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# MECHANISM OF ACTION OF ANDROGENS ON THE ANEMIA ASSOCIATED WITH EXPERIMENTAL CHRONIC RENAL FAILURE IN THE MOUSE

by
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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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To Joseph, May and Dima,

# ABSTRACT

Anemia is a hallmark of chronic renal failure (CRF) which, if left untreated, is a major contributing factor to the high morbidity and mortality of this condition. This characteristically hypoproliferative anemia is due primarily to decreased erythropoietin (EPO) production by the diseased kidney. Currently, correction of the anemia with recombinant human EPO (r-HuEPO) constitutes the mainstay of management in patients with end-stage renal disease (ESRD). However, the use of r-HuEPO is severely limited by its elevated cost. In addition, a few patients demonstrate hyporesponsiveness to r-HuEPO and require unusually large doses of this drug.

An alternative approach to the treatment of the anemia of CRF is the administration of androgens. Androgen use was widespread in ESRD patients prior to the clinical introduction of r-HuEPO. Several recent studies have indicated a renewed interest in the use of androgens in this patient population, with results that rival those seen with r-HuEPO. Despite this long-standing clinical experience with androgens, their mechanism of action remains obscure.

My project utilized a well characterized mouse model of surgically-induced renal failure to study the mechanism(s) of androgen effect on the anemia of CRF. Recent experiments in this model revealed a dose-response to r-HuEPO similar to that in humans, absent EPO gene expression in the liver and full correction of the anemia of CRF by the administration of a combination of subtherapeutic doses of r-HuEPO and insulin-like growth factor-I (IGF-I), a known regulator of erythropoiesis in the normal physiological state.

My hypothesis followed two lines of investigation: androgens may act to increase EPO production or they may increase production of IGF-I, thereby increasing extra-renal erythropoietic activity by either of the two hormones. To test my hypothesis, I developed a model of androgen-treated mice with renal failure, utilizing a range of doses of nandrolone decanoate (0.3, 1.0, 3.0, 9.0 mg/kg BW/wk) starting one week after the surgical induction of renal failure, and continuing for 5 consecutive weeks, with sacrifice / evaluation performed at 6 weeks of renal failure.

Androgen treatment increased the expression of EPO mRNA in the remnant kidney of CRF mice with a concomitant significant increase in serum EPO levels (at the two higher androgen doses). Surprisingly, androgen treatment did not modify the absent EPO production observed in the liver of untreated CRF mice. The rise in hemoglobin induced by androgen treatment was not significant, but it exhibited a consistently increasing trend commensurate with the androgen dose. The degree of renal failure had no effect on the changes in EPO production and serum EPO levels induced by androgens, but the changes in hemoglobin concentrations were dampened in mice with the more severe renal failure. Serum IGF-I levels were not significantly different between untreated and androgen-treated CRF mice and normal controls.

The above studies represent a comprehensive approach to understanding selected aspects of the action of androgens on the anemia of CRF mice. The results demonstrate that increased production of EPO by the remnant kidney is the principal mechanism of action of androgens on erythropoiesis during CRF.

# RÉSUMÉ

L'anémie est une manifestation primordiale de l'insuffisance rénale chronique (CRF) qui, si laissée non-traitée, contribue de façon importante à la morbidité et à la mortalité de cette condition. Cette anémie, typiquement non-régénératrice, est due essentiellement à une diminution de la production d'érythropoiétine (EPO) par le rein malade. Actuellement, la correction de l'anémie avec l'érythropoiétine humaine de recombinaison (r-HuEPO) constitue le traitment principal des malades en insuffisance rénale terminale (ESRD). Toutefois, l'utilisation de la r-HuEPO est limitée de façon importante par son coût élevé. De plus, certains malades présentent une réponse atténuée à la r-HuEPO et nécessitent des doses inhabituellement élevées de ce médicament.

Une approche alternative du traitement de l'anémie de CRF est l'administration d'androgènes. L'utilisation d'androgènes était étendue chez les maladies en ESRD avant l'introduction clinique de la r-HuEPO. Plusieurs études récentes ont indiqué un intérêt renouvelé dans l'utilisation des androgènes chez cette population de malades, avec des résultats rivalisant avec ceux observés avec la r-HuEPO. Malgré cette expérience clinique de longue date avec les androgènes, leur mécanisme d'action demeure obscur.

Mon projet a utilisé un modèle bien caractérisé d'insuffisance rénale induite chirurgicalement chez la souris pour étudier le(s) mécanisme(s) de l'effet des androgènes sur l'anémie de CRF. Des expériences récentes dans ce modèle ont révélé une réponse reliée à la dose de la r-HuEPO similaire à celle des malades, l'absence d'expression du gène de l'EPO dans le foie et la correction complète de l'anémie de CRF par l'administration d'une combinaison de doses sous-thérapeutiques de la r-HuEPO et du facteur I de croissance ressemblant à l'insuline (IGF-I), un facteur connu de la régulation de l'érythropoièse dans l'état physiologique normal.

Mon hypothèse a suivi deux voies d'investigation: les androgènes peuvent agir en augmentant la production d'EPO ou ils peuvent augmenter la production de l'IGF-I, augmentant ainsi l'activité érythropoiétique extra-rénale par l'une ou l'autre de ces deux hormones. Pour tester mon hypothèse, j'ai développé un modèle de souris avec insuffisance rénale traitées par les androgènes utilisant un choix de doses du décanoate de nandrolone (0.3, 1.0, 3.0, et 9.0 mg/kg poids corporel/semaine) en commençant une semaine après l'induction chirurgicale de l'insuffisance rénale, et continuant pour 5 semaines consécutives, avec le sacrifice / évaluation pratiqués à 6 semaines d'insuffisance rénale.

Le traitment avec les androgènes a augmenté l'expression du mRNA de l'EPO dans le rein restant des souris avec CRF ainsi qu'une augmentation concomitante des niveaux sériques d'EPO (aux deux doses les plus élevées). Etonnamment, le traitment avec les androgènes n'a pas modifié la production absente d'EPO observée dans le foie des souris avec CRF non-traitées. L'augmentation de l'hémoglobine induite par le traitement avec les androgènes n'était pas significative, mais elle exhibait une tendance consistente d'augmentation proportionnée avec la dose d'androgène. Le degré d'insuffisance rénale n'a pas eu d'effet sur les changements de production d'EPO et les niveaux sériques d'EPO induits par les androgènes, mais les changements des concentrations de l'hémoglobine ont été amoindris chez les souris avec l'insuffisance rénale la plus sévère. Les niveaux sériques de l'IGF-I n'étaient pas significativment différents entre les souris avec CRF non-traitées et traitées avec les androgènes et les souris témoins normales.

Les études ci-dessus représentent une approche d'ensemble à la compréhension d'aspects sélectifs de l'action des androgènes sur l'anémie des souris avec CRF. Les résultats démontrent que la production augmentée d'EPO par le rein restant est le mécanisme d'action principal des androgènes sur l'érythropoièse durant CRF.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ALP | alkaline phosphatase |
| AND | androgens |
| ATP | adenosine triphosphate |
| BFU-E | burst-forming unit erythroid |
| bp | base pair |
| BW | body weight |
| °C | degrees centigrade |
| CAPD | continuous ambulatory peritoneal dialysis |
| CBC | complete blood count |
| cDNA | complementary deoxyribonucleic acid |
| CFU-E | colony forming unit erythroid |
| cm | centimeter |
| $CO_2$ | carbon dioxide |
| CORR | Canadian Organ Replacement Register |
| CRF | chronic renal failure |
| dCTP | deoxycytosine triphosphate |
| DEPC | diethyl pyrocarbonate |
| dNTP | deoxynucleotide triphosphate |
| dpm | decays per minute |
| EPO | erythropoietin |
| e.g. | exempli gratia (for example) |
| ESRD | end-stage renal disease |
| fl | femtoliter |
| g | gram |
| g | gravitational force |
| GM-CSF | granulocyte/monocyte colony stimulating factor |
| H&E | hematoxylin and eosin |
| Hb | hemoglobin |
| Hct | hematocrit |
| HMP | hexose monophosphate |
| hr | hour |
| i.m. | intramuscular |
| i.p. | intraperitoneal |
| i.v. | intravenous |
| IGFBP | insulin-like growth factor binding protein |
| IGF-I | insulin-like growth factor-I |
| IL- | interleukin- |
| IU | international unit |
| kb | kilobase pair |
| kg | kilogram |
| KIE | kidney-inducibility element |
| l | liter |

| | |
|---|---|
| LIE | liver-inducibility element |
| MCH | mean corpuscular hemoglobin |
| MCV | mean corpuscular volume |
| MCHC | mean corpuscular hemoglobin concentration |
| mg | milligram |
| µg | microgram |
| mg/kg BW/wk | milligram per kilogram body weight per week |
| MGH | Montreal General Hospital |
| min | minute |
| ml | milliliter |
| µl | microliter |
| µm | micrometer |
| mm | millimeter |
| mM | millimolar |
| µM | micromolar |
| mmol | millimole |
| µmol | micromole |
| mRNA | messenger ribonucleic acid |
| mU | milliunit |
| n | sample size |
| ng | nanogram |
| nm | nanometer |
| NRE | negative regulatory element |
| Plt | platelets |
| PTH | parathyroid hormone |
| r-HuEPO | recombinant human erythropoietin |
| RBC | red blood cell |
| RF | renal failure |
| RIA | radioimmunoassay |
| RNA | ribonucleic acid |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| RVH | Royal Victoria Hospital |
| s.c. | subcutaneous |
| SCF | stem cell factor (Steel factor) |
| SD | standard deviation |
| SDS | sodium dodecylsulfate |
| sec | second |
| SSC | sodium chloride/sodium citrate |
| TGF | transforming growth factor |
| TNF | tumor necrosis factor |
| U | unit |
| UND | undetectable |
| WBC | white blood cell |
| wk | week |

# HYPOTHESIS

Androgen treatment alleviates the anemia of chronic renal failure in the mouse by one of two mechanisms:

1) Increasing erythropoietin production

OR

2) Increasing bioavailable insulin-like growth factor-I

# INTRODUCTION

Anemia is the most characteristic hematologic abnormality in chronic renal failure (CRF). The anemia of CRF is principally caused by deficient erythropoietin (EPO) production from the diseased kidneys. Currently, the mainstay of therapy for this anemia in end-stage renal disease (ESRD) patients is recombinant human EPO (r-HuEPO). Prior to the advent of EPO, androgens were used in the treatment of the anemia of CRF. Therapeutic approaches with early formulations of androgens were often accompanied by undesirable side effects and yielded contradictory and inconsistent results. In addition, no dose-response to androgens was established nor was their mechanism of action determined.

An animal model of CRF provides a tool to address several problems associated with CRF. A well-characterized mouse model of surgically-induced renal failure, developed by Gagnon et al. in 1983 (44), has been used extensively in the study of the regulation of erythropoiesis by EPO and insulin-like growth factor-I (IGF-I). Recent experiments in this model revealed absent extra-renal EPO gene expression in the liver and bone marrow and full correction of the anemia, which correlates with the degree of renal failure, by the administration of EPO as well as a combination of subtherapeutic EPO and IGF-I. Therefore, this model can be manipulated to understand the mechanisms that might intervene during states of EPO deficiency.

Although the use of recombinant human EPO corrects the anemia of CRF, its use is limited by several factors. Treatment with r-HuEPO is highly costly and thus not all patients can receive this form of therapy. In addition, cases are documented of

hyporesponsiveness to r-HuEPO even when administered at doses 2-3 times the recommended dosages. In view of recent data demonstrating equivalent responses of ESRD patients to r-HuEPO and androgens, there has been a renewed interest in the use of androgens to alleviate the anemia of CRF.

Since the mouse model of CRF can faithfully reproduce the main features of severe renal failure in man, including correction of the anemia with r-HuEPO, I propose to use this murine model for the study of the mechanism(s) of action of androgens in the treatment of the anemia of CRF.

## 1. The Anemia of Chronic Renal Failure

### *a) Main characteristics*

Anemia is one of the main and most consistent manifestations of CRF. The association of anemia with renal failure has been recognized for over 150 years since Richard Bright first described the manifestations of CRF (11). Seventy-five percent of uremic patients requiring dialysis have a hematocrit level which is less than 30% (62). Anemia develops when the creatinine clearance has decreased to between 30 and 40 ml/min/1.73 $m^2$, and its severity increases with further deterioration of excretory renal function (70). Anemia in CRF is usually normochromic and normocytic and is characteristically hyporegenerative with an inappropriate low reticulocyte response. The impact of anemia in CRF on physical and mental abilities is considerable and represents a major obstacle for the rehabilitation of ESRD patients (62, 70).

The anemia of CRF can be caused by several factors. These include inadequate production of EPO, shortened life span of red cells, moderate hemolysis, bleeding secondary to qualitative platelet defects, and perhaps inhibitors of hematopoiesis such as TNF, TGFβ, and IL-1α and β. Iron and folate deficiency may each aggravate the anemia in CRF (1, 62, 70). Uremic toxins, such as spermine and spermidine, may also be present (3).

Hemolysis is primarily caused by circulating uremic toxins acting on membrane adenosine triphosphate (ATP) as well as on the enzymes of the hexose monophosphate (HMP) shunt pathway of erythrocytes. Decreased function of the HMP shunt pathway renders hemoglobin and the red cell membrane sensitive to oxidant drugs or chemicals (36).

Secondary hyperparathyroidism in CRF may reduce red blood cell survival by one half to two thirds because intact parathyroid hormone increases the osmotic fragility of human red blood cells, thereby reducing their survival (36).

CRF is accompanied by prominent morphological changes in the bone marrow. Osteitis fibrosa cystica, secondary to severe secondary hyperparathyroidism, is a well-known complication of CRF in man. This fibrosis destroys the bone marrow and once prominent hematopoietic progenitors are replaced by fibrous tissue. Loss of the hematopoietic progenitors leads to anemia and compensatory extra-medullary erythropoiesis.

The inadequate production of EPO is the primary etiological factor of anemia in CRF. This EPO deficiency results from the progressive destruction of EPO-producing

sites in the kidney by the underlying renal disease. In comparison to anemic patients without renal disease, patients with the anemia of renal failure display an inadequate rise in the serum concentration of this hormone (1, 70). The importance of the role of EPO deficiency in the pathogenesis of the anemia of CRF has also been demonstrated in the clinical situation by the remarkable rise in hemoglobin following administration of r-HuEPO to patients with ESRD undergoing regular hemodialysis (37).

The strategies to improve the anemia of CRF are based on the aforementioned etiological factors. A number of therapeutic approaches have been applied. Blood transfusions are only of temporary benefit and carry significant risks such as exposure to the hepatitis virus and human acquired immune deficiency syndrome. Renal transplantation is usually not available before a waiting period of variable length and not every regular dialysis patient is eligible for kidney transplantation. Dialysis, whether hemodialysis or peritoneal dialysis, removes the uremic toxins and improves the anemia by removal endogenous inhibitors of erythropoiesis and endogenous hemolytic toxins. However, those patients on hemodialysis frequently remain moderately anemic due to hemolysis and the loss of blood during the hemodialysis procedure. Patients treated with continuous ambulatory peritoneal dialysis (CAPD) are relatively less anemic due to the smaller blood loss associated with this form of dialysis.

***b) Treatment with r-HuEPO***

The efficacy of r-HuEPO in the therapy of renal anemia has been proven in many multi-center trials conducted worldwide. The median dose of r-HuEPO required to maintain a target hematocrit of 33% to 38% is 75 U/kg s.c. three times per week.

However, 17% of the patients will require more than 150 U/kg s.c. three times weekly. Adjustments in dosages are usually made every 2 to 6 weeks if the rise in hematocrit is steady and not greater than 1% to 2% per week. If there is no response, the dose is usually increased by 25%. The rise in hemoglobin concentration as a consequence of r-HuEPO administration is associated with a rise in maximal oxygen uptake ($V0_2$ max) (30). In addition, coagulation parameters improve; platelet aggregation, factor VIII, and fibrinogen levels improve due to the increase in the red cell mass (132). Prior to r-HuEPO therapy, as many as 25% of dialysis patients were transfusion-dependent. Since r-HuEPO became available, the need of transfusion therapy has no longer been necessary, except for acute and unavoidable problems such as gastrointestinal bleeding (51). Sexual function, especially in males, improves (30) as does the nutritional status, upon r-HuEPO administration.

However, uncorrectable resistance to r-HuEPO in patients with ESRD does exist and is usually associated with red cell substrate depletion (iron, vitaminB12 and folate deficiency in particular), intercurrent infection, aluminum overload, or poorly controlled hyperparathyroidism. Aluminum overload may diminish bone marrow response to EPO. There is, therefore, a direct relationship between the aluminum body pool and the doses of r-HuEPO necessary to reach a comparable degree of correction of anemia in hemodialysis patients (117). Aluminum overload is no longer a factor due to the adequate monitoring of patients to prevent this condition.

Due to the elevated cost of EPO therapy, the percentage of ESRD patients receiving r-HuEPO treatment in different countries is a function of the budgetary

allocations for this form of treatment (129). The Canadian statistics regarding dialysis patients are reported annually in the Canadian Organ Replacement Register (CORR). According to the 1996 report, only approximately 55% of total dialysis patients were receiving EPO in 1994 (Figures 2a and 2b). Although this percentage represents an increase from 1991, many anemic patients remain deprived of this form of therapy.

Since the advent of r-HuEPO, the use of androgens in treating the anemia of renal failure has diminished. However, several recently observed complications with the use of r-HuEPO (severe arterial hypertension and seizures) have prompted a resurgent interest in androgens. Newer formulations of androgens, particularly nandrolone decanoate, with fewer side effects are quite effective in alleviating the anemia of CRF.

Despite their use, many questions regarding androgens remain unanswered. Firstly, there has been no documented optimal dose for this type of therapy as no dose-response study has ever been established. Secondly, their mechanism of action is unclear. Hypotheses range from androgens stimulating stem cell proliferation in the bone marrow to androgens increasing the production of EPO. Our well-characterized mouse model of CRF provides a means to perform, on a large scale, studies on the mechanism of action of androgens.

## 2. The Regulation of Erythropoiesis

The essential purpose of erythropoiesis is to provide a vehicle for hemoglobin to transport oxygen. The complex process of erythropoiesis is regulated to maintain the

production of erythrocytes at a rate determined by the body's demands. This process requires an intact integrated system of regulations.

***a) The origin of red blood cells***

During fetal life, the main site of hematopoiesis is the liver, while the spleen plays an accessory role. The bone marrow gradually replaces the liver and spleen during the third trimester of pregnancy. In the post-natal period, the bone marrow becomes the sole site of hematopoiesis, although extramedullry hematopoiesis may occur in diseases in which the bone marrow becomes destroyed or fibrosed.

As shown in Figure 3, all blood cells originate from a population of totipotent stem cells. These cells are maintained only by self-replication. Most of these cells are in the resting state, and only a small proportion of them are in proliferation at any given time. One of the direct progeny of totipotent stem cells is the pluripotent hematopoietic stem cell. Its own progeny become progressively more committed to either the erythroid, granulocytic or megakaryocytic lines. The first functional detectable precursors of erythropoiesis are erythroid-burst forming units (BFU-E); these are defined by in vitro culture and require stimulation factors containing burst-forming activity. BFU-E have large potential proliferative capacity and can produce several thousand red blood cells. They are maintained mainly by influx from early progenitor cells and also by self-replication under the influence of IL-3 and stem cell factor (SCF). BFU-E, under the influence of EPO, give rise to the erythroid colony-forming units (CFU-E). CFU-E generate a small cluster of erythroid cells which immediately mature, under the influence of EPO and IGF-I, to pronormoblasts, basophilic normoblasts, polychromatophilic

normoblasts, orthochromic normoblasts, reticulocytes, and finally to circulating red blood cells. Erythrocytes are delivered into the circulation at the reticulocyte stage.

***b) The factors influencing erythropoiesis***

Erythropoiesis is regulated by the interaction of BFU-E with circulating hormones and auxiliary cells which regulate their growth and differentiation. These auxiliary cells include macrophages, T-lymphocytes, and cells of the hematopoietic microenvironment which consist of fat cells, reticulum cells, mast cells, plasma cells, fibroblasts and endothelial cells. T-cells are an important source of interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF), both of which stimulate BFU-E formation from the earlier pluripotent precursor.

