased in the group of osteoarthritis patients in line with its pro-fibrotic role with disease progression of osteoarthritis. This significantly higher level for the anabolic cytokine TGF-β in the osteoarthritis patients resulted in a group of patients with a cytokine profile close to normal, but with a pronounced increase in one cytokine. The expression of the H-subunit and L-subunit of ferritin was thus investigated separately for this group and will be discussed in the last section on osteoarthritis patients.

6) EXPRESSION OF THE H-SUBUNIT AND L-SUBUNIT OF FERRITIN IN THE BONE MARROW MACROPHAGE AND CELLS OF THE ERYTHRON IN PATIENTS WITH A PRO-INFLAMMATORY IMMUNE STATUS COMPARED TO PATIENTS WITH NO PRONOUNCED IMMUNE ACTIVATION

The expression of the H-subunit and L-subunit and the relationship between these subunits depend not only on the type of cell, but also on the role of the cell in iron homeostasis and various other factors that can influence the expression of the H-subunit and L-subunit of ferritin. Since the role that the macrophage on the one hand, and the

cells of the erythron on the other, play in iron homeostasis is to a great extent different, it is expected that these subunits would be expressed differently. In this study the expression of the H-subunit and L-subunit of ferritin was determined in the bone marrow macrophage and in the cells of the erythron in patients.

6.1) H-subunit and L-subunit expression in the macrophage in the Kalafong patients with a pro-inflammatory immune status

Both the group of Kalafong patients with elevated C-reactive protein levels and the group of Kalafong patients with elevated neopterin levels displayed a predominantly pro-inflammatory state orchestrated by classically activated macrophages. Both groups showed significantly higher levels for the pro-inflammatory (T-helper cell type-1) cytokines, but not similar increases for the T-helper cell type-2 cytokines. In the group of Kalafong patients with elevated neopterin some of the T-helper cell type-2 cytokines were increased whereas for the group of Kalafong patients with elevated C-reactive protein these cytokines were mostly not significantly different from the group of Kalafong patients with normal C-reactive protein.

When the expression of the H-subunit and L-subunit of ferritin was determined in the bone marrow macrophage it was found that the expression of the H-subunit of ferritin was marginally higher (p-value = 0.074) in the group of Kalafong patients with elevated C-reactive protein (96.9 ± 40.6 counts/μm^2; n = 25) than in the group of Kalafong patients with normal C-reactive protein (76.5 ± 30 counts/μm^2; n = 19). No significant difference was shown for the expression of the L-subunit of ferritin between the group of Kalafong patients with elevated C-reactive protein (117.8 ± 45.9 counts/μm^2; n = 26) and the group of Kalafong patients with normal C-reactive protein (107.7 ± 26.8 counts/μm^2; n = 19). The H-subunit/L-subunit ratio in the macrophage was marginally higher (p-value = 0.086) in the group of Kalafong patients with elevated C-reactive

protein (0.99 ± 0.58; n = 25) than in the group of Kalafong patients with normal C-reactive protein (0.73 ± 0.29; n = 19). Therefore, for the group of Kalafong patients with increased C-reactive protein, for every one H-subunit there was one L-subunit. However, for the group of Kalafong patients with normal C-reactive protein, for every one H-subunit there was almost one and a half times as many L-subunits. This probably reflects isoferritins containing a higher quantity of H-subunits in the group of Kalafong patients with increased C-reactive protein compared to the group of Kalafong patients with normal C-reactive protein with isoferritins containing a lower quantity of H-subunits.

With the subdivision based on C-reactive protein levels there were indications of an increase in the expression of the H-subunit in the macrophage of the bone marrow during chronic pro-inflammatory stimulation. With the subdivision based on neopterin levels, the increase in the expression of the H-subunit of ferritin in the macrophage was pronounced for the group of patients with chronic pro-inflammatory immune stimulation. In the subdivision according to neopterin the expression of the H-subunit of ferritin in the bone marrow macrophage was significantly higher (p-value = 0.037) in the group of Kalafong patients with elevated neopterin (94.7 ± 37.3 counts/μm^2; n = 31) than in the group of Kalafong patients with normal neopterin (72.4 ± 34.0 counts/μm^2; n = 13). As was the case for the subdivision according to C-reactive protein, no difference was shown for the expression of the L-subunit in the bone marrow macrophage between the group of Kalafong patients with elevated neopterin (108.1 ± 34.3 counts/μm^2; n = 32) and the group of Kalafong patients with normal neopterin (126.8 ± 47.5 counts/μm^2; n = 13). The H-subunit/L-subunit ratio in the macrophage was significantly higher (p-value = 0.023) in the group of Kalafong patients with elevated neopterin (0.97 ± 0.49; n = 31) than in the group of Kalafong patients with normal neopterin (0.66 ± 0.43; n =

13). Therefore, similar to the C-reactive protein subdivision the group of Kalafong patients with elevated neopterin had one L-subunit for every H-subunit. Furthermore, in comparison to the C-reactive protein subdivision the group of Kalafong patients with normal neopterin had one and a half as many L-subunits for every one H-subunit. Similar to the C-reactive protein subdivision this probably reflects isoferritins containing a higher quantity of H-subunits in the group of Kalafong patients with elevated neopterin compared to the group of Kalafong patients with normal neopterin with isoferritins containing a lower quantity of H-subunits.

6.2) H-subunit and L-subunit expression in cells of the erythron in the Kalafong patients with a pro-inflammatory immune status

The expression of the H-subunit and L-subunit of ferritin was not only determined in the macrophages of the bone marrow but also in the cells of the erythron. For the C-reactive protein subdivision there was no significant difference for the expression of the H-subunit of ferritin in the cells of the erythron between the group of Kalafong patients with elevated C-reactive protein (144.4 ± 57.6 counts/μm^2; n = 25) and the group of Kalafong patients with normal C-reactive protein (128.1 ± 48.7 counts/μm^2; n = 19). In addition, there was no significant difference for the expression of the L-subunit of ferritin in the cells of the erythron between the group of Kalafong patients with elevated C-reactive protein (212.3 ± 76.6 counts/μm^2; n = 26) and the group of Kalafong patients with normal C-reactive protein (215.6 ± 54.4 counts/μm^2; n = 19). Furthermore, there was no significant difference for the H-subunit/L-subunit ratio in the cells of the erythron between the group of Kalafong patients with elevated C-reactive protein (0.78 ± 0.47; n = 25) and the group of Kalafong patients with normal C-reactive protein (0.61 ± 0.24; n = 19).

Similar to the C-reactive protein subdivision, the neopterin subdivision also showed no significant difference for the expression of the H-subunit of ferritin in the cells of the erythron between the group of Kalafong patients with elevated neopterin (141.4 ± 54.6 counts/μm^2; n = 31) and the group of Kalafong patients with normal neopterin (127.8 ± 53.3 counts/μm^2; n = 13). In addition, there was no significant difference for the expression of the L-subunit of ferritin in the cells of the erythron between the group of Kalafong patients with elevated neopterin (200.7 ± 54.9 counts/μm^2; n = 31) and the group of Kalafong patients with normal neopterin (245.7 ± 85.7 counts/μm^2; n = 13). However, different from the C-reactive protein subdivision the H-subunit/L-subunit ratio in the cells of the erythron was marginally higher (p-value = 0.062) in the group of Kalafong patients with elevated neopterin (0.77 ± 0.42; n = 31) than in the group of Kalafong patients with normal neopterin (0.57 ± 0.31; n = 13). This could possibly indicate isoferritins in cells of the erythron with marginally increased H-subunits in the group of Kalafong patients with elevated neopterin.

6.3) Discussion of the expression of the H-subunit and L-subunit of ferritin in patients with a pro-inflammatory immune status

Several *in vitro* studies which point towards the differential expression of the H-subunit and L-subunit of ferritin in a number of tissues in conditions marked by the excessive production of toxic oxygen radicals, and by infectious and/or inflammatory processes have been published. The present study focuses on the intracellular expression of the H-subunit and L-subunit of ferritin. In no previous study was the expression of the H-subunit and L-subunit of ferritin measured quantitatively in the macrophage of the bone marrow in combination with the inflammatory status of patients. A few studies have none the less been published that relate to the present study. One study exists in which the expression of the H-subunit and L-subunit of ferritin in liver biopsies from three

patients were investigated by ultrastructural immunolocalisation. However, no attempt was made to quantify the level of expression of these two subunits (53). In this particular study it was shown that the denatured H-ferritin subunit is a major constituent of haemosiderin in the liver of patients with iron overload. This is similar to the finding of another study which showed that antibodies directed against denatured human H-ferritin stain only reticuloendothelial cells within the bone marrow (54). However, ultrastructural immunolocalisation was not employed for the study. In a third related study the expression of the two subunits with different disease states was investigated in bone marrow aspirates with fluorescent immunolocalisation (55). However, due to the nature of the fluorescent immunolocalisation technique it was not possible to quantify the expression of the two subunits.

In the bone marrow, iron is differently metabolised by the cells of the erythron including erythroblasts, reticulocytes and red blood cells on the one hand and the macrophage on the other. Furthermore, iron is shuttled between the cells of the erythron and the macrophage to support erythropoiesis. Not only is iron metabolised differently by these various kinds of cells, but during conditions of chronic immune stimulation iron is retained by the macrophage resulting in an increase in storage iron and hypoferraemia – the so-called iron transfer block (56). In order to investigate the role of ferritin and more specifically the subunits of ferritin in the handling of iron by cells of the bone marrow it would be necessary to investigate the expression of the two subunits of ferritin, the H-subunit and L-subunit, at the single cell level.

The results of this study showed in patients with a predominantly pro-inflammatory immune status, governed by pro-inflammatory cytokines, the expression of the H-subunit of ferritin in the macrophage to be higher and the expression of the L-subunit of ferritin in the macrophage to be unaffected. In contrast, the expression of both the H-

subunit and the L-subunit in cells of the erythron was unaffected in the patients with a pro-inflammatory immune status.

The question now arises why the level of significance was higher with regard to the expression of the H-subunit of ferritin in the macrophage for the subdivision according to neopterin than for the subdivision according to C-reactive protein. Although both C-reactive protein and neopterin are commonly used as indicators of pro-inflammatory activity it is obvious that there are differences. One important difference is the time-points at which C-reactive protein and neopterin peak during an immune reaction. C-reactive protein peaks early in an immune reaction and neopterin peaks only later in the immune response (28). Furthermore, with regard to the cytokine profiles of these groups it could not be said that the group of Kalafong patients with elevated neopterin had purely a pro-inflammatory immune response since some of the T-helper cell type-2 cytokines were also increased.

The findings in the present study of the increased expression of the H-subunit of ferritin and unaffected expression of the L-subunit of ferritin in the macrophage are consistent with previous results in other cell types. Various pro-inflammatory cytokines have been reported to have the ability to induce ferritin expression including Il-1α, Il-1β, Il-2, Il-6, TNF-α and IFN-γ (Table 1, Chapter 1). These cytokines modulate ferritin expression by both transcriptional and translational mechanisms (57), but largely by an increase in the rate of transcription of the ferritin gene (19, 58). The expression of the ferritin subunits are, furthermore, differentially regulated by cytokines, and it is mostly the H-subunit of ferritin that is increased by cytokine induction at variance with the L-subunit (19). *In vitro* experiments with various cell types showed an increase in H-subunit expression relative to L-subunit expression upon cytokine activation (Table 1, Chapter 1).

The cytokine-induced increase in the expression of the H-subunit of ferritin is probably even higher than observed since two other processes induced by cytokines during an immune response can reduce the levels of the H-subunit of ferritin. Firstly, pro-inflammatory cytokines not only induce the expression of intracellular ferritin as part of the acute phase response, but also brings about an increase in the secretion of ferritin. For instance, the secretion of ferritin was shown to be stimulated by cytokines in a primary human hepatocyte culture where IL-1α and Il-6 induced a transient secretion of ferritin at 24 hours, followed by a decline to baseline, and TNF-α treatment resulted in a sustained increase in ferritin secretion (58). Therefore, in the present study, secretion of ferritin could very well have resulted in partially masking the response of an increase in the expression of the H-subunits of ferritin in macrophages during pro-inflammatory immune activation.

The second process, that could have brought about a lower observed increase in the H-subunit of ferritin, also induced by pro-inflammatory cytokines, is the degradation of ferritin (haemosiderin formation). It has been shown that activation of macrophages by cytokines such as TNF-α and Il-1β can result in the slower release of iron compared to the release by non-stimulated macrophages, thus supporting the proposed role of cytokines in ferritin-mediated iron sequestration by macrophages (59, 60). This increased formation of haemosiderin during immune stimulation with the subsequent with-holding of iron will be addressed in a following section.

The role of ferritin in inflammatory conditions can be summarised by saying that pro-inflammatory cytokines increase the production of ferritin early in the inflammatory response and that H-subunit rich ferritins are preferentially up-regulated at variance with L-subunit rich ferritins. At this stage this process not only protects the body against

reactive oxygen species generation but, in addition reduces the bio-availability of iron needed by pathogenic microorganisms.

The results of this study showed the expression of the H-subunit and L-subunit of ferritin in the cells of the erythron not to be influenced by the cytokines as was the case for the macrophage. The increase in the expression of the H-subunit of ferritin in the macrophage with immune stimulation is consistent with the role of ferritin in with-holding of iron. The accumulation of iron with chronic immune stimulation has been shown to occur in the macrophages (56). This brings about haemosiderosis of the macrophage and a reduction in serum iron. Due to this hypoferraemic state less iron will reach the cells of the erythron resulting in the subsequent depletion of iron in the cells of the erythron for the production of haemoglobin. In the present study it seems that in the cells of the erythron the H-subunit of ferritin is not up-regulated in order to contribute to the withholding of iron from haemoglobin production during a pro-inflammatory immune reaction.

However, the H-subunit/L-subunit ratio in the cells of the erythron was higher in the group of Kalafong patients with elevated neopterin than in the group of Kalafong patients with normal neopterin. This possible increase in H-subunit rich isoferritins in cells of the erythron in the group of Kalafong patients with elevated neopterin could play a role in the regulation of bio-available iron for haemoglobin production. It has been shown in a previous study that an increase in H-subunit rich ferritins in erythroid cells could result in chelation of the labile iron pool (61).

6.4) H-subunit and L-subunit expression in the macrophage and cells of the erythron in the osteoarthritis patients

The expression of the H-subunit and L-subunit of ferritin in the macrophage and cells of the erythron in the osteoarthritis patients was subsequently compared to the expression of these subunits in the Kalafong patients. Comparisons were made for both the C-reactive protein subdivision and the neopterin subdivision.

Osteoarthritis patients and the subdivision according to C-reactive protein

When the osteoarthritis patients were compared to the group of Kalafong patients with normal C-reactive protein the osteoarthritis patients showed a significantly higher (p-value = 0.02) H-subunit expression in the macrophage (118.8 ± 56.1 counts/μm^2; n = 7). There was no difference for the expression of the H-subunit in the macrophage between the osteoarthritis patients and the group of Kalafong patients with high C-reactive protein. When the expression of the L-subunit in the macrophage of the osteoarthritis patients (136.2 ± 32.8 counts/μm^2; n = 6) was compared to the group of Kalafong patients with normal C-reactive protein it was also seen that the osteoarthritis group had significantly higher (p-value = 0.042) L-subunit counts. The expression of the L-subunit of ferritin in the macrophage in the osteoarthritis patients was not significantly different from the group of Kalafong patients with elevated C-reactive protein. There was no significant difference for the H-subunit/L-subunit ratio in the macrophage between the group of osteoarthritis patients (0.81 ± 0.26; n = 6) and either the group of Kalafong patients with normal C-reactive protein or the group of Kalafong patients with elevated C-reactive protein.

The expression of the H-subunit of ferritin in the cells of the erythron for the osteoarthritis patients (148.8 ± 41.2 counts/μm^2; n = 7) was similar to that of the group of Kalafong patients with elevated C-reactive protein and to the group of Kalafong

patients with normal C-reactive protein. The expression of the L-subunit of ferritin in the cells of the erythron for the osteoarthritis patients (198.8 ± 66.8 counts/μm^2; n = 6) was also similar to the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with normal C-reactive protein. However, the osteoarthritis patients (0.83 ± 0.31; n = 6) showed a marginally higher H-subunit/L-subunit ratio (p-value = 0.075) in the cells of the erythron compared to the group of Kalafong patients with normal C-reactive protein. No difference was shown for the H-subunit/L-subunit ratio in the cells of the erythron between the osteoarthritis patients and the group of Kalafong patients with elevated C-reactive protein.

Osteoarthritis patients and the subdivision according to neopterin

When the expression of the H-subunit of ferritin in the macrophage in the osteoarthritis patients was compared to that of the Kalafong patient groups, subdivided according to their neopterin values, a very similar outcome was found as for the C-reactive protein subdivision. The expression of the H-subunit of ferritin in the macrophage was significantly higher (p-value 0.033) in the osteoarthritis patients (118.8 ± 56.1 counts/μm^2; n = 7) than in the group of Kalafong patients with normal neopterin and not significantly different from that in the group of Kalafong patients with elevated neopterin. There was no significant difference between the expression of the L-subunit of ferritin in the macrophages of the osteoarthritis patients and the Kalafong patients with normal neopterin. A marginally higher (p-value = 0.073) expression of the L-subunit of ferritin was seen in the macrophage of the osteoarthritis patients (136.2 ± 32.8 counts/μm^2; n = 6) compared to the group of Kalafong patients with elevated neopterin. There was no significant difference between the osteoarthritis patients and the group of Kalafong patients with normal neopterin for the expression of the L-subunit of ferritin in the macrophage. No difference was shown between the H-subunit/L-subunit ratio in

the macrophages of the osteoarthritis patients (0.81 ± 0.26; n = 6) and either of the group of Kalafong patients with elevated neopterin or the group of Kalafong patients with normal neopterin.

When the H-subunit and L-subunit expression in cells of the erythron of the osteoarthritis patients were compared to that of the Kalafong patient groups subdivided according to neopterin levels there was no significant difference between the expression of the H-subunit of ferritin in the cells of the erythron in the osteoarthritis patients (148.8 ± 41.2 counts/μm^2; n = 7) and that of either the group of Kalafong patients with elevated neopterin or the group of Kalafong patients with normal neopterin. Furthermore, no significant difference was seen between the expression of the L-subunit of ferritin in the cells of the erythron in the osteoarthritis patients (198.8 ± 66.8 counts/μm^2; n = 6) and that of either the group of Kalafong patients with elevated neopterin or the group of Kalafong patients with normal neopterin. However, the H-subunit/L-subunit ratio in the cells of the erythron was significantly increased (p-value = 0.035) in the osteoarthritis patients (0.83 ± 0.31; n = 6) compared to that of the group of Kalafong patients with normal neopterin. No significant difference was shown for the H-subunit/L-subunit ratio in the cells of the erythron between the osteoarthritis patients and the group of Kalafong patients with elevated neopterin.

In summary on the osteoarthritis patients

- their cytokine profile largely corresponded to that of the group of Kalafong patients with normal C-reactive protein and to that of the group of Kalafong patients with normal neopterin, therefore they did not show overt inflammatory activity.

- their TGF-β was significantly higher than in the group of Kalafong patients with normal C-reactive protein and significantly higher than in the group of Kalafong patients with elevated C-reactive protein.
- their TGF-β was significantly higher than in the group of Kalafong patients with normal neopterin but not significantly different from the group of Kalafong patients with normal neopterin.
- the expression of the H-subunit of ferritin in the bone marrow macrophage was significantly higher than that for the Kalafong patient groups with no pronounced pro-inflammatory activity – in fact, it corresponded to that of the Kalafong patient groups with overt pro-inflammatory activity.
- the expression of the H-subunit and the L-subunit of ferritin in the erythron was similar to all the groups of Kalafong patients, irrespective of immune status.
- the H-subunit/L-subunit ratio in the cells of the erythron was higher than that for the Kalafong patient groups with no pronounced pro-inflammatory activity – in fact, it corresponded to that of the Kalafong patient groups with overt pro-inflammatory activity.