The maintenance of a relatively constant circulating red cell mass requires an erythropoietic stimulus which ensures that, under physiological conditions, the rate of production of new erythrocytes equals the rate of destruction. There must also be an increased production rate in response to anoxia, high altitude, hemorrhage or hemolysis, anemia and hypoventilation. The most important factor ensuring homeostasis of the circulating red cell mass under physiological and pathological conditions is EPO.

Erythropoiesis is also influenced by nutrients such as iron, vitamin B12 and folate. Other essential factors are vitamin B6 (pyroxidine), riboflavin, vitmain E (α-tocopherol), copper, as well as proteins and carbohydrates. The absence of any of these elements will cause anemia (82).

Several other hormones also play a role, such as thyroxine, androgens, corticosteroids, estrogens, growth hormone, and cytokines such as GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-9, tumor necrosis factor (TNF), and steel factor (c-kit ligand or SCF) (82).

IL-3, GM-CSF and EPO are necessary, in vitro, to induce the formation of the BFU-E. They both have a synergistic role in the stimulation of the burst formation, and IL-3 seems to have more burst-forming activity than GM-CSF. IL-3 is produced by activated T-cells, whereas GM-CSF originates from macrophages. These factors exert their influence at the level of the stem cells and BFU-E, but are largely ineffective in influencing the development of later erythroid precursors.

Androgens, corticosteroids, and growth hormone are thought to enhance the EPO response of red cell precursors. Corticosteroids also have a direct stimulatory effect on erythropoiesis. Thyroid hormones stimulate EPO production indirectly by increasing the general metabolism, resulting in increased oxygen requirement. On the other hand, estrogens inhibit erythropoiesis both by reducing EPO production and by antagonizing its effect (80, 83).

Recent evidence suggests that several cytokines have a significant effect at various stages of erythropoiesis either directly or indirectly. TNF is produced by activated macrophages in response to infection. TNF suppresses red cell proliferation and differentiation (2). IL-1a is the mediator of TNF action (2). IL-9 supports the development of BFU-E in cultures supplemented with EPO. IL-9 likely interacts with an early population of IL-3 responsive BFU-E (82). Steel factor or c-kit ligand (SCF) interacts with EPO to stimulate BFU-E. C-kit is a proto-oncogene which encodes a

tyrosine kinase transmembrane receptor. IL-4 enhances the proliferation of murine BFU-E and CFU-E in the presence of EPO. IL-2 exerts a dose-dependent inhibition of BFU-E in the presence of T-cells with IL-2 receptors. This inhibition may be mediated in part by release of interferon-γ from IL-2 responsive T-cells, since T-cells increase interferon dramatically in the presence of IL-2 and specific interferon-γ antibody abrogate this inhibition.

c) ***New developments in the regulation of erythropoiesis***

Recently, Brox et al. (15, 24) have described two anephric dialysis patients demonstrating normal hemoglobin concentrations despite the absence of circulating EPO. In this study, a low-molecular-weight peptide, capable of supporting erythroid colony formation, was identified to be insulin-like growth factor-I (IGF-I). It was concluded that IGF-I may serve as an important stimulator of erythropoiesis in anephric patients. In addition, experiments with subtherapeutic doses of r-HuEPO and IGF-I (16) yielded significant increases in hemoglobin concentrations in CRF mice. Therefore, IGF-I may have a role in the regulation of erythropoiesis but its exact mechanism of action remains elusive.

## 3. Androgens in the treatment of the anemia of CRF

***a) Review of androgen use in CRF***

Owing to the well-established correlation of red blood cell (RBC) count, age and sex in mammals, it has long been suspected that androgens stimulate normal erythropoiesis (115, 130). Hemoglobin and hematocrit values increase with age in

children between 2 and 14 years (124), and between the ages of 14 and 20 years, they steadily increase in males and decrease in females (58). These differences persist throughout adulthood and are not attributable to iron deficiency, pregnancy or blood loss (128). Furthermore, clinical disorders characterized by excess androgen production such as Cushing's syndrome and congenital adrenal hyperplasia may be complicated by polycythemia (52).

Such findings prompted testosterone administration to females of the species as early as 1941. The result was augmented erythrocyte production (69). At approximately the same time, androgens were used to treat patients with anemia associated with a variety of endocrinologic and hematologic disorders (49, 87). While serious side effects such as intense masculinization and jaundice were observed with high doses of methyltestosterone, reticulocytosis and increased RBC counts were also noted. Subsequent studies showed that androgens were effective agents for treatment of anemia associated with other disease states, including chronic renal failure (106).

Although the efficacy of androgens in the treatment of the anemia due to primary bone marrow failure has been debated (18, 50), their usefulness in the therapy of anemia of ESRD has been confirmed in a number of studies (17, 20, 28, 35, 40, 71, 90, 112). A review of the above studies underscored the potential usefulness of androgens in the treatment of the anemia of ESRD.

DeGowin et al. treated two patients with chronic renal failure (one anephric) with 200 mg testosterone enthanate twice weekly (28). This group found increased hematocrit and plasma erythropoietin levels in both patients, eliminating the need for transfusions in

the nephric patient. The anephric patient still required transfusions, albeit significantly less than when he did not receive androgens.

The group of Shaldon, in 1971, treated 25 male and female long-term dialysis patients with 250-500 mg per week of testosterone for six months (112). All patients had a significant increase in hemoglobin concentration and this response diminished when administration of testosterone was ceased. The treatment was also effective in bilaterally nephrectomized patients. Eschbach et al. treated 8 female and 6 male hemodialyzed, chronic renal failure, patients with fluoxymesterone (35). Female patients received 10 mg/day while male patients received 30 mg/day for at least 6 months. Clinically, significant improvement in erythropoiesis was seen within 3 to 6 months of androgen therapy in 13 of the 14 patients, as reflected by a reduction in transfusion requirements and an increase in hematocrit value. The response did not depend on the presence of residual renal tissue. Fried et al. treated 11 male (5 anephric) chronic renal failure patients on maintenance hemodialysis with 150 mg testosterone propionate twice weekly for 5 months (40). Fried found that the six patients with native kidneys responded to therapy with a significant rise in both packed cell volume and plasma erythropoietin titer. None of the five anephric patients had an increase in either of these variables.

Koch et al. reported on 28 patients who received 250 mg testosterone ester mixture per week for 6 months (71). Patients receiving androgens had a mean hematocrit of 36.8% compared to 25.4% in patients not receiving treatment and dialyzed for the same time. Koch found that androgens stimulated erythropoiesis in bilaterally nephrectomized patients but the effect was less pronounced and higher doses were necessary than in patients with kidneys. Buchwald et al. performed another study with 17

male and 4 female patients on maintenance hemodialysis (17). Male patients were treated with 200 mg of nandrolone decanoate, while the female patients received 100 mg per week for 4 months. Control patients received an injection of sesame oil. The results of this study confirmed that nandrolone decanoate increased serum erythropoietin levels, packed cell volume, hemoglobin concentrations and red cell mass of patients. In a study conducted by Richardson et al., 15 male patients on maintenance dialysis were given courses of testosterone enthanate in weekly doses of 400 to 600 mg for 5 to 44 weeks (103). 13 of the patients exhibited a mean increment in hematocrit (5.6%), red cell volume (353 ml) and red cell volume per kilogram body weight (4.4 ml/kg). The treatment eliminated transfusion requirements in most of their patients; of note most patients on androgens developed acne while one patient developed a mild increase in serum bilirubin and enzyme values, suggesting intra-hepatic cholestasis.

In addition, a study with 24 male, regular dialysis patients receiving 250 mg testosterone ester mixture demonstrated a significant increase in hemoglobin concentration from 6.8 to 8.2 g/dl in six months of treatment (131). Side-effects were essentially mild and acceptable but one patient developed priapism and another a large hematoma. Rishpon-Meyerstein et al. described a 39-year old male with chronic renal disease (104), who was treated with 100 mg testosterone propionate, twice per week, for three weeks. Accordingly, his plasma EPO titer, expressed as the percentage $Fe^{59}$ uptake into newly formed RBC's in a mouse assay, increased to 6% $Fe^{59}$ incorporation from a baseline of 1%. Upon increasing the dose of testosterone to 200 mg, the EPO titer again rose to 16%.

*b) Androgen use in the EPO era*

It was not until 1987 that r-HuEPO became available for the treatment of the anemia of CRF. Because of the key role of r-HuEPO in the management of the anemia of renal disease, androgen use diminished considerably. Despite widespread use of r-HuEPO, the cost of treatment, development of complications (hypertension, seizures and occasional cases of hyporesponsiveness) prompted a resurgent interest in androgens.

Upon the premise that androgens increase the sensitivity of erythroid progenitors to EPO, Ballal et al. examined the effect of androgens on the action of exogenous r-HuEPO (7). Seven adult male hemodialysis patients (mean age 48 years) received 2000U EPO three times per week for 14 weeks. At the same time, 8 adult male hemodialysis patients (mean age 54 years) received a combination of 2000U EPO three times per week and 100 mg of nandrolone decanoate once weekly, also for 14 weeks. Results showed that the group receiving a combination of the two therapies had a significant increase in their hematocrit (from a mean of 24.4% to 32.9%) compared to the group receiving r-HuEPO alone. No side effects were seen with nandrolone in this short term study.

Pascual et al. demonstrated the effect on blood pressure of three different forms of therapy for correction of the anemia of chronic renal failure. Patients were treated with either r-HuEPO (50 U/kg, 8 males, 4 females), intravenous (i.v.) iron (1 g divided into 8 doses, 5 males, 5 females), or nandrolone decanoate (200 mg/wk, 7 males, 2 females) for 6 months. This group noted that some patients being treated with r-HuEPO needed antihypertensive medication before there was any clear rise in hematocrit. In addition,

they demonstrated an absence of any blood pressure increment after partial correction of anemia with i.v. iron or nandrolone at similar rates and hematocrit levels.

More recently, in 1996, the group of Teruel demonstrated an equivalent response of patients to nandrolone decanoate and r-HuEPO (120). 18 male patients, aged over 50 years, received 200 mg/wk nandrolone for 6 months, while 22 male (<50 yrs) and female patients received 2000 IU r-HuEPO three times per week for 6 months. Increases in hemoglobin concentrations were similar for both groups. The group treated with androgens had additional increases in serum albumin and dry weight, suggesting an appreciable anabolic effect. Patients treated with nandrolone did not report any severe side effects.

This latter study has several strengths and weaknesses. Its strengths include: 1) the administered androgens produce a similar rise in hemoglobin as r-HuEPO alone, 2) androgen therapy costs much less than r-HuEPO therapy, 3) the androgens have an appreciable anabolic effect and 4) patients did not experience an increase in blood pressure as did those on r-HuEPO. However, its weaknesses include a selection bias as the groups chosen by Teruel et al. may not represent a global picture of the dialysis population.

Finally, in the study of Davies et al., 26 patients received 100 mg/day oxymetholone while 29 patients received a placebo for three months (27). At the end of the 3-month period, the 26 patients had their oxymetholone replaced by a placebo and the 29 patients started receiving oxymetholone instead of the placebo. 8 of the 55 patients were anephric. 39 of the 55 patients completed the trial. Davies showed no significant

change in hemoglobin and hematocrit in these 39 patients. Davies et al. concluded that oxymetholone, at this dosage, had little or no role in treating the anemia of renal failure.

### *c) Proposed mechanisms of action of androgens*

Although androgens seem to increase serum EPO levels many of the aforementioned studies were conducted prior to accurate EPO measurements. Despite major advances in understanding the mechanism of action of androgens, many questions remain concerning their molecular action on EPO production and whether or not these steroids or their metabolites directly stimulate the bone marrow. Since androgen use in CRF began in the early 1950s, investigators have hypothesized on the possible mechanism of action of androgens.

A direct action of androgens on the blood forming organs has been supported by the findings that testosterone stimulates erythropoiesis (33, 102) and DNA synthesis (102) in bone marrow cultures. A pronounced shift from early erythroid cells (pronormoblasts) to late erythroid cells (basophilic and polychromatic normoblasts) was noted when testosterone (0.1 mg/ml) was combined with EPO (0.165 units/ml). Potentiation of the erythropoietic effects of EPO has also been demonstrated by Naets et al. (93). This group found that the response of polycythemic mice to exogenous EPO or hypoxic stimulation was markedly enhanced by testosterone, whereas no erythropoietic response was elicited in polycythemic mice treated with androgens alone. Necheles et al. (94) postulated that androgens produced an increase in the number of EPO-responsive stem cells in the bone marrow thus providing a larger number of EPO-responsive stem cells for EPO to exert its primary effect. More recently, Ballal et al. found that patients

treated with a combination of nandrolone decanoate and r-HuEPO had a significantly higher hematocrit than patients on EPO alone after 14 weeks of treatment (7). Based on their results, this group concluded that the mechanism of action of nandrolone decanoate had to involve increased sensitivity of erythroid progenitors after androgen treatment.

Fried et al. observed that the anemia of patients with chronic renal failure becomes more severe and more refractory to androgen therapy after nephrectomy (40). They explain this by stating that androgens stimulate erythropoiesis in patients with CRF, largely by increasing the amount of EPO produced by the kidney remnants. Similarly, von Hartitzsch et al. postulated that the mode of action of androgens includes an increase in EPO production from the remnant kidneys (131). The nephrectomized patients in their group showed no response to androgens. This is in keeping with the experience of most authors that observe a poorer response than in patients who retained their kidneys, thereby requiring larger doses of androgen. von Hartitzsch gave little credence to the possibility of androgens having a direct effect on the bone marrow or on extra-renal sites of EPO production. Koch et al. also observed a less pronounced effect of androgens on nephrectomized patients, thus requiring higher doses of androgen to produce a hematological effect (71). However, Koch hypothesized that because of positive results in nephrectomized patients, androgens not only increased extra-renal EPO production or secretion but also increased the number of EPO-sensitive bone marrow cells. Eschbach at al. believed that the probable mechanism of androgen action is an EPO-dependent increase in erythropoiesis (35). Through stimulation of extra-renal EPO-producing sites, erythropoiesis may be increased in some anephric patients, however the rate of erythropoiesis is less than optimal.

Shahidi et al. stressed the importance of kidneys for the production of EPO during androgen therapy (111). They found that in laboratory animals, the kidneys seem to play a major part in the stimulation of EPO, because the response to androgens is completely abolished in nephrectomized rodents. They added that in man, because of the extra-renal source of EPO, the presence of kidneys does not seem essential for androgens to stimulate erythropoiesis. Shaldon et al. have demonstrated a remarkable erythropoietic response after androgen therapy in nephrectomized patients on chronic hemodialysis (112). Furthermore, DeGowin et al. suggested that such patients might show an even greater erythropoietic response if one of the kidneys were still present (28). Dainiak also proposed that androgens may stimulate erythropoiesis indirectly by enhancing EPO production (26). Contrary to other clinical observations, he has observed that nephrectomized patients appear to respond as well to androgens as do patients with their native kidneys still in place.

While the existence of significant EPO production outside the kidneys is well established, the extent and localization of such production is less certain, especially in CRF. The potential for extrarenal production of EPO is an important consideration in the anemia of CRF, but might be altered as a consequence of uremia. In a mouse model of chronic renal failure (44), Zhang et al. examined EPO mRNA expression in renal as well as extra-renal sites (138). In a surprising finding, there was absent EPO mRNA expression in the liver and the bone marrow - two of the proposed sites of EPO production during kidney disease. EPO mRNA expression was minimal in the kidney remnant.

*d) Synthetic androgens and their activity*

The steroid hormones can be described as classes based on the carbon number in their structures. For example, sex hormones can be distinguished easily by the carbon number, C-19 being androgens, C-18 being estrogens, and C-21 being progestational or adrenal steroids. Within the androgen class of C-19 compounds, there are several sub-classes:

1) **Testosterone esters** which include testosterone enanthate (Delatestryl®) and testosterone propionate (Oreton-P®);

2) **17α-alkylated compounds** such as fluoxymesterone (Android-F®), oxymetholone (Anadrol-50®) and methyl testosterone (Oreton-Methyl®); and

3) **Norsteroids** such as nandrolone decanoate (Deca-Durabolin®) and nandrolone phenylpropionate (Durabolin®).

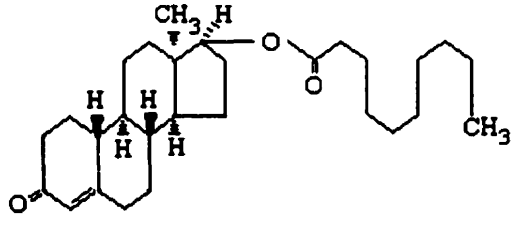
The formation of esters, such as fluoxymesterone and oxymetholone, protects against metabolic inactivation and brings about a protracted biologic effect. The introduction of the 17α-alkyl substituent, such as in the 17α-alkylated compounds, prevents metabolic inactivation through oxidation of the 17-hydroxy group to a 17-keto group. The presence of the alkyl group is also responsible for oral activation of these compounds.

In the case of the norsteroids, the C19 methyl group of testosterone has been replaced by a hydrogen atom. In 19-nortestosterone derivatives, there is an important dissociation in anabolic and androgenic activity, however, it is incomplete and variable. Testosterone can be, experimentally, induced to undergo a number of configuration

changes, including the elimination of the C19 methyl group, the loss of the -OH group at C17 and the addition of an $-OCO(CH_2)_8CH_3$ at C16, to form nandrolone decanoate.

Today, androgens of all types have been used in treating the anemia associated with renal insufficiency. While they are effective in nearly one half of cases, the hematocrit of these patients usually remains under 36% and rarely reaches normal levels (26). Certain agents appear to be more effective, such as those that are administered parenterally, suggesting that the route of administration may be important in obtaining a favorable response. Agents such as oxymetholone have shown little efficacy as assessed by reduction in blood transfusion requirements (27). Because norsteroids with low androgenic-anabolic ratios (1.0:2.5), nandrolone decanoate for example, are often successful (17, 20, 90), it is possible that the anabolic effect rather than the androgenic activity of the hormone preparation is critical to its erythropoietic action. It has been suggested that androgens having an angular configuration (5β-epimers) stimulate stem cell proliferation to a greater degree than those whose configuration is planar (5α-epimers), which may act primarily by enhancing EPO production (35). The configuration of nandrolone is planar and thus could be involved in increasing EPO production, from yet unknown sites.

The pharmaceutical name for nandrolone (3-oxo-estr-4-en-17β-yl decanoate) is Deca-Durabolin, and it is manufactured by Organon, Spain. It is a derivative of testosterone, with the molecular formula $C_{28}H_{44}O_3$ (see left). It has a molecular weight



*Nandrolone Decanoate*

of 428.66 daltons. It contains no less than 97.0% and no more than 103.0% of $C_{28}H_{44}O_3$, calculated with reference to the dried substance.

Deca Durabolin® is a white to creamy white crystalline powder and its odor is faint. It is practically insoluble in water, freely soluble in chloroform, in ethanol (96%), in ether, in fixed oils and in esters. The preparation must be preserved in tightly closed, light-resistant containers, and be stored in a refrigerator. In a 2% w/v solution in 1,4 dioxan, its specific rotation is +32° to +36°, calculated with reference to the dried substance.

## 4. A Mouse Model of CRF

A mouse model of chronic renal failure has been developed in young adult female mice of the C57BL/6 inbred strain (43, 44, 45). Renal failure was induced surgically by two procedures set two weeks apart. First, electrocoagulation of the surface of the exposed right kidney is performed followed by a left nephrectomy. Detailed analysis of the resulting model six weeks after the onset of renal failure demonstrated the full spectrum of characteristics observed in humans with severe CRF: expected retention of nitrogenous compounds together with hyperkalemia and acidosis; growth retardation; lipid abnormalities and accelerated atherosclerosis in defined mouse strains such as the one used in this study; immunological deficits particularly increased susceptibility to infection; florid bone disease; and normochromic-normocytic anemia whose severity is proportional to degree of renal failure. Additionally, the mouse is a species which has been used extensively in previous studies of non-renal anemias. Thus, this model

appeared highly suitable for the planned investigations on the mechanism of action of androgens in the correction of the anemia of CRF.

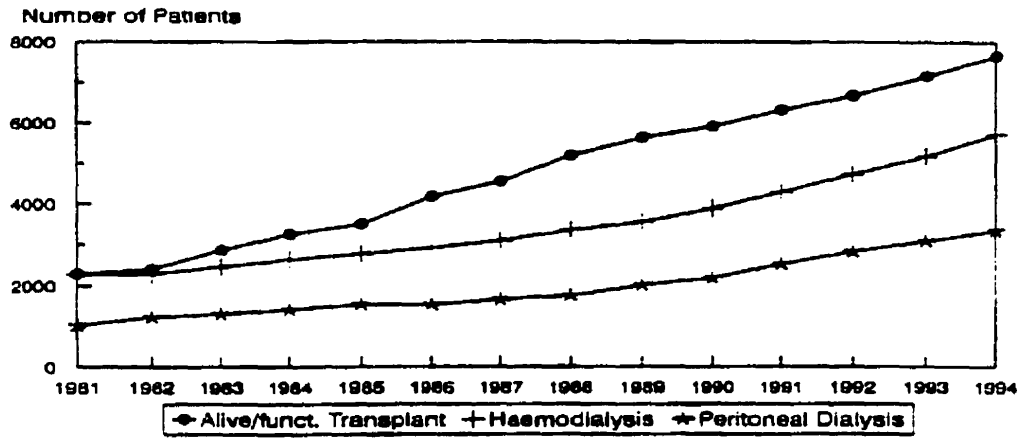



**Figure 1.** Percentage distribution of renal failure patients by treatment, Canada, December 31, 1994.

Peritoneal dialysis includes continuous ambulatory peritoneal dialysis (CAPD), intraperitoneal dialysis (IPD), Continuous cycling peritoneal dialysis (CCPD) and peritoneal dialysis used in combination with hemodialysis.

*Annual Report 1996, Volume 1: Dialysis and Renal Transplantation, Canadian Organ Replacement Register (CORR)*, Canadian Institute for Health Information, Ottawa, Ontario, March 1996. The data reported here have been supplied by the Canadian Organ Replacement Register, a Registry of the Canadian Institute for Health Information. The analysis and interpretation of these data are the responsibility of the author and do not necessarily reflect official policy or interpretation of CORR.

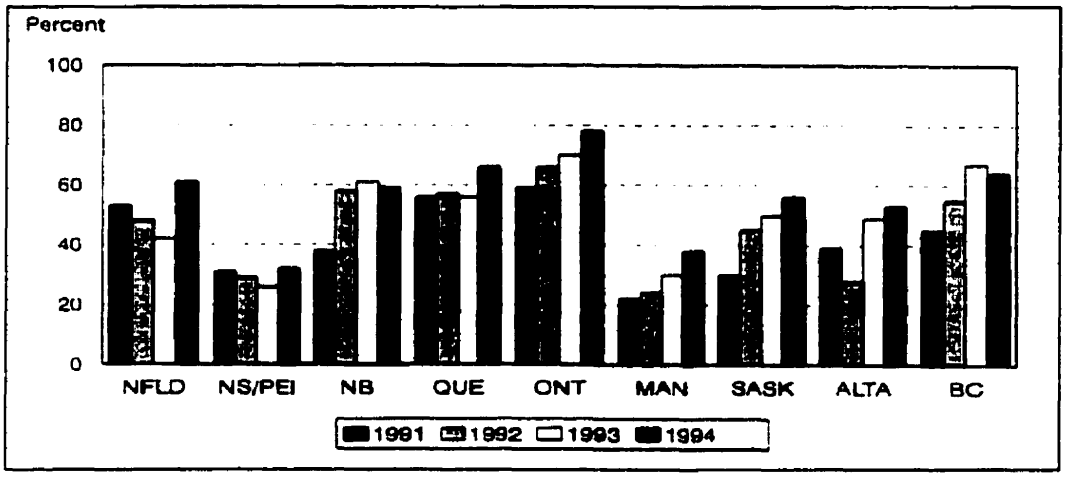



**Figure 2a**

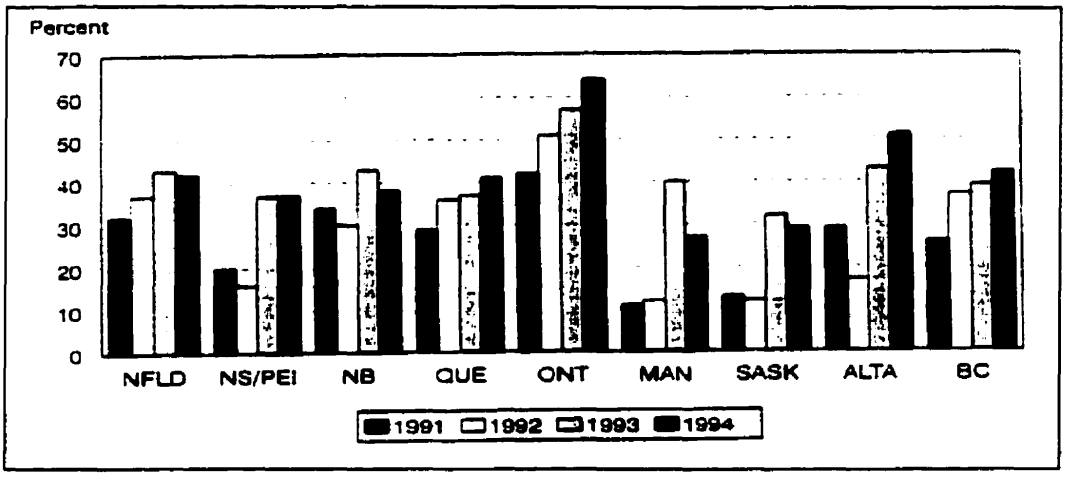



**Figure 2b**

**Figure 2a.** Hemodialysis patients receiving erythropoietin (EPO) by province and year.
**Figure 2b.** Peritoneal dialysis patients receiving erythropoietin (EPO) by province and year.

*Annual Report 1996, Volume 1: Dialysis and Renal Transplantation, Canadian Organ Replacement Register (CORR)*, Canadian Institute for Health Information, Ottawa, Ontario, March 1996. The data reported here have been supplied by the Canadian Organ Replacement Register, a Registry of the Canadian Institute for Health Information. The analysis and interpretation of these data are the responsibility of the author and do not necessarily reflect official policy or interpretation of CORR.

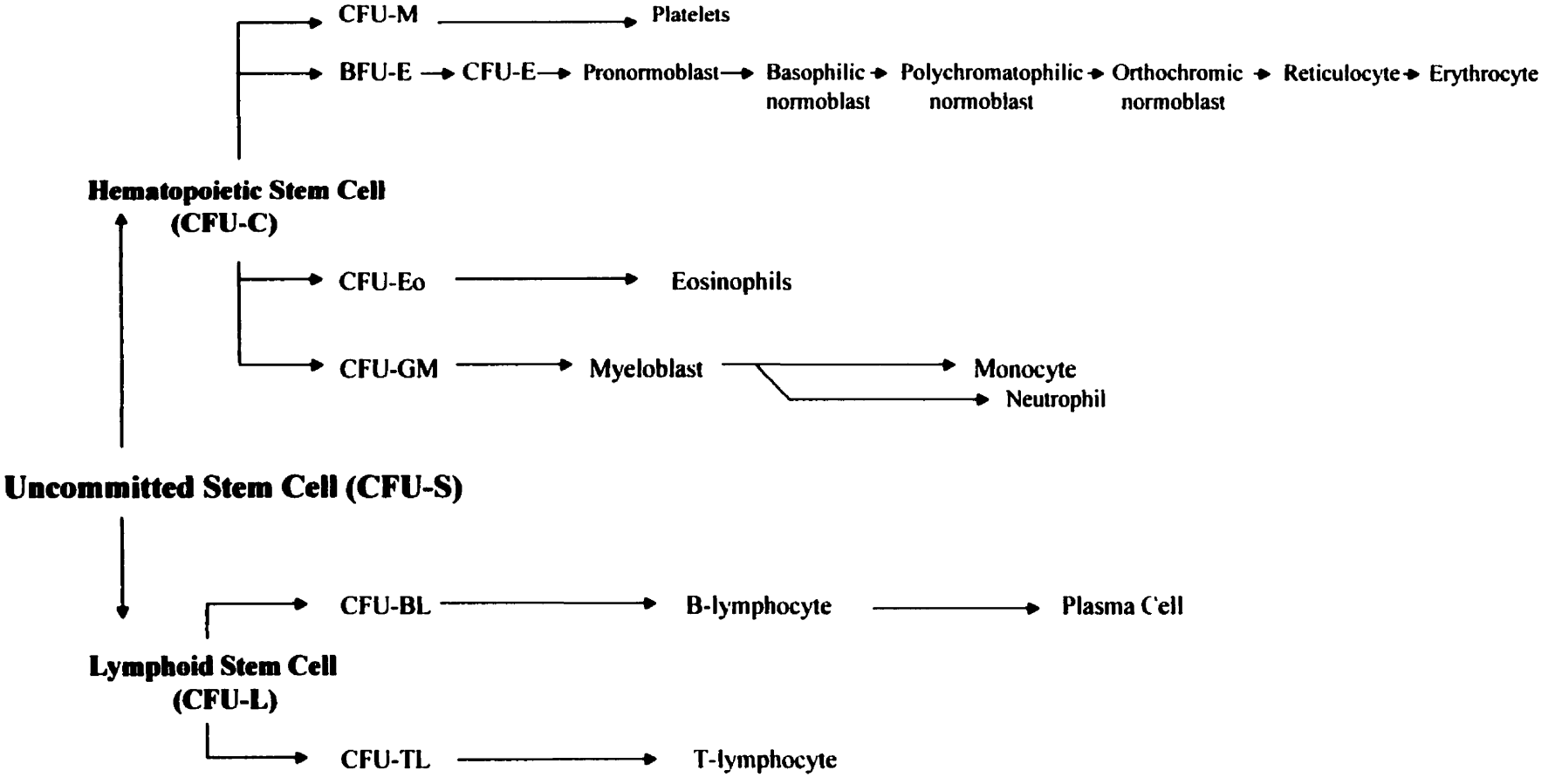



**Figure 3.** Hierarchy of hematopoiesis

CFU-S: Colony-forming units-spleen; CFU-L=CFU-lymphocyte; CFU-C=CFU-culture; CFU-BL=CFU-B lymphocyte; CFU-TL=CFU-T lymphocyte CFU-GM=CFU-granulocyte/macrophage; CFU-Eo=CFU-eosinophil; BFU-E=burst forming unit-erythroid; CFU-E=CFU-erythroid; CFU-M=CFU-megakaryocyte.

# AIMS OF THE STUDY

1. To further characterize this mouse model of renal failure particularly with regards to bone and bone marrow morphology.
2. To delineate the effects of CRF on circulating hematological parameters.
3. To establish a dose-response curve to nandrolone decanoate.
4. To determine whether nandrolone treatment is effective in increasing serum EPO concentrations and, by extension, hemoglobin concentrations significantly.
5. To confirm earlier original observations of the effects of CRF on the gene expression of EPO in the selected tissues of the kidney and liver using RT-PCR.
6. To observe, upon treatment with androgens, the EPO mRNA expression in CRF mice.
7. To examine the mechanism of action of androgens on the anemia of CRF.

# MATERIALS AND METHODS

## 1. Animals

### *a) Selection*

Female mice of the C57BL/6 inbred strain were purchased from Harlan-Sprague Dawley Inc. (Indianapolis, USA) and Charles River Canada (St. Constant, Québec). The mice, bought in groups of 40 animals, weighed approximately 20 g, corresponding to 5-7 weeks of age. Excluded from this study were runt mice, mice with low body weight (<17 g) and, because of the need for bleeding from the retro-orbital venous plexus, those with congenital eye abnormalities. This mouse strain has been successfully used previously in this laboratory to induce a reproducible, stable, severe renal failure by surgical means in young adults (43). This strain has also been used elsewhere extensively in studies of erythropoiesis (72). Female mice were used because of their docile character to reduce animal infighting during the planned long-term experiments. Female mice were also selected because of the known hepatotoxicity to a number of chemical agents (mostly used for anesthesia) demonstrated by male mice of the C57BL/6 strain (114).

### *b) Maintenance*

The mice were maintained under standard conditions of husbandry in the Animal Facilities of the Royal Victoria Hospital (RVH), Montreal. Groups of 3-5 mice were housed in standard, polycarbonate plastic shoe box cages with filter tops (Microbarrier®, Allentown) on woodshaving bedding. The mice had free access to autoclaved, untreated tap water and rat chow (Charles River Autoclaved Rodent Chow 18% 9F). Following each of the two surgical procedures to induce renal failure, powdered rat chow was placed in a small petri dish at the bottom of the cage for easier access. After arrival in the RVH

animal facilities, the mice were left for one week to acclimatize. The mice were then weighed on a flatbed scale (DeltaRange®, Mettler BB2440) and they were ear-labeled for future identification. The mice were inspected daily for level of activity and hair grooming, and, when appropriate, healing of surgical wounds. Dead animals were immediately removed from the cages, and the date of death was recorded. Whenever possible, an autopsy examination was performed. All animal procedures except the sacrifice were performed in the RVH animal facilities. Sacrifice was performed in the laboratory of Dr. Alan Brox, RVH Research Institute, because of ease of access to storage at -80°C for the various biological samples harvested.

*c) Induction of renal failure*

Renal failure was induced surgically in the mice by two procedures set two weeks apart: electrocoagulation of the surface of the right kidney followed by left nephrectomy. The right kidney was approached through a 2 cm-long skin incision in the right flank. Following exposure, the kidney was freed from the perirenal fat and adrenal gland by fine tissue dissection and brought up to the skin surface. Using a hand-operated pencil cauterizer (Valleylab Inc., Boulder, CO, USA), the entire surface of the kidney was electrocoagulated except for a 2 mm rim of intact tissue around the hilum (Plate 1). After the electrocoagulation, the kidney was guided back into the right renal fossa. Great care was taken to avoid damage to the right ureter from either direct trauma or elongation / distortion during mobilization of the kidney. The deep layer of the surgical wound was closed with a running suture using 4-0 silk and a few drops of antibiotic (Neosporin®,

1 ml /1000 ml saline, Burroughs Wellcome Inc., Canada) were applied to the wound surface before closing the skin with stainless steel surgical autoclips.

A left nephrectomy was performed two weeks later. The left kidney was dissected and exposed through a left flank incision as described for the right kidney and the vascular pedicle was ligated at the hilum using 6-0 silk. The vascular tissue was sectioned distal to the suture and the kidney was removed. The surgical wound was closed as above. Anesthesia was provided through a s.c. injection of atropine (0.03 mg/kg) followed 10 min later by an i.p. injection of a mixture of ketamine (Ketaset®, 100 mg/ml) and xylazine (Rompin®, 20 mg/ml). Methoxyflurane (Metofane®, Janssen Pharmaceutica, Mississauga, Ontario) was administered through a nose cone when deemed necessary. The administration of the Metofane® was strictly controlled because of its well-known hepato-renal toxicity. The skin clips were removed 5-6 days after each surgical procedure. The time of the left nephrectomy marks the onset of the renal failure. To assess the renal status, the blood urea level was determined at various times (1-6 weeks) after the nephrectomy. Mice with renal failure of 6 weeks' duration were designated as having chronic renal failure (CRF).

Selected experiments were also performed with normal, unoperated mice. These mice were housed and maintained under the same conditions as the operated mice. Bone and bone marrow histology sections were also performed on these mice. Lastly, phlebotomized mice were used as controls in molecular biology experiments. Both normal and phlebotomized control mice were of the same age as the CRF mice. The phlebotomized controls consist of normal mice bled, from the retro-orbital venous plexus,

of a volume of 0.4 ml for 3 consecutive days before sacrifice. Previous studies have demonstrated that this protocol induces severe anemia with all the predictable physiological consequences of this acute state, especially increased EPO production from both the kidney and liver (138).

***d) Blood collection and processing***

Before the start of the treatment periods, the mice were bled from the retro-orbital venous plexus using heparinized, 75 mm capillary tubes (Microhematocrit®, Fisher Scientific, Pittsburgh, PA). 75 µl of blood was collected and the hemoglobin concentration was measured by spectrophotometric analysis at 520 nm (UltroSpec 3000®, Pharmacia Biotech, Cambridge, England).

At the time of sacrifice, 150 µl of blood were drawn from the retro-orbital venous plexus again using Microhematocrit® capillary tubes and sent to the RVH Division of Hematology for a complete blood count (CBC). Subsequently, the mice were exsanguinated using a subdiaphragmatic cardiac puncture. By this method, an average blood volume of 0.8 ml was obtained from each mouse. The blood samples were placed separately in polypropylene tubes (Microtainer®, Fisher Scientific, Pittsburgh, PA) and allowed to stand at room temperature for at least 2 hr. After clot formation and retraction, the tubes were centrifuged at 7500 x g for 3 min at 4°C in a microcentrifuge (Savant Instruments Inc., SFR13K, Farmingdale, NY, USA). The sera were then transferred to appropriately labeled micro-centrifuge, polypropylene tubes (VWR Scientific Products, USA) and stored at -80°C until use.

*e) Harvesting and processing of tissues*

At the time of sacrifice, samples of the liver and kidneys were collected sequentially, snap-frozen and stored at -80°C for subsequent determination of mRNA expression. In selected experiments, the knee joint together with the tibia and femur of a hind leg were also obtained for characterization of bone disease (see Section 6 below: Bone and Bone Marrow Histology). Sacrifice of the animals, not in the fasting state, was always performed in early morning. The time required for the collection and intial processing of all tissues did not exceed 5 min per mouse.

Immediately following cardiac puncture, the mice were pinned down to a Styrofoam board, in dorsal decubitus.

1) Liver: The skin of the mice was opened from the chest downward to expose the underlying tissues of the abdomen and hind legs. The peritoneal cavity was opened aseptically to expose the liver and 4-5 small samples (0.2 x 0.2 cm) of the left lobe of the liver were resected.

2) Kidney: In mice with renal failure, the single remaining electrocoagulated right kidney was removed following fine tissue dissection of any adhesions with surrounding tissues, especially the liver. In selected experiments including control mice with normal renal function and phlebotomized mice, both kidneys were removed following dissection of the retroperitoneal space.

## 2. Routine Blood Tests

*a) Urea*

Urea concentrations in serum were measured by means of the Beckman-developed enzymatic conductivity rate method using Synchron CX® Delta Systems (Beckman Instruments, Inc.). A volume of 10 µl was placed into a reaction cup containing a urease solution (Synchron Cx BUN reagent). The ratio used was one part sample to 100 parts reagent. The reaction converts the non-ionic species (urea) to an ionic form (ammonium carbonate). During the reaction period, the timed rate of increase of solution conductivity is directly proportional to the concentration of urea present in the reaction cup.

***b) Complete blood count***

Hemoglobin concentrations were measured by spectrophotometric analysis at the beginning of treatment and at sacrifice at selected times (1, 3, and 6 weeks of renal failure). At sacrifice, a CBC was performed on 120 µl of heparinized blood using the Technicon H-3 RTX® counter (Miles, USA) in the RVH Division of Hematology-Oncology. To compare the hemoglobin values obtained by Technicon counter to those obtained by spectrophotometric analysis, 25 blood samples were drawn and subjected to measurements by each of the two methods. There was no significant difference in hemoglobin concentration between the two methods. The CBC determination provided the following relevant results: white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (Hct), mean corpuscular volume (MCV), and platelet count (Plt).

*c) Biochemical tests for characterization of bone disease*

Serum levels of urea, creatinine, total calcium, magnesium, phosphate, and alkaline phosphatase were measured by automated methods (BM/Hitachi 911 counter, Boehringer Mannheim). Testing of each parameter requires addition of the specific reagent for that parameter to a certain amount of serum. To measure serum creatinine levels, 350 µl of sodium hydroxide and 350 µl of picric acid (a starter reagent) were added to 20 µl of serum. Creatinine forms a colored complex with picrate in alkaline solution. The rate of complex formation was then measured. To measure total serum calcium levels, 250 µl of buffer and 100 µl of chromogen (a starter reagent) were added to 10 µl of serum. The calcium ion, $Ca^{2+}$, forms a violet complex with o-cresolpthalein complexone in an alkaline medium. Serum magnesium levels were measured by adding 200 µl of buffer / EGTA (ethylenebis [oxyethylenenitrilo]-tetraacetic acid) and 200 µl of xylidyl blue (a starter reagent) to 6 µl of serum. Magnesium ions form a purple-red complex with xylidyl blue in alkaline solution. The magnesium concentration was measured bichromatically as the decrease in absorbance of xylidyl blue at 600 nm. Interference by calcium is prevented by EGTA contained in the buffer. Phosphate levels were measured by adding 250 µl of reagent serving as a blank and 110 µl of phosphate reagent (a starter reagent) to 5 µl of serum. Inorganic phosphates react with ammonium molybdate in sulfuric acid solution to form an ammonium phosphomolybdate complex. To measure alkaline phosphatase levels, 250 µl of buffer/$Mg^{2+}$ reagent and 50 µl of p-nitrophenyl phosphate were added to 11 µl of serum. The formation of p-nitrophenol

and phosphate from p-nitrophenylphosphate and water, a reaction catalyzed by alkaline phosphatase, was measured spectrophotometrically at a wavelength of 660 nm.