6.5) Discussion of the expression of the H-subunit and L-subunit of ferritin in osteoarthritis patients

Osteoarthritis is a disease of articular joints and results in destruction of articular cartilage, sclerosis of underlying bone and osteophyte formation (46). Cytokines are implicated in the disease progression of osteoarthritis. Il-1β and TNF-α have been shown to be involved in primary cartilage damage, whereas TGF-β is implicated in the promotion of the repair and integrity of cartilage with osteoarthritis (46). However, in the present study the only cytokine elevated in the osteoarthritis patients compared to the group of Kalafong patients with normal C-reactive protein and to the group of Kalafong

patients with normal neopterin was TGF-β. The results of this study could thus implicate TGF-β in the increased expression of the H-subunit of ferritin in the macrophages of the osteoarthritis patients. However, the significance of this possible stimulation of the expression of the H-subunit in the macrophage by TGF-β is not known and can only be speculated on.

TGF-β as for many other cytokines has pleiotropic functions in many tissues and can play a role in various physiological processes. It is known that TGF-β plays an anabolic role in cartilage formation and it has been shown that TGF-β supplementation enhances cartilage repair. It is therefore sometimes used as a therapeutic tool. However, application of TGF-β provides problems in other tissues of the joints and results in fibrosis and osteophyte formation (52). The TGF-β family consists of over 35 members and includes, besides TGF-βs, activins and bone morphogenetic proteins (BMPs). They regulate cell proliferation, differentiation, apoptosis and migration, as well as control extracellular matrix synthesis and degradation of various tissues (52). Furthermore, they mediate cell and tissue responses to injury and modulate immune functions (52).

Due to the role that TGF-β plays in the differentiation of various cell types and the up-regulation of the expression of the H-subunit of ferritin upon cellular differentiation it is possible that TGF-β could cause an increase in the expression of the H-subunit of ferritin. In various cell types including pre-adipocytes, erythroid cells and neuronal cells the expression of the H-subunit of ferritin was shown to be up-regulated upon differentiation (62, 63, 64). This increase in the expression of the H-subunit of ferritin was also found with differentiation of monocytes to macrophages. Similarly differentiation of, for instance colon carcinoma cells is, as for normal cells, accompanied by an increased expression of the H-subunit of ferritin (65). TGF-β was first identified

for promoting the transformation of non-neoplastic cells (43). For instance, TGF-β has been shown to suppress erythropoiesis, not by inhibiting the proliferation of red blood cell precursors, but by initiating differentiation of the red blood cell precursors (66). It is thus speculated that TGF-β could possibly bring about differentiation of cells by up-regulating the expression of the H-subunit of ferritin. This is supported by the results of a previous study, which showed that TGF-β selectively increased the expression of the H-subunits of ferritin in malignant H-ras transformed cells (67).

TGF-β has been shown to have both stimulatory and inhibitory effects upon monocytes/macrophages. Stimulatory effects include chemotaxis of monocytes, induction of FcγRIII and induction of the transcription and translation of Il-1β, TNF-α, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). The inhibitory effects include a reduction in TGF-β receptors and deactivation of hydrogen peroxide and superoxide anion production (43). Therefore, monocytes/macrophages are responsive to TGF-β at different time-points during an immune reaction and it is possible that some of these effects of TGF-β on the monocyte/macrophage could be brought about by an up-regulation of the expression of the H-subunit of ferritin.

The suppression of proliferation and induction of differentation are regulated by intracellular iron (labile iron pool). Iron is a necessary element for cellular proliferation and it is generally accepted that rapidly dividing cells require more iron for their growth and metabolism than resting cells. It is also known that cells normally display an increase in cellular proliferation upon an increase in the labile iron pool (13). The reason for the high need for iron is that iron is necessary for the functioning of different enzymes involved in cellular proliferation, including ribonucleotide reductase, which controls a rate-limiting step in DNA synthesis, and for various mitochondrial enzymes involved in

the metabolism of the cell (68, 69). One way in which to bring about an increase in the cellular labile iron pool is by suppression of ferritin synthesis. The expression of the H-subunit gene is shown to be down-regulated by c-MYC and to be essential for the control of cellular proliferation and transformation by c-MYC (70). This is in agreement with the fact that the H-subunit is responsible for controlling the labile iron pool and that down-regulation of H-subunit expression would result in an increase in the labile iron pool. Therefore, in line with TGF-β's role in cellular growth it could be speculated that TGF-β could regulate proteins involved in iron homeostasis such as the H-subunit of ferritin. Furthermore, has it been shown that administration of BMP-2 (member of the TGF-β family) increases hepcidin expression and decreases serum iron levels *in vivo* (71). This increased expression of hepcidin has been shown to occur by the activation of the TGF-β/SMAD4 signalling pathway (72).

In the present section of the study it was seen that an increase in the expression of the H-subunit of ferritin occurred in the bone marrow macrophage in groups of Kalafong patients with a pro-inflammatory immune status and also in a group of osteoarthritis patients with no overt pro-inflammatory activity, but with high TGF-β levels. It is now of interest to investigate whether this increase in the expression of the H-subunit of ferritin could play a role in entrapment of iron in the macrophage and in the resulting reduction in serum iron.

7) PREVALENCE OF THE IRON TRANSFER BLOCK IN PATIENTS WITH A PRO-INFLAMMATORY IMMUNE STATUS COMPARED TO PATIENTS WITH NO PRONOUNCED IMMUNE ACTIVATION

In the next sections the possibility that the H-subunit and L-subunit of ferritin may play a role in an increase in macrophage storage iron and in the resulting hypoferraemia, are investigated. As pro-inflammatory cytokines are involved in the development of an iron

transfer block the first step was to investigate the prevalence of an iron transfer block in the groups of Kalafong patients with high pro-inflammatory activity.

7.1) Iron transfer block

A decrease in serum iron is often found in patients with immune activation. This hypoferraemic state is predominantly orchestrated by cytokines. Various iron homeostatic processes are affected by these cytokines with the combined actions resulting in entrapment of iron in the macrophage and a decrease in serum iron. This process is known as the iron transfer block (73).

The so-called iron transfer block is generally associated with chronic inflammatory conditions. Inflammation, mediated by cytokines, produces a shift in iron handling by the macrophage in favour of iron storage, in time leading to haemosiderosis of the macrophage (74, 75, 76), hypoferraemia (56, 76, 77) and the anaemia of chronic disease. The latter is, however, only partly due to a decrease in iron available for haemotopoiesis. Ferritin plays a major part in the establishment and maintenance of an iron transfer block and thus in the hypoferraemic state of the inflammatory reaction. TNF-α, Il-1β, Il-6 and Il-10 have all been shown to directly stimulate the transcription and translation of ferritin (73, 78). Furthermore, has it been shown that ferritin up-regulation precedes the reduction in serum iron. However, other iron homeostasis proteins also contribute to the reduction in serum iron. These include not only proteins responsible for the handling of macrophage iron but also many others responsible for body iron homeostasis. These proteins involved in the acquisition, storage and release of iron are all influenced, to different extents, at different time-points during the inflammatory reaction, by both pro- and anti-inflammatory cytokines. With an inflammatory reaction the cytokine Il-6 has been shown to stimulate the hepatic expression of the acute-phase protein hepcidin. In turn hepcidin inhibits duodenal absorption of iron by down-

regulating ferroportin expression thus reducing serum iron (79). By increasing the expression of the divalent metal transporter 1, IFN-γ has been shown to stimulate the uptake of ferrous iron by macrophages. However, not only pro-inflammatory cytokines are involved in the establishment of an iron transfer block, but it has also been shown that the anti-inflammatory cytokines, Il-4 and Il-10, can up-regulate transferrin receptor expression, resulting in an increase in transferrin receptor-mediated uptake of iron by the macrophage (73, 78). Furthermore, an increase in the phagocytosis and degradation of senescent erythrocytes is known to occur with an inflammatory reaction. This process is directly up-regulated by TNF-α stimulation as a result of the increase in the expression of C3bi (CD11b/CD18) receptors responsible for the recognition and uptake of damaged erythrocytes. In addition, it has been shown that TNF-α can also indirectly up-regulate this process by damaging circulating erythrocytes. These damaged erythrocytes are then phagocytosed upon binding to C3bi (CD11b/CD18) receptors followed by degradation (73, 78). Not only is haemoglobin iron obtained by degradation of red blood cells, but free plasma haemoglobin is taken up by the haemoglobin scavenger receptor, CD163. It has been shown that Il-10 and Il-6 contribute to macrophage haemoglobin acquisition by stimulating the expression of the haemoglobin scavenger receptor, CD163 (78). INF-γ down-regulates the expression of ferroportin, the major transmembrane protein responsible for the release of macrophage iron, thus inhibiting iron export from macrophages (78), a process that is also affected by hepcidin (80). Down-regulation of ferroportin occurs only later in the inflammatory response after the onset of hypoferremia. It is therefore suggested that the down-regulation of ferroportin is not responsible for the development of the iron transfer block, but that it plays a major role in the maintenance of the iron transfer block (78). In addition, many of these pro-inflammatory mediated effects on iron homeostasis are counterbalanced by anti-inflammatory cytokines such as IL-4 and IL-13 (78).

7.2) Diagnosis of iron transfer block

In the present study iron transfer block was diagnosed by examining various factors known to be associated with an iron transfer block. In general, the presence of an iron transfer block is diagnosed by haematologists as a reduction in the amount of sideroblasts relative to the amount of macrophage storage iron seen with Prussian Blue iron staining of a bone marrow aspirate. However, various serum iron markers can also be employed to assess the presence or absence of an iron transfer block. These include serum iron, transferrin, transferrin saturation, ferritin and soluble transferrin receptor. The transferrin/log ferritin ratio and the soluble transferrin receptor/log ferritin ratio also show changes characteristic of an iron transfer block. The evaluation of various red blood cell indices, influenced negatively by an iron transfer block, also contributes to the evaluation of a patient's iron status.

Normal serum iron levels range from 10-30 μmol/l and normal transferrin saturation between 15-60%. Both serum iron and transferrin saturation are decreased with true iron deficiency and are increased with iron overload (81). These changes relate to the changes in storage iron and depend on the size of the storage iron compartment. In the presence of an iron transfer block, serum iron and transferrin saturation are also decreased. Serum iron and transferrin saturation are decreased to below the normal ranges for these serum iron markers. However, the decrease in these values in the presence of an iron transfer block depends on the size of the storage iron compartment and the duration of the iron transfer block. Therefore, similar to the Prussian blue iron stains, the serum iron concentration and transferrin saturation are decreased relative to the size of the storage iron compartment. For the quantitative, non-invasive determination of iron stores, the plasma ferritin concentration is determined (81). It has been well established that each microgram of ferritin per litre of serum represents 8-10 milligrams of stored iron. In healthy iron-replete adult males the plasma ferritin is about

100 ± 60 µg/l, reflecting an average store of about 1000 mg iron. When the body iron reserve is completely exhausted the plasma ferritin is less than 12 µg/l. In women the average store is about one-third of that in men (82). However, with iron transfer block ferritin is elevated, since ferrtin is an acute phase protein and is therefore increased in various chronic diseases and thus do not reflect the size of the iron stores anymore. Therefore, in the presence of an iron transfer block only a Prussian blue iron stain of a bone marrow aspirate could give a reliable evaluation of the size of the storage iron compartment. Serum transferrin, the protein responsible for the transport of iron in the blood, is usually increased in iron deficiency and decreased in iron overload. Normal serum transferrin levels are between 2-3.5 g/l (81). In the presence of iron transfer block, serum transferrin levels will be decreased. Transferrin, in contrast to ferritin is a negative acute phase protein and decreased with inflammation. In order to determine to what extent the functional iron compartment is reduced the soluble transferrin receptor is determined. The soluble transferrin receptor is increased when the amount of iron reaching the red blood cell precursors is decreased (83). In addition, since haemoglobin contains the greatest part of the functional iron, can the haemoglobin concentration be determined to give an indication of the decrease in the functional iron compartment (82). Of the soluble transferrin receptor and haemoglobin concentration it is the increase in soluble transferrin receptor that would occur before the subsequent reduction in haemoglobin concentration. The haemoglobin concentration for males ranges between 130-180 g/l and for females between 120-160 g/l (81). These various factors are usually measured together to give the best indication of the iron status of the patient. Furthermore, two ratios, transferrin/log ferritin and soluble transferrin receptor/log ferritin can also be employed to assist in the differentiation between an iron transfer block and true iron deficiency. These ratios employ two factors, i.e., transferrin and transferrin receptor. Transferrin is decreased in the face of an inflammatory reaction and increased with true iron deficiency. Whereas, the soluble transferrin receptor is markedly

increased with true iron deficiency and with inflammation the soluble transferrin receptor is only slightly increased or normal (84). The denominator of these ratios, ferritin, is increased with an inflammatory reaction and decreased with true iron deficiency. Therefore, these ratios are expected to decrease with an inflammatory reaction and iron transfer block, whereas it is expected to increase with true iron deficiency.

As pro-inflammatory cytokines are generally presumed to be major role players in the development and maintenance of an iron transfer block, and as it was shown in the previous section of this study that the groups of patients with high pro-inflammatory activity have increased levels of the H-subunit of ferritin in the bone marrow macrophage, the prevalence of iron transfer block was investigated in these groups of Kalafong patients. Thereafter, the expression of the H-subunit and L-subunit of ferritin was examined in a group of Kalafong patients with iron transfer block, irrespective of their cytokine profiles.

7.3) Iron status of the C-reactive protein and neopterin subdivisions

The presence or absence of an iron transfer block was evaluated for individual Kalafong patients of both the subdivision according to C-reactive protein levels and the subdivision according to neopterin levels. In an attempt to identify the presence of an iron transfer block and to differentiate between an iron transfer block and true iron deficiency, bone marrow iron stains of the aspirate and the core, various serum iron markers and various red blood cell indices were assessed. This categorisation is discussed in volume 2, chapter 6.

7.3.1) Body iron stores as evaluated by Prussian blue iron stains of the bone marrow aspirates and cores of the C-reactive protein and neopterin subdivisions

To assess total body iron stores a Prussian blue iron stain was performed on the bone marrow aspirates and bone marrow cores. The magnitude of blue iron deposits formed in the bone marrow reticuloendothelial cells are known to correspond to total body iron stores. The findings for these iron stains are presented in volume 2, chapter 6. Iron stores for the group of Kalafong patients with elevated C-reactive protein were normal for 22% of the patients, increased for 59% and for 19% of the patients in this group the iron stores were reduced. Iron stores for the group of Kalafong patients with elevated neopterin were normal for 15% of the patients, increased for 52% and for 33% of the patients in this group the iron stores were reduced. Iron stores for the group of Kalafong patients with normal C-reactive protein were normal for 21% of the patients, increased for 16% and for 63% of the patients in this group the iron stores were reduced. Iron stores for the group of Kalafong patients with normal neopterin were normal for 38% of the patients, increased for 15% and for 46% of the patients in this group the iron stores were reduced.

7.3.2) Serum iron markers and determination of the iron status of the C-reactive protein and neopterin subdivisions

In the following section the iron status for the C-reactive protein and neopterin subdivisions was assessed by means of their serum iron markers. A summary of the findings is in this section provided in small print in order to facilitate the reading of the chapter.

Serum iron was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (9 ± 6.9 µmol/l; n = 29) and the group of

Kalafong patients with normal C-reactive protein (11.3 ± 10.6 µmol/l; n = 19) and
- not significantly different between the group of Kalafong patients with elevated neopterin (10.4 ± 9.6 µmol/l; n = 35) and the group of Kalafong patients with normal neopterin (8.7 ± 4.5 µmol/l; n = 13).
- The laboratory values for normal serum iron are between 10-30 µmol/l.

Serum iron was low-normal for the group of Kalafong patients with normal C-reactive protein and low for the group of Kalafong patients with normal neopterin. For these two patient groups the Prussian blue iron stains showed that the majority of the patients had low or absent iron stores. Therefore, the low mean serum iron for these groups was as a result of true iron deficiency. In the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin the mean serum iron was also low. However, in these patient groups the Prussian blue iron stains showed that the iron stores for these patients were mostly normal or elevated. This situation is characteristic of an iron transfer block.

Serum transferrin was
- significantly lower (p-value < 0.0001) in the group of Kalafong patients with elevated C-reactive protein (1.3 ± 0.5 g/l; n = 28) compared to the group of Kalafong patients with normal C-reactive protein (2.5 ± 0.83 g/l; n = 19) and
- significantly lower (p-value = 0.0008) in the group of Kalafong patients with elevated neopterin (1.5 ± 0.70 g/l; n = 34) compared to the group of Kalafong patients with normal neopterin (2.5 ± 0.84 g/l; n = 13).
- The laboratory values for normal serum transferrin are between 2-3.6 g/l.

In the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin the transferrin was normal as a result of the storage iron compartments that were low, normal and increased for these patients. The decrease in serum transferrin in the presence of low/deficient serum iron in the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin are characteristic of an iron transfer block. Transferrin is a negative acute phase protein and is decreased with an iron transfer block.

Percentage transferrin saturation was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (34.2 ± 47.7%; n = 26) and the group of Kalafong patients with normal C-reactive protein (24.3 ± 26.5%; n = 19) and
- marginally lower (p-value = 0.058) in the group of Kalafong patients with normal neopterin (17.41 ± 15.1%; n = 13) compared to the group of Kalafong patients with elevated neopterin (35.2 ± 45.8%; n = 32).
- The laboratory values for normal percentage transferrin saturation are between 15-50% in females and between 20-50% in males.

For the group of Kalafong patients with normal neopterin the average transferrin saturation of 17.41% is as a result of the decrease in serum iron together with an increase in transferrin. Since, the percentage transferrin saturation is determined not only by serum iron but also by serum transferrin levels. The group of Kalafong patients with normal C-reactive protein showed similar transferrin levels but higher serum iron levels than the group of Kalafong patients with normal neopterin. This resulted in a smaller decrease in transferrin saturation in this group of patients. In the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin serum transferrin saturation was thus normal for both groups. This is as a result of the low serum iron and low transferrin.

Serum ferritin was

- significantly higher (p-value = 0.033) in the group of Kalafong patients with elevated C-reactive protein (3903.1 ± 8942.6 µg/l; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (166.8 ± 270.3 µg/l; n = 19) and
- significantly higher (p-value = 0.03) in the group of Kalafong patients with elevated neopterin (3277.4 ± 8236 µg/l; n = 35) compared to the group of Kalafong patients with normal neopterin (126.8 ± 164.5 µg/l; n = 13).
- The laboratory values for normal serum ferritin are between 11-306.8 µg/l in females and between 23.9-336.2 µg/l in males.

Serum ferritin is an acute phase protein and was markedly increased in the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin. This increase in serum ferritin is characteristic of an iron transfer block.

The transferrin/log ferritin ratio was

- significantly lower (p-value = 0.002) in the group of Kalafong patients with elevated C-reactive protein (0.51 ± 0.31; n = 28) compared to the group of Kalafong patients with normal C-reactive protein (2.2 ± 2; n = 19) and
- significantly lower (p-value = 0.0008) in the group of Kalafong patients with elevated neopterin (0.96 ± 1.4; n = 34) compared to the group of Kalafong patients with normal neopterin (1.8 ± 1.7; n = 13).

The transferrin/log ferritin ratio was less than one for both the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin. A transferrin/log ferritin ratio of less than one is characteristic of patients with an iron transfer block. This confirms the iron transfer block in patients with a pro-inflammatory, T-helper cell type-1 immune reaction.

Soluble transferrin receptor was

- significantly lower (p-value = 0.023) in the group of Kalafong patients with elevated C-reactive protein (9.5 ± 7.4 μg/ml; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (17.3 ± 12.9 μg/ml; n = 19) and
- not significantly different between the group of Kalafong patients with elevated neopterin (11.8 ± 10.2 μg/ml; n = 35) and the group of Kalafong patients with normal neopterin (14.8 ± 11.5 μg/ml; n = 13).
- The ELISA kit's values for normal soluble transferrin receptor are between 2.9-8.3 μg/ml.

Soluble transferrin receptor was significantly lower in the group of Kalafong patients with elevated C-reactive protein than in the group of Kalafong patients with normal C-reactive protein. However, soluble transferrin receptor was higher for both groups compared to the normal range for soluble transferrin receptor. It has been shown in previous studies that soluble transferrin receptor is normal or slightly increased in the presence of an iron transfer block and markedly elevated with true iron deficiency. For patients with a possible iron transfer block together with true iron deficiency soluble transferrin receptor will be significantly elevated and not normal or slightly increased (73). A different result was shown for the neopterin subdivision. Soluble transferrin receptor was not significantly different between the group of Kalafong patients with

elevated neopterin and the group of Kalafong patients with normal neopterin. Nevertheless, similar to the C-reactive protein subdivision, soluble transferrin receptor was higher for both the group of Kalafong patients with elevated neopterin and the group of Kalafong patients with normal neopterin compared to the normal range for soluble transferrin receptor.