## 3. Serum Erythropoietin

Serum EPO levels were measured by a commercial radioimmunoassay designed for the quantitative measurement of human serum EPO (Diagnostic Systems Laboratories Inc, cat. no. DSL1100, Webster, Texas, USA). 100 µl of each of the serum samples were assayed in duplicate together with the recommended standards and kit controls. Upon addition of the radiolabelled $^{125}$I-EPO antibody, the samples were allowed to stand for 18 hr at room temperature. A precipitating reagent was then added to all tubes. Following incubation for 30 min and centrifugation for 30 min, the radioactivity in each of the samples was counted using a gamma counter.

## 4. Serum Insulin-like Growth Factor-I

Levels of free serum IGF-I were measured by a commercial radioimmunoassay designed for the quantitative measurement of human serum IGF-I (Nichols Institute Inc., cat. No. 40-2100, Los Angeles, California, USA). The procedure to measure free serum IGF-I began with an acid-ethanol extraction. 100 µl of serum were mixed in 1 ml acid ethanol solution (12.5% acid, 87.5% ethanol) and incubated at room temperature. After centrifugation for 30 min, 200 µl of the supernatant were added to 100 µl of Tris base solution and incubated for 30 min at room temperature. The mixture was again centrifuged for 30 min. Subsequently, 100 µl of the resulting supernatant were added to

1.4 ml of phosphate buffer. The final dilution of the serum sample was 1/225. 50 µl of the diluted sample was mixed with rabbit anti-IGF-I and incubated for 1 hr. Radiolabelled $^{125}$I-IGF was added to the mixture which was then incubated for 16-18 hr at 4°C. Goat-anti-rabbit precipitant was added to the radiolabelled mixture. Normal rabbit serum was also added to help precipitate the antigen-antibody complexes. The mixture was incubated at room temperature for 20 min. Subsequently, it was centrifuged and the supernatant decanted to collect the pellet. Radioactivity in the pellet was counted using a gamma counter. All samples were measured together with the recommended kit standards and controls.

## 5. Expression of Erythropoietin mRNA

The gene expression of EPO in selected tissues from mice with renal failure and selected controls (normal and phlebotomized mice) were compared by determining mRNA tissue levels. At the same time β-actin mRNA was detected from each of the same samples as an internal control. RT-PCR was used to reverse transcribe the total mRNA in the test samples into cDNAs, followed by the amplification of these cDNAs. These products were analyzed by Southern blotting using appropriate mouse EPO and β-actin probes.

***a) RNA extraction for RT-PCR***

To prepare for RT-PCR, RNA must be extracted from each tissue. First, each tissue was placed in 1 ml TRIZOL® Reagent (Gibco BRL) and blended using a homogenizer (Polytron®, Brinkmann Instruments, Westbury, NY). After incubation of the homogenized samples at room temperature, 0.2 ml of chloroform was added to

separate the mixture into aqueous and organic layers. The samples were centrifuged at 12000 x g for 15 min and the aqueous layer, which contained the RNA, was removed. The RNA was then precipitated from the aqueous phase using isopropanol. After incubation at room temperature for 10 min, the samples were centrifuged at 12000 x g for 10 min. The RNA precipitate, usually not visible to the naked eye before centrifugation, formed a gel-like pellet on the walls and bottom of the tube.

The supernatant was then decanted and the RNA pellet was washed with 75% ethanol. The sample was then vortexed and centrifuged at 7500 x g for 5 min. At the end of the procedure, the RNA pellet was allowed to air-dry for 5 to 10 min and then was redissolved in diethylpyrocarbonate-treated (DEPC) water and incubated for 10 min at 55°C. To test the purity of the RNA extraction, absorbances at 230 nm, 260 nm, and 280 nm were measured using a spectrophotometer. The ratio of absorbance at 260 nm to that at 280 nm should always be greater than 1.5, indicating that the extraction is free of any contaminating proteins.

Subsequently, 1 µl of the extracted RNA, mixed with 1 µl of loading buffer [0.25% Bromophenol Blue, 0.25% Xylene Cyanol FF, 0.2M EDTA, 40% (w/v) sucrose in water] and 4 µl of distilled water, were resolved by 1% agarose-TAE buffer electrophoresis. The purpose of this step was essentially to verify whether the extraction was pure and not degraded. Optimally, we should see three bands corresponding to the 18S and 28S RNA and the tRNA.

*b) Reverse transcription and polymerase chain reaction*

Total cell RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (21) and any residual contaminating genomic DNA was eliminated by digestion with 1 unit of DNAse I (Pharmacia) at 37°C for 10 min. The reaction was stopped by heating samples at 95°C for 5 min. RNA was reverse transcribed and murine EPO, and β-actin sequences were amplified by the polymerase chain reaction (RT-PCR), using reagents supplied in the GeneAmp thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer Cetus, Norwalk, LT), following procedures recommended by the manufacturer. The reaction mixtures for reverse transcription were carried out in 0.5 ml microcentrifuge tubes and included 250 ng (β-actin) or 50 ng (EPO) of RNA, 1 x reverse transcriptase buffer, 200 μM dNTPs, 5 units rTth polymerase and, 0.15 μM downstream primer in a total volume of 20 μl overlaid with 100 μl of mineral oil. Negative controls (mixtures in which sample RNA was omitted) were included in each experiment. Samples were incubated at 70°C for 5 min and the reaction was stopped by cooling samples on ice. PCR reaction mixtures were prepared by adding 80 μl of solution containing 1 x chelating buffer, $MgCl_2$ (1 mM final concentration), and the upstream primer (0.15 μM final concentration) to the reverse transcriptase mixtures in a total volume of 100 μl. Temperature cycling was performed in a Perkin-Elmer Cetus DNA Thermal Cycler using the following protocol: 94°C for 5 min x 1 cycle, 60°C for 5 min x 1 cycle, (72°C for 1.5 min, 94°C for 45 sec, 60°C for 45 sec) x 35 cycles and 72°C for 10 min x 1 cycle. The sequences for the primers used were as follows:

EPO-upstream: 5'-AGGAGGCAGAAAATGTCACGATG-3' (bridges the second and third exons),

EPO-downstream: 5'-TGTTCGGAGTGGAGCAG-3' (exon 5);

β-actin-upstream: 5' - TGTGATGGTGGGAATGGGTCAG-3';

β-actin-downstream: 5'-TTTGATGTCACGCACGATTTCC -3'.

Oligonucleotides for EPO and β-actin were synthesized by and obtained from Sheldon Biotechnologies. The predicted size of the amplified fragments is 379 bp for EPO and 245 bp for β-actin.

***c) Southern blotting***

20 µl aliquots from the PCR samples were resolved by 2% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed before transfer to nylon membrane (BMC, Canada) by the method of Southern using standard procedures (100). Membranes were pre-hybridized at 42°C for 2 hr in 6 x SSC, 1 x Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS, Terochem Scientific, Canada) and 50 µg/ml herring sperm DNA (BMC). cDNA probes were labelled with [$\alpha^{32}$P]-dCTP to a high specific activity (typically 1-2 x $10^9$ dpm/µg), by the random primer method using a commercial kit (Multiprime, Amersham). Hybridization was carried out overnight at 42°C in 50% formamide (Gibco-BRL), 5 x SSC, 10% dextran sulfate (Pharmacia), 0.5% SDS and 50 µg/ml herring sperm DNA. Membranes were subsequently washed twice in 6 x SSC, 0.1% SDS for 15 min at room temperature; twice in 1 x SSC, 0.5% SDS for 15 min at 37°C; once in 0.1% SSC, 0.5% SDS for 30 min at

65°C, and exposed to Kodak X-AR5 film (Eastman Kodak Co, Rochester, NY, USA). The probe used (kindly donated by Dr. Prem Ponka) was as follows: 1.1 kb *Pst*I insert from the plasmid pUC19 containing murine genomic EPO sequences including exons 2, 3 and part of 4.

## 6. Bone and Bone Marrow Histology

The bone samples were processed according to standard histological procedures. The entire knee joint of a hind leg with attached femur and tibia was dissected from selected groups of mice. Immediately after resection, the entire knee joint was placed in a fixative solution (1 part formalin: 1 part 0.5% sucrose) and stored at 4°C for 24 hr (volume of fixative was at least 10 times the volume of the bone sample). The samples were then dehydrated in progressively increasing concentrations of propanol, starting at 50% and ending at 100%. The dehydration process took place over a period of 2 days in a volume of alcohol that was also 10 times the volume of the bone sample. The samples were then infiltrated and embedded in a glycol methacrylate-methyl methacrylate (GMA-MMA) solution for 24 hr. Subsequently, polymerization of the sample was allowed to occur at 4°C. 24 hr later, the bone samples were cut on a Reichert-Jung Supercut 2050® microtome at a thickness of 2 µm and placed on microscope glass slides. The resulting undecalcified bone sections with preserved bone marrow were then allowed to dry and were stained, according to standard procedures, with the appropriate histological stain (hematoxylin & eosin or acid phosphatase).

## 7. Treatment with Androgens

### *a) Drug preparation and administration*

A commercial injectable formulation of nandrolone decanoate (Deca-Durabolin®, Organon Canada Ltd., Whitby, Ontario, 100 mg/ml) was used for this study. A recent study by Teruel et al. (120) demonstrated the correction of anemia in dialysis patients with androgen treatment using a dosage of 200 mg/wk. In a 70 kg man, this dosage of nandrolone decanoate corresponds to approximately 3.0 mg/kg BW/wk. Based on this previous clinical experience, a dose-response curve was established, in CRF mice, using logarithmically escalating doses of nandrolone including the optimal dose, in humans, of 3.0 mg/kg BW/wk.

A 9.0 mg/kg dose was prepared first using appropriate amounts of nandrolone and saline, used as the vehicle. Subsequently, the 9.0 mg/kg solution was diluted with saline to yield additional doses of 3.0, 1.0, and 0.3 mg/kg in a 0.1 ml final volume.

Due to the low miscibility of the lipid-based nandrolone in water, special measures were used to ensure proper emulsification of the androgen preparation in saline. This procedure was performed using two syringes attached by a stopcock (Three Way Stopcock with Male Luer Slip Adapter®, Baxter Healthcare Corp., USA). The mixture of nandrolone and saline was repeatedly passed from one syringe to the other through the stopcock for approximately 5 min until the oily and aqueous phases were completely mixed.

The freshly prepared nandrolone solutions were administered by i.m. injections into the quadriceps muscle of the mice in the conscious state. Control untreated mice with renal failure received no injections.

***b) Experimental protocol***

In a previous study using this mouse model of CRF (43), the hemoglobin concentration of mice was monitored from the first to the fifteenth week following the onset of renal failure (time of left nephrectomy). This experiment demonstrated that the anemia reached its nadir by the third week and remained stable thereafter. Based on this previous work, androgen treatment was initially begun three weeks after the onset of renal failure. The nandrolone was given once per week, for three weeks with sacrifice-evaluation of the mice performed after 6 weeks of renal failure. During this intial experiment, however, the androgens did not increase the hemoglobin concentrations significantly. Based on the well-known slow action of androgens on erythropoiesis in man, it was thought that the 3-week treatment period had not been long enough to induce a hemoglobin change. For this reason, treatment in the subsequent experiments began one week after the left nephrectomy. Accordingly, in the definitive protocol, mice were treated for a period of 5 weeks while, in a time-course study, mice were treated for periods of 1, 2, and 5 weeks. The 3 different protocols used in the course of this study are shown schematically in Figure 4.

Immediately before treatment, the mice were bled from the retro-orbital venous plexus to determine their hemoglobin concentration, which formed the basis for their randomization to the different androgen-treated groups and to the untreated control group.

The mice were then sacrificed at selected times after the onset of renal failure. In the definitive protocol (Figure 4), from which the majority of the results of this thesis were obtained, the sacrifice was scheduled after 5 weeks of androgen treatment; the mice were bled under $CO_2$ narcosis and various tissue samples were procured for assessment. The following protocol was employed for the definitive treatment study:

| Time (weeks) | Procedure |
|---|---|
| 0 | Arrival of the mice and acclimatization to animal facilities |
| 1 | Electrocoagulation of the surface of the right kidney |
| 3 | Left nephrectomy (time of onset of renal failure) |
| 4 | Determination of hemoglobin concentration and randomization of mice into different experimental groups |
| 4 - 9 | Treatment with various concentrations of androgens by once-weekly i.m. injections |
| 9 | Sacrifice and evaluation |

For the purpose of comparing the serum EPO levels of androgen-treated CRF mice (above protocol) to those of CRF mice treated with r-HuEPO, a small additional experiment was conducted according to a closely similar protocol. Induction of renal failure, by electrocoagulation and left nephrectomy, occurred as in the experimental protocol for androgen treatment. However, treatment with r-HuEPO began three weeks after the left nephrectomy (induction of renal failure) and continued for 3 weeks before sacrifice / evaluation. r-HuEPO was administered s.c., at a dose of 10 U per mouse, 3 times weekly. Sacrifice / evaluation was performed in the same manner as for those mice treated with androgens.

## 8. Data Analysis

The statistical analysis and graphical presentation of all data were performed using Microsoft Excel for Windows 95, version 7.0 (Microsoft Corporation, Seattle, Washington). The response of the hemoglobin concentration to androgen treatment was

expressed as hemoglobin (Hb) change during the treatment period, calculated using the following equation: $\frac{\text{post Hb - pre Hb}}{\text{pre Hb}} \times 100$

The percent change of the mean Hb concentrations was also calculated. In addition, the mean and standard deviation of all reported parameters for each group of mice were calculated. Paired t-tests were performed between treated and untreated animals for all selected parameters. One level of significance ($p<0.05$) was retained for presentation of the data. Correlation and regression analysis were applied to selected parameters such as serum EPO and hemoglobin concentrations. The F-test was used to determine the significance of the correlations. The data are presented in the results section, first in the text and, in turn, in a series of Tables, Figures and Plates. In certain cases, a Table was inserted in a Figure in order to highlight an important aspect of the Figure.

**Initial protocol** **Definitive protocol** **Time-course study**

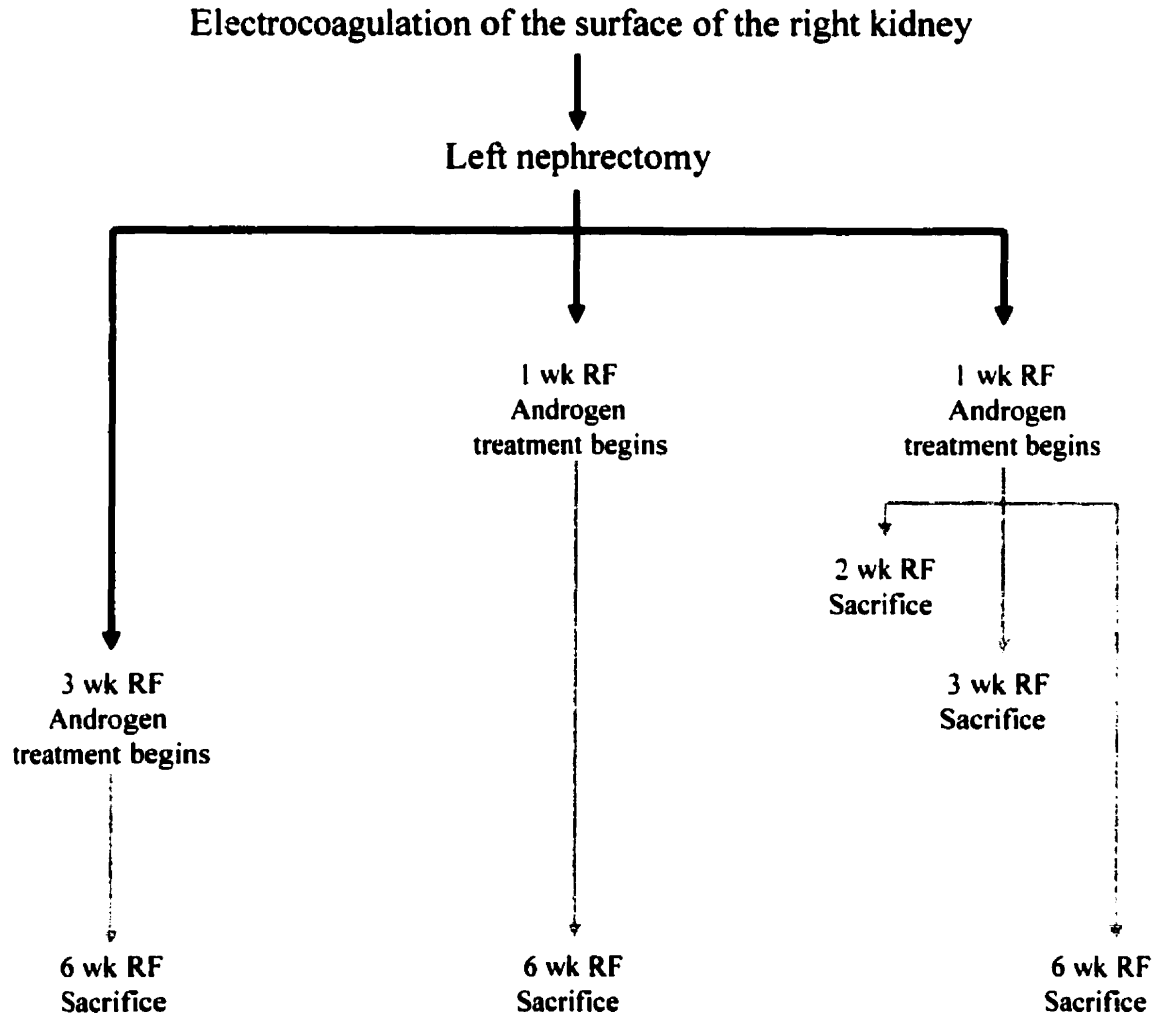



**Figure 4.** Evaluation of the effect of androgen treatment on the anemia of mice with renal failure: Outline of three experimental protocols.

The evaluation of the effect of androgen treatment on the anemia of mice with renal failure was conducted according to three separate, different experimental protocols. All 3 protocols shared the two early surgical steps: electrocoagulation of the right kidney and left nephrectomy 2 weeks later (onset of renal failure). A full evaluation was performed in all mice of each group at the time of sacrifice. The results obtained in the "definitive protocol" constitute the main part of the thesis.

The shaded portion of the vertical arrows indicates the duration of androgen treatment. RF = renal failure

**Plate 1.** The technique of electrocoagulation of the right kidney utilized as the first of two surgical procedures for the induction of renal failure in mice.