Soluble transferrin receptor in anaemia of chronic disease and iron deficiency anaemia

Transferrin receptors are expressed on the cell surface of many cell types and can be detected in serum (soluble transferrin receptors) as a result of cleavage from the cell surface (83). Although different cell types contribute to the serum soluble transferrin receptor concentration, erythroblasts provide more than 80% of the total concentration of serum soluble transferrin receptor (83). The erythroid marrow is the major site for iron uptake to support haemoglobin production with transferrin receptor numbers and iron uptake by developing erythroid cells maximal at the basophilic normoblast stage. When the erythroid cells cease dividing and mature into reticulocytes the transferrin receptor numbers decrease, and with the attainment of the circulating erythrocyte stages all transferrin receptors will have disappeared (85). Two factors can influence the amount of transferrin receptors expressed on the cell surface of the erythroid cells. Firstly, the rate of proliferation, with an increase in the rate of proliferation resulting in an up-regulation of transferrin receptors on the membranes of the erythroid cells. The second factor is the amount of available intracellular iron (85). The translation of transferrin receptor mRNA is regulated by the iron-responsive protein/iron-responsive element (IRP/IRE) with an increase in intracellular iron resulting in the down-regulation of transferrin receptors and a decrease in intracellular iron resulting in an increase in transferrin receptors. Since the proliferation of cells is dependent on the availability of intracellular iron, the effects of the increase in the proliferative rate are possibly a

consequence of the increased iron requirements with proliferation and thus another manifestation of the regulation of transferrin receptor synthesis through iron availability (86). Therefore, the serum soluble transferrin receptor concentration largely reflects the size of the erythroid marrow and the transferrin receptor number on the erythroblast surface (83).

In both the anaemia of chronic disease (ACD) and iron deficiency anaemia (IDA) sideropenia is shown with a bone marrow iron stain (83). Therefore, in both ACD and IDA the iron delivered to the erythroid marrow is suboptimal. However, with ACD this can be in the face of either normal or increased macrophage storage iron. Due to the suboptimal intracellular iron in the erythroblasts, an increase in the soluble transferrin receptor concentration is expected. Clearly it becomes increased earlier in the development of functional iron depletion than any of the red blood cell related parameters (84). However, this is only true for IDA. With ACD soluble transferrin receptor concentrations are normal or only slightly increased. This is as a result of the general suppression of erythropoiesis in ACD. With the suppression of the proliferative rate of the erythroblasts a decrease in transferrin receptor expression occurs. However, the coexistence of true iron deficiency with chronic disease is associated with an increase in both the efficiency and number of erythroblast transferrin receptors and a highly significant rise in soluble transferrin receptor numbers. Therefore, with ACD in the presence of true iron deficiency soluble transferrin receptor concentrations are increased (83). It has been suggested that soluble transferrin receptor levels can distinguish between ACD and IDA. Despite low serum iron levels, in both ACD and IDA, serum soluble transferrin receptor values remain largely within the normal range for ACD but are elevated in IDA, even in the presence of inflammatory disease (83, 84).

In this study soluble transferrin receptor/log ferritin ratio was

- significantly lower (p-value = 0.025) in the group of Kalafong patients with elevated C-reactive protein (3.4 ± 2.9; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (18.1 ± 26.1; n = 19) and
- marginally lower (p-value = 0.065) in the group of Kalafong patients with elevated neopterin (8.5 ± 19; n = 35) compared to the group of Kalafong patients with normal neopterin (11.2 ± 14.9; n = 13).

With a pro-inflammatory immune reaction ferritin is known to be indiscriminately increased. Together with this increase in ferritin it is known that the soluble transferrin receptor levels are normal or slightly elevated. Therefore, with a pro-inflammatory immune reaction the soluble transferrin receptor/log ferritin ratio would decrease. In contrast, with true iron deficiency this ratio is supposed to increase. The soluble transferrin receptor/ferritin ratio was lower in both the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin compared to the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin, respectively. This lower soluble transferrin receptor/log ferritin ratio in the groups of patients with a pro-inflammatory immune reaction is characteristic for an iron transfer block.

7.3.3) Red blood cell indices of the C-reactive protein and neopterin subdivisions

The evaluation of various red blood cell indices, influenced negatively by an iron transfer block, also contributes to the evaluation of a patient's iron status.

The red blood cell count was

- significantly lower (p-value = 0.0008) in the group of Kalafong patients with elevated C-reactive protein (2.6 ± 1.1 x 10^{12}/l; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (2.9 ± 1.4 x 10^{12}/l; n = 19) and
- significantly lower (p-value = 0.002) in the group of Kalafong patients with elevated neopterin (2.3 ± 0.95 x 10^{12}/l; n = 35) compared to the group of Kalafong patients with normal neopterin (3.7 ± 1.2 x 10^{12}/l; n = 13).
- The laboratory values for normal red blood cell count are between 4.13-5.67 x 10^{12}/l in females and between 4.89-6.11 x 10^{12}/l in males.

Anaemia was present in the majority of Kalafong patients. The anaemias had various etiologies including anaemia due to blood loss, haemolytic anaemia, iron deficiency anaemia, megaloblastic anaemia and anaemia of inflammation. Furthermore, the etiologies of anaemia in some of the Kalafong patients were multi-factorial.

The haemoglobin was

- significantly lower (p-value = 0.004) in the group of Kalafong patients with normal C-reactive protein (7.2 ± 3.8 g/dl; n = 19) compared to the group of Kalafong patients with elevated C-reactive protein (7.6 ± 3.5 g/dl; n = 29) and
- significantly lower (p-value = 0.005) in the group of Kalafong patients with elevated neopterin (6.4 ± 2.9 g/dl; n = 35) compared to the group of Kalafong patients with normal neopterin (10.2 ± 3.9 g/dl; n = 13).
- The laboratory values for normal haemoglobin are 12.1-16.3 g/dl for females and 14.3-18.3 g/dl for males.

Most of the Kalafong patients showed a decrease in haemoglobin. The patients had multifactorial causes for the reduction in haemoglobin.

The haematocrit was

- significantly lower (p-value = 0.001) in the group of Kalafong patients with elevated C-reactive protein (0.23 ± 0.1 l/l; n = 29) compared to the group of Kalafong patients with normal C-reactive protein (0.24 ± 0.12 l/l; n = 19) and
- significantly lower (p-value = 0.002) in the group of Kalafong patients with elevated neopterin (0.2 ± 0.08 l/l; n = 35) compared to the group of Kalafong patients with normal neopterin (0.33 ± 0.12 l/l; n = 13).
- The laboratory values for normal haematocrit are 0.370-0.490 l/l in females and 0.430-0.550 l/l in males.

Most of the Kalafong patients showed a decrease in haematocrit.

The mean corpuscular volume was

- significantly higher in the group of Kalafong patients with elevated C-reactive protein (91.3 ± 15.6 fl; n = 29) compared to that of the group of Kalafong patients with normal C-reactive protein (80.3 ± 14.9 fl; n = 19) and
- not significantly different between the group of Kalafong patients with elevated neopterin (86.8 ± 16.2 fl; n = 35) and the group of Kalafong patients with normal neopterin (87.3 ± 16.3 fl; n = 13).
- The laboratory values for normal mean corpuscular volume are 79.1-98.9 fl.

In the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin the inclusion of patients with different iron storage compartments and different red blood cell pathologies such as macrocytic anaemias resulted in a normal average mean corpuscular volume. The average for the mean corpuscular volume for both the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin showed the presence of normocytic red blood cells. It is known that most patients with an iron transfer block have a normocytic, normochromic anaemia (73, 87). The patients with an iron transfer block are mostly seen in medical care facilities at the time when they display the presence of a normocytic, normochromic anaemia. With further development of anaemia of chronic disease the patients develop a microcytic, hypochromic anaemia.

The mean corpuscular haemoglobin was

- significantly lower in the group of Kalafong patients with normal C-reactive protein (24.5 ± 5.9 pg; n = 19) compared to the group of Kalafong patients with elevated C-reactive protein (29.8 ± 6.1 pg; n = 29), and
- not significantly different between the group of Kalafong patients with elevated neopterin (27.9 ± 6.3 pg; n = 35) and the group of Kalafong patients with normal neopterin (27.2 ± 7.2 pg; n = 13).
- The laboratory values for normal mean corpuscular haemoglobin are between 27-32 pg.

The mean corpuscular haemoglobin concentration was

- significantly lower (p-value = 0.002) in the group of Kalafong patients with normal C-reactive protein (30.3 ± 2.4 g/dl; n = 19) compared to the group of Kalafong patients with elevated C-reactive protein (32.5 ± 2 g/dl; n = 29) and
- not significantly different between the group Kalafong patients with elevated neopterin (31.9 ± 2.2 g/dl; n = 35) and the group of Kalafong patients with normal neopterin (30.8 ± 3 g/dl; n = 13).
- The laboratory values for normal mean corpuscular haemoglobin concentration are between 32-36 g/dl.

The red blood cell distribution width (RDW) was

- not significantly different between the group of Kalafong patients with elevated C-reactive protein (20.2 ± 6.6%; n = 29) and the group of Kalafong patients with normal C-reactive protein (21.1 ± 8.2%; n = 19) and
- not significantly different between the group of Kalafong patients with elevated neopterin (20.6 ± 7.5%; n = 35) and the group of Kalafong patients with normal neopterin (20.3 ± 6.8%; n = 13).
- The laboratory values for normal red blood cell distribution width are between 11.6-14%.

The red blood cell distribution width is a measure of the variation of red blood cell width with an increase in the red blood cell distribution width known as anisocytosis. A greater variation in size occurs with iron deficient anaemia and anaemia of chronic inflammation. The red blood cells produced with iron deficiency anaemia initially show highly variable sizes therefore a markedly increased RDW, but with the development of a pronounced microcytic, hypochromic anaemia the RDW will decrease (88).

The reticulocyte production index (RPI) was

- significantly higher (p-value = 0.039) in the group of Kalafong patients with elevated C-reactive protein (0.81 ± 0.65; n = 11) compared to the group of Kalafong patients with normal C-reactive protein (0.47 ± 0.8; n = 11) and
- significantly higher (p-value = 0.047) in the group of Kalafong patients with elevated neopterin (0.75 ± 0.8; n = 17) compared to the group of Kalafong patients with normal neopterin (0.28 ± 0.23; n = 5).

A decrease in the reticulocyte production index to below 2.5 is as a result of hypoproliferative anaemias or anaemias due to ineffective erythropoiesis. The majority of patients for which the reticulocyte production index were determined had a value of less than 2.5. The decrease in the reticulocyte production index was as a result of various factors such as suppression of erythropoiesis by cytokines and/or nutritional deficiencies.

7.3.4) Prevalence of iron transfer block in patients with a pro-inflammatory immune status

In a previous section it was shown that the cytokine profiles of both the elevated neopterin and elevated C-reactive protein groups were predominantly pro-inflammatory (T-helper cell type-1). When the results of the Prussian blue iron stains of the bone marrow aspirate and core, the serum iron markers and the red blood cell indices were evaluated for each individual patient (volume 2, chapter 6) the prevalence of an iron transfer block in the subdivision according to C-reactive protein and the subdivision according to neopterin was as follows.

Iron status categorisation of patients for the C-reactive protein subdivision showed that

- 20 of the 29 patients (69%) with elevated C-reactive protein had an iron transfer block while
- 14 of the 19 patients (74%) with normal C-reactive protein did not have an iron transfer block, only five of the 19 patients (26%) with normal C-reactive protein had an iron transfer block.

Iron status categorisation of patients for the neopterin subdivision showed that

- 23 of the 35 patients (66%) with elevated neopterin had an iron transfer block while
- 11 of the 13 patients (85%) with normal neopterin did not have an iron transfer block, only two of the 13 patients (15%) with normal neopterin had an iron transfer block.

The above results supported the notion that T-helper cell type-1/pro-inflammatory cytokines are major role players in the development of an iron transfer block. However, it also showed that T-helper cell type-1/pro-inflammatory cytokines are not the only mediators for the development of an iron transfer block. In view of the pleiotropic nature of cytokines, the intracellular signalling cross-talk between cytokines, the many interactions between factors such as transmembrane iron transporters, hepcidin and others, it is difficult to predict the development of an iron transfer block merely on the basis of isolated factors such as the pro-inflammatory status. Despite the predominant role of pro-inflammatory cytokines in the development of an iron transfer block it was, for instance, previously shown that T-helper cell type-2 cytokines can also play a role in the development of an iron transfer block (73, 78). It was thus not a surprising finding that all patients with a pro-inflammatory cytokine profile did not have an iron transfer block. Neither was it a surprising finding that a small percentage of patients with a

relatively normal cytokine profile had an iron transfer block. Furthermore, although the cytokine profiles were very similar for the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin, there was one notable exception. Together with the increase in the T-helper cell type-1 cytokines it was found that Il-5 was significantly higher in the group of Kalafong patients with elevated neopterin. Il-5 is, in essence, a T-helper cell type-2 cytokine, but belongs to the GM-CSF/Il-3 subgroup (89). In contrast to GM-CSF and Il-3 no effect on monocytes for Il-5 has been shown (90). However, it has been shown that Il-5, as for GM-CSF and Il-3, displayed proliferative effects on both microglia and macrophage cell lines (91). Furthermore, both Il-5 and Il-3 have been shown to bind and activate the GM-CSF receptor present on monocytes (44). It is therefore suggested that Il-5 could have contributed to the establishment of inflammation. Other confounding factors in the prediction of whether an iron transfer block will develop or not, are the activity of ferroportin and hepcidin (80). It has been shown that hepcidin production is increased in inflammation and that hepcidin plays a major role in iron transfer block (79). Hepcidin was shown to bind to ferroportin (major iron transporter involved in the export of cellular iron) resulting in the internalization of ferroportin and a decrease in the release of macrophage iron. However, it has also been shown that hepcidin's role in the iron transfer block involves, not the development of the block, but the maintenance of the iron transfer block (78).

In summary on the iron status of the groups of Kalafong patients with a pro-inflammatory immune status and the groups of Kalafong patients with no pronounced immune activation

The group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin displayed a pro-inflammatory cytokine profile. For these two groups of patients a high prevalence of an iron transfer block was found

when evaluating their Prussian blue iron stains for the bone marrow aspirate and core, their serum iron markers and their red blood cell indices. The prevalence of an iron transfer block was 69% for the group of Kalafong patients with elevated C-reactive protein and 66% for the group of Kalafong patients with elevated neopterin. The majority of the patients in these two groups of Kalafong patients had serum iron profiles characteristic of patients with an iron transfer block. A decrease in serum iron, an increase in ferritin, a decrease in transferrin, a small increase in soluble transferrin receptor, a decrease in transferrin/log ferritin ratio and a decrease in soluble transferrin receptor/log ferritin ratio all characteristic of the presence of an iron transfer block (73). On the other hand, for the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin, no excessive pro-inflammatory immune activation was found. As was expected most of the patients in the group of Kalafong patients with normal C-reactive protein and in the group of Kalafong patients with normal neopterin did not have serum iron profiles characteristic of an iron transfer block. However, these groups of patients did not have normal iron status but a high incidence of true iron deficiency. For these groups of patients a decreased serum iron, normal ferritin, normal transferrin, a marked increase in soluble transferrin receptor, an increase in transferrin/log ferritin ratio and an increase in the soluble transferrin receptor/log ferritin ratio were shown. In the presence of an iron transfer block and in the presence of true iron deficiency a marked decrease in red blood cell production is known to occur (84). In the presence of an iron transfer block a negative effect on red blood cell production occurs as a result of the decrease in bio-available iron for haemoglobin production and various other factors resulting in a decrease in red blood cell production. These factors include suppression of the proliferation of erythroid progenitor cells, decrease in the synthesis of erythropoietin, decrease in the sensitivity of erythroblasts to erythropoietin and a decrease in red blood cell life span (73). The anaemia of inflammation, is therefore on the one hand a result of a decrease in

iron reaching the erythron and on the other as a result of the suppression of red blood cell synthesis (92, 93, 94). Il-1, TNF-α and TGF-β have all been shown to inhibit erythropoietin synthesis and action during an inflammatory reaction (92).

In the next few sections important observations derived from the investigation of the prevalence of an iron transfer block in the subdivision of the Kalafong patients based on C-reactive protein and the subdivision of the Kalafong patients based on neopterin are discussed.

7.4) Loss of the relationship between storage iron, bio-available iron and red blood cell production in patients with a pro-inflammatory, T-helper cell type-1 immune response

Normally a close relationship exists between bio-available iron and red blood cell production. In the present study, this close relationship has been shown to exist for the groups of Kalafong patients with no pronounced immune activation. In the following paragraphs it will be shown that this relationship was disturbed in the group of patients with a pro-inflammatory, T-helper cell type-1 immune response.

Serum iron and percentage transferrin saturation reflect the amount of iron that is available to bind to the transferrin receptors of the red blood cell precusors. Therefore, a decrease in serum iron and a decrease in percentage transferrin saturation are usually associated with a decrease in haemoglobin production. This was seen in both the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.

In the group of Kalafong patients with normal C-reactive protein a decrease in percentage transferrin saturation was shown to correlate with

- a decrease in mean corpuscular volume ($r = 0.62$, p-value = 0.005),
- a decrease in mean corpuscular haemoglobin ($r = 0.64$, p-value = 0.003) and
- a decrease in mean corpuscular haemoglobin concentration ($r = 0.49$, p-value = 0.033).

In the group of Kalafong patients with normal neopterin a decrease in percentage transferrin saturation was shown to correlate with

- a decrease in mean corpuscular haemoglobin concentration (r = 0.65, p-value = 0.015).

In the group of Kalafong patients with normal neopterin a decrease in serum iron was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.55, p-value = 0.053),
- a decrease in mean corpuscular haemoglobin (r = 0.57, p-value = 0.040) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.49, p-value = 0.091).

When the available iron is reduced in the plasma due to decreased iron stores transferrin will normally be up-regulated. An increase in serum transferrin is known to occur in patients where the iron stores are deficient. An increase in transferrin in these patients was related to iron-deficient iron stores resulting in iron-deficient erythropoiesis. This was seen in both the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.

In the group of Kalafong patients with normal C-reactive protein an increase in serum transferrin was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.62, p-value = 0.005),
- a decrease in mean corpuscular haemoglobin (r = 0.74, p-value = 0.0003) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.79, p-value = 0.0001).

In the group of Kalafong patients with normal neopterin an increase in serum transferrin was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.70, p-value = 0.008),
- a decrease in mean corpuscular haemoglobin (r = 0.80, p-value = 0.001) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.84, p-value = 0.0003).

Furthermore, in the group of Kalafong patients with normal C-reactive protein an increase in the transferrin/log ferritin ratio was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.66, p-value = 0.002),
- a decrease in mean corpuscular haemoglobin (r = 0.72, p-value = 0.0005) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.70, p-value = 0.001).

In the group of Kalafong patients with normal neopterin an increase in the transferrin/log ferritin ratio was shown to correlate with

- a decrease in mean corpuscular haemoglobin concentration (r = 0.68, p-value = 0.01).

With a decrease in iron reaching the red blood cell precursors these cells normally produce more transferrin receptors in attempt to take up more iron. This was seen in both the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.

In the group of Kalafong patients with normal C-reactive protein an increase in soluble transferrin receptor was shown to correlate with

- a decrease in serum iron (r = 0.52, p-value = 0.024),
- an increase in serum transferrin (r = 0.59, p-value = 0.007), an increase in serum transferrin indicates decreased iron stores as a result too little iron reached the erythroblasts for red blood cell production,
- a decrease in mean corpuscular volume (r = 0.65, p-value = 0.002),
- a decrease in mean corpuscular haemoglobin (r = 0.75, p-value = 0.0002) and
- a decrease in mean corpuscular heamaglobin concentration (r = 0.75, p-value = 0.0002).