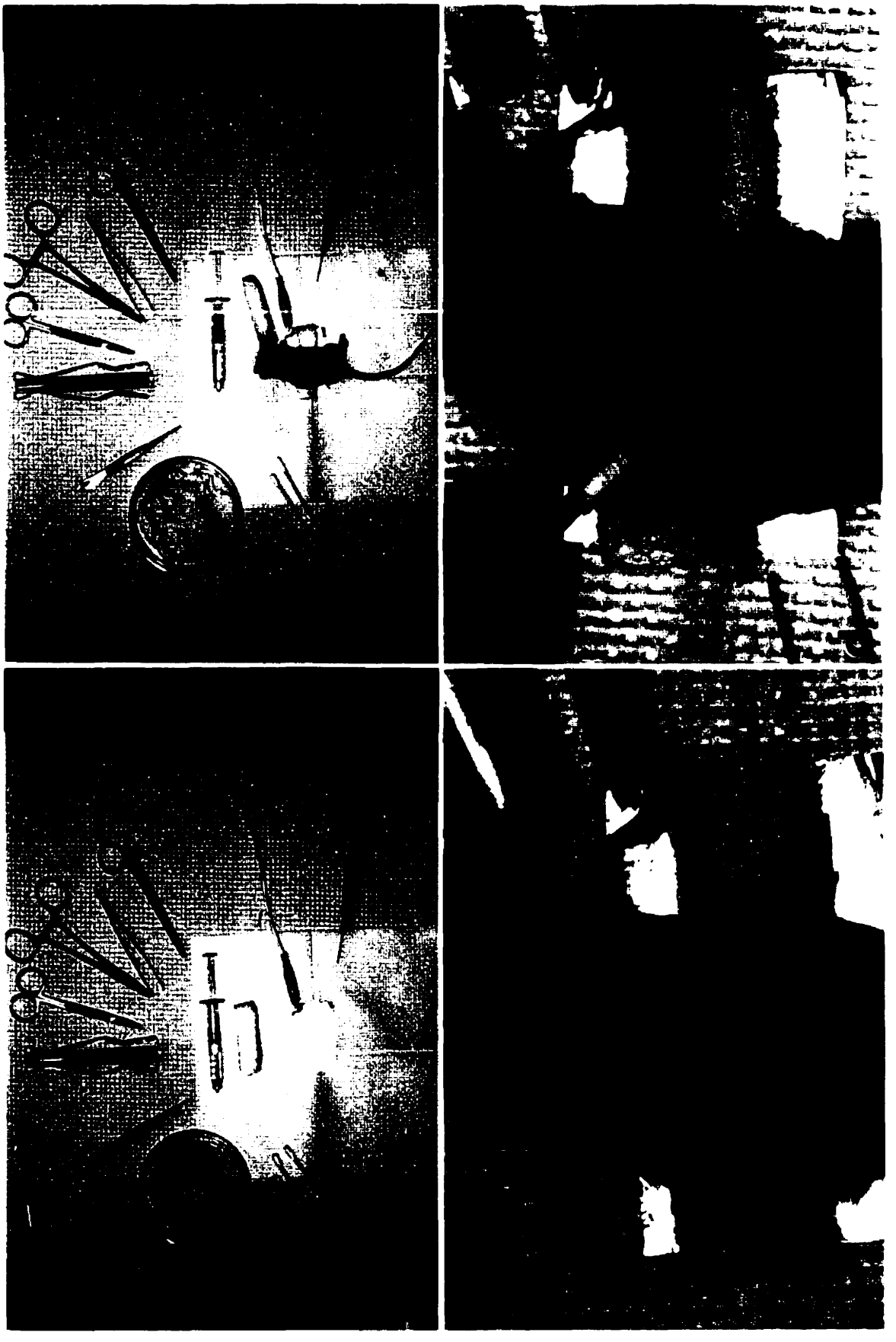
a) General view of the physical setup for the first surgical procedure i.e. electrocoagulation of the surface of the right kidney. As a modification of the previous technique, each mouse was placed on a smaller electrocoagulation metal plate during the electrocoagulation procedure.

b) Placement of a mouse on the electrocoagulation metal plate taking care that the head, legs, and tail rested outside the plate in order to avoid electrical burning of these fragile extremities.

c) The right kidney was brought to the surface after being freed from perirenal fat and the adrenal gland by fine tissue dissection. The mid-section of the mouse was propped up by a gauze pack placed under the left flank in order to facilitate the isolation of the right kidney. The kidney is now ready for electrical burning of its surface.

d) The kidney was held in position with a saline-dipped cotton bud while the electrocoagulator was applied gently to its surface, taking care not to burn the hilar structures and the inner aspect of the lower pole in order not to cause trauma to the ureter. Note the minimal amount of blood lost during the entire procedure (on cotton bud). Two weeks following this procedure on the right kidney, a left nephrectomy was performed, marking the onset of renal failure.

# Plate 1



# RESULTS

## General data

The survival of all mice was monitored throughout the experiments. As expected, the mice tolerated well the first surgical procedure (electrocoagulation of the surface of the right kidney), however large animal losses were observed during the first week after the second procedure (left nephrectomy). As previously shown (45), death of animals occurred during the second post-operative period because of severe renal failure due to insufficient residual functional renal parenchyma. The technique of electrocoagulation of the surface of the right kidney is operator-dependent and excessive renal tissue destruction occasionally occurs. This situation is not overt while the left kidney is still in place but becomes readily apparent immediately following the left nephrectomy. Overall, animal losses in the order of 15-30% were observed in the experiments, most of those occurring during the first week following the left nephrectomy.

The body weight of all the mice was obtained at sacrifice; additional measurements were made at the start of androgen treatment in order to determine the percent body weight change during treatment. The individual values of body weight of all animals are presented (Tables 2, 5, 6) together with circulating levels of urea, hemoglobin and EPO; where applicable, pre-treatment hemoglobin values are also included. As previously demonstrated (43), mice with renal failure gained weight following the surgical induction of renal failure (Tables 4 and 5), despite moderately severe renal impairment. In the CRF mice treated with androgens, the body weight served as a measure of the anabolic effect of androgens. Surprisingly, in none of the treatment experiments was there any consistent effect of androgens on the body weight of

CRF mice. A few mice had low body weight (<18 g) prior to treatment and were treated nonetheless. However, these small mice rarely responded to androgens compared to larger animals and their inclusion in these experiments is therefore questionable.

## Biochemical characterization

The blood urea level of normal 14-week old C57BL/6 female mice was 5.5 ± 1.2 mmol/l (Tables 1 and 2b) which is comparable to many previous determinations in normal mice in this laboratory (range 4 - 10 mmol/l) and in the literature (132). Six weeks after the surgical induction of renal failure, the blood urea levels of mice were significantly higher than normal control mice (Tables 1 and 2a). The urea levels of CRF mice were increased approximately six-fold (39.3 ± 9.2 mmol/l) (Table 1) indicating a corresponding six-fold decrease in renal function to 15% of normal.

This mouse model has previously been characterized extensively after 6 weeks of renal failure. In the present study, an evaluation of the changes of selected parameters with the duration of renal failure up to 6 weeks was conducted. In this time-course study, a marked increase in blood urea levels was observed at the three selected times of observation (1, 3, and 6 weeks of renal failure) (Table 2a, Figure 5). In addition, after six weeks of renal failure the mice exhibited significant increases in serum creatinine, calcium, phosphate, and alkaline phosphatase and decreases in serum magnesium (Table 3). Several of these parameters e.g. urea, creatinine, phosphate and alkaline phosphatase showed the greatest changes, from normal, in the early phase of renal failure (1-3 weeks). Of note, important species differences exist between the mouse and man for creatinine, phosphate, magnesium, and alkaline phosphatase.

## Hematological assessment

The results of the hematological tests performed by Technicon® counter on the peripheral blood of normal and CRF mice are presented in Table 1. This assessment also included serum EPO levels which were determined by radioimmunoassay.

Mice with renal failure of 6-week duration demonstrated marked hematological changes in the peripheral blood affecting predominantly the red cell series (Table 1). The CRF mice developed a significant anemia with hematocrit (28.6 ± 3.2 %), hemoglobin concentration (95.3 ± 24.0 g/l) and red blood cell count (6.4 ± 3.9 x $10^{12}$/fl) all significantly decreased compared to normal control mice (45). Furthermore, there was a negative correlation between the hemoglobin concentration and serum urea levels (Figure 6).

The mean corpuscular volume (MCV) was significantly decreased in CRF mice (38.6 ± 1.3 fl) compared to normal (44.1 ± 1.1 fl). The MCV in mice is, of note, much lower than in man (normal range: 80 - 100 fl) (82). The CRF mice had neither significantly different circulating white blood cell counts nor significantly different number and mean volume of platelets than normal. Platelet counts in normal mice (782.4 ± 184.5 x $10^9$/l) are noticeably higher than in man (normal range: 150 - 440 x $10^9$/l) (82).

The serum EPO levels of normal (18.8 ± 3.9 mU/ml) and CRF (23.7 ± 3.9 mU/ml) mice were not significantly different. The serum EPO level in CRF mice was therefore inappropriately low for the degree of anemia, as is characteristically observed in man with severely impaired renal function.

To further characterize the changes occurring with time in this animal model, mice were sacrificed at 1, 3, and 6 weeks of renal failure (Table 2a) with the above

hematological tests performed. The anemia expectedly worsened with the duration of renal failure. After one week of renal failure the hemoglobin was already decreased to 112.7 ± 6.0 g/l with further reduction observed at 3 weeks (102.9 ± 9.5 g/l) and at 6 weeks (90.7 ± 7.8 g/l) of renal failure (Figure 7). Serum EPO values of mice with renal failure were not significantly different than the normal controls at any point during this time-course study (Figure 8).

## Bone and bone marrow evaluation

Sections of undecalcified bone from the tibial plateau were obtained from mice with renal failure of different duration and normal controls. As these were undecalcified sections, some showed the expected fragmentation of the bony lamellae induced by the microtome.

Evidence of severe bone disease with marked changes in the bone marrow were observed in mice with renal failure. The most marked changes were observed after 6 weeks of renal failure (Plate 2, panels c and d), and are typical of osteitis fibrosa cystica, a consequence of severe secondary hyperprathyroidism. Surprisingly, significant changes in bone and marrow morphology were already present after only one week of renal failure (Plate 3, panel a).

Hematoxylin and eosin (H&E) staining of normal mouse bone (Plate 2, panel a) demonstrated a sharp interface between bone and marrow. There were large marrow spaces with prominent adipocytes and easily identifiable hematopoietic progenitors. Also evident was the preserved architecture of the cartilage and bone surrounding the marrow. Acid phosphatase staining of normal bone (Plate 2, panel b) was used to demonstrate the number, size and location of osteoclasts (bone resorbing cells) indicated in red. Few of

these red-stained cells were identified in normal bone along the bone margin. In addition, a clear, smooth delineation was evident between the spacious marrow and the surrounding bone.

H&E staining of CRF bone (Plate 2, panel c) demonstrated considerable changes from normal bone. There was obvious extensive damage to the bone and marrow with prominent remodeling. There was extensive fibrosis of the marrow and an irregular interface between the bone and marrow; areas of distinct scalloping of the bone margins could easily be identified. Remodeled marrow spaces with loss of marrow progenitor cells and reduced numbers of adipocytes were also readily apparent. The acid phosphatase staining of CRF bone (Plate 2, panel d) revealed an increased number and size of osteoclasts which were also seen at a distance from the bone margins, within the fibrotic tissue of the marrow. A few markedly enlarged osteoclasts were easily identifiable. The profoundly altered marrow spaces were filled with fibrosis lined with irregular bone surface.

In order to document the progression of renal osteodystrophy in the mouse, bone and marrow sections were obtained at 1, 3, and 6 weeks after the onset of renal failure (Plate 3). Surprisingly, significant bone marrow remodeling was already present as early as at one week of renal failure. All these changes are more evident in the subcartilaginous bone. Although no morphometric analysis was performed, progressively increasing osteoclastic activity was observed with time. At 6 weeks of renal failure compared to one week, there are increased numbers of osteoclasts which are now present in all marrow spaces.

As renal failure progresses, there is a differential localization of the acid phosphatase-positive cells (osteoclasts) in the bone marrow. Initially, the cells are located in the center of the marrow (1 week renal failure); with progressively longer renal failure, the cells are observed mostly in the periphery of the marrow (3 weeks renal failure) and eventually at the bone / marrow interface (6 weeks renal failure).

## Treatment with androgens

We next examined the effectiveness of treatment with androgens to alleviate the anemia of CRF. In treating CRF mice with androgens, several endpoints were evaluated in order to support or refute our hypothesis. The anabolic effect of the androgens on the mice was assessed by body weight measurement. Hemoglobin concentrations were measured as an indication of the response of the anemia to androgen treatment whereas serum EPO and IGF-I concentrations were measured as an indication of the possible mode of action of androgens on the anemia of CRF. RT-PCR was performed on the remnant kidney and liver of CRF mice to determine whether EPO message RNA (mRNA) was expressed in these tissues as a result of androgen treatment. Statistical differences between the results of androgen-treated and untreated CRF mice were sought. In addition, the effects of the different androgen doses were compared.

In the first set of experiments, nandrolone decanoate was administered to CRF mice starting three weeks after the onset of renal failure (time of left nephrectomy) (Figure 4: Initial protocol). The initial protocol of androgen treatment consisted of weekly i.m. injections of nandrolone decanoate (Deca-Durabolin®) for a period of three weeks starting three weeks after the onset of renal failure. Four different androgen doses were chosen (0.3, 1.0, 3.0, and 9.0 mg/kg BW/wk) while untreated control CRF mice

received no injections. These androgen doses comprised the range of dosages currently used in patients with ESRD on dialysis for the purpose of anemia correction averaging 100-200 mg/wk for a 70 kg man corresponding to 1.5-3.0 mg/kg BW/wk. Before the start of treatment, the CRF mice were randomized into five experimental groups (4 treatment groups and one untreated control group) according to their hemoglobin concentration. The response of CRF mice to the administered androgens is presented in Table 4 and Figure 9. Androgen-treated CRF mice showed a mild, not significant, fall in hemoglobin during the three-week treatment period which was no different than in untreated CRF controls. The lack of response to androgens was attributed to the relatively short treatment period of 3 weeks since androgens are recognized to require long periods of administration before inducing measurable hematological effects (20).

Accordingly, in the next series of experiments, treatment of the mice consisted in the administration of androgens for 5 consecutive weeks starting one week after the left nephrectomy (Figure 4: Definitive protocol).

Results obtained in the modified experimental protocol are presented in Table 5 and Figure 10. Untreated CRF mice and those treated with low-dose androgen (0.3 mg/kg BW/wk) showed a not significant decrease in hemoglobin concentration at the time of assessment, after 6 weeks of renal failure. In contrast, mice treated with 1.0 mg/kg BW/wk of nandrolone were able to maintain their hemoglobin concentrations, whereas CRF mice treated with 3.0 and 9.0 mg/kg BW/wk demonstrated an increase, albeit not significant, in hemoglobin concentration. Although there was no difference in the change in hemoglobin between the different animal groups, there was a consistently increasing trend in percent hemoglobin change with increasing androgen doses.

Furthermore the proportion of mice with a positive change in hemoglobin was clearly different between the androgen-treated groups (Table in Figure 10) with a higher proportion of mice showing a positive change in the 3 groups treated with the higher androgen doses compared to the lowest dose of 0.3 mg/kg BW/wk. Androgen treatment had no measurable effect on the other routine hematological parameters.

The effect of androgen treatment of CRF mice on serum EPO concentration was also evaluated (Table 5 and Figure 11). Compared to untreated CRF animals (22.9 ± 3.1 mU/ml), a significant effect of androgens on EPO concentration was evident at the higher dosages of 3.0 (42.5 ± 13.9 mU/ml) and 9.0 mg/kg BW/wk (45.3 ± 15.9 mU/ml) with no discernible effect observed at the lower dosage of 0.3 mg/kg BW/wk (25.6 ± 7.9 mU/ml) and a mild, albeit not significant, rise at 1.0 mg/kg BW/wk (36.8 ± 11.5 mU/ml). The significant increase in serum EPO concentration observed during treatment of CRF mice with the higher doses of nandrolone (3.0 and 9.0 mg/kg BW/wk) occurred in the absence of a corresponding increase in hemoglobin concentration (Figure 12)

An important part of this study was to seek correlations between serum EPO and hemoglobin concentrations, according to the androgen doses. For the purpose of data analysis, we identified a combined value of serum EPO (>25 mU/ml) and hemoglobin concentrations (>120 g/l) which was never observed in untreated CRF mice but was reached in nearly half of the androgen-treated animals (Figure 13). The use of this combined target value discriminating between treated and untreated mice also facilitated the identification of trends in the response to androgen treatment. Further analysis of the correlation between serum EPO and hemoglobin concentrations with androgen treatment (Figures 14 and 15) shows that an increasing proportion of treated mice reached the

combined target value with increasing androgen dose. Another pattern emerges in the analysis of the response to graded doses of androgen (Figure 15). The majority of animals in the untreated group and those treated with the lowest androgen dose have a serum EPO below 25 mU/ml and a hemoglobin inferior to 120 g/l. All mice treated with the 3 higher androgen doses have serum EPO values above 25 mU/ml, and the proportion of mice with a hemoglobin superior to 120 g/l increased with the androgen dose.

In a separate, related experiment, CRF mice treated with r-HuEPO (10U, s.c., 3 times weekly for 3 weeks) exhibited serum EPO levels of 249 180 mU/ml at sacrifice / evaluation. This mean value is significantly greater than the highest serum EPO level attained by any of the androgen-treated CRF mice. this marked increase in serum EPO levels (more than 10 times normal values) in the r-HuEPO-treated CRF mice was observed in conjunction with a significant increase in hemoglobin concentrations, resulting in full correction of the anemia in 50% of the animals (137).

The possible modification of the therapeutic effect of androgens by the degree of renal failure was also examined. Figure 16 presents the change in hemoglobin concentrations resulting from androgen treatment of CRF mice with different degrees of renal failure (as determined by serum urea levels). Although the number of animals in each subgroup was small, several different trends were observed. As expected, a direct correlation was found between the anemia (hemoglobin concentration) and degree of renal failure (urea level) of untreated CRF mice (value on the far left of each row). In mice with mild renal failure (back row: urea 11-30 mmol/l) no significant effect of androgens was detected. In mice with moderate renal failure (middle row: urea 31-50 mmol/l), androgen treatment induced an increase in hemoglobin up to the level in the

mild CRF group. The higher androgen dose (9.0 mg/kg BW/wk) was ineffective perhaps for reasons of toxicity. In mice with severe renal failure (front row: urea 51-70 mmol/l), having the lowest hemoglobin concentration in the absence of treatment, the androgens had only a limited effect, readily reaching a plateau, suggesting the presence of inhibitors of erythropoiesis. A graphic presentation of the correlation between serum EPO and hemoglobin concentrations according to the degree of renal failure is shown in Figure 17 with the androgen doses identified by different symbols. Again, the poor response of the hemoglobin to increased serum EPO levels is observed in severe renal failure, suggesting the presence of uremic inhibitors preventing erythropoiesis.

The previous sets of experiments evaluated the effect of androgens on the anemia of renal failure using a range of doses administered during a fixed period of 5 weeks. In the last experiment, the effect of the demonstrably effective, and non-toxic, dose of 3.0 mg/kg BW/wk during 5 weeks was administered for different periods of time (1, 2, and 5 weeks) and its effect on selected hematological parameters was examined. The hemoglobin concentration did not change significantly during the 3 different durations of androgen treatment (Table 6, Figure 18). The serum EPO concentration however increased significantly after 2 and 5 weeks of treatment compared to one week (Figure 19). To reiterate, a significant increase in serum EPO concentration was induced by androgen treatment without change in hemoglobin concentration, suggesting that the observation period may not have been sufficiently long for hemoglobin concentration to increase significantly in response to EPO stimulation (Figure 20). Prolongation of the androgen treatment for longer periods would therefore be expected to achieve a significant rise in hemoglobin commensurate to the increased EPO concentrations.

## Serum levels of IGF-I

The serum IGF-I levels of normal mice and of untreated and androgen-treated CRF mice were compared (Table 7). In all experiments, there was no discernible difference between the serum level of IGF-I of untreated CRF mice and normal control animals. Furthermore, IGF-I levels of mice with renal failure did not change significantly with the duration of renal failure (1, 3, and 6 weeks). Lastly, the IGF-I levels of CRF mice, as determined by radioimmunoassay, did not change significantly with androgen treatment. Of note, the IGF-I levels in the CRF mice were significantly higher than in man (normal range in this laboratory: 200 to 400 ng/ml). This difference may be explained by the fact that the mice were not fasting whereas all normal human samples were collected after an overnight fast.

## Expression of EPO mRNA

The results of the mRNA expression for EPO in the kidney and liver of untreated and androgen-treated CRF mice as well as selected controls (normal and phlebotomized mice) are summarized in Table 8. There was no correlation between the degree of renal failure (as determined by serum urea level) and the expression of EPO mRNA in either the kidney or the liver. Illustrative autoradiographs of amplified cDNA of EPO are presented in Figures 21 to 24. All bands seen hybridizing with the EPO probe were of a size approximately 0.38 kb which is the expected size for EPO (379 bp). Surprisingly, another band of lower molecular weight (approximately 250 bp) was observed to hybridize with the EPO probe. Upon further examination, it was revealed that the second band was evident in all mice tested suggesting the presence of as yet unsequenced bacterial contamination.