In the group of Kalafong patients with normal neopterin an increase in soluble transferrin receptor was shown to correlate with

- a decrease in the red blood cell count (r = 0.63, p-value = 0.021),
- a decrease in haemoglobin (r = 0.90, p-value < 0.0001),
- a decrease in haematocrit (r = 0.84, p-value = 0.0003) and
- a decrease in mean corpuscular heamoglobin concentration (r = 0.79, p-value = 0.001).

Furthermore, in the group of Kalafong patients with normal C-reactive protein an increase in the soluble transferrin receptor/log ferritin ratio was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.60, p-value = 0.007),
- a decrease in mean corpuscular haemoglobin (r = 0.65, p-value = 0.003) and
- a decrease in mean corpuscular haemoglobin concentration(r = 0.61, p-value = 0.005).

In the group of Kalafong patients with normal neopterin an increase in the soluble transferrin receptor/log ferritin ratio was shown to correlate with

- a decrease in mean corpuscular volume (r = 0.53, p-value = 0.06),
- a decrease in mean corpuscular haemoglobin (r = 0.63, p-value = 0.021) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.75, p-value = 0.003).

These relationships between red blood cell production and iron was not shown to exist in the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin. Only the following correlations were indicated. In the group of Kalafong patients with elevated C-reactive protein a decrease in the red blood cell count was shown to correlate with an increase in the red blood cell distribution width (r = 0.57, p-value = 0.001). For the group of patients with elevated neopterin an increase in serum transferrin correlated with a decrease in mean corpuscular haemoglobin concentration (r = 0.56, p-value = 0.0006).

The results of the present study showed that in the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin (patients with relatively normal pro-inflammatory activation) the availability of iron was

the most important factor for regulating red blood cell production. In contrast, in the groups of Kalafong patients with a pronounced pro-inflammatory cytokine profile (group of Kalafong patients with elevated C-reactive protein and group of Kalafong patients with elevated neopterin) this role of bio-available iron in the regulation of proper red blood cell production was lost.

7.5) Possible role of the anti-inflammatory cytokine, transforming growth factor-β, in resolving the iron transfer block

No direct correlation of any of the pro-inflammatory cytokines was shown between any of the serum iron markers or between any of the red blood cell indices in either the group of Kalafong patients with elevated C-reactive protein or the group of Kalafong patients with elevated neopterin. However, in the group of Kalafong patients with elevated C-reactive protein a possible role for the anti-inflammatory cytokine TGF-β was shown in resolving the iron transfer block. In the group of Kalafong patients with elevated C-reactive protein an increase in TGF-β correlated with an increase in serum transferrin ($r = 0.73$, p-value < 0.0001) and an increase in the transferrin/log ferritin ratio ($r = 0.67$, p-value $= 0.0001$). TGF-β is an anti-inflammatory cytokine and could perhaps contribute (directly or indirectly) to the increase in the negative acute phase protein transferrin.

7.6) Relationship between storage iron, bio-available iron, expression of the H-subunit and L-subunit of ferritin and red blood cell production in the group of Kalafong patients with normal neopterin

In the group of Kalafong patients with normal neopterin indications for a possible regulatory role for the H-subunit and L-subunit of ferritin was shown in the production of red blood cells. In the group of Kalafong patients with normal neopterin an increase in the H-subunit/L-subunit ratio in the macrophage correlated with a decrease in mean

corpuscular volume (r = 0.58, p-value = 0.037). A correlation was also seen between an increase in the H-subunit/L-subunit ratio in the macrophage with a decrease in mean corpuscular haemoglobin (r = 0.51, p-value = 0.072). Therefore, with an increase in the amount of H-subunits relative to the amount of L-subunits in the macrophage (increase in H-subunit rich ferritins) a decrease in efficient red blood cell production was shown. It has already been established by previous studies that a reduced amount of iron reaches the red blood cell precursors with an iron transfer block (95). An increase in the H-subunit/L-subunit ratio in the macrophage could contribute to the reduction in iron that reached the red blood cell precursors since H-subunit rich ferritins are more prone to conversion into haemosiderin. Once H-subunit rich ferritins are converted into haemosiderin, the iron that was contained in ferritin is trapped. In support of the trapping of iron in these patients it was shown that with an increase in the expression of the L-subunit of ferritin in the macrophage the mean corpuscular volume (r = 0.85, p-value = 0.0002) and the mean corpuscular haemoglobin (r = 0.76, p-value = 0.003) were increased. The L-subunit of ferritin is more likely to be influenced by iron and therefore is indicative of an increase in the labile iron pool of the macrophage. An increase in the labile iron pool of the macrophage is likely to result in more iron being delivered to the red blood cell precursors. Furthermore, an increase in the L-subunit of ferritin in the cells of the erythron correlated with an increase in the mean corpuscular volume (r = 0.69, p-value = 0.01) and an increase in the mean corpuscular haemoglobin (r = 0.65, p-value = 0.016). An increase in the L-subunit of ferritin in cells of the erythron most probably reflects an increase in the labile iron pool of these cells. It has been shown in previous studies that when iron accumulates in erythroid cells due to an increase in the cellular uptake of iron, the L-subunit rich ferritins seem to increase and to be closely related to the iron status of the cells (55, 96). An increase in the labile iron pool would furnish the cells of the erythron with more iron for haemoglobin synthesis. This increase in the intracellular labile iron pool in the cells of the erythron reflected by an increase in

the expression of the L-subunit of ferritin was most likely as a result of an increase in bio-available iron since the L-subunit of ferritin in the cells of the erythron was shown to correlate with an increase in serum iron ($r = 0.66$, p-value = 0.014) and an increase in the percentage transferrin saturation ($r = 0.68$, p-value = 0.01).

7.7) Relationship between the H-subunit/L-subunit ratio in cells of the erythron and the mean corpuscular volume in the group of Kalafong patients with normal neopterin

Similar to what was shown for the H-subunit/L-subunit ratio in the macrophage an increase in the H-subunit/L-subunit ratio in the cells of the erythron correlated with a decrease in the mean corpuscular volume ($r = 0.52$, p-value = 0.069) in the group of Kalafong patients with normal neopterin. The mean corpuscular volume of red blood cells is closely related to the amount of haemoglobin in these cells. Therefore, a reduction in the mean corpuscular volume reflects a decrease in the amount of iron reaching the red blood cell precursors (97). The importance of an increase in the H-subunit/L-subunit ratio in the cells of the erythron in terms of iron bio-availability is not known. It could be that an increase in the H-subunit/L-subunit ratio in the cells of the erythron, similar to the macrophage, can result in the un-availability of cellular iron for red blood cell production. Red blood cell precursors obtain iron for haemoglobin production possibly by the following two routes – transferrin iron via the transferrin receptor and as ferritin during the process of rhopheocytosis (98). Once the iron reaches the intracellular labile iron pool it is either incorporated into ferritin or used for haemoglobin production. The expression of the H-subunit and L-subunit of ferritin plays an important role in the sequestration and the release of bio-avalable iron for the production of haemoglobin. The erythroid cells contain mainly H-subunit rich ferritins, which play a major role in the intracellular transport and donation of iron for the active synthesis of haem (99), particularly in immature erythroid cell precursors such as

proerythroblasts and basophilic erythroblasts (55). However, it has been shown that an increase in H-subunit rich isoferritins can result in the rapid chelation of labile iron in erythroid cells (61). Therefore, the increase in the H-subunit/L-subunit ratio in the red blood cell precursors could result in the reduction in the red blood cell volume as a result of the withholding of iron by these H-subunit rich ferritins.

7.8) Relationship between the H-subunit of ferritin in the cells of the erythron and the mean corpuscular haemoglobin concentration in the group of Kalafong patients with elevated C-reactive protein

In the group of Kalafong patients with elevated C-reactive protein a decrease in the H-subunit of ferritin in the cells of the erythron correlated with a decrease in the mean corpuscular haemoglobin concentration ($r = 0.51$, p-value = 0.007). The intracellular labile iron pool of the red blood cell precursors is regulated by the expression of the H-subunit and L-subunit of ferritin (61). It is suggested by previous studies that in the red blood cell precursors the expression of the H-subunit of ferritin plays an important role in the release of bio-available iron for the production of haemoglobin. It has been shown that H-subunit rich ferritins accumulate and release iron faster than do L-subunit rich ferritins (2, 3, 76, 100) and it is suggested that the H-subunit rich ferritins permit more dynamic intracellular traffic of iron (3, 101). Therefore, this correlation of a decrease in the expression of the H-subunit in the cells of the erythron together with the decrease in the mean corpuscular haemoglobin concentration points to a role for the decrease in H-subunit resulting in a reduction in the release of bio-available iron. It was shown, *in vitro*, that a too great proportion of H-subunits in the ferritin protein shell result in ferritin aggregation. This may be due to the inadequacy of the ferritin protein shell to retain the formed Fe^{3+} resulting in the loss of Fe^{3+} and hydrolysis of Fe^{3+} on the outside of the ferritin molecule (4). Upon ferritin aggregation, the iron contained in the

ferritin protein shell may be trapped and therefore no longer available to support haemoglobin production.

7.9) Pro-hepcidin and caeruloplasmin levels for the C-reactive protein and neopterin subdivisions

Pro-hepcidin is the precursor of the hormone hepcidin that plays a role in the mediation of the homeostasis of extracellular iron. Hepcidin synthesis is physiologically increased by elevated plasma iron, decreased by erythropoietic activity and pathologically increased by inflammation. Hepcidin acts by regulating iron influx into plasma from duodenal enterocytes, hepatocytes and macrophages. Hepcidin binds to the transmembrane iron-transporter, ferroportin, responsible for the movement of iron from the cytoplasm of these cells to the plasma. An increase in hepcidin results in the internalization of ferrroportin and a reduction in the release of iron from enterocytes, hepatocytes and macrophages. Due to the role of hepcidin in the regulation of the release of iron from macrophages, it has been suggested that hepcidin could play a role in the development of the iron transfer block with inflammation (79).

At the time of the study the newly developed hepcidin ELISA from DRG Diagnostics, Germany was not avaliable for purchase and therefore only pro-hepcidin could be measured. In this study pro-hepcidin was significantly decreased (p-value = 0.039) for the group of Kalafong patients with elevated C-reactive protein (135.2 ± 48.2 ng/ml; n = 28) compared to the group of Kalafong patients with normal C-reactive protein (159.3 ± 38.6 ng/ml; n = 19). Similar to what was found for the C-reactive protein subdivision pro-hepcidin was significantly decreased (p-value = 0.02) in the group of Kalafong patients with elevated neopterin (135.6 ± 40 ng/ml; n = 34) compared to the group of Kalafong patients with normal neopterin (169.2 ± 52.2 ng/ml; n = 13).

Although it was previously shown that pro-hepcidin is increased during an inflammatory reaction (102), the results of the present study showed the opposite to occur in the groups of Kalafong patients with a pro-inflammatory immune reaction. Nevertheless, pro-hepcidin might be reduced in this study due to an increase in the conversion to hepcidin. This could, however, only be clarified by the measurement of hepcidin.

Caeruloplasmin is a multi-copper binding protein that acts as a serum ferroxidase and is essential for the mobilization of iron from storage tissues (103). Caeruloplasmin is situated on the extracellular side of the cell membrane and oxidizes Fe^{2+} to Fe^{3+} to facilitate the binding to transferrin. Therefore, caeruloplasmin plays an important role in the export of iron from tissue storage sites. In the present study, there was no significant difference for caeruloplasmin between the group of Kalafong patients with elevated C-reactive protein (0.47 ± 0.15 g/l; n = 27) and the group of Kalafong patients with normal C-reactive protein (0.46 ± 0.16 g/l; n = 19). There was also no difference for caeruloplasmin between the group of Kalafong patients with elevated neopterin (0.47 ± 0.16 g/l; n = 33) and the group of Kalafong patients with normal neopterin (0.47 ± 0.14 g/l; n = 13).

8) EXPRESSION OF THE H-SUBUNIT AND L-SUBUNIT OF FERRITIN IN A GROUP OF KALAFONG PATIENTS WITH IRON TRANSFER BLOCK COMPARED TO A GROUP OF KALAFONG PATIENTS WITH NO IRON TRANSFER BLOCK

In order to investigate the possibility that the H-subunit and L-subunit of ferritin may play a role in the development and maintenance of an iron transfer block the Kalafong patients were subdivided in a group of patients with iron transfer block and a group of patients with no iron transfer block.

8.1) Iron status of the iron transfer block subdivision of the Kalafong patients

For the next section the presence or absence of an iron transfer block was evaluated for all Kalafong patients irrespective of their immune status. In an attempt to identify the presence of an iron transfer block and to differentiate between an iron transfer block and true iron deficiency, bone marrow iron stains of the aspirate and the core, various serum iron markers and various red blood cell indices were assessed. This categorisation was discussed in the results chapter (volume 2, chapter 6) and will be summarised in the following section.

8.1.1) Body iron stores as evaluated by Prussian blue iron stains of the bone marrow aspirates and cores of the iron transfer block subdivision of the Kalafong patients

The iron stores for the group of Kalafong patients with no iron transfer block were normal for 7 of 23 (30%) of the patients, increased for 3 of 23 (13%) of the patients and for 13 of 23 (57%) of the patients in this group the iron stores were reduced. The iron stores for the group of Kalafong patients with iron transfer block were normal for 3 of 23 (13%) of the patients, increased for 16 of 23 (70%) of the patients and for 4 of 23 (17%) of the patients in this group the iron strores were reduced. Not all patients with an iron transfer block had increased iron stores. This can be ascribed to the concomitant existence of true iron deficiency.

8.1.2) Serum iron markers and determination of the iron status of the iron transfer block subdivision of the Kalafong patients

The following serum iron markers were evaluated; serum iron, transferrin, transferrin saturation, ferritin, soluble transferrin receptor, transferrin/log ferritin and soluble transferrin receptor/log ferritin.

Serum iron was

- not significantly different between the group of Kalafong patients with iron transfer block (9.1 ± 7.5 µmol/l; n = 25) and the group of Kalafong patients without iron transfer block (10.8 ± 9.6 µmol/l; n = 23).
- The laboratory values for normal serum iron are between 10-30 µmol/l.

The group of Kalafong patients without iron transfer block had low serum iron due to the inclusion of many patients with true iron deficiency.

Serum transferrin was

- significantly lower (p-value < 0.0001) in the group of Kalafong patients with iron transfer block (1.3 ± 0.42 g/l; n = 24) compared to the group of Kalafong patients without iron transfer block (2.3 ± 0.92 g/l; n = 23).
- The laboratory values for normal serum transferrin are between 2-3.6 g/l.

Transferrin is a negative acute phase protein and a decrease in transferrin is characteristic of the presence of iron transfer block.

Percentage transferrin saturation was

- not significantly different between the group of Kalafong patients with iron transfer block (27.7 ± 21.7%; n = 22) and the group of Kalafong patients without iron transfer block (32.3 ± 52.5%; n = 23).
- The laboratory values for normal percentage transferrin saturation are between 15-50% in females and between 20-50% in males.

The percentage transferrin saturation is determined not only by serum iron but also by serum transferrin levels. In the group of Kalafong patients with iron transfer block the percentage transferrin saturation was thus normal because of the low serum iron and low transferrin.

Serum ferritin was

- significantly higher (p-value < 0.0001) in the group of Kalafong patients with iron transfer block (2709.6 ± 4210.9 µg/l; n = 25) compared to the group of Kalafong patients without iron transfer block (2113.8 ± 9465 µg/l; n = 23).
- The laboratory values for normal serum ferritin are between 11-306.8 µg/l in females and between 23.9-336.2 µg/l in males.

Serum ferritin is an acute phase protein and is elevated in the presence of an inflammatory reaction. The high mean for serum ferritin in the group of Kalafong patients with no iron transfer block was as a result of a patient included in this group with a serum ferritin value of more than 45500 µg/l.

Transferrin/log ferritin ratio was

- significantly lower (p-value = 0.002) in the group of Kalafong patients with iron transfer block (0.48 ± 0.28; n = 24) compared to the group of Kalafong patients without iron transfer block (2 ± 1.9; n = 23).

A decrease in the transferrin/log ferritin ratio is characteristic of an iron transfer block.

The soluble transferrin receptor was

- significantly lower (p-value < 0.0001) in the group of Kalafong patients with iron transfer block (7.1 ± 4.4 µg/ml; n = 25) compared to the group of Kalafong patients without iron transfer block (18.5 ± 12.1 µg/ml; n = 23).
- The ELISA kit's values for normal soluble transferrin receptor are between 2.9-8.3 µg/ml.

The soluble transferrin receptor is normal or only slightly increased in the presence of an iron transfer block but with true iron deficiency it is markely elevated (83). In the group of Kalafong patients without the iron transfer block a pronounced elevation of the soluble transferrin receptor was shown indicating the presence of true iron deficiency. This underlines the fact that many of the Kalafong patients without iron transfer block did not have normal iron status, but true iron deficiency.

The soluble transferrin receptor/log ferritin ratio was

- significantly lower (p-value = 0.011) in the group of Kalafong patients with iron transfer block (2.6 ± 1.9; n = 25) compared to the group of Kalafong patients without iron transfer block (16.5 ± 23.9; n = 23).

A decrease in the soluble transferrin receptor/log ferritin ratio is characteristic of an iron transfer block, whereas the tremendously increased soluble transferrin receptor/log ferritin ratio is characteristic for true iron deficiency. This once again indicated the

presence of true iron deficiency in many of the Kalafong patients without iron transfer block.

8.1.3) Red blood cell indices of the iron transfer block subdivision of the Kalafong patients

The evaluation of various red blood cell indices, influenced negatively by an iron transfer block, also contributes to the evaluation of a patient's iron status.

The red blood cell count was

- not significantly different between the group of Kalafong patients with iron transfer block (2.5 ± 0.95 x 10^{12}/l; n = 25) and the group of Kalafong patients without iron transfer block (2.9 ± 1.4 x 10^{12}/l; n = 23).
- The red blood cell counts were low compared to the normal ranges of 4.13-5.67 x 10^{12}/l for females and 4.89-6.11 x 10^{12}/l for males.

The low red blood cell counts was as a result of not only functional iron deficiency, with or without the presence of an iron transfer block, but was also the result of many other pathologies found in the Kalafong patients.

Haemoglobin was

- not significantly different between the group of Kalafong patients with iron transfer block (7.3 ± 3.2 g/dl; n = 25) and the group of Kalafong patients without iron transfer block (7.5 ± 4.1 g/dl; n = 23).
- The laboratory values for normal haemoglobin are between 12.1-16.3 g/dl for females and between 14.3-18.3 g/dl for males.

Haematocrit was

- not significantly different between the group of Kalafong patients with iron transfer block (0.22 ± 0.09 l/l; n = 25) and the group of Kalafong patients without iron transfer block (0.24 ± 0.13 l/l; n = 23).
- The laboratory values for normal haematocrit are between 0.370-0.490 l/l for females and between 0.430-0.550 l/l for males.

The mean corpuscular volume was

- not significantly different between the group of Kalafong patients with iron transfer block (89.8 ± 13 fl; n = 25) and the group of Kalafong patients without iron transfer block (83.9 ± 18.6 fl; n = 23).
- The laboratory values for normal mean corpuscular volume are between 79.1-98.9 fl.

The mean corpuscular volume for the group of Kalafong patients was normal when compared to the normal laboratory values. It is known that the anaemia of chronic disease is mostly normocytic and normochromic (73, 87).

The mean corpuscular haemoglobin was

- not significantly different between the group of Kalafong patients with iron transfer block (29.1 ± 5 pg; n = 25) and the group of Kalafong patients without iron transfer block (26.2 ± 7.7 pg; n = 23).
- The laboratory values for normal mean corpuscular haemoglobin are between 27-32 pg.

The mean corpuscular haemoglobin was normal for the group of Kalafong patients with iron transfer block, but below normal for the group of Kalafong patients without iron transfer block. Many patients with true iron deficiency were included in the group of Kalafong patients with no iron transfer block. The anaemia of true iron deficiency is mostly a microcytic, hypochromic anaemia with the anaemia of chronic disease mostly a normocytic, normochromic anaemia.

The mean corpuscular haemoglobin concentration was

- significantly higher (p-value = 0.024) in the group of Kalafong patients with iron transfer block (32.4 ± 1.9 g/dl; n = 25) compared to the group of Kalafong patients without iron transfer block (30.8 ± 2.7 g/dl; n = 23).
- The normal ranges for the mean corpuscular haemoglobin concentration are between 32-36 g/dl.