In the kidney, a band of mild to moderate intensity was observed in normal and CRF mice while the band in phlebotomized mice was very intense (Figure 21). This finding suggests that mRNA expression for EPO in phlebotomized mice was amplified, as would be expected in the experimental situation of severe anemia secondary to bleeding. When the liver was assayed using the EPO probe, there was very intense expression in the phlebotomized mice while no expression could be seen in normal as well as untreated CRF mice (Figure 22). The latter finding of absent message in the liver of untreated CRF mice was evident in all mice tested. This observation, as yet incompletely understood, corroborates previous findings from our laboratory (137).

RT-PCR, Southern blotting and subsequent hybridization with the EPO probe was again performed on kidney and liver samples from mice treated with androgens (Figure 23). Hybridization of the kidney with the EPO probe showed the intensity of the message to increase as the dose of nandrolone increased compared to the faint expression in untreated mice. Mice treated with 1.0, 3.0 and 9.0 mg/kg BW/wk presented similar intensities of expression. Surprisingly, the analysis of the liver of these same mice revealed no EPO message. This is the first demonstration that treatment with androgens (over a wide range of doses) does not cause an increase in EPO mRNA expression in the liver.

Mice with renal failure treated with a fixed dose of 3.0 mg/kg BW/wk for periods of 1, 2 and 5 weeks were additionally assayed (Figure 24). Only 3 of the 5 mice treated for 5 weeks showed moderate mRNA expression in the kidney. None of the other mice demonstrated EPO mRNA message in either organ assayed.

**Table 1.** Comparison of hematological parameters in normal and CRF mice.

| Parameters | Normal | | n | CRF | | n |
|---|---|---|---|---|---|---|
| Hemoglobin (g/l) | 150.6 ± 5.9 | (151) | 11 | 95.3 ± 24.0* | (105) | 21 |
| Hct (%) | 38.8 ± 1.8 | (37) | 11 | 28.6 ± 3.2* | (27) | 16 |
| RBC ($10^{12}$/l) | 8.7 ± 1.6 | (8.3) | 11 | 6.4 ± 3.9* | (6.1) | 16 |
| MCV (fl) | 44.1 ± 1.1 | (44) | 11 | 38.6 ± 1.3* | (37) | 16 |
| WBC ($10^9$/l) | 5.23 ± 1.9 | (4.9) | 11 | 7.4 ± 2.2 | (8.1) | 16 |
| Platelets ($10^9$/l) | 782.4 ± 184.5 | (813) | 11 | 413.2 ± 201.6 | (526) | 16 |
| EPO (mU/ml) | 18.8 ± 3.9 | (17) | 11 | 23.7 ± 3.9 | (23) | 11 |
| Urea (mmol/l) | 5.5 ± 1.2 | (5.5) | 11 | 39.3 ± 9.2* | (38) | 17 |

Results of routine hematological testing in 14-week old female C57BL/6 mice at the time of sacrifice, six weeks after the onset of renal failure. Serum EPO levels were measured by radioimmunoassay. Serum urea levels are presented at the bottom of the Table to indicate the degree of renal function. The results are compared to those of normal 14-week old female C57BL/6 mice.

Hct = hematocrit; RBC = red blood cell; MCV = mean corpuscular volume;
WBC = white blood cell; EPO = erythropoietin.

Data represent mean ± SD, with median indicated in parentheses.
Significant differences between normal and CRF mice (*$p<0.05$).

**Table 2a.** Comparison of selected parameters between mice with renal failure of different duration.

| Characteristics of mice | Mouse # | Urea (mmol/l) | Body weight (g) | | | Hemoglobin (g/l) | | | EPO (mU/ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | First assessment | Sacrifice | % Δ | First assessment | Sacrifice | % Δ | |
| *9 wk old* | 1 | 50.7 | | 19.0 | | | 111.0 | | 20 |
| *1 wk renal failure* | 2 | 47.2 | | 18.5 | | | 107.0 | | 22 |
| | 3 | 46.9 | | 20.0 | | | 106.0 | | 23 |
| | 4 | 35.8 | | 19.9 | | | 122.0 | | 26 |
| | 5 | 42.3 | | 20.8 | | | 118.0 | | 25 |
| | 6 | 27.6 | | 21.8 | | | 109.0 | | 25 |
| | 7 | 31.2 | | 18.3 | | | 116.0 | | 27 |
| | **Mean** | **40.2** | | **19.8** | | | **112.7** | | **24.0** |
| | **SD** | **8.8** | | **1.3** | | | **6.0** | | **2.4** |
| *11 wk old* | 1 | 59.6 | 19.3 | 19.7 | 2.1 | 122.0 | 101.0 | -17.2 | 24 |
| *3 wk renal failure* | 2 | 55.8 | 19.4 | 20.2 | 4.1 | 112.0 | 88.0 | -21.4 | 26 |
| | 3 | 67.1 | 20.3 | 19.9 | -2.0 | 109.0 | 97.0 | -11.0 | 23 |
| | 4 | 33.9 | 19.2 | 22.9 | 19.3 | 128.0 | 119.0 | -7.0 | 26 |
| | 5 | 39.3 | 18.8 | 21.7 | 15.4 | 126.0 | 108.0 | -14.3 | 26 |
| | 6 | 39.3 | 19.6 | 18.6 | -5.1 | 122.0 | 103.0 | -15.6 | 25 |
| | 7 | 31.2 | 19.6 | 22.3 | 13.8 | 117.0 | 104.0 | -11.1 | 30 |
| | **Mean** | **46.6** | **19.5** | **20.8** | **6.8** | **119.4** | **102.9** | **-14.0** | **25.7** |
| | **SD** | **14.0** | **0.5** | **1.6** | **9.4** | **7.1** | **9.5** | **4.7** | **2.2** |
| *14 wk old* | 1 | 35.6 | 18.3 | 18.4 | 0.5 | 119.0 | 93.0 | -21.8 | 26 |
| *6 wk renal failure* | 2 | 36.8 | 19.8 | 19.1 | -3.5 | 117.0 | 97.0 | -17.1 | 28 |
| | 3 | 40.3 | 20.2 | 19.6 | -3.0 | 108.0 | 82.0 | -24.1 | 23 |
| | **Mean** | **37.6** | **19.4** | **19.0** | **-2.0** | **114.7** | **90.7** | **-21.0** | **25.7** |
| | **SD** | **2.4** | **1.0** | **0.6** | **2.2** | **5.9** | **7.8** | **3.6** | **2.5** |

Note: Mice with one week renal failure were only weighed at sacrifice.

**Table 2b.** Selected parameters in normal mice.

| Characteristics of mice | Mouse # | Urea (mmol/l) | Body weight (g) | Hemoglobin (g/l) | EPO (mU/ml) |
|---|---|---|---|---|---|
| *14 wk old* | 1 | 5.4 | 18.5 | 150 | 24 |
| *Normal* | 2 | 5.7 | 19.3 | 159 | 24 |
| | 3 | 4.8 | 18.9 | 148 | 24 |
| | 4 | 6.3 | 18.5 | 152 | 14 |
| | 5 | 7.5 | 18.7 | 154 | 17 |
| | 6 | 3.3 | 19.3 | 141 | 17 |
| | 7 | 4.7 | 18.8 | 145 | 16 |
| | 8 | 6.9 | 19.3 | 152 | 22 |
| | 9 | 5.5 | 20.6 | 159 | 15 |
| | 10 | 4.9 | 19.9 | 146 | 16 |
| | 11 | 6.2 | 21.3 | 151 | 18 |
| | **Mean** | **5.5** | **19.2** | **150.6** | **18.8** |
| | **SD** | **1.2** | **0.7** | **5.9** | **3.9** |

Results of body weight and selected hematological and biochemical parameters in female C57BL/6 mice with 1, 3, and 6 weeks of renal failure (Table 2a).
Similar results in normal 14-week old female C57BL/6 mice presented for comparison in Table 2b.
Data from individual mice presented together with mean ± SD.

**Table 3.** Comparison of selected biochemical parameters in normal mice and in mice with renal failure of different duration.

| Characteristics of mice | Urea (mmol/l) | Creatinine (μmol/l) | Calcium (mmol/l) | Phosphate (mmol/l) | ALP (U/l) | Magnesium (mmol/l) |
|---|---|---|---|---|---|---|
| Renal failure | | | | | | |
| 1 week | 42.4 | 89 | 2.40 | 5.32 | 532 | 1.60 |
| | 40.0 | 94 | 2.60 | 4.31 | 512 | 2.04 |
| | 39.2 | 89 | 2.64 | 5.57 | 516 | 2.42 |
| **Mean** | **40.5** | **90.7** | **2.5** | **5.1** | **520** | **2.0** |
| **SD** | **1.7** | **2.9** | **0.1** | **0.7** | **11** | **0.4** |
| 3 weeks | 48.1 | 97 | 2.41 | 5.43 | 666 | 1.88 |
| | 49.0 | 89 | 2.65 | 5.65 | 384 | 1.87 |
| | 55.5 | 95 | 2.42 | 5.83 | 562 | 1.76 |
| **Mean** | **50.9** | **93.7** | **2.5** | **5.6** | **537** | **1.8** |
| **SD** | **4.0** | **4.2** | **0.1** | **0.2** | **143** | **0.1** |
| 6 weeks | 36.0 | 79 | 2.69 | 4.49 | 273 | 1.74 |
| | 30.6 | 74 | 2.74 | 3.76 | 251 | 1.67 |
| | 37.7 | 87 | 2.67 | 4.94 | 392 | 1.82 |
| **Mean** | **34.8** | **80.0** | **2.7** | **4.4** | **305** | **1.7** |
| **SD** | **3.7** | **6.6** | **0.0** | **0.6** | **76** | **0.1** |
| Normal (n=10) | | | | | | |
| **Mean** | **7.7** | **43** | **2.1** | **3.8** | **222** | **2.0** |
| **SD** | **1.3** | **2.2** | **0.1** | **0.6** | **23** | **0.2** |
| **Range** | **6.0 - 8.9** | **40 - 47** | **1.8 - 2.2** | **3.6 - 4.3** | **199 - 251** | **1.84 - 2.38** |
| Man | | | | | | |
| **Normal range** | **3.0 - 8.9** | **65 - 120** | **2.11 - 2.51** | **0.8 - 1.5** | **37 - 117** | **0.75 - 1.05** |

Results of selected biochemical parameters in female C57BL/6 mice with renal failure of different duration: 1, 3, and 6 weeks.

Data from individual mice presented together with mean ± SD.

The results are compared to those of normal 14-week old C57BL/6 mice shown below.

Also shown below are the ranges of normal values in C57BL/6 mice and man.

Serum PTH levels in all mice was tested but were found to be undetectable (<0.01) using the current assay. ALP = alkaline phosphatase

**Table 4.** Hematological response of CRF mice to 3-week treatment with graded doses of nandrolone decanoate: Initial experimental protocol.

| Characteristics of mice | Mouse # | Body weight (g) | | | Hemoglobin (g/l) | | |
|---|---|---|---|---|---|---|---|
| | | pre-treatment | post-treatment | % Δ | pre-treatment | post-treatment | % Δ |
| | 1 | 19.8 | 20.3 | 2.5 | 100 | 92 | -8.0 |
| *14 wk old* | 2 | 14.3 | 17.0 | 18.9 | 118 | 60 | -49.2 |
| *6 wk RF* | 3 | 16.1 | 18.4 | 14.3 | 89 | 71 | -20.2 |
| *Untreated* | 4 | 18.2 | 18.4 | 1.1 | 107 | 107 | 0.0 |
| | 5 | 21.8 | 22.1 | 1.4 | 94 | 100 | 6.4 |
| | **Mean** | **18.0** | **19.2** | **7.6** | **101.6** | **86.0** | **-14.2** |
| | **SD** | **3.0** | **2.0** | **8.3** | **11.4** | **19.8** | **21.9** |
| | 1 | 21.0 | 24.0 | 14.3 | 94 | 80 | -14.9 |
| *14 wk old* | 2 | 20.5 | 21.7 | 5.9 | 99 | 89 | -10.1 |
| *6 wk RF* | 3 | 20.3 | 22.4 | 10.3 | 125 | 115 | -8.0 |
| *3 wk AND treatment* | 4 | 20.4 | 20.1 | -1.5 | 105 | 88 | -16.2 |
| *0.3 mg/kg BW/wk* | 5 | 19.6 | 19.3 | -1.5 | 84 | 81 | -3.6 |
| | **Mean** | **20.4** | **21.5** | **5.5** | **101.4** | **90.6** | **-10.6** |
| | **SD** | **0.5** | **1.9** | **7.0** | **15.3** | **14.2** | **5.1** |
| | 1 | 20.5 | 19.8 | -3.4 | 90.1 | 86 | -4.6 |
| *14 wk old* | 2 | 21.2 | 20.5 | -3.3 | 96 | 92 | -4.2 |
| *6 wk RF* | 3 | 20.0 | 22.1 | 10.5 | 97 | 88 | -9.3 |
| *3 wk AND treatment* | 4 | 23.3 | 22.8 | -2.1 | 116 | 117 | 0.9 |
| *1.0 mg/kg BW/wk* | 5 | 18.7 | 22.4 | 19.8 | 105 | 59 | -43.8 |
| | **Mean** | **20.7** | **21.5** | **4.3** | **100.8** | **88.4** | **-12.2** |
| | **SD** | **1.7** | **1.3** | **10.5** | **10.0** | **20.6** | **18.0** |

**Table 4** cont'd.

| Characteristics of mice | Mouse # | Body weight (g) | | | Hemoglobin (g/l) | | |
|---|---|---|---|---|---|---|---|
| | | pre-treatment | post-treatment | % Δ | pre-treatment | post-treatment | % Δ |
| | 1 | 19.5 | 21.7 | 11.3 | 103 | 96 | -6.8 |
| *14 wk old* | 2 | 15.5 | 19.0 | 22.6 | 91 | 63 | -30.8 |
| *6 wk RF* | 3 | 21.6 | 22.4 | 3.7 | 114 | 72 | -36.8 |
| *3 wk AND treatment* | 4 | 20.1 | 22.6 | 12.4 | 82 | 101 | 23.2 |
| *3.0 mg/kg BW/wk* | 5 | 16.2 | 20.2 | 24.7 | 97 | 79 | -18.6 |
| | **Mean** | **18.6** | **21.2** | **14.9** | **97.4** | **82.2** | **-14.0** |
| | **SD** | **2.6** | **1.5** | **8.7** | **12.1** | **16.0** | **23.8** |
| | 1 | 19.9 | 20.8 | 4.5 | 96.5 | 80 | -17.1 |
| *14 wk old* | 2 | 16.9 | 19.5 | 15.4 | 84 | 76 | -9.5 |
| *6 wk RF* | 3 | 21.3 | 21.4 | 0.5 | 70 | 72 | 2.9 |
| *3 wk AND treatment* | 4 | 19.8 | 20.9 | 5.6 | 114 | 104 | -8.8 |
| *9.0 mg/kg BW/wk* | 5 | 15.0 | 19.4 | 29.3 | 103 | 64 | -37.9 |
| | **Mean** | **18.6** | **20.4** | **11.1** | **93.5** | **79.2** | **-14.1** |
| | **SD** | **2.6** | **0.9** | **11.6** | **17.0** | **15.1** | **15.1** |

**Results of body weight and selected hematological and biochemical parameters in female C57BL/6 mice with 6 weeks of renal failure, treated with graded doses of nandrolone decanoate.**

**Treatment was for 3 weeks starting 3 weeks after the time of onset of renal failure (left nephrectomy). Treatment groups received i.m. injections of 0.3, 1.0, 3.0, or 9.0 mg/kg BW once weekly. Untreated mice received no injections.**

**Baseline and final hemoglobin concentrations were not statistically significant between the groups.**

**Data from individual mice presented together with mean ± SD.**

**Table 5.** Hematological response of CRF mice to 5-week treatment with graded doses of nandrolone decanoate: Definitive experimental protocol.

| Characteristics of mice | Mouse # | Urea (mmol/l) | Body weight (g) | | | Hemoglobin (g/l) | | | EPO (mU/ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | pre | post | % Δ | pre | post | % Δ | |
| | 1 | 27.5 | 20.3 | 22.7 | 11.8 | 136.0 | 129.0 | -5.1 | 23.0 |
| *14 wk old* | 2 | 24.7 | 19.4 | 20.7 | 6.7 | 96.0 | 121.0 | 26.0 | 25.0 |
| *6 wk RF* | 3 | 37.6 | 19.2 | 19.8 | 3.1 | 113.0 | 109.0 | -3.5 | 28.0 |
| *Untreated* | 4 | 38.7 | 18.6 | 19.5 | 4.7 | 130.0 | 110.0 | -15.4 | 22.0 |
| | 5 | 36.6 | 18.8 | 20.9 | 11.2 | 126.0 | 105.0 | -16.7 | 20.0 |
| | 6 | 55.2 | 17.9 | 20.0 | 11.7 | 117.0 | 68.0 | -41.9 | 18.0 |
| | 7 | 36.5 | 18.5 | 20.5 | 10.8 | 128.0 | 115.0 | -10.2 | 25.0 |
| | 8 | 32.4 | 19.6 | 21.1 | 7.7 | 115.0 | 54.0 | -53.0 | 22.0 |
| | **Mean** | **36.2** | **19.0** | **20.7** | **8.5** | **120.1** | **101.4** | **-15.0** | **22.9** |
| | **SD** | **9.2** | **0.7** | **1.0** | **3.4** | **12.6** | **26.3** | **24.2** | **3.1** |
| | 1 | 34.6 | 18.9 | 21.1 | 11.6 | 96.0 | 110.0 | 14.6 | 15.0 |
| *14 wk old* | 2 | 41.2 | 19.3 | 21.3 | 10.4 | 132.0 | 84.0 | -36.4 | 34.0 |
| *6 wk RF* | 3 | 50.7 | 20.3 | 20.6 | 1.5 | 107.0 | 91.0 | -15.0 | 40.0 |
| *5 wk AND treatment* | 4 | 34.2 | 20.7 | 21.1 | 1.9 | 135.0 | 120.0 | -11.1 | 22.0 |
| *0.3 mg/kg BW/wk* | 5 | 38.7 | 20.6 | 21.3 | 3.4 | 130.0 | 122.0 | -6.2 | 24.0 |
| | 6 | 28.2 | 20.1 | 22.8 | 13.4 | 128.0 | 113.0 | -11.7 | 26.0 |
| | 7 | 35.7 | 19.5 | 20.7 | 6.2 | 125.0 | 119.0 | -4.8 | 24.0 |
| | 8 | 24.3 | 21.4 | 21.4 | 0.0 | 114.0 | 89.0 | -21.9 | 20.0 |
| | **Mean** | **36.0** | **20.1** | **21.3** | **6.0** | **120.9** | **106.0** | **-11.6** | **25.6** |
| | **SD** | **8.0** | **0.8** | **0.7** | **5.2** | **13.8** | **15.5** | **14.6** | **7.9** |

**Table 5** cont'd.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 62.0 | 17.8 | 18.2 | 2.2 | 129.0 | 92.0 | -28.7 | 55.0 |
| *14 wk old* | 2 | 30.4 | 20.7 | 21.3 | 2.9 | 106.0 | 136.0 | 28.3 | 55.0 |
| *6 wk RF* | 3 | 68.4 | 19.0 | 19.3 | 1.6 | 95.0 | 82.0 | -13.7 | 30.0 |
| *5 wk AND treatment* | 4 | 39.0 | 19.3 | 21.0 | 8.8 | 128.0 | 120.0 | -6.3 | 32.0 |
| *1.0 mg/kg BW/wk* | 5 | 27.3 | 20.4 | 18.7 | -8.3 | 129.0 | 115.0 | -10.9 | 28.0 |
| | 6 | 22.8 | 19.4 | 23.5 | 21.1 | 124.0 | 124.0 | 0.0 | 29.0 |
| | 7 | 32.1 | 19.8 | 21.7 | 9.6 | 116.0 | 122.0 | 5.2 | 35.0 |
| | 8 | 29.4 | 16.0 | 21.5 | 34.4 | 116.0 | 118.0 | 1.7 | 30.0 |
| | **Mean** | **38.9** | **19.1** | **20.7** | **9.0** | **117.9** | **113.6** | **-3.0** | **36.8** |
| | **SD** | **16.9** | **1.5** | **1.8** | **13.3** | **12.3** | **17.8** | **16.6** | **11.5** |
| | 1 | 43.4 | 20.0 | 21.9 | 9.5 | 105.0 | 114.0 | 8.6 | 57.0 |
| *14 wk old* | 2 | 64.0 | 16.0 | 16.0 | 0.0 | 119.0 | 87.0 | -26.9 | 40.0 |
| *6 wk RF* | 3 | 32.4 | 20.0 | 22.4 | 12.0 | 80.0 | 119.0 | 48.8 | 70.0 |
| *5 wk AND treatment* | 4 | 23.4 | 20.2 | 21.4 | 5.9 | 126.0 | 112.0 | -11.1 | 35.0 |
| *3.0 mg/kg BW/wk* | 5 | 37.9 | 17.3 | 16.3 | -5.8 | 129.0 | 129.0 | 0.0 | 32.0 |
| | 6 | 44.1 | 18.3 | 18.8 | 2.7 | 124.0 | 128.0 | 3.2 | 32.0 |
| | 7 | 40.5 | 17.6 | 18.0 | 2.3 | 118.0 | 125.0 | 5.9 | 32.0 |
| | 8 | 18.6 | 21.8 | 23.2 | 6.4 | 115.0 | 126.0 | 9.6 | 42.0 |
| | **Mean** | **38.0** | **18.9** | **19.8** | **4.1** | **114.5** | **117.5** | **4.8** | **42.5** |
| | **SD** | **14.0** | **1.9** | **2.8** | **5.6** | **15.8** | **13.9** | **21.6** | **13.9** |
| | 1 | 45.5 | 19.2 | 18.5 | -3.6 | 113.0 | 89.0 | -21.2 | 64.0 |
| *14 wk old* | 2 | 22.6 | 19.9 | 21.5 | 8.0 | 79.0 | 130.0 | 64.6 | 54.0 |
| *6 wk RF* | 3 | 39.2 | 17.3 | 20.2 | 16.8 | 100.0 | 121.0 | 21.0 | 65.0 |
| *5 wk AND treatment* | 4 | 29.0 | 18.4 | 20.0 | 8.7 | 129.0 | 128.0 | -0.8 | 30.0 |
| *9.0 mg/kg BW/wk* | 5 | 10.5 | 19.5 | 22.3 | 14.4 | 116.0 | 122.0 | 5.2 | 44.0 |
| | 6 | 27.7 | 20.0 | 23.5 | 17.5 | 126.0 | 132.0 | 4.8 | 28.0 |
| | 7 | 21.3 | 22.3 | 26.8 | 20.2 | 124.0 | 130.0 | 4.8 | 32.0 |
| | **Mean** | **28.0** | **19.5** | **21.8** | **11.7** | **112.4** | **121.7** | **11.2** | **45.3** |
| | **SD** | **11.6** | **1.5** | **2.7** | **8.1** | **17.7** | **15.0** | **26.6** | **15.9** |

Results presented as per Table 3. Treatment was for 5 weeks starting 1 week post-left nephrectomy (onset of renal failure).