Similar to the mean corpuscular haemoglobin the mean corpuscular haemoglobin concentration was low-normal for the group of Kalafong patients with iron transfer block and below normal for the group of Kalafong patients with no iron transfer block.

The red blood cell distribution width was

- not significantly different between the group of Kalafong patients with iron transfer block (19.7 ± 5.5%; n = 24) and the group of Kalafong patients without iron transfer block (22.3 ± 7.5%; n = 23).
- The laboratory values for normal red blood cell distribution width are between 11.6-14 %.

The red blood cell distribution width was above normal for both the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block. An increase in the red blood cell distribution width occurs for both anaemia of chronic disease and for the anaemia of true iron deficiency.

The RPI was

- not significantly different between the group of Kalafong patients with iron transfer block (0.41 ± 0.41; n = 10) and the group of Kalafong patients without iron transfer block (0.84 ± 0.89; n = 12).

The reticulocyte production index was below 2.5 for both the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block. A reticulocyte production index of below 2.5 indicates a suppressed bone marrow response.

In this section the Prussian blue iron stains of the bone marrow aspirates and cores, the serum iron markers and the red blood cell indices were used to subdivide the Kalafong patients into a group of patients with iron transfer block and a group of patients without iron transfer block. This was done irrespective of their immune status. This subdivision will be used to investigate the expression of the H-subunit and L-subunit of ferritin in bone marrow macrophages and the cells of the erythron with an iron transfer block.

8.2) Loss of the relationship between storage iron, bio-available iron and red blood cell production in the group of Kalafong patients with an iron transfer block

In the group of Kalafong patients with no iron transfer block the role that iron play in red blood cell production was clearly shown.

An increase in serum iron correlated with

- an increase in mean corpuscular volume (r = 0.57, p-value = 0.004),

- an increase in mean corpuscular haemoglobin (r = 0.59, p-value = 0.003) and
- an increase in mean corpuscular haemoglobin concentration (r = 0.50, p-value = 0.016).

An increase in transferrin correlated with

- a decrease in mean corpuscular volume (r = 0.58, p-value = 0.004),
- a decrease in mean corpuscular haemoglobin (r = 0.69, p-value = 0.0003) and
- a decrease in mean corpuscular haemoglobin concentration (r = 0.82, p-value < 0.0001).

An increase in soluble transferrin receptor correlated with

- an increase in haemoglobin (r = 0.71, p-value = 0.0001),
- an increase in haematocrit (r = 0.66, p-value = 0.0006),
- an increase in mean corpuscular volume (r = 0.46, p-value = 0.027),
- an increase in mean corpuscular haemoglobin (r = 0.53, p-value = 0.01) and
- an increase in mean corpuscular haemoglobin concentration (r = 0.64, p-value = 0.001).

A decrease in transferrin/log ferritin ratio correlated with

- an increase in mean corpuscular haemoglobin concentration (r = 0.69, p-value = 0.0003).

A decrease in the red blood cell distribution width correlated with

- an increase in haemoglobin (r = 0.70, p-value = 0.0002) and
- an increase in haematocrit (r = 0.68, p-value = 0.0003).

In the group of Kalafong patients with an iron transfer block no direct role was indicated for iron in red blood cell production as was shown for the group of Kalafong patients with no iron transfer block. The results showed that with an iron transfer block the iron status is only one of the factors that contribute to the decrease in red blood cell production. The other factors that play a role include suppression of the proliferation of erythroid progenitor cells, decrease in the synthesis of erythropoietin, decrease in the sensitivity of erythroblasts to erythropoietin and a decrease in red blood cell life span (73). Iron status is therefore not the major determinant of proper red blood cell production in patients with an iron transfer block.

8.3) Expression of the H-subunit and L-subunit of ferritin in macrophages and cells of the erythron in the group of Kalafong patients with an iron transfer block compared to the group of Kalafong patients with no iron transfer block

In this study the expression of the H-subunit in the macrophages of the bone marrow in the group of Kalafong patients with an iron transfer block (99.5 ± 41 counts/μm^2; n = 22) was marginally higher (p-value = 0.06) than in the group of Kalafong patients without an iron transfer block (78.5 ± 30.8 counts/μm^2; n = 23). For the expression of the L-subunit in the macrophages there was no difference between the group of Kalafong patients with an iron transfer block (113.1 ± 39.4 counts/μm^2; n = 21) and the group of Kalafong patients without an iron transfer block (113.6 ± 40.3 counts/μm^2; n = 23). There was no significant difference for the H-subunit/L-subunit ratio in the macrophages between the group of Kalafong patients with an iron transfer block (1 ± 0.57; n = 21) and the group of Kalafong patients without an iron transfer block (0.76 ± 0.39; n =23).

There were no significant differences for the expression of the H-subunit of ferritin in the cells of the erythron between the group of Kalafong patients with an iron transfer block (141.5 ± 50.9 counts/μm^2; n = 22) and the group of Kalafong patients without an iron transfer block (135.1 ± 57.1 counts/μm^2; n = 23). For the expression of the L-subunit of ferritin in the cells of the erythron there was no significant difference between the group of Kalafong patients with iron transfer block (211.5 ± 59.6 counts/μm^2; n = 21) and the group of Kalafong patients without an iron transfer block (217.5 ± 76.1 counts/μm^2; n = 23). There was no significant difference for the H-subunit/L-subunit ratio in the cells of the erythron between the group of Kalafong patients with iron

transfer block (0.74 ± 0.44; n = 21) and the group of Kalafong patients without an iron transfer block (0.68 ± 0.36; n = 23).

The increase in only the H-subunit of ferritin in the macrophage in the group of Kalafong patients with iron transfer block was similar to the group of Kalafong patients with elevated C-reactive protein and to the group of Kalafong patients with elevated neopterin. Both the group of Kalafong patients with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin showed a pro-inflammatory, T-helper cell type-1 response. The immune status for the the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block is discussed in a following section.

8.4) Relationship between the H-subunit/L-subunit ratio in cells of the erythron and the mean corpuscular volume and the mean corpuscular haemoglobin in the group of Kalafong patients with an iron transfer block

In the group of Kalafong patients with an iron transfer block an increase in the H-subunit/L-subunit ratio in cells of the erythron correlated with a decrease in mean corpuscular volume ($r = 0.55$, p-value = 0.008) and a decrease in mean corpuscular haemoglobin ($r = 0.42$, p-value = 0.049). This suggests that an increase in H-subunit rich ferritins in the cells of the erythron might play a role in the amount of iron available for haemoglobin synthesis.

Iron taken up by the red blood cell precursors either as ferritin with rhopheocytosis or as transferrin via the transferrin receptor pathway is incorporated into ferritin in the red blood cell precursors before this iron is used for haemoglobin production (9, 104). In the present study it was shown that with an increase in H-subunit rich ferritins in red

blood cell precursors less haemoglobin was produced. Therefore, it could be said that these H-subunit rich ferritins withholds iron from haemoglobin production.

8.5) Relationship between the L-subunit of ferritin in cells of the erythron and the mean corpuscular volume and the mean corpuscular haemoglobin in the group of Kalafong patients with an iron transfer block

In the group of Kalafong patients with an iron transfer block a positive correlation was shown between the L-subunit of ferritin in the cells of the erythron and the mean corpuscular volume ($r = 0.53$, p-value = 0.014) and the mean corpuscular haemoglobin ($r = 0.53$, p-value = 0.013). This correlation might reflect an increase in iron reaching the cells of the erythron. It is known that the expression of the L-subunit of ferritin is closely related to the intracellular labile iron pool. Translation of the L-subunit mRNA is increased by binding of iron to the iron responsive protein resulting in an unoccupied iron responsive element site on the L-subunit mRNA which is then available for binding of the translation complex (58). This results in an increase in the expression of the L-subunit of ferritin with an increase in the labile iron pool.

8.6) Relationship between the soluble transferrin receptor and the red blood cell distribution width in the group of Kalafong patients with an iron transfer block

Different factors play a role in the anaemia of chronic disease including an iron transfer block (73). That the iron transfer block could be a contributing factor in the anaemia associated with chronic inflammation was shown for the group of Kalafong patients with an iron transfer block. A correlation was shown between an increase in soluble transferrin receptor with an increase in the red blood cell distribution width ($r = 0.79$, p-value < 0.0001) for the group of Kalafong patients with an iron transfer block. An increase in the soluble transferrin receptor is known to occur with a reduction in the

amount of iron reaching the red blood cell precursors (83). The resulting increase in the red blood cell distribution width occurs as a result of differences in the amount of haemoglobin in the red blood cells and the subsequent variation in the red blood cell's volume (88).

8.7) Cytokine levels of the iron transfer block subdivision of the Kalafong patients

When the pro-inflammatory immune status as reflected by C-reactive protein and neopterin was compared between the group of Kalafong patients with iron transfer block and the group of Kalafong patients without iron transfer block it was found that the group of Kalafong patients with iron transfer block showed a significantly higher level of pro-inflammatory activity. C-reactive protein was significantly higher (p-value = 0.0004) in the group of Kalafong patients with an iron transfer block (84.1 ± 83.2 mg/l; n = 25) compared to the group of Kalafong patients without an iron transfer block (14.5 ± 22.3 mg/l; n = 23). Neopterin was also significantly higher (p-value = 0.0004) in the group of Kalafong patients with an iron transfer block (31.7 ± 24.5 ng/ml; n = 25) compared to the group of Kalafong patients without an iron transfer block (9.2 ± 14.5 ng/ml; n = 23). Furthermore, it was found that an increase in neopterin correlated with a decrease in transferrin (r = 0.47, p-value = 0.019). Therefore with an increase in cellular immune activation (T-helper cell type-1 response) a decrease in transferrin was shown. A decrease in transferrin is characteristic of iron transfer block.

This pronounced pro-inflammatory immune status indicated by the significantly elevated C-reactive protein and neopterin levels for the group of Kalafong patients with iron transfer block was reflected in the cytokine profile of these patients. Cytokines have previously been shown to play a significant role in the establishment of an iron transfer block (56, 74, 75, 76, 77). Various cytokines are known to contribute to the

establishment and maintenance of an iron transfer block whereas some cytokines are known to play a major part in resolving the iron transfer block (78). Cytokines make use of different intracellular pathways but also overlapping pathways resulting in cross-talk between different cytokines where specific cytokines can augment or down-regulate the effects of other cytokines (34). Therefore, the establishment and the maintenance of an iron transfer block depend not only on the type of cytokines but also on the combination of these cytokines. In the group of Kalafong patients with an iron transfer block compared to the group of Kalafong patients with no iron transfer block the pro-inflammatory cytokines that were significantly higher included INF-γ, Il-6, Il-8 and Il-2. No significant increases were shown for Il-12 and GM-CSF in the group of Kalafong patients with iron transfer block compared to the group of Kalafong patients with no iron transfer block.

INF-γ was

- significantly higher (p-value = 0.0007) in the group of Kalafong patients with iron transfer block (14.8 ± 33.7 pg/ml; n = 24) compared to the group of Kalafong patients with no iron transfer block (16.5 ± 76.2 pg/ml; n = 23),
- significantly higher (p-value = 0.043) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (0.1 ± 0 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

Il-6 was

- significantly higher (p-value = 0.002) in the group of Kalafong patients with iron transfer block (525.4 ± 1782.4 pg/ml; n = 25) compared to the group of Kalafong patients with no iron transfer block (453.1 ± 2025.5 pg/ml; n = 23),
- significantly higher (p-value = 0.0005) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (5 ± 2.1 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

Il-8 was

- significantly higher (p-value = 0.0001) in the group of Kalafong patients with iron transfer block (321.1 ± 893.6 pg/ml; n = 25) compared to the group of Kalafong patients with no iron transfer block (89.4 ± 246.5 pg/ml; n = 23),
- significantly higher (p-value = 0.0007) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (18.9 ± 6.4; n = 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

Il-2 was

- not significantly different between the group of Kalafong patients with iron transfer block (11 ± 6.9 pg/ml; n = 25) and the group of Kalafong patients with no iron transfer block (9.6 ± 7.3 pg/ml; n = 23),
- significantly higher (p-value = 0.047) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (6.7 ± 4.9 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

Il-12 was

- not significantly different between the group of Kalafong patients with iron transfer block (4 ± 3.4 pg/ml; n = 25) and the group of Kalafong patients with no iron transfer block (5.4 ± 4.3 pg/ml; n = 23),
- significantly higher (p-value = 0.0009) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (1.1 ± 1.3 pg/ml; n = 10) and
- significantly higher (p-value = 0.0001) in the group of Kalafong patients with no iron transfer block compared to the osteoarthritis patients.

GM-CSF was

- not significantly different between the group of Kalafong patients with iron transfer block (15.2 ± 48.6 pg/ml; n = 24) and the group of Kalafong patients with no iron transfer block (10.9 ± 17.4 pg/ml; n = 23),
- not significantly different between the group group of Kalafong patients with iron transfer block and the osteoarthritis patients (3.6 ± 6.2; n = 10) and
- marginally higher (p-value = 0.085) in the group of Kalafong patients with no iron transfer block compared to the osteoarthritis patients.

These pro-inflammatory cytokines could all contribute to the establishment and maintenance, either directly or indirectly, of the iron transfer block. The role of Il-6 in the establishment and maintenance of an iron transfer block is complicated. Il-6 is generally seen as a pro-inflammatory cytokine although Il-6 does not bring about NO production and can bring about an anti-inflammatory macrophage phenotype (40). Il-6 utilises the JAK/Stat signalling pathway involving the Janus family of tyrosine kinases (JAK kinases). Stimulation of this pathway by Il-6, similar to Il-1β, results in activation of the Stat3 transcription factor (105). Stat3 results in transcription of genes of inhibitory pathways, which brings about an anti-inflammatory macrophage phenotype (106). However, Il-6 possibly plays a role in the establishment of the iron transfer block by the stimulation of the secretion of hepcidin by hepatocytes (79). A major role for hepcidin has been proposed in causing an iron transfer block during an inflammatory reaction (79). Hepcidin results in an increase in iron retainment in macrophages and enterocytes

by decreasing the availability of the transmembrane protein, ferroportin, on the cellular membranes. Ferroportin is responsible for the efflux of iron from macrophages and enterocytes and thus when ferroportin is not available on the cell membrane less iron is released into the circulation. In the macrophage this will significantly contribute to the increased amounts of storage iron during an inflammatory reaction (79).

The pro-inflammatory cytokine, TNF-α, was not significantly different between the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block, but marginally higher in the group of Kalafong patients with iron transfer block when compared to the osteoarthritis patients. TNF-α is able to mediate macrophage activation along one of two pathways. One pathway plays a role in wound repair and is characterised by the induction of insulin-like growth factor. The second pathway is involved in macrophage cytocidal activation and is characterised by the induction of the inducible form of nitric oxide synthase (iNOS). It is this second pathway, which would predominate during a pro-inflammatory immune reaction with the production of iNOS. However, TNF-α only activates this pathway in the presence of INF-γ whereas the first pathway can be activated by TNF-α without the presence of INF-γ (107). Therefore, TNF-α is perhaps redundant for the establishment and maintenance of an iron transfer block since INF-γ is the most potent activator of iNOS (40).

TNF-α was

- not significantly different between the group of Kalafong patients with iron transfer block (3.8 ± 2.9 pg/ml; n = 25) and the group of Kalafong patients with no iron transfer block (3.2 ± 1.9 pg/ml; n = 23),
- marginally higher (p-value = 0.071) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (2.5 ± 0.96 pg/ml; n = 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

The cytokine, Il-1β, was not significantly different between any of the groups. Il-1β is also generally known as a pro-inflammatory cytokine. However, Il-1β has been shown to bring about an anti-inflammatory macrophage phenotype. Il-1β utilises the JAK/Stat signalling pathway involving the Janus family of tyrosine kinases (JAK kinases). Stimulation of this pathway by Il-1β, similar to Il-6, results in activation of the Stat3 transcription factor (105). Stat3 results in transcription of genes of inhibitory pathways, which brings about an anti-inflammatory macrophage phenotype (40, 106). Therefore, it is very likely that Il-1β did not contribute to the establishment and maintenance of an iron transfer block in the Kalafong patients.

Il-1β was

- not significantly different between the group of Kalafong patients with iron transfer block (5.5 ± 10.3 pg/ml; n = 25), the group of Kalafong patients without iron transfer block (3.4 ± 6.1 pg/ml; n = 23) and the osteoarthritis patients (1.6 ± 3.3 pg/ml; n = 10).

The cytokine, Il-5, was the only T-helper cell type-2 cytokine that was significantly higher in the group of Kalafong patients with an iron transfer block compared to the group of Kalafong patients with no iron transfer block. Il-5 is, in essence, a T-helper cell type-2 cytokine, but belongs to the GM-CSF/Il-3 subgroup (89). In contrast to GM-CSF and Il-3 no effect on monocytes for Il-5 has been shown (90). However, it has been shown that Il-5, as for GM-CSF and Il-3, displayed proliferative effects on both microglia and macrophage cell lines (91). Furthermore, both Il-5 and Il-3 has been shown to bind and activate the GM-CSF receptor present on monocytes (44). It is therefore suggested that Il-5 could have contributed to the establishment of the iron transfer block.

Il-5 was

- significantly higher (p-value = 0.045) in the group of Kalafong patients with iron transfer block (4.2 ± 4.3 pg/ml; n = 25) compared to the group of Kalafong patients with no iron transfer block (2.3 ± 1.3 pg/ml; n = 23),
- marginally higher (p-value = 0.07) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (2.4 ± 1.6 pg/ml; n= 10) and
- not significantly different between the group of Kalafong patients with no iron transfer block and the osteoarthritis patients.

The T-helper cell type-2 cytokine, Il-10, was not significantly different between the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block, but was significantly higher for both these groups compared to the osteoarthritis group. The anti-inflammatory cytokine, Il-10, results in decreased production of pro-inflammatory cytokines (transcriptional repression perhaps through the up-regulation of the formation of p50 homodimers of NF-κB and/or the expression of Bcl-3, which preferentially recruits p50 homodimers of NF-κB – negative effect on pro-inflammatory gene transcription) into the nucleus (41). Therefore, the role that Il-10 plays in the establishment and maintenance of the iron transfer block as an anti-inflammatory cytokine is perhaps not clear. However, in previous studies it has been shown that Il-10 can up-regulate the transferrin receptor on the cell membrane of the macrophage resulting in an increase in transferrin receptor-mediated uptake of iron (73, 78). Furthermore, has it been shown that Il-10 contributes to macrophage haemoglobin acquisition by stimulating the expression of the haemoglobin scavenger receptor, CD163 (78).

Il-10 was

- not significantly different between the group of Kalafong patients with iron transfer block (15.2 ± 19.9 pg/ml; n = 25) and the group of Kalafong patients with no iron transfer block (23.5 ± 68 pg/ml; n = 23),
- significantly higher (p-value = 0.005) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (2.7 ± 1.5 pg/ml; n = 10) and
- significantly higher (p-value = 0.014) in the group of Kalafong patients with no iron transfer block compared to the osteoarthritis patients.

The anti-inflammatory cytokine Il-4 is able to markedly suppress transcriptional activation of INF-γ responsive genes and the promoter sequences required for both INF-γ and Il-4 sensitivity are identical. INF-γ activates Stat1 and Il-4 activates Stat6, where Stat1 promotes transcription and Stat6 is inactive. Therefore, Il-4 appears to suppress INF-γ-inducible pro-inflammatory gene expression through the ability of Stat6 to compete with Stat1 for occupancy of promoter sites necessary for INF-γ-induced

transcriptional initiation (108). In the present study, Il-4 was neither higher in the group of Kalafong patients with iron transfer block, nor in the group of Kalafong patients with no iron transfer block compared to the osteoarthritis patients.

Il-4 was

- not significantly different between the group of Kalafong patients with iron transfer block (2.7 ± 1.9 pg/ml; n = 25), the group of Kalafong patients with no iron transfer block (2.2 ± 2 pg/ml; n = 23) and the osteoarthritis patients (2.5 ± 2.2 pg/ml; n = 10).

There was no significant difference for TGF-β between the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block. However, TGF-β was significantly lower for both these groups when compared to the osteoarthritis patients. This significant increase of TGF-β in the osteoarthritis patients was discussed in a previous section.

TGF-β was

- not significantly different between the group of Kalafong patients with iron transfer block (10.4 ± 5.6 ng/ml; n = 24) and the group of Kalafong patients with no iron transfer block (12.8 ± 8.3 ng/ml; n = 23),
- significantly lower (p-value = 0.0001) in the group of Kalafong patients with iron transfer block compared to the osteoarthritis patients (18.3 ± 4.1 ng/ml; n = 10) and
- significantly lower (p-value = 0.016) in the group of Kalafong patients with no iron transfer block compared to the osteoarthritis patients.

The question now remains whether any relationship could be expected between specific cytokines and any of the serum iron markers or red blood cell indices. In the group of Kalafong patients with an iron transfer block no correlations was shown for any of the pro-inflammatory cytokines and serum iron markers or red blood cell indices. Although no direct role for any of the T-helper cell type-1 cytokines was shown, an increase in TGF-β (anti-inflammatory cytokine) correlated with an increase in transferrin (r = 0.48, p-value = 0.02). The opposite of what occurs with an iron transfer block.

8.8) Pro-hepcidin and caeruloplasmin levels for the iron transfer block subdivision

For pro-hepcidin there was no significant difference between the group of Kalafong patients with iron transfer block (194 ± 281 ng/ml; n = 25) and the group of Kalafong patients without iron transfer block (151.6 ± 44.7 ng/ml; n = 23). As was previously mentioned, pro-hepcidin levels not necessarily reflect the hepcidin levels. The pro-hepcidin/hepcidin relationship can only be established when a suitable commercial ELISA kit is available.

There was no significant difference for caeruloplasmin between the group of Kalafong patients with iron transfer block (0.44 ± 0.16 g/l; n = 23) and the group of Kalafong patients without iron transfer block (0.49 ± 0.14 g/l; n = 23).

8.9) In summary on the expression of the H-subunit and L-subunit of ferritin in the iron transfer block subdivision of the Kalafong patients

The expression of the H-subunit of ferritin in the bone marrow macrophage was marginally higher in the group of Kalafong patients with iron transfer block compared to the group of Kalafong patients with no iron transfer block. The expression of the L-subunit of ferritin in the bone marrow macrophage was not significantly different between the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block. Furthermore, the expression of the H-subunit and L-subunit of ferritin in the cells of the erythron was not significantly different between the group of Kalafong patients with iron transfer block and the group of Kalafong patients with no iron transfer block. These findings were similar to the C-reactive protein and neopterin subdivisions. The cytokine profile of the iron transfer block subdivision was similar to that of the neopterin subdivision – a pro-inflammatory

T-helper cell type-1 immune response, but for a significant increase in the T-helper cell type-2 cytokine, Il-5.

No correlation was shown between the expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and any of the serum iron markers or red blood cell indices in iron transfer block. However, correlations were shown between the expression of the H-subunit and L-subunit of ferritin in the cells of the erythron and red blood cell indices in iron transfer block. An increase in the H-subunit/L-subunit ratio in cells of the erythron was shown to correlate with a decrease in the mean corpuscular volume ($r = 0.67$, p-value = 0.0009) and a decrease in the mean corpuscular haemoglobin ($r = 0.56$, p-value = 0.008) in the group of Kalafong patients with an iron transfer block. An increase in the H-subunit/L-subunit ratio showed a possible increase in H-subunit rich ferritins. These H-subunit rich ferritins could have resulted in withholding of the iron from haemoglobin production in the cells of the erythron. Furthermore, an increase in the L-subunit of ferritin in the cells of the erythron was shown to correlate with an increase in the mean corpuscular volume ($r = 0.53$, p-value = 0.014) and an increase in the mean corpuscular haemoglobin ($r = 0.53$, p-value = 0.013) in the group of Kalafong patients with an iron transfer block. The expression of the L-subunit of ferritin has been shown to be closely related to the amount of intracellular iron. Therefore, the increase in the expression of the L-subunit of ferritin in the cells of the erythron could have indicated an increase in intracellular iron available for haemoglobin production.

9) INCREASE IN THE EXPRESSION OF THE H-SUBUNIT OF FERRITIN IN THE MACROPHAGES OF OSTEOARTHRITIS PATIENTS AND IMPLICATIONS

In the group of osteoarthritis patients the expression of the H-subunit of ferritin in the bone marrow macrophage was similarly increased as for the group of Kalafong patients

with elevated C-reactive protein and the group of Kalafong patients with elevated neopterin. This increase in the expression of the H-subunit of ferritin in the macrophage was found despite the osteoarthritis patients not showing any pronounced pro-inflammatory activity as reflected by their C-reactive protein and neopterin levels. Furthermore, no increases were found for any of the pro-inflammatory cytokines. The only cytokine that was significantly increased for the group of osteoarthritis patients was the anti-inflammatory cytokine TGF-β. In this section the possible role that an increase in the H-subunit of ferritin in the macrophage can play in iron metabolism and possibly iron transfer block in osteoarthritis is addressed.

9.1) Iron status of the osteoarthritis patients

In order to investigate the possible role of the H-subunit of ferritin in an iron transfer block the iron status of the osteoarthritis patients were determined by Prussian blue iron stains for bone marrow cores, serum iron markers and red blood cell indices.

9.1.1) Body iron stores as evaluated by Prussian blue iron stains of the bone marrow cores of the osteoarthritis patients

Prussian blue iron stains were done for only six of the ten osteoarthritis patients. Four of these osteoarthritis patients had reduced iron stores, one had absent iron stores and one had normal iron stores. The body iron stores for the osteoarthritis patients were markedly low.

9.1.2) Serum iron markers and determination of the iron status of the osteoarthritis patients

The results on the serum iron markers are given in small print followed by a summary on the serum iron markers in osteoarthritis patients.

- Serum iron was low-normal for the group of osteoarthritis patients (12.9 ± 5.9 µmol/l; n = 10) and not significantly different from the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.
- Serum transferrin was low-normal for the group of osteoarthritis patients (2 ± 0.57 g/l; n = 10) and not significantly different from the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.
- Percentage transferrin saturation was normal for the osteoarthritis patients (30.3 ± 14.4%; n = 10) and not significantly different from the group of Kalafong patients with normal C-reactive protein but significantly higher (p-value = 0.049) compared to the group of Kalafong patients with normal neopterin.
- Ferritin was normal for the osteoarthritis patients (94.2 ± 69.6 µg/l; n = 10) and not significantly different from the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin.
- The transferrin/log ferritin ratio was significantly lower (p-value = 0.044) in the osteoarthritis patients (1.2 ± 0.58; n = 10) compared to the group of Kalafong patients with normal C-reactive protein and significantly higher (p-value = 0.005) compared to the group of Kalafong patients with elevated C-reactive protein. When compared to the neopterin subdivision the transferrin/log ferritin ratio was significantly higher (p-value = 0.008) in the osteoarthritis patients compared to the group of Kalafong patients with elevated neopterin and not significantly different from the group of Kalafong patients with normal neopterin.
- The soluble transferrin receptor was significantly lower (p-value = 0.0002) in the osteoarthritis patients (3.3 ± 1.4 µg/ml; n = 10) compared to the group of Kalafong patients with normal C-reactive protein and significatly lower (p-value = 0.0001) compared to the group of Kalafong patients with elevated C-reactive protein. Compared to the neopterin subdivision the soluble transferrin receptor in the osteoarthritis patients was significantly lower (p-value = 0.004) compared to the group of Kalafong patients with normal neopterin and significantly lower (p-value < 0.0001) compared to the group of Kalafong patients with elevated neopterin.
- The soluble transferrin receptor/log ferritin ratio was significantly lower (p-value = 0.015) in the osteoarthritis patients (2 ± 1.3; n = 10) compared to the group of Kalafong patients with normal C-reactive protein and significantly lower (p-value = 0.04) compared to the group of Kalafong patients with elevated C-reactive protein. Compared to the neopterin subdivision the soluble transferrin receptor/log ferritin ratio in the osteoarthritis patients was significantly lower (p-value = 0.045) compared to the group of Kalafong patients with normal neopterin and significantly lower (p-value = 0.042) compared to the group of Kalafong patients with elevated neopterin.

In summary on the serum iron markers of the osteoarthritis patients

The osteoarthritis patients showed low/reduced body iron stores and low-normal serum iron. In this situation a marked increase in transferrin would be expected. However, the osteoarthritis patients showed low-normal transferrin. Furthermore, the osteoarthritis patients showed a transferrin/log ferritin ratio significantly lower than that in the group of Kalafong patients with normal C-reactive protein but still significantly higher than that in the group of Kalafong patients with elevated C-reactive protein. The transferrin/log

ferritin ratio was lower for the group of osteoarthritis patients when compared to the group of Kalafong patients with normal C-reactive protein. This decrease in the transferrin/log ferritin ratio is characteristic of iron transfer block. A marked decrease for soluble transferrin receptor was seen in the osteoarthritis patients. The soluble transferrin receptor was abnormally low in the face of low serum iron in the osteoarthritis patients. The soluble transferrin receptor was not only significantly lower in the osteoarthritis patients than in the groups of Kalafong patients with normal C-reactive protein and normal neopterin but was also significantly lower than in the group of Kalafong patients with elevated C-reactive protein and elevated neopterin. Such a reduced soluble transferrin receptor is expected in patients with an iron transfer block. In addition, the soluble transferrin receptor/log ferritin ratio in the osteoarthritis patients was not only significantly lower than in the groups of Kalafong patients with normal C-reactive protein and normal neopterin, but also significantly lower than in the groups of Kalafong patients with elevated C-reactive protein and elevated neopterin.

The group of osteoarthritis patients thus displayed some characteristics that could be expected for patients with a combination of true iron deficiency and iron transfer block. These factors were low-normal serum iron, reduced transferrin/log ferritin ratio, reduced soluble transferrin receptor and reduced soluble transferrin receptor/log ferritin ratio.

9.1.3) Red blood cell indices of the osteoarthritis patients

The results on the red blood cell indices are given in small print followed by a summary on the red blood cell indices in osteoarthritis patients.

- The red blood cell count was low-normal for the osteoarthritis patients (4.8 ± 0.67 x 10^{12}/l; n = 9) and significantly higher (p-value < 0.0001) compared to the group of Kalafong patients with normal C-reactive protein and marginally higher (p-value = 0.057) compared to the group of Kalafong patients with normal neopterin.

- The haemoglobin was normal in the osteoarthritis patients (14.9 ± 1.5 g/dl; n = 10) and significantly higher (p-value < 0.0001) compared to the group of Kalafong patients with normal C-reactive protein and significantly higher (p-value = 0.001) compared to the group of Kalafong patients with normal neopterin.
- The haematocrit was normal in the osteoarthritis patients (0.45 ± 0.05 l/l; n = 10) and significantly higher (p-value < 0.0001) compared to the group Kalafong patients with normal C-reactive protein and significantly higher (p-value = 0.003) compared to the group of Kalafong patients with normal neopterin.
- The mean corpuscular volume was normal in the osteoarthritis patients (94.2 ± 5.7 fl; n = 9) and significantly higher (p-value = 0.002) compared to the group Kalafong patients with normal C-reactive protein and marginally higher (p-value = 0.082) compared to the group of Kalafong patients with normal neopterin.
- The mean corpuscular haemoglobin was normal in the osteoarthritis patients (31.1 ± 2.2 pg; n = 9) and significantly higher (p-value = 0.0002) compared to the group of Kalafong patients with normal C-reactive protein and marginally higher (p-value 0.053) compared to the group of Kalafong patients with normal neopterin.
- The mean corpuscular haemoglobin concentration was normal in the osteoarthritis patients (33 ± 1.2 g/dl; n = 9) and significantly higher (p-value = 0.0003) compared to the group of Kalafong patients with normal C-reactive protein and significantly higher (p-value = 0.03) compared to group of the Kalafong patients with normal neopterin.
- The red blood cell distribution width was normal in the osteoarthritis patients (12.9 ± 0.64%; n = 9) and significantly lower (p-value = 0.0004) compared to the group of Kalafong patients with normal C-reactive protein and significantly lower (p-value = 0.002) compared the group of Kalafong patients with normal neopterin.

In summary on the red blood cell indices of the osteoarthritis patients

The red blood cell production was low-normal for the osteoarthritis patients. This was most probably as a result of the low body iron stores and serum iron. None of the red blood cell indices indicated that insufficient amounts of iron were reaching the erythron for haemoglobin production.

9.1.4) Iron status of the osteoarthritis patients

When the results of the Prussian blue iron stains of the bone marrow core, the serum iron markers and the red blood cell indices were evaluated for each individual osteoarthritis patient (volume 2, chapter 6) an iron transfer block was shown for some of the osteoarthritis patients. For 40% (four of ten patients) of the osteoarthritis patients an iron transfer block was shown and for 60% (six of ten patients) no iron transfer block was shown. The most pronounced changes shown for the osteoarthritis patients characteristic of an iron transfer block were low-normal serum iron, reduced

transferrin/log ferritin ratio, reduced soluble transferrin receptor and reduced soluble transferrin receptor/log ferritin ratio. In addition, a decrease in transferrin was related to an increase in the red blood cell distribution width (r = 0.67, p-value = 0.05). This is characteristic for an iron transfer block.

9.2) Relationship between storage iron, bio-available iron, expression of the H-subunit and L-subunit of ferritin and red blood cell production in the osteoarthritis patients

The soluble transferrin receptor/transferrin complex is responsible for the acquiring of iron for various cell types and especially so for the cells of the erythron (85). In the group of osteoarthritis patients the soluble transferrin receptor was closely related to the transferrin levels (r = 0.79, p-value = 0.006), negatively related to the percentage transferrin saturation (r = 0.74, p-value = 0.014) and negatively related to the ferritin levels (r = 0.75, p-value = 0.013). Therefore, with a decrease in body iron (decrease in ferritin, decrease in transferrin saturation and increase in transferrin) the amount of soluble transferrin receptors was probably increased in an attempt by the erythron to acquire more iron.

Hepcidin (formed from pro-hepcidin) decreases ferroportin availability in cell membranes. Ferroportin transports iron out of cells and is the major iron transmembrane channel for the movement of iron from the cytosol to the blood in enterocytes and macrophages. As body iron increases, hepcidin is induced, which then inhibits the efflux of iron through ferroportin down-regulation (109). This blocks intestinal iron absorption and iron recycling from erythrocytes by macrophages. In this study pro-hepcidin was closely related to the serum iron in the group of osteoarthritis patients (r = 0.67, p-value = 0.034).

The expression of the H-subunit of ferritin in the cells of the erythron was closely related to the serum iron (r = 0.80, p-value = 0.031). This might further be suggestive of the role that this subunit plays in rapid turnover of iron in the cells of the erythron. Due to the H-subunit rich ferritin's more dynamic ability of iron uptake and release it would appear to be largely found in cells having high iron requirements for metabolic activities and a non-existent role in iron storage (96).

The H-subunit/L-subunit ratio in the bone marrow macrophages was closely related to the serum iron level (r = 0.82, p-value = 0.047) in the osteoarthritis patients. Therefore, with an increase in serum iron and body iron the macrophages responded by increasing the expression of the H-subunits relative to that of the L-subunits. It is known that the H-subunit to L-subunit ratio of a specific type of cell does not remain constant and the proportion of the H-subunits and L-subunits present in the ferritin shell changes during differentiation and in various pathological states (110, 111, 112, 113).

9.3) Possible role for the anti-inflammatory cytokine, transforming growth factor-β, in resolving the iron transfer block in osteoarthritis patients

The osteoarthrtitis patients had a relatively normal cytokine profile similar to the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin. However, an increase in TGF-β was found for the osteoarthritis patients. TGF-β is an anti-inflammatory cytokine (41) and could have brought about an increase in soluble transferrin receptor and transferrin (both are decreased by pro-inflammatory cytokines). This was found in the osteoarthritis patients where an increase in TGF-β correlated with an increase in soluble transferrin receptor (r = 0.67, p-value = 0.034) and an increase in transferrin (r = 0.63, p-value = 0.05).

9.4) Cytokines and the expression of the H-subunit and L-subunit of ferritin in osteoarthritis patients

It is generally assumed that cytokines play a major role in the expression of the H-subunit of ferritin not only by the up-regulation of transcription but also by inducing secretion and ferritin aggregation (58, 59, 60). In the present study on the osteoarthritis patients some cytokines were shown to correlate with a decrease in the expression of the subunits of ferritin. An increase in Il-8 correlated with a decrease in the H-subunit of ferritin ($r = 0.81$, p-value = 0.026) and a decrease in the H-subunit/L-subunit ratio in the macrophage ($r = 0.84$, p-value = 0.037). An increase in Il-6 correlated with a decrease in the L-subunit of ferritin in the macrophage ($r = 0.85$, p-value = 0.034). This is consistent with the role that ferritin plays in inflammation with possibly first the up-regulation of specifically the H-subunit of ferritin followed by a decrease in the H-subunit and L-subunit as a result of an increase in haemosiderin formation or secretion of ferritin. An increase in Il-2 correlated with a decrease in the H-subunit of ferritin in the cells of the erythron ($r = 0.73$, p-value = 0.064). An increase in TNF-α ($r = 0.94$, p-value = 0.006) and an increase in Il-10 ($r = 0.85$, p-value = 0.03) correlated with a decrease in the L-subunit of ferritin in the cells of the erythron. This negative correlation between different cytokines and the decrease in the expression of the ferritin subunits might point to a role for cytokines in the possible decrease of the ferritin subunits in osteoarthritis patients.

9.5) Pro-hepcidin and caeruloplasmin levels for the osteoarthritis patients

Pro-hepcidin was not significantly different between the osteoarthritis patients (140.6 ± 30.2 ng/ml; $n = 10$) and any of the groups of Kalafong patients with elevated C-reactive protein, normal C-reactive protein, elevated neopterin and normal neopterin. However, in the group of osteoarthritis patients an increase in pro-hepcidin correlated with an increase in serum iron ($r = 0.67$, p-value = 0.034). This finding is similar to the results of

previous studies where it has been shown that the production of pro-hepcidin is regulated by serum iron (79). Caeruloplasmin was not significantly different between the osteoarthritis patients (0.45 ± 0.14 g/l; n = 10) and any of the groups of Kalafong patients with elevated C-reactive protein, normal C-reactive protein, elevated neopterin and normal neopterin.

9.6) In summary on the osteoarthritis patients

The pro-inflammatory indicators, C-reactive protein and neopterin, of the osteoarthritis patients were not significantly different from the groups of Kalafong patients with no overt pro-inflammatory activity. The cytokine profiles of the osteoarthritis patients were also similar to the groups of Kalafong patients with no overt pro-inflammatory activity except for the significant increase in TGF-β levels in the osteoarthritis patients. However, the osteoarthritis patients had some serum iron markers characteristic of an iron transfer block including low-normal serum iron, reduced transferrin/log ferritin ratio, reduced soluble transferrin receptor and reduced soluble transferrin receptor/log ferritin ratio. In addition, a decrease in transferrin was related to an increase in the red blood cell distribution width. However, an increase in TGF-β correlated with an increase in soluble transferrin receptor and an increase in transferrin, both opposite for what occurs with an iron transfer block.

The expression of the H-subunit of ferritin in the bone marrow macrophage in the osteoarthritis patients was showed to be increased, similar to the group of Kalafong patients with a pronounced increase in pro-inflammatory activity. However, it is thought that this increase in the expression of the H-subunit of ferritin in the macrophage was caused by the increase in TGF-β in the osteoarthritis patients. A very interesting finding for the group of osteoarthritis patients was the correlation of the decrease in the expression of the H-subunit and L-subunit of ferritin with an increase in some cytokines.

Cytokines has been shown, in previous studies, to not only induce the transcription of mostly the H-subunit of ferritin, but also to cause an increase in the formation of haemosiderin and the secretion of ferritin (58, 59, 60).