**Table 6.** Response of mice with renal failure of different duration to treatment with a fixed dose of nandrolone decanoate (3.0 mg/kg BW/wk).

| Characteristics of mice | Mouse # | Urea (mmol/l) | Body weight (g) | | | Hemoglobin (g/l) | | | EPO (mU/ml) |
|---|---|---|---|---|---|---|---|---|---|
| | | | pre | post | % Δ | pre | post | % Δ | |
| *9 wk old* | 1 | 45.9 | 19.8 | 20.0 | 1.0 | 119.0 | 97.0 | -18.5 | 17 |
| *2 wk RF* | 2 | 44.1 | 18.9 | 18.7 | -1.1 | 110.0 | 98.0 | -10.9 | 31 |
| *1 wk AND treatment* | 3 | 53.4 | 17.6 | 17.8 | 1.1 | 108.0 | 115.0 | 6.5 | 26 |
| | 4 | 32.1 | 17.5 | 17.4 | -0.6 | 116.0 | 116.0 | 0.0 | 26 |
| | 5 | 32.1 | 14.6 | 15.8 | 8.2 | 133.0 | 119.0 | -10.5 | 26 |
| | **Mean** | **41.5** | **17.7** | **17.9** | **1.7** | **117.2** | **109.0** | **-6.7** | **25.2** |
| | **SD** | **9.3** | **2.0** | **1.6** | **3.7** | **9.9** | **10.6** | **9.9** | **5.1** |
| *11 wk old* | 1 | 35.1 | 19.3 | 19.7 | 2.1 | 108.0 | 99.0 | -8.3 | 42 |
| *3 wk RF* | 2 | 42.9 | 17.6 | 18.5 | 5.1 | 93.0 | 90.0 | -3.2 | 40 |
| *2 wks AND treatment* | 3 | 40.8 | 18.7 | 19.3 | 3.2 | 123.0 | 93.0 | -24.4 | 40 |
| | 4 | 37.4 | 18.9 | 19.8 | 4.8 | 118.0 | 114.0 | -3.4 | 44 |
| | 5 | 39.3 | 19.4 | 20.1 | 3.6 | 110.0 | 100.0 | -9.1 | 41 |
| | **Mean** | **39.1** | **18.8** | **19.5** | **3.8** | **110.4** | **99.2** | **-9.7** | **41.4** |
| | **SD** | **3.0** | **0.7** | **0.6** | **1.2** | **11.5** | **9.3** | **8.7** | **1.7** |
| *14 wk old* | 1 | 45.6 | 19.1 | 18.7 | -2.1 | 94.0 | 91.0 | -3.2 | 42 |
| *6 wk RF* | 2 | 32.4 | 23.2 | 25.0 | 7.8 | 87.0 | 115.0 | 32.2 | 47 |
| *5 wks AND treatment* | 3 | 68.4 | 14.6 | 16.0 | 9.6 | 118.0 | 79.0 | -33.1 | 14 |
| | 4 | 41.6 | 16.4 | 16.1 | -1.8 | 108.0 | 77.0 | -28.7 | 42 |
| | **Mean** | **47.0** | **18.3** | **19.0** | **3.4** | **101.8** | **90.5** | **-8.2** | **36.3** |
| | **SD** | **15.3** | **3.7** | **4.2** | **6.2** | **13.9** | **17.5** | **30.0** | **15.0** |

Results presented as per Table 3.

CRF mice received 3.0 mg/kg BW/wk nandrolone decanoate for periods of 1, 2, and 5 weeks.

Data for individual mice presented together with mean ± SD.

**Table 7.** Comparison of serum IGF-I levels in normal mice and in untreated and androgen-treated mice with renal failure of different duration

| Characteristics of mice | n | Final hemoglobin (g/l) | EPO (mU/ml) | IGF-I (ng/ml) |
|---|---|---|---|---|
| *14 wk old, normal* | 11 | 150.6 ± 5.9 | 18.8 ± 3.9 | 433.3 ± 44.9 |
| *9 wk old, 1 wk RF Untreated* | 7 | 112.7 ± 6.0* | 24.0 ± 2.4 | 623.0 ± 63.6 |
| *11 wk old, 3 wk RF Untreated* | 7 | 102.9 ± 9.5* | 25.7 ± 2.2 | 492.3 ± 74.8 |
| *14 wk old, 6 wk RF Untreated* | 16 | 94.6 ± 7.6* | 23.6 ± 2.8 | 497.4 ± 58.4 |
| *14 wk old, 6 wk RF 0.3 mg/kg BW/wk* | 13 | 106 ± 15.5 | 25.6 ± 7.9 | 440.5 ± 54.2 |
| *14 wk old, 6 wk RF 1.0 mg/kg BW/wk* | 13 | 113.6 ± 17.8 | 36.8 ± 11.5 | 400.0 ± 22.1 |
| *14 wk old, 6 wk RF 3.0 mg/kg BW/wk* | 13 | 117.5 ± 13.9 | 42.5 ± 13.9** | 449.0 ± 64.8 |
| *14 wk old, 6 wk RF 6 wk RF 9.0 mg/kg BW/wk* | 12 | 121.7 ± 15.0 | 45.3 ± 15.9** | 476.4 ± 42.6 |
| *9 wk old, 2 wk RF 1 wk 3.0 mg/kg BW/wk* | 5 | 109.0 ± 10.6 | 25.2 ± 5.1 | 505.6 ± 52.7 |
| *11 wk old, 3 wk RF 2 wk 3.0 mg/kg BW/wk* | 5 | 99.2 ± 9.3 | 41.4 ± 1.7§ | 470.8 ± 41.3 |
| *14 wk old, 6 wk RF 5 wk 3.0 mg/kg BW/wk* | 4 | 90.5 ± 17.5 | 36.3 ± 15.0§ | 384.8 ± 66.0 |

Results of testing performed in female C57BL/6 mice.
Levels of IGF-I and EPO were determined by radioimmunoassay.

IGF-I = insulin-like growth factor-1. Data represent mean ± SD.
Significant differences between *normal and untreated CRF mice, between **treated and untreated CRF mice ($p<0.05$), and between §mice with renal failure of different duration treated with a fixed dose of nandrolone.

**Table 8.** Comparison of EPO mRNA expression in normal and phlebotomized mice and in untreated and androgen-treated CRF mice.

| Characteristics of mice | n | Urea (mmol/l) | EPO | |
|---|---|---|---|---|
| | | | Kidney | Liver |
| *14 wk old, normal* | 7 | 3 | ** | UND |
| *14 wk old, normal phlebotmized* | 5 | 4 | **** | ** |
| *9 wk old, 1 wk RF* | 2 | 47, 51 | UND | UND |
| *Untreated* | | | | |
| *11 wk old, 3 wk RF* | 1 | 34 | * | UND |
| *Untreated* | 1 | 39 | UND | UND |
| *14 wk old, 6 wk RF* | 3 | 34, 38, 41 | * | UND |
| *Untreated* | 6 | 35,38,37,38, 39,40 | UND | UND |
| *14 wk old, 6 wk RF* | 3 | 34, 36, 51 | ** | UND |
| *0.3 mg/kg BW/wk* | 2 | 35, 41 | UND | UND |
| *14 wk old, 6 wk RF* | 1 | 68 | ***** | UND |
| *1.0 mg/kg BW/wk* | 2 | 30,39 | UND | UND |
| *14 wk old, 6 wk RF* | 1 | 32 | **** | UND |
| *3.0 mg/kg BW/wk* | 2 | 43,64 | UND | UND |
| *14 wk old, 6 wk RF* | 1 | 39 | *** | UND |
| *9.0 mg/kg BW/wk* | 2 | 23,29 | UND | UND |
| *9 wk old, 2 wk RF* | 2 | 23, 46 | UND | UND |
| *1 wk 3.0 mg/kg BW/wk* | | | | |
| *11 wk old, 3 wk RF* | 2 | 37, 43 | UND | UND |
| *2 wk 3.0 mg/kg BW/wk* | | | | |
| *14 wk old, 6 wk RF* | 3 | 33, 41, 42 | ** | UND |
| *5 wk 3.0 mg/kg BW/wk* | 2 | 32, 44 | UND | UND |

Results of mRNA expression were obtained by RT-PCR followed by Southern blotting in two selected tissues from female C57BL/6 mice at the time of sacrifice. Data expressed as * to ***** represent the qualitative visual assessment of the intensity of the message. UND = undetected mRNA. Illustrative gels are presented in Figures 21 - 24.

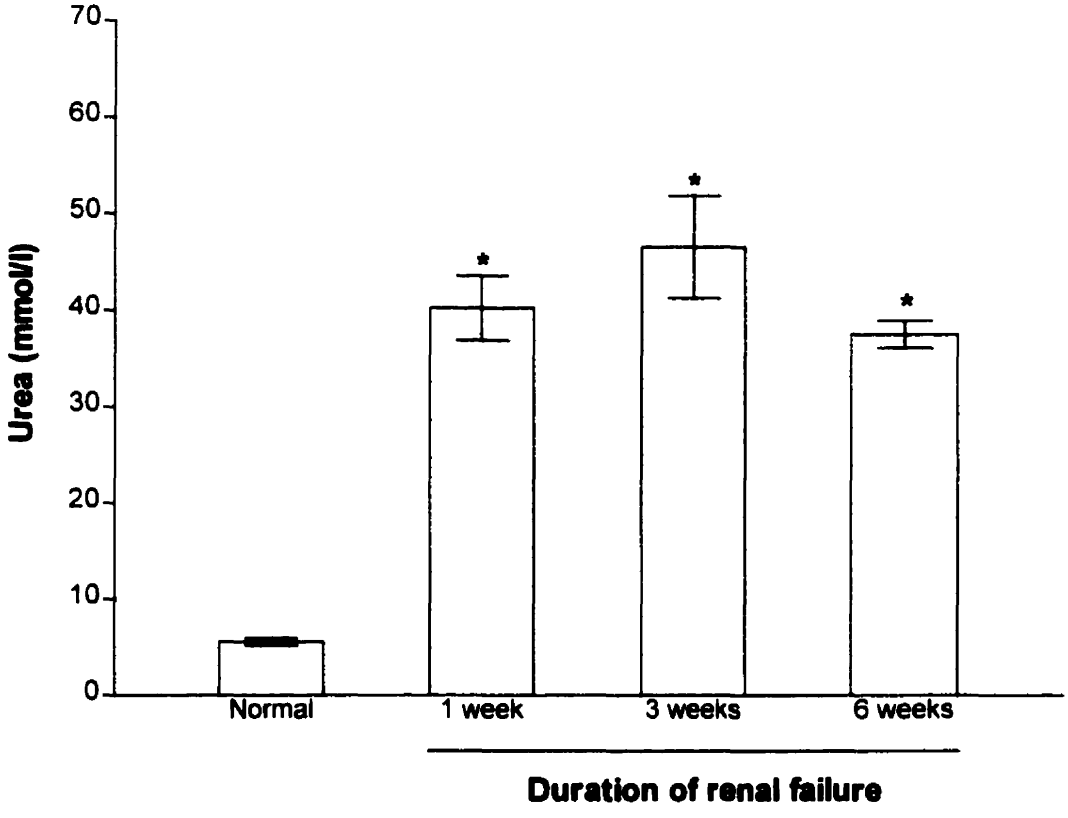



**Figure 5.** Comparison of urea levels in normal mice (n=11) and in mice with renal failure of different duration: 1 week (n=7), 3 weeks (n=7) and 6 weeks (n=11).

Data presented as mean ± SD.
Significant differences between normal and renal failure mice (*$p<0.05$).

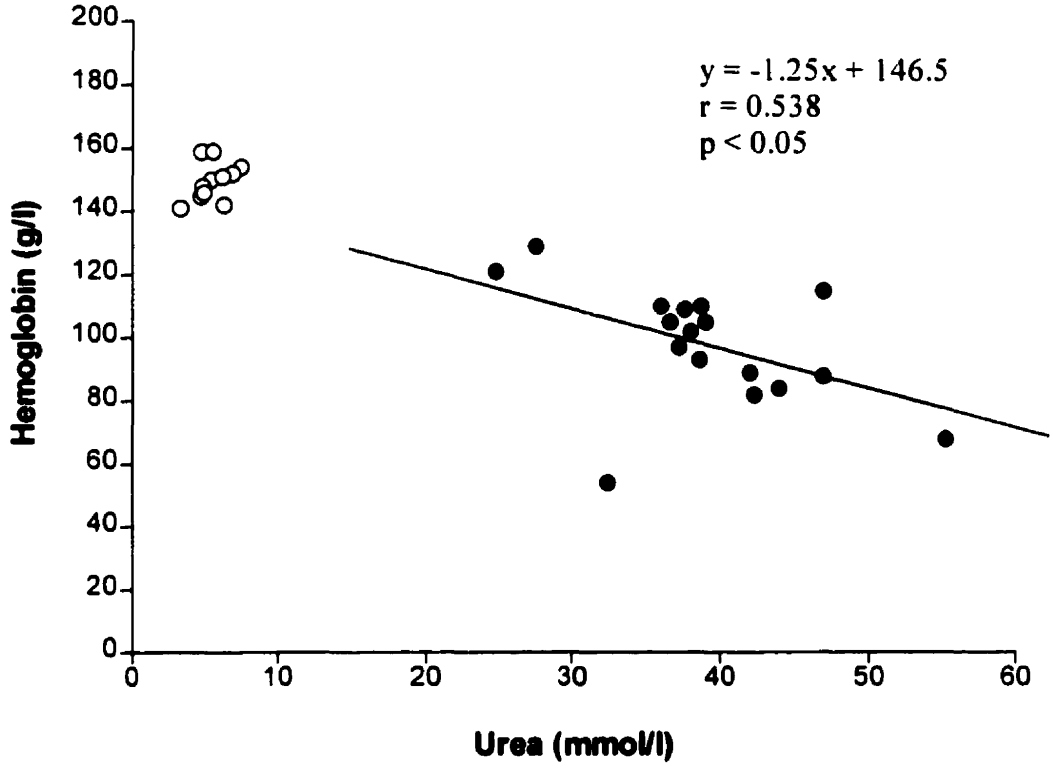



**Figure 6.** Correlation between serum urea levels and hemoglobin concentrations in CRF mice (•, n=17). For comparison, results of normal mice (o, n=11) are shown at the top left.

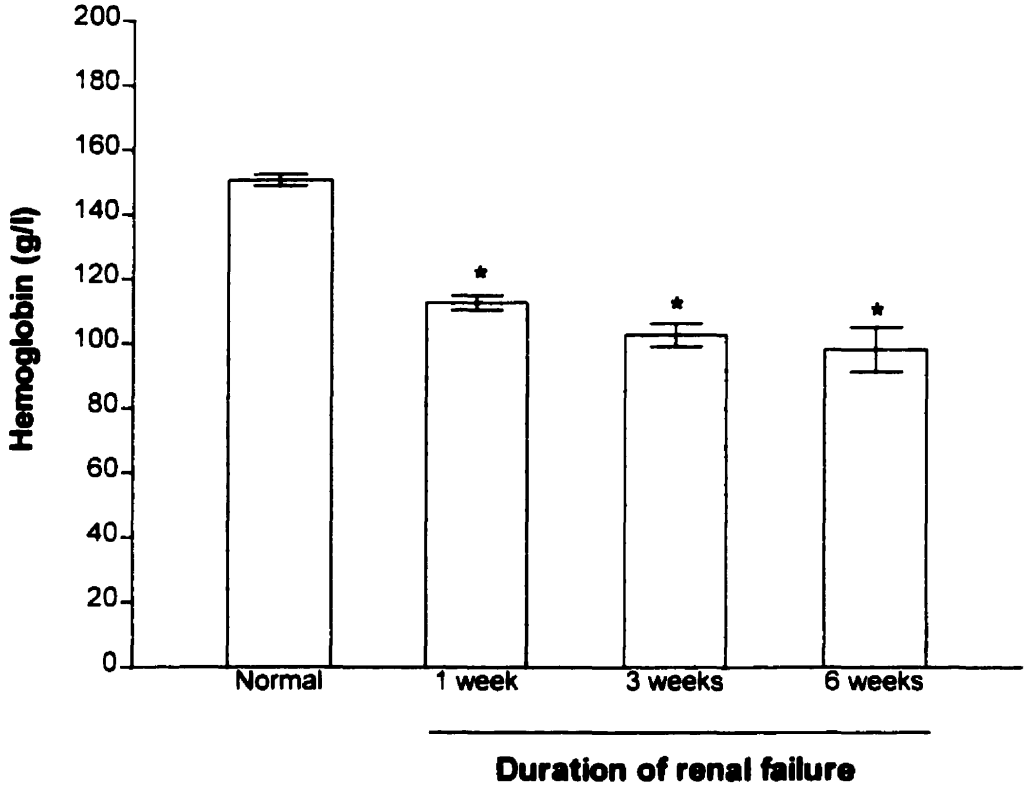



**Figure 7.** Comparison of hemoglobin concentrations between normal mice (n=11) and mice with renal failure of different duration: 1 week (n=7), 3 weeks (n=7) and 6 weeks (n=11).

Data represent mean ± SD
Significant differences between normal and renal failure mice (*$p<0.05$).