In this chapter the results were discussed in five main sections a) subdivision of the patients according to their immune status where the Kalafong patients were subdivided firstly based on C-reactive protein levels and secondly on neopterin levels, b) the expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with a pro-inflammatory immune status where the expression of the H-subunit and L-subunit of ferritin was investigated in the groups of Kalafong patients with high C-reactive protein and high neopterin, respectively, c) the prevalence of iron transfer block in patients with a pro-inflammatory immune status where the iron status of the Kalafong patients and the possible role that the H-subunit and L-subunit of ferritin in macrophages and cells of the erythron play in iron transfer block were investigated, d) the expression of the H-subunit and L-subunit of ferritin in a group of Kalafong patients with iron transfer block and a group of Kalafong patients with no iron transfer block where the possible role that the H-subunit and L-subunit of ferritin in macrophages and cells of the erythron play in the establishment and maintenance of iron transfer block were investigated and finally e) a section on the osteoarthritis patients where the expression of the H-subunit and L-subunit of ferritin in macrophages and cells of the erythron were investigated. The next and final chapter will present a summation of the results, conclusions and suggestions for further study.

10) References

1) Chasteen ND. Ferritin. Uptake, storage, and release of iron. Metal Ions in Biological Systems 1998; 35: 479-514.

2) Arosio P, Levi S, Santambrogio P, Cozzi A, Luzzago A, Cesareni G *et al.* Structural and functional studies of human ferritin H and L chains. Current Studies in Hematology and Blood Transfusion 1991; 58: 127-131.

3) Chiancone E, Stefanini S. Heterogeneity of ferritin. I. Structural and functional aspects. In: Albertini A, Arosio P, Chiancone E, Drysdale J editors. Ferritins and isoferritins as biochemical markers. Amsterdam: Elsevier Science Publishers; 1984. p. 23-32.

4) Harrison PM, Arosio P. The ferritins: molecular properties, iron storage function and cellular regulation. Biochimica *et* Biophysica Acta 1996; 1275: 161-203.

5) Rogers J, Lacroix L, Durmowitz G, Kasschau K, Andriotakis J, Bridges KR. The role of cytokines in the regulation of ferritin expression. Advances in Experimental Medicine and Biology 1994; 356: 127-132.

6) Wixom RL, Prutkin L, Munro HN. Hemosiderin: Nature, Formation, and Significance. International Reviews in Experimental Pathology 1980; 22: 193-225.

7) Surguladze N, Patton S, Cozzi A, Fried MG, Connor JR. Characterization of nuclear ferritin and mechanism of translocation. Biochemical Journal 2005; 388: 731-740.

8) Levi S, Corsi B, Bosisio M, Invernizzi R, Volz A, Sanford D *et al.* A human mitochondrial ferritin encoded by an intronless gene. Journal of Biological Chemistry 2001; 270(27): 24437-24440.

9) Meyron-Holtz EG, Fibach E, Gelvan D, Konijn AM. Binding and uptake of exogenous isoferritins by cultured human erythroid precursor cells. British Journal of Haematology 1994; 86: 635-641.

10) Broxmeyer HE, Gentile P, Listowsky I, Cavanna F, Feickert HJ, Dorner MH *et al.* Acidic isoferritins in the regulation of hematopoiesis in vitro and in vivo. In: Albertini A, Arosio P, Chiancone E, Drysdale J editors. Ferritins and isoferritins as biochemical markers. Amsterdam: Elsevier Science Publishers; 1984. p. 97-111.

11) Torti SV, Torti FM. Iron and ferritin in inflammation and cancer. Advances in Inorganic Biochemistry 1994; 10: 119-137.

12) Tsuji Y, Ayaki H, Whitman SP, Morrow CS, Torti SV, Torti FM. Coordinate transcriptional and translational regulation of ferritin in response to oxidative stress. Molecular and Cellular Biology 2000; 20(16): 5818-5827.

13) Kakhlon O, Gruenbaum Y, Cabantchik ZI. Repression of ferritin expression increase the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. Blood 2001; 97(9): 2853-2871.

14) Bevilacqua MA, Costanzo F, Buonaguro L, Cimino F. Ferritin H and L mRNAs in human neoplastic tissues. Italian Journal of Biochemistry 1988; 37(1): 1-7.

15) Connor JR, Snyder BS, Arosio P, Loeffler DA, LeWitt P. A quantitative analysis of isoferritins in select regions of aged, parkinsonian, and alzheimer's diseased brains. Journal of Neurochemistry 1995; 65: 717-724.

16) Higgy NA, Salicioni AM, Russo IH, Zhang PL, Russo J. Differential expression of human ferritin H chain gene in immortal human breast epithelial MCF-10F cells. Molecular Carcinogenesis 1997; 20(4): 332-339.

17) Jones BM, Worwood M, Jacobs A. Serum ferritin in patients with cancer: determination with antibodies to HeLa cell and spleen ferritin. Clinica Chimica Acta 1980; 106: 203-214.

18) Lin F, Girotti AW. Elevated ferritin production, iron containment, and oxidant resistance in hemin-treated leukaemia cells. Archives of Biochemistry and Biophysics 1997; 346(1): 131-141.

19) Miller LL, Miller SC, Torti SV, Tsuji Y, Torti FM. Iron-independent induction of ferritin H chain by tumor necrosis factor. Proceedings of the National Academy of Sciences of the United States of America 1991; 88(11): 4946-4950.

20) Tripathi PK, Chatterjee SK. Elevated expression of ferritin H-chain mRNA in metastatic ovarian tumor. Cancer Investigation 1996; 14(6): 518-526.

21) Vernet M, Renversez JC, Lasne Y, Revenant MC, Charlier-de-Bressing C, Guillemin C *et al.* Comparison of six serum ferritin immunoassays and isoferritin spectrotypes in malignancies. Annales de Biologie Clinique 1995; 53: 419-427.

22) Whittaker D, Torrance JD, Kew MC. Isolation of ferritin from human hepatocellular carcinoma. Scandinavian Journal of Haematology 1984; 33(5): 432-439.

23) Rosalki SB. C-reactive protein. International Journal of Clinical Practice 2001; 55(4): 269-270.

24) Volanakis JE. Human C-reactive protein: expression, structure and function. Molecular Immunology 2001; 38(2-3): 189-197.

25) Black S, Kushner I, Samols D. C-reactive protein. Journal of Biological Chemistry 2004; 279(47): 48487-48490.

26) Marnell L, Mold C, Du Clos TW. C-reactive protein: ligands, receptors and role in inflammation. Clinical Immunology 2005; 117(2): 104-111.

27) Murr C, Widner B, Wirleitner B, Fuchs D. Neopterin as a marker for immune system activation. Current Drug Metabolism 2002; 3:175-187.

28) Huber C, Batchelor JR, Fuchs D, Hausen A, Lang A, Niederwieser D *et al.* Immune response-associated production of neopterin. Journal of Experimental Medicine 1984; 160: 310-316.

29) Sghiri R, Feinberg J, Thabet F, Dellagi K, Boukadida J, Casanova AB *et al.* Gamma interferon is dispensable for neopterin production. Clinical and Diagnostic Laboratory Immunology 2005; 12(12): 1437-1441.

30) Sheldon J, Riches PG, Soni N, Jurges E, Gore M, Dadian G *et al.* Plasma neopterin as an adjunct to C-reactive protein in assessment of infection. Clinical Chemistry 1991; 37(12): 2038-2042.

31) Mosser DM. The many faces of macrophage activation. Journal of Leukocyte Biology 2003; 73: 209-212.

32) McGrath MS, Kodelja V. Balanced macrophage activation hypothesis: a biological model for development of drugs targeted at macrophage functional states. Pathobiology 1999; 67: 277-281.

33) Correll PH, Morrison AC, Lutz MA. Receptor tyrosine kinases and the regulation of macrophage activation. Journal of Leukocyte Biology 2004; 75: 731-737.

34) Ohmori Y, Hamilton TA. Regulation of macrophage gene expression by T-cell derived lymphokines. Pharmaco Therapy 1994; 63: 235-264.

35) Montavani A, Sica A, Locati M. New vistas on macrophage differentiation and activation. European Journal of Immunology 2007; 37: 14-16.

36) Duffield JS. The inflammatory macrophage: a story of Jekyll and Hyde. Clinical Science 2003; 104: 27-38.

37) Keshav S, Chung L-P, Gordon S. Macrophage products in inflammation. Diagnostic Microbiology and Infectious Diseases 1990; 13: 439-447.

38) Bogdan C, Nathan C. Modulation of macrophage function by transforming growth factor-β, interleukin-4, and interleukin-10. Annals of the New York Academy of Sciences 1993; 685: 713-739.

39) Stoy N. Macrophage biology and pathobiology in the evolution of immune responses: a functional analysis. Pathobiology 2001; 69: 179-211.

40) Mills CD. Macrophage arginine metabolism to ornithine/urea or nitric oxide/citrulline: a life or death issue. Critical Reviews in Immunology 2001; 21(4): 399-426.

41) Butchar JP, Parsa KVL, Marsh CB, Tridandapani S. Negative regulators of Toll-like receptor 4-mediated macrophage inflammatory response. Current Pharmaceutical Design 2006; 12: 4143-4153.

42) Ashcroft GS. Bidirectional regulation of macrophage function by TGF-β. Microbes and Infection 1999; 1: 1275-1282.

43) Wahl SM, McCartney-Francis N, Allen JB, Dougherty EB, Dougherty SF. Macrophage production of TGF-β and regulation by TGF-β. Annals of the New York Academy of Sciences 1990; 593: 188-196.

44) Woodcock JM, Bagley CJ, Lopez AF. The functional basis of granulocyte-macrophage colony stimulating factor, interleukin-3 and interleukin-5 receptor activation, basic and clinical implications. International Journal of Biochemistry and Cell Biology 1999; 31: 1017-1025.

45) Fleetwood AJ, Cook AD, Hamilton JA. Functions of granulocyte-macrophage colony-stimulating factor. Critical Reviews in Immunology 2005; 25(5): 405-428.

46) Blom AB, van der Kraan PM, van den Berg WB. Cytokine targeting in osteoarthritis. Current Drug Targets 2007; 8: 283-292.

47) Leheita O, Abed Elrazek NY, Younes S, Mahmoud AZ. Lymphocyte subsets in osteoarthritis versus rheumatoid arthritis. Egyptian Journal of Immunology 2005; 12(2): 113-124.

48) Rollin R, Marco F, Jover JA, Garcia-Asenjo JA, Rodriguez L, Lopez-Duran L *et al.* Early lymphocyte activation in the synovial microenvironment in patients with osteoarthritis: comparison with rheumatoid arthritis patients and healthy controls. Rheumatology International 2008; 28(8): 757-764.

49) Zangerle PF, De Groote D, Lopez M, Meuleman RJ, Vrindts Y, Fauchet F *et al.* Direct stimulation of cytokines (Il-1 beta, TNF-alpha, Il-6, Il-2, IFN-gamma and GM-CSF) in whole blood: II. Application to rheumatoid arthritris and osteoarthritis. Cytokine 1992; 4(6): 568-572.

50) Roman-Blas JA, Stokes DG, Jimenez SA. Modulation of TGF-β signaling by proinflammatory cytokines in articular cartilage. Osteoarthritis and Cartilage 2007; 15: 1367-1377.

51) Iannone F, Lapadula G. The pathophysiology of osteoarthritis. Aging Clinical and Experimental Research 2003; 15(5): 364-372.

52) Blaney-Davidson EN, van der Kraan PM, van den Berg WB. TGF-β and osteoarthritis. Osteoarthritis and Cartilage 2007; 15: 597-604.

53) Miyazaki E, Kato J, Kobune M, Okumura K, Sasaki K, Shintani N *et al.* Denatured H-ferritin subunit is a major constituent of haemosiderin in the liver of patients with iron overload. Gut 2002; 50(3): 413-419.

54) Ruggeri G, Santambrogio P, Bonfiglio, Levi S, Bugari G, Verardi R *et al.* Antibodies for denatured human H-ferritin stain only reticuloendothelial cells within the bone marrow. British Journal of Haematology 1992; 81: 118-124.

55) Invernezzi R, Cazzola M, De Fazio P, Rosti V, Ruggeri G, Arosio P. Immunocytochemical detection of ferritin in human bone marrow and peripheral blood cells using monoclonal antibodies specific for the H and L subunit. British Journal of Haematology 1990; 76(30): 427-432.

56) Alvarez-Hernández X, Licéaga J, McKay IC, Brock JH. Induction of hypoferremia and modulation of macrophage iron metabolism by tumor necrosis factor. Laboratory Investigation 1989; 61(3): 319-322.

57) Piñero DJ, Hu J, Cook BM, Scaduto Jr RC, Connor JR. Interleukin-1β increases binding of the iron regulatory protein and the synthesis of ferritin by increasing the labile iron pool. Biochimica *et* Biophysica Acta 2000; 1497: 279-288.

58) Torti FM, Torti SV. Regulation of ferritin genes and protein. Blood 2002; 99(10): 3505-3516.

59) Alvarez-Hernández X, Felstein MV, Brock JH. The relationship between iron release, ferritin synthesis and intracellular iron distribution in mouse peritoneal

macrophages. Evidence for a reduced level of metabolically available iron in elicited macrophages. Biochimica *et* Biophysica Acta 1986; 886: 214-222.

60) Savarino A, Pescarmona GP, Boelaert JR. Iron metabolism and HIV infection: reciprocal interactions with potentially harmful consequences. Cell Biochemistry and Function 1999; 17: 279-287.

61) Picard V, Renaudie F, Porcher C, Hentze MW, Grandchamp B, Beaumont C. Overexpression of the ferritin H subunit in cultured erythroid cells changes the intracellular iron distribution. Blood 1996; 87(5): 2057-2064.

62) Festa M, Ricciardelli G, Mele G, Pietropaolo C, Ruffo A, Colonna A. Overexpression of H ferritin and up-regulation of iron regulatory protein genes during differentiation of 3T3-L1 pre-adipocytes. Journal of Biological Chemistry 2000; 275(47): 36708-36712.

63) Marziali G, Perrotti E, Ilari R, Testa U, Coccia EM, Battistini A. Transcriptional regulation of the ferritin heavy-chain gene: the activity of the CCAAT binding factor NF-Y is modulated in heme-treated Friend leukemia cells and during monocyte-to-macrophage differentiation. Molecular and Cellular Biology 1997; 17(3): 1387-1395.

64) Vanlandingham JW, Levenson CW. Effect of retinoic acid on ferritin H expression during brain development and neuronal differentiation. Nutritional Neuroscience 2003; 6(1): 39-45.

65) Bevilacqua MA, Faniello MC, D'Agostino P. Transcriptional activation of the H-ferritin gene in differentiated Caco-2 cells parallels a change in the activity of the nuclear factor Bbf. Biochemical Journal 1995; 311(Pt 3): 769-773.

66) Zermati Y, Fichelson S, Valensi F, Freyssinier JM, Rouyer-Fessard P, Cramer E *et al.* Transforming growth factor inhibits erythropoiesis by blocking proliferation and accelerating differentiation of erythroid progenitors. Experimental Hematology 2000; 28(8): 885-894.

67) Lo J, Hurta RAR. Transforming growth factor β_1 selectively regulates ferritin gene expression in malignant H-ras-transformed fibrosarcoma cell lines. Biohemistry and Cell Biology 2000; 78(4): 527-535.

68) Shterman N, Kupfer B, Moroz C. Comparison of transferrin receptors, iron content and isoferritn profile in normal and malignant human breast cell lines. Pathobiology 1991; 59(1): 19-25.

69) Yang DC, Wang F, Elliott RL, Head JF. Expression of transferrin receptor and ferritin H-chain mRNA are associated with clinical and histopathological prognostic indicators in breast cancer. Anticancer Research 2001; 21(1B): 541-549.

70) Wu KJ, Polack A, Dalla-Favera R. Coordinated regulation of iron-controlling genes, H-ferritin and IRP-2, by c-MYC. Science 1999; 283(5402): 676-679.

71) Babitt JL, Huang FW, Xia Y, Sidis Y, Andrews NC, Lin HY. Modulation of bone morphogenetic protein signaling *in vivo* regulates systemic iron balance. Journal of Clinical Investigation 2007; 117(7): 1933-1939.

72) Wang R-H, Li C, Xu X, Zheng Y, Xiao C, Zerfas P. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metabolism 2005; 2: 399-409.

73) Weiss G, Goodnough LT. Anemia of chronic disease. New England Journal of Medicine 2005; 352(10): 1011-1023.

74) Finch CA, Huebers HA, Cazzola M, Bergamaschi G, Bellotti V. Storage iron. In: Albertini A, Arosio P, Chiancone E, Drysdale J editors. Ferritins and isoferritins as biochemical markers. Amsterdam: Elsevier Science Publishers; 1984. p. 3-21.

75) Lipschitz DA, Simon MO, Lynch SR, Dugard J, Bothwell TH, Charlton RW. Some factors affecting the release of iron from reticuloendothelial cells. British Journal of Haematology 1971; 21: 289-303.

76) Worwood M. Ferritin. Blood Reviews 1990; 4: 259-269.

77) Fahmy M, Young SP. Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA. Biochemical Journal 1993; 296: 175-181.

78) Ludwiczek S, Aigner E, Theurl I, Weiss G. Cytokine-mediated regulation of iron transport in human monocytic cells. Blood 2003; 101(10): 4148-4154.

79) Ganz T. Hepcidin in iron metabolism. Current Opinion in Hematology 2004; 11: 251-254.

80) Deicher R, Hörl WH. New insights into the regulation of iron homeostasis. European Journal of Clinical Investigation 2006; 36: 301-309.

81) Sherwood RA, Pippard MJ, Peters TJ. Iron homeostasis and the assessment of iron status. Annals of Clinical Biochemistry 1998; 35: 693-708.

82) Charlton RW, Bothwell TH. Definition, prevalence and prevention of iron deficiency. Clinics in Haematology 1982; 11(2): 309-325.

83) Mclintock LA, Fitzsimons EJ. Erythroblast iron metabolism in sideroblastic and sideropenic states. Hematology 2002; 7(3): 189-195.

84) Baynes RD. Iron deficiency. In: Brock JH, Halliday JW, Pippard MJ, Powell LW editors. Iron metabolism in health and disease. London: W.B. Saunders Company Ltd; 1994. p. 189-225.

85) Brittenham GM. The red cell cycle. In: Brock JH, Halliday JW, Pippard MJ, Powell LW editors. Iron metabolism in health and disease. London: W.B. Saunders Company Ltd; 1994. p. 31-62.

86) Harford JB, Rouault TA, Klausner RD. The control of cellular iron homeostasis. In: Brock JH, Halliday JW, Pippard MJ, Powell LW editors. Iron metabolism in health and disease. London: W.B. Saunders Company Ltd; 1994. p. 123-149.

87) Brock JH. Iron in infection, immunity, inflammation and neoplasia. In: Brock JH, Halliday JW, Pippard MJ, Powell LW editors. Iron metabolism in health and disease. London: W.B. Saunders Company Ltd; 1994. p. 353-389.

88) Uchida T. Change in red blood cell distribution width with iron deficiency. Clinical and Laboratory Haematology 1989; 11(2): 117-121.

89) Scott CL, Begley CG. The beta common chain (βc) of the granulocyte-colony stimulating factor, interleukin-3 and interleukin-5 receptors. International Journal of Biochemistry and Cell Biology 1999; 31: 1011-1015.

90) Miyajima A, Mui AL, Ogorochi T, Sakamaki K. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood 1993; 82: 1960-1974.

91) Ringheim GE. Mitogenic effects of interleukin-5 on microglia. Neuroscience Letters 1995; 201(2): 131-134.

92) Pieracci FM, Barie PS. Diagnosis and management of iron-related anemias in critical illness. Critical Care Medicine 2006; 34(7): 1898-1905.

93) Fuchs D, Hausen A, Reibnegger G, Werner ER, Werner-Felmayer G, Dierich MP *et al.* Immune activation and the anaemia associated with chronic inflammatory disorders. European Journal of Haematology 1991; 46: 65-70.

94) Jongen-Lavrencic M, Peeters HRM, Vreugdenhil G, Swak AJG. Interaction of inflammatory cytokines and erythropoietin in iron metabolism and erythropoiesis in anaemia of chronic disease. Clinical rheumatology 1995; 14(5): 519-525.

95) Jurado RL. Iron, infections, and anemia of inflammation. Clinical Infectious Diseases 1997; 25: 888-895.

96) Cazzola M, Dezza L, Bergamaschi G, Barosi G, Bellotti V, Caldera D *et al.* Biologic and clinical significance of red cell ferritin. Blood 1983; 62(5): 1078-1087.

97) Perkins S. Diagnosis of anaemia. In: Kjeldsberg CR editor. Practical diagnosis of hematologic disorders. Chicago IL: American Society for Clinical Pathology Press; 2006. p. 1-16.

98) Gelvan D, Fibach E, Meyron-Holtz EG, Konijn AM. Ferritin uptake by human erythroid precursors is a regulated iron uptake pathway. Blood 1996; 88(8): 3200-3207.

99) Coccia EM, Profita V, Fiorucci G, Romeo G, Affabris E, Testa U *et al.* Modulation of ferritin H-chain expression in Friend erythroleukemia cells: transcriptional and translational regulation by hemin. Molecular and Cellular Biology 1992; 12(7): 3015-3022.

100) Wagstaff M, Worwood M, Jacobs A. Iron and isoferritins in iron overload. Clinical Science 1982; 62(5): 529-540.

101) Speyer BE, Fielding J. Ferritin as a cytosol iron transport intermediate in human reticulocytes. British Journal of Haematology 1979; 42: 255-267.

102) Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 2003; 102: 783-788.

103) Sharp P. The molecular basis of copper and iron interactions. Proceedings of the Nutrition Society 2004; 63: 563-569.

104) Konijn AM, Meyron-Holtz EG, Fibach E, Gelvan D. Cellular ferritin uptake: a highly regulated pathway for iron assimilation in human erythroid precursor cells. Advances in Experimental Medicine and Biology 1994; 356: 189-197.

105) Tietzel I, Mosser DM. The modulation of macrophage activation by tyrosine phosphorylation. Frontiers in Bioscience 2002; 7: d1494-d1502.

106) Lang R. Tuning of macrophage responses by Stat3-inducing cytokines: molecular mechanisms and consequences in infection. Immunobiology 2005; 210: 63-76.

107) Winston BW, Krein PM, Mowat C, Huang Y. Cytokine-induced macrophage differentiation: a tale of 2 genes. Clinical and Investigative Medicine 1999; 22(6): 236-255.

108) Hamilton TA, Ohmori Y, Tebo JM, Kishore R. Regulation of macrophage gene expression by pro- and anti-inflammatory cytokines. Pathobiology 1999; 67: 241-244.

109) Hugman A. Hepcidin: an important new regulator of iron homeostasis. Clinical and Laboratory Haematology 2006; 28: 75-83.

110) Arosio P, Yokota M, Drysdale JW. Structural and immunological relationships of isoferritins in normal and malignant cells. Cancer Research 1976; 36: 1735-1739.

111) Boyd D, Vecoli C, Belcher DM, Jain SK, Drysdale JW. Structural and functional relationships of human ferritin H and L chains deduced from cDNA clones. Journal of Biological Chemistry 1985; 260(21): 11755-11761.

112) Ponka P, Beaumont C, Richardson DR. Function and regulation of transferrin and ferritin. Seminars in Hematology 1998; 35(1): 35-54.

113) Theil EC. The ferritin family of iron storage proteins. Advances in Enzymology and Related Areas in Molecular Biology 1990; 63: 421-449.

CHAPTER 5

FINAL SUMMARY AND CONCLUSIONS

The primary aim of the present study was to quantitatively measure the expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with chronic immune stimulation – more specifically chronic T-helper cell type-1 stimulation. The expression of the H-subunit and L-subunit of ferritin has not previously been measured quantitatively in the bone marrow macrophage and cells of the erythron in relation to patients' immune status. A second aim was to investigate the possible role that the expression of the H-subunit and L-subunit of ferritin may have in the establishment and maintenance of an iron transfer block in patients with chronic immune stimulation.

The study subjects included 48 patients with chronic diseases from the Department of Internal Medicine, Kalafong Hospital and 10 patients with osteoarthritis, scheduled for hip replacement at the Department of Orthopaedics, Pretoria Academic Hospital. Bone marrow and blood samples were collected from each patient. The diagnoses of the patients from Kalafong Hospital were diverse and resulted in an extremely heterogenous group of patients. For the purpose of this study the immune status and iron status were used to group these patients.

The first objective of the study was to subdivide patients according to their immune status. Both neopterin and C-reactive protein levels are often used to assess the pro-

inflammatory status of patients. The Kalafong patients were thus subdivided, firstly based on C-reactive protein levels and secondly on neopterin levels. Although a significant correspondence was seen between the patients in whom high C-reactive protein and patients in whom high neopterin levels were recorded, it was found that there were patients in whom neopterin levels gave a different picture in terms of their inflammatory status from that assumed from their C-reactive protein values. This, for reasons explained in the relevant sections of the discussion, was not an unexpected finding. In view of potential subtle differences in immune status, the subdivision according to C-reactive protein and that according to neopterin were henceforth tested independently and were, despite the large overlap in patients, referred to as the C-reactive protein and neopterin groups or subdivisions.

The pro-inflammatory status of the C-reactive protein and neopterin subdivisions was subsequently examined by their cytokine profiles. The cytokine profiles were investigated in terms of the macrophage phenotype-associated cytokines, i.e., those related to the classically activated macrophage which is an effector in T-helper cell type-1 cellular immune responses, those related to the type-2 or anti-inflammatory macrophage that preferentially effects T-helper cell type-2 humoral responses, and those related to the alternatively activated macrophage predominantly involved in immunosuppression and tissue repair. The cytokine profiles confirmed that the subdivisions according to high C-reactive protein and high neopterin, respectively, did represent patient groups with high pro-inflammatory activity.

The osteoarthritis group showed no pronounced pro-inflammatory activity, similar to the group of Kalafong patients with normal C-reactive protein and the group of Kalafong patients with normal neopterin, but in contrast to these groups the osteoarthritis patients had a significantly higher plasma TGF-β level. The high TGF-β level in the

osteoarthritis group is not unexpected since TGF-β has, for instance, been shown to play an anabolic role in cartilage formation and to modulate cell and tissue responses to injury (1). TGF-β has, in addition, been shown to play a role in cell proliferation, differentiation, apoptosis and migration and is, despite its ability to contribute to fibrosis and osteophyte formation, sometimes used as a therapeutic tool in osteoarthritis (1). In view of their particular cytokine profile, the osteoarthritis patients were, for further analyses, subsequently treated as a separate group.

The expression of the H-subunit and L-subunit of ferritin in the bone marrow macrophage and cells of the erythron in patients with chronic pro-inflammatory activity, i.e., immune activity related to the classically activated macrophage was investigated separately for the subdivisions of Kalafong patients according to high C-reactive protein and high neopterin. The osteoarthritis patient group was treated as separate. The Kalafong patients with chronic immune stimulation, the subdivision according to C-reactive protein and that according to neopterin levels, showed similar changes in expression of the H-subunit and L-subunit of ferritin. The one major difference from normal found in expression of the ferritin subunits in the patient groups with overt pro-inflammatory activity was that of a significantly higher H-subunit expression in the macrophage of the bone marrow. The expression of the L-subunit in the bone marrow macrophage in the groups of Kalafong patients with high pro-inflammatory activity did not differ markedly from those with normal immune activity. The H-subunit/L-subunit ratio in the macrophage was also higher in the groups of Kalafong patients with pronounced pro-inflammatory activity possibly reflecting an increase in H-subunit rich isoferritins. These findings of increased H-subunit, but normal L-subunit expression of ferritin in the macrophage of the bone marrow are in line with previous indications that pro-inflammatory cytokines have the ability to modulate ferritin expression through both transcriptional and translational mechanisms, but that it is mostly the expression of the

H-subunit of ferritin that is increased by pro-inflammatory cytokines (2). From a functional point of view the observed increase in the expression of the H-subunit of ferritin in inflammatory conditions could have a physiological advantage as it may offer protection against increased oxidative onslaughts to the cell. The latter statement is based on the fact that pro-inflammatory conditions stimulate the production of reactive oxygen species (3) and that the H-subunit of ferritin can offer protection against oxidative damage through the withdrawal of iron (4).

There were no significant differences between the pro-inflammatory and normal Kalafong groups for either the H-subunit or L-subunit expression in the cells of the erythron. However, in the group of Kalafong patients with elevated neopterin the H-subunit/L-subunit ratio in the cells of the erythron was higher than in the group of Kalafong patients with normal neopterin. This could indicate that the expression of the H-subunit of ferritin in the cells of the erythron might be influenced by cytokines, but not to the same extent as for the macrophage. The cells of the erythron also handle large amounts of iron, but they do not play the same major role in the storage of iron as the macrophage.

When the expression of the subunits was examined in the osteoarthritis patients, a somewhat surprising observation was made. As previously discussed, the pro-inflammatory status of the osteoarthritis group was similar to that of the Kalafong patients with normal inflammatory status whether examined by C-reactive protein, neopterin or pro-inflammatory cytokines. The only marked cytokine difference from normal was seen in their TGF-β levels that were significantly higher in the group of osteoarthritis patients than in the other groups. However, when the ferritin subunits were examined, the osteoarthritis group's subunit profile corresponded to that of the Kalafong patient groups with overt pro-inflammatory activity. From these findings it is

tempting to imply a role for TGF-β in the enhanced expression of the H-subunit. However, it is obvious that further investigations are required to confirm or refute this possibility and that the results found here merely suggest further investigation into the relationship between TGF-β and H-subunit expression. Before embarking on such work one should perhaps ask whether a possible advantage exists for an increase in H-subunit expression in these patients or whether the co-occurrence of an increase in H-subunit expression and in TGF-β is merely an epiphenomenon.

The second aim of this study was to investigate a possible role for the expression of the H-subunit of ferritin in the macrophage in iron transfer block. The rationale for such an investigation can be found firstly, in the fact that inflammatory conditions can underlie iron retention by bone marrow macrophages, and – as seen from the results of this study – increased H-subunit expression, and secondly, that the H-subunit of ferritin is known to reduce the efflux of iron from cells (5, 6). As pro-inflammatory cytokines may not be the only factors involved in the development and maintenance of the iron transfer block the first step was to investigate the prevalence of the iron transfer block in the Kalafong patients – against the background of their pro-inflammatory activity. The second step in investigating a possible role for the H-subunit of ferritin in the macrophage in iron transfer block was to subdivide the Kalafong patients on the basis of the presence or absence of an iron transfer block – irrespective of their immune status.

In examining the prevalence of an iron transfer block in the presence of chronic pro-inflammatory activity the results showed almost 70% of the patients with high pro-inflammatory activity to exhibit the characteristics of an iron transfer block. However, between 15% and 26% of patients, depending on whether C-reactive protein or neopterin was used as indicator, who did not show overt pro-inflammatory activity, also had an iron transfer block. These results thus confirmed the strong association between

the development and maintenance of an iron transfer block on the one hand, and pro-inflammatory activity on the other. It does, however, also show that the chronic pro-inflammatory state cannot summarily be assumed to be accompanied by an iron transfer block and consequently with the associated anaemia of chronic disease. Whether the observation of an iron transfer block in a small number of patients without pronounced pro-inflammatory activity was due to the stage of the disease and therefore to the phase of the immune response, or some other factor, cannot at this point be deduced from the results. Other potential role players in the development and maintenance of the iron transfer block were discussed in the previous chapter.

In the second phase of investigating the possibility that the H-subunit of ferritin may play a role in the iron transfer block, the Kalafong patients were subdivided according to the presence or absence of an iron transfer block – irrespective of their immune status. The subdivision into patients with and patients without an iron transfer block was made on the grounds of assessment of the iron stores of Prussian blue stains of the bone marrow aspirates and cores, serum indicators of iron status and red blood cell profiles. The subdivision based solely on aspirates was made virtually impossible due to many of the patients, in addition to iron transfer block, also suffering from true iron deficiency. However, in addition to serum iron, serum transferrin, percentage serum transferrin saturation, transferrin/log ferritin ratio, other factors such as soluble transferrin receptor and soluble transferrin receptor/log ferritin ratio, as well as the red blood cell profiles, were employed in order to group the patients. A marginally higher expression of the H-subunit of ferritin was seen in the patients with an iron transfer block than in those without an iron transfer block. Although not the primary aim of this study, the results thus pointed towards a role for increased expression of the H-subunit in the bone marrow macrophage in iron transfer block.

The preceding paragraphs summarized the outcome of the main aims of the study. However, a number of important observations were made that warrant reporting and may point the way for further investigation. These are touched upon in the following paragraphs.

In investigating the relationship between the presence of an iron transfer block and immune activity the normal, expected relationships between storage iron, bio-available iron and red blood cell profile was seen. This normal relationship was present in the Kalafong groups with normal immune status, the Kalafong group with no iron transfer block and in the osteoarthritis patients. However, these relationships were largely lost in the immune-stimulated Kalafong patients. It would thus appear that, in conditions with chronic pro-inflammatory activity, iron availability loses its primary role in the establishment of the circulating red blood cell profile.

The study also provided indications that the H-subunit and L-subunit of ferritin may, in patients with normal immune status, play a role in the bio-availability of iron for haemoglobin synthesis. In the group of Kalafong patients with normal neopterin the possibility that the H-subunit and L-subunit of ferritin may play a role in the bio-availability of iron for haemoglobin synthesis was supported by a) the negative correlation between the H-subunit/L-subunit ratio in the macrophage and the MCV and MCH of the red blood cells, b) the negative correlation between the H-subunit/L-subunit ratio (increase in H-subunit rich isoferritins) in the cells of the erythron and the red blood cell MCV, c) the positive correlation between the expression of the L-subunit of ferritin in the macrophage (reflecting an increase in the labile iron pool in the macrophage and therefore a reduction in the shunting of iron to more stable pools) and the red blood cell MCV and MCH and d) the positive correlation between the bio-available serum iron (serum iron and percentage transferrin saturation) and the

expression of the L-subunit of ferritin in the cells of the erythron. Similar correlations were found in the Kalafong patients with an iron transfer block where it was seen that a) the H-subunit/L-subunit ratio in cells of the erythron correlated negatively with the MCV and MCH of the red blood cells and b) the expression of the L-subunit of ferritin in the cells of the erythron correlated positively with the MCV and MCH of the red blood cells. According to these results it would appear that an increase in H-subunit rich ferritins in the macrophage may result in less iron being released by the macrophage due to the possible shunting of iron to more stable pools and that an increase in the L-subunit of ferritin in the macrophage may reflect an increase in the labile iron pool in the macrophage and therefore a reduction in the shunting of iron to more stable pools and an increase in the bio-availability of iron. Although the possibility that the H-subunit and L-subunit of ferritin may play a role in the bio-availability of iron for haemoglobin synthesis has not before been investigated *in vivo,* there are indications from investigations in cell cultures that would appear to support the above correlations. The first of the above deductions is in line with the results of Picard *et al.* on cultured erythroid cells where it was shown that an increase in H-subunit rich isoferritins can result in the rapid chelation of the labile iron pool (7) and the second deduction with that of a study by Ruggeri *et al.* where it was shown that the concentration of L-subunit rich ferritins in liver, serum and cultured cells is related to iron levels (8). An, as yet, inexplicable, but opposing, relationship was found in the immune-stimulated Kalafong patients where a positive correlation was found between the expression of the H-subunit of ferritin in the cells of the erythron and red blood cell MCHC.

In the osteoarthritis patients the correlations between the H-subunit and L-subunit of ferritin and the bio-availability of iron was directly opposite to that of the Kalafong patients with normal immune status. The H-subunit/L-subunit ratio in the bone marrow macrophage and the H-subunit of ferritin in the cells of the erythron were closely related

to the serum iron level. This contradiction in results between the immunological normal Kalafong group and the osteoarthritis group needs further investigation. In fact, further studies on the H-subunit and L-subunit of ferritin, in general, would be of interest in osteoarthritis patients as several of the osteoarthritis patients showed characteristics of an iron transfer block while their circulating cytokine profiles were, except for TGF-β, relatively normal.

In the group of osteoarthritis patients a role for various cytokines in the regulation of the levels of the H-subunit and L-subunit of ferritin was shown. Some cytokines were shown to correlate with a decrease in the expression of the subunits of ferritin. An increase in Il-8 correlated with a decrease in the H-subunit of ferritin and a decrease in the H-subunit/L-subunit ratio in the macrophage. An increase in Il-6 correlated with a decrease in the L-subunit of ferritin in the macrophage. This is consistent with the role that ferritin plays in inflammation (9) with possibly first the up-regulation of specifically the H-subunit of ferritin followed by a decrease in the H-subunit and L-subunit as a result of an increase in haemosiderin formation or secretion of ferritin.

Some correlations were also found between the expression of the H-subunit and L-subunit of ferritin in cells of the erythron and cytokines. An increase in Il-2 correlated with a decrease in the H-subunit of ferritin in the cells of the erythron. An increase in TNF-α and an increase in Il-10 correlated with a decrease in the L-subunit of ferritin in the cells of the erythron. In view of the pleiotropic nature of cytokines, it would be unwarranted to make definitive assumptions on the role that cytokines play in the expression of the H-subunit and L-subunit of ferritin without further investigations.

Suggestions for further investigations

In no previous study was the expression of the H-subunit and L-subunit of ferritin measured quantitatively in bone marrow macrophages and cells of the erythron in combination with the inflammatory status of patients. This study investigated the influence of an overt pro-inflammatory state on the expression of the H-subunit and L-subunit of ferritin. The expression/levels of the H-subunit and L-subunit of ferritin are determined by various processes including transcription of the H-subunit and L-subunit genes, translation of the H-subunit and L-subunit mRNA, aggregation of ferritin and secretion of ferritin. It is suggested that the regulation of H-subunit/L-subunit levels during inflammatory processes be further investigated by measuring the mRNA levels, the amount of ferritin aggregation and the secretion of ferritin. By determining the mRNA levels the change in transcription could be evaluated. Measuring mRNA with ultrastructural *in situ* hybridisation is theoretically possible and was tried. However, the copies of mRNA transcripts were very few and even completely absent due to the harsh treatments of fixation, dehydration and embedding. It is suggested that *in situ* hybridisation of tissue prepared by cryo-fixation could be tried in order to retain more mRNA copies. The process of secretion can also contribute to the levels of the H-subunit and L-subunit of ferritin. By determining the plasma levels of the H-subunit and L-subunit of ferritin this process of secretion could be investigated.

References

1) Blaney-Davidson EN, van der Kraan PM, van den Berg WB. TGF-β and osteoarthritis. Osteoarthritis and Cartilage 2007; 15: 597-604.

2) Miller LL, Miller SC, Torti SV, Tsuji Y, Torti FM. Iron-independent induction of ferritin H chain by tumor necrosis factor. Proceedings of the National Academy of Sciences of the United States of America 1991; 88(11): 4946-4950.

3) Closa D, Folch-Puy E. Oxygen free radicals and the systemic inflammatory response. IUBMB Life 2004; 56(4): 185-191.

4) Lin F, Girotti AW. Elevated ferritin production, iron containment, and oxidant resistance in hemin-treated leukaemia cells. Archives of Biochemistry and Biophysics 1997; 346(1): 131-141.

5) Alvarez-Hernández X, Licéaga J, McKay IC, Brock JH. Induction of hypoferremia and modulation of macrophage iron metabolism by tumor necrosis factor. Laboratory Investigation 1989; 61(3): 319-322.

6) Fahmy M, Young SP. Modulation of iron metabolism in monocyte cell line U937 by inflammatory cytokines: changes in transferrin uptake, iron handling and ferritin mRNA. Biochemical Journal 1993; 296: 175-181.

7) Picard V, Renaudie F, Porcher C, Hentze MW, Grandchamp B, Beaumont C. Overexpression of the ferritin H subunit in cultured erythroid cells changes the intracellular iron distribution. Blood 1996; 87(5): 2057-2064.

8) Ruggeri G, Iacobello C, Albertini A, Brocchi E, Levi S, Gabri E *et al.* Studies of human isoferritins in tissues and body fluids. In: Albertini A, Arosio P, Chiancone E, Drysdale J editors. Ferritins and isoferritins as biochemical markers. Amsterdam: Elsevier Science Publishers; 1984. p. 67-78.

9) Alvarez-Hernández X, Felstein MV, Brock JH. The relationship between iron release, ferritin synthesis and intracellular iron distribution in mouse peritoneal

macrophages. Evidence for a reduced level of metabolically available iron in elicited macrophages. Biochimica *et* Biophysica Acta 1986; 886: 214-222.