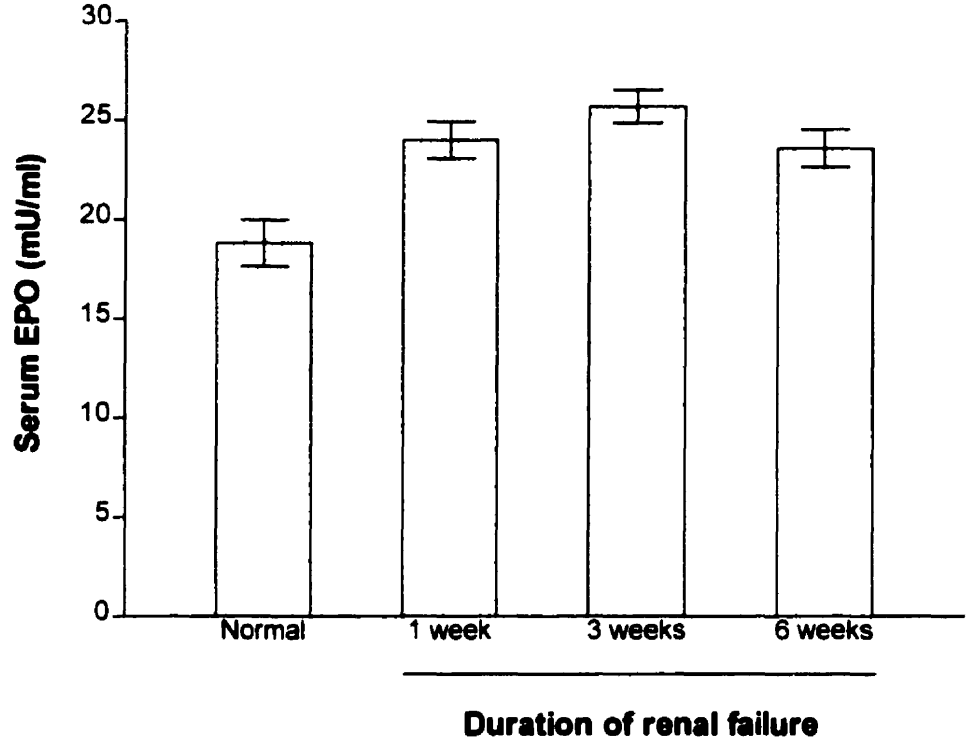



**Figure 8.** Comparison of serum EPO concentrations between normal mice and mice with renal failure of different duration: 1 week (n=7), 3 weeks (n=7) and 6 weeks (n=11).

Data expressed as mean ± SD.

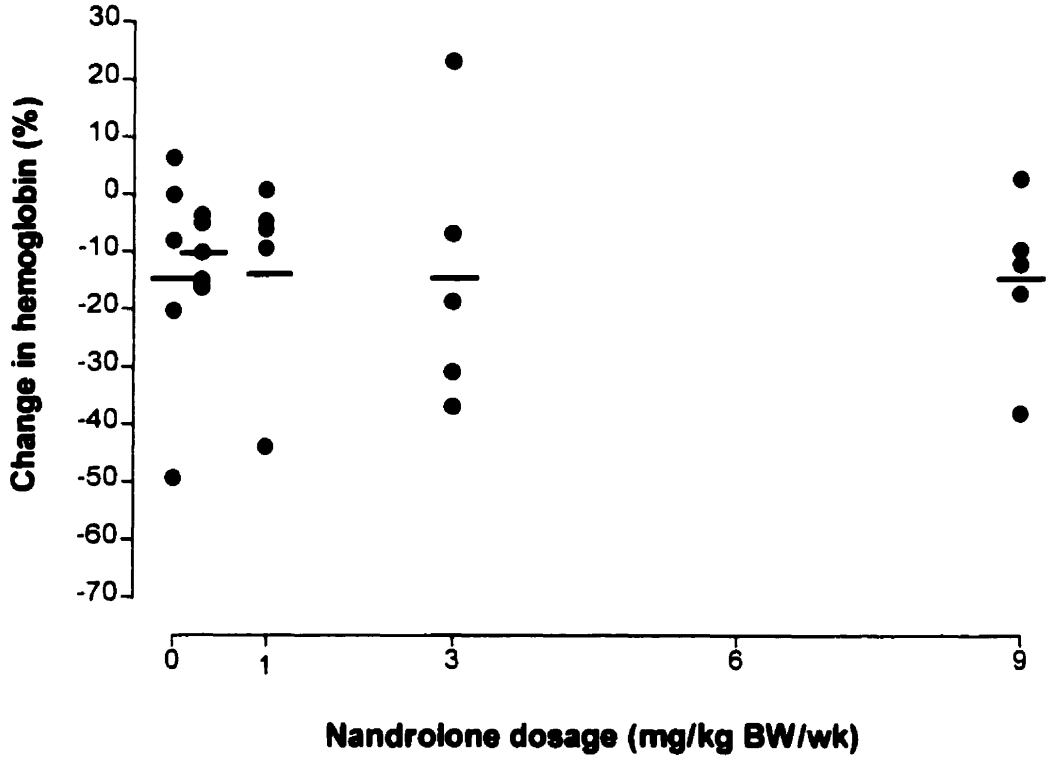



**Figure 9.** The effect of androgen treatment on hemoglobin concentrations in CRF mice: Initial experimental protocol (See Table 4).

Treatment was for 3 weeks starting 3 weeks after the onset of renal failure (left nephrectomy). Treatment groups received i.m. injections of 0.3, 1.0, 3.0, or 9.0 mg/kg BW once weekly. Untreated mice received no injections. Baseline and final hemoglobin concentrations were not statistically significant between the groups. Individual data shown with means indicated by horizontal bars. The Table below displays important information relevant to the Figure (hemoglobin concentrations expressed as mean ± SD).

| Dose of nandrolone (mg/kg BW/wk) | Hemoglobin (g/l) | | Proportion of mice with positive change in Hb |
|---|---|---|---|
| | Pre-treatment | Post-treatment | |
| 0 (n=5) | 101.6 ± 11.4 | 86.0 ± 19.8 | 1 / 5 |
| 0.3 (n=5) | 101.4 ± 15.3 | 90.6 ± 14.2 | 0 / 5 |
| 1.0 (n=5) | 100.8 ± 10.0 | 88.4 ± 20.6 | 1 / 5 |
| 3.0 (n=5) | 97.4 ± 12.1 | 82.2 ± 16.0 | 1 / 5 |
| 9.0 (n=5) | 93.5 ± 17.0 | 79.2 ± 15.1 | 1 / 5 |

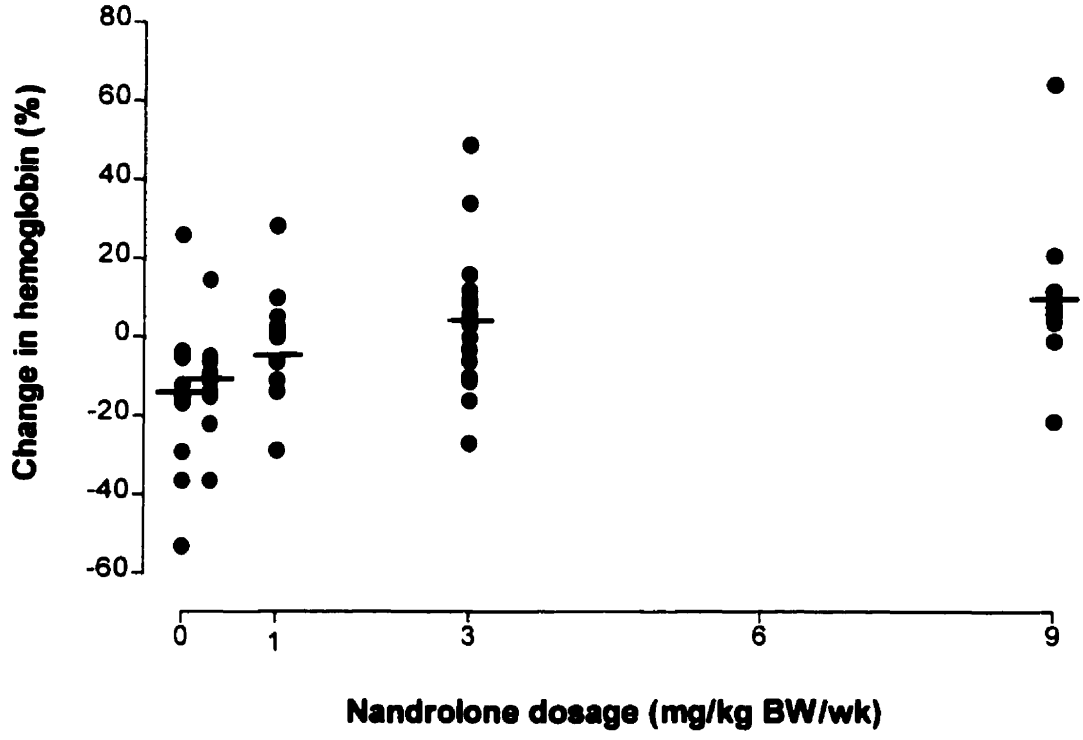



**Figure 10.** The effect of androgen treatment on hemoglobin concentrations in CRF mice: Definitive experimental protocol (See Table 5).

Treatment was for 5 weeks starting one week after the onset of renal failure (left nephrectomy). Treatment groups received i.m. injections of 0.3, 1.0, 3.0, or 9.0 mg/kg BW once weekly. Untreated mice received no injections. Baseline and final hemoglobin concentrations were not statistically significant between the groups. Individual data shown with means indicated by horizontal bars. The Table below displays important information relevant to the Figure (hemoglobin concentrations expressed as mean ± SD).

| Dose of nandrolone (mg/kg BW/wk) | Hemoglobin (g/l) | | Proportion of mice with positive change in Hb |
|---|---|---|---|
| | Pre-treatment | Post-treatment | |
| 0 (n=11) | 120.1 ± 12.6 | 101.4 ± 26.3 | 2 / 11 |
| 0.3 (n=11) | 120.9 ± 13.8 | 106.0 ± 15.5 | 1 / 11 |
| 1.0 (n=10) | 117.9 ± 12.3 | 113.6 ± 17.8 | 6 / 10 |
| 3.0 (n=15) | 114.5 ± 15.8 | 117.5 ± 13.9 | 8 / 15 |
| 9.0 (n=8) | 112.4 ± 17.7 | 121.7 ± 15.0 | 6 / 8 |

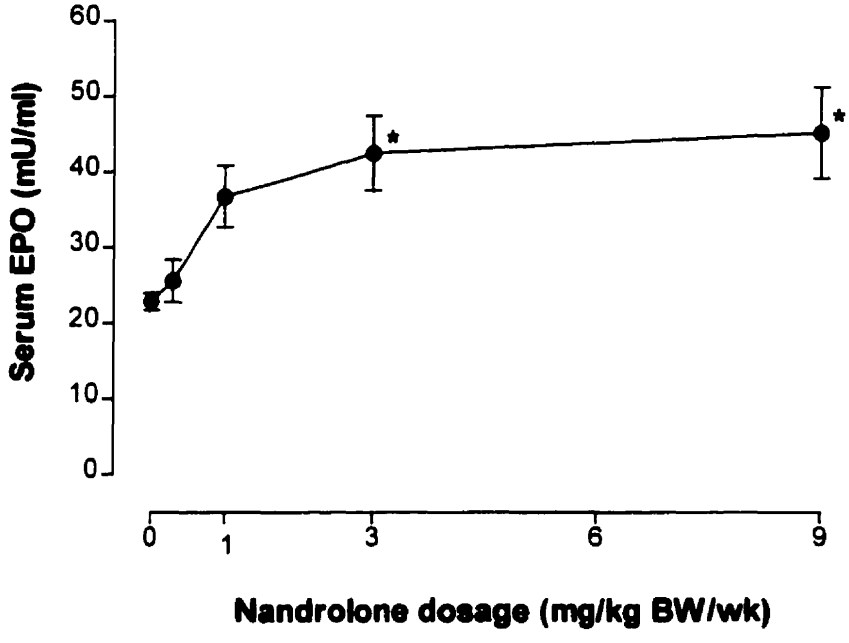



**Figure 11.** The effect of androgen treatment on serum EPO concentrations: Definitive experimental protocol (See Table 5).

Data expressed as mean ± SD.
Significant differences between treated and untreated CRF mice (*$p<0.05$).

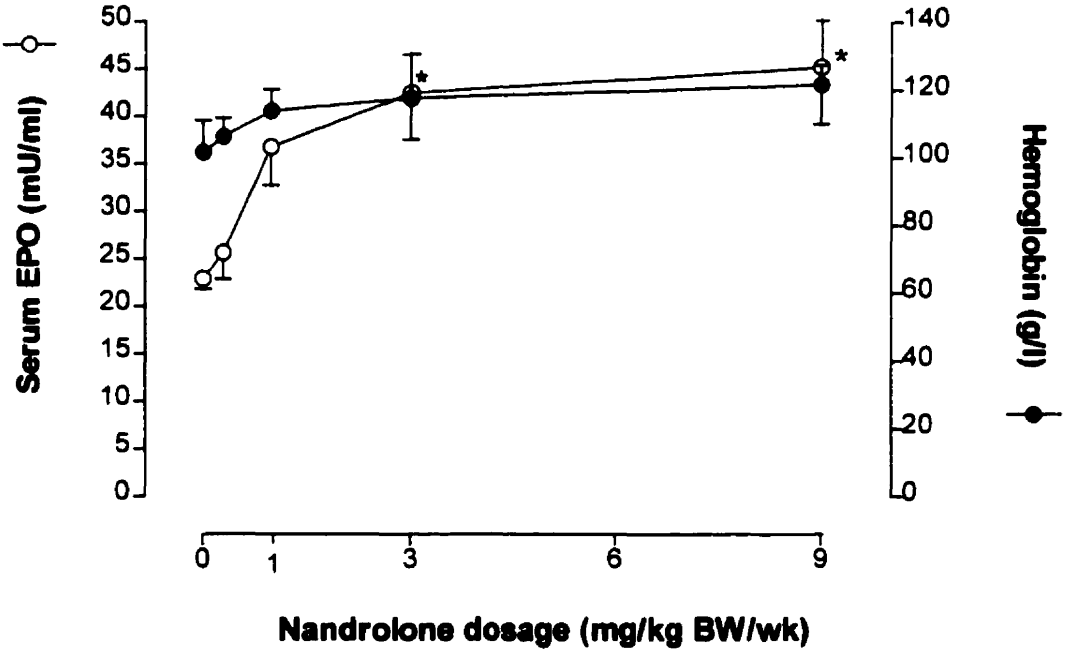



**Figure 12.** Relation between the changes in serum EPO and hemoglobin concentrations of CRF mice in response to different androgen doses (0.3, 1.0, 3.0 and 9.0 mg/kg BW/wk during 5 weeks): Definitive experimental protocol (See Table 5).

Data expressed as mean ± SD.
Significant differences in serum EPO concentrations between treated and untreated CRF mice (*$p<0.05$).

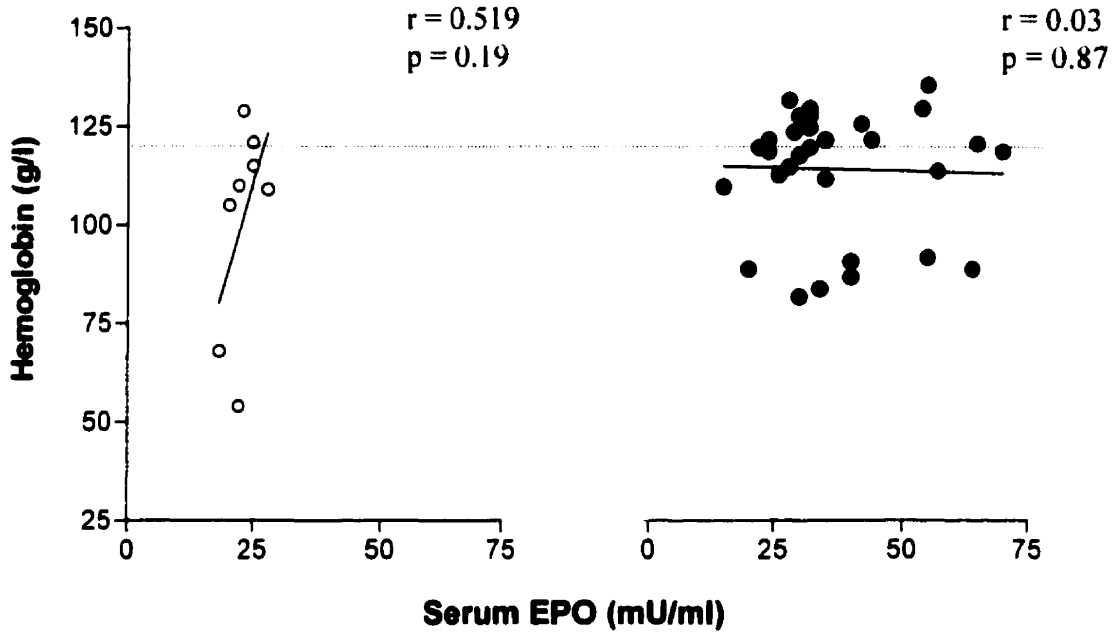



**Figure 13.** Correlation between serum EPO and hemoglobin concentrations in untreated (o) and androgen-treated (•) CRF mice: Definitive experimental protocol (See Table 5)

The serum EPO level of 25 mU/ml and the hemoglobin concentration of 120 g/l are indicated by the dashed lines.

| Proportion of mice with serum EPO >25 mU/ml and hemoglobin >120 g/l | |
|---|---|
| CRF, untreated: | 0 / 8 |
| CRF, androgen-treated: | 13 / 31 |

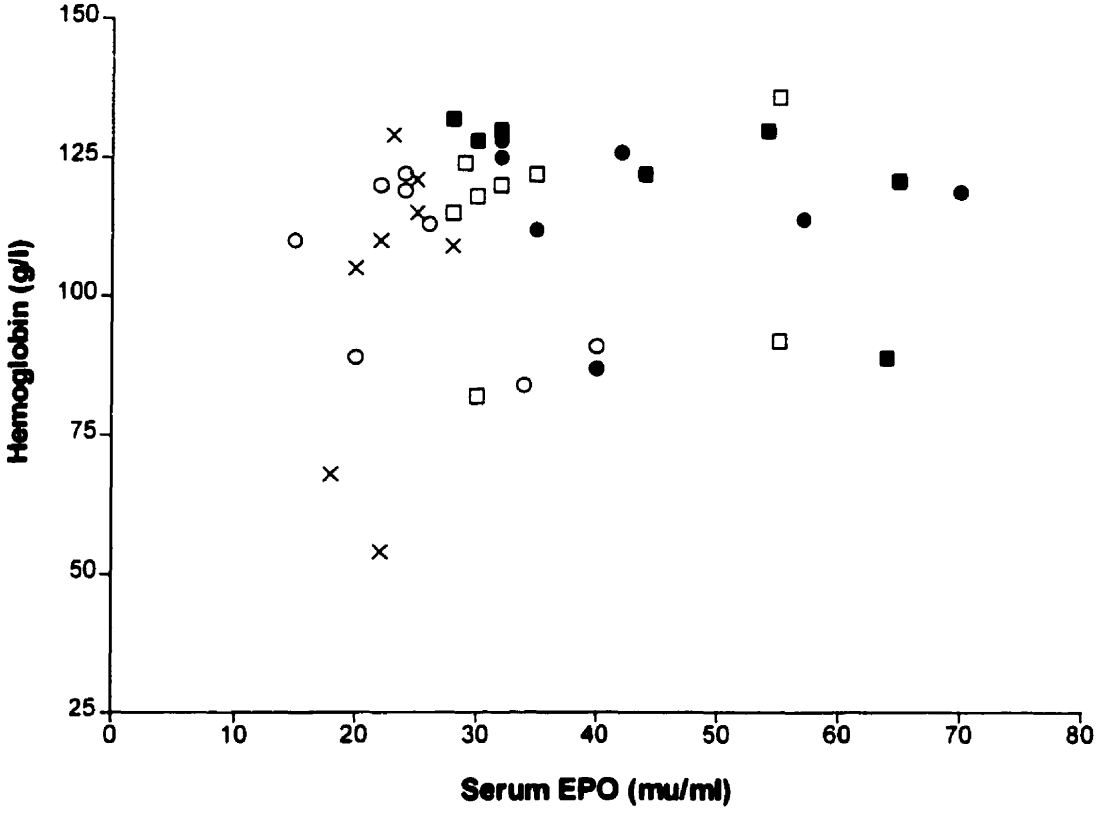



**Figure 14.** Correlation between serum EPO and hemoglobin concentrations in untreated and androgen-treated CRF mice, according to treatment dose.

The serum EPO level of 25 mU/ml and the hemoglobin concentration of 120 g/l are indicated by the dashed lines.

X = untreated; O = 0.3 mg/kg BW/wk; □ = 1.0 mg/kg BW/wk;
● = 3.0 mg/kg BW/wk; ■ = 9.0 mg/kg BW/wk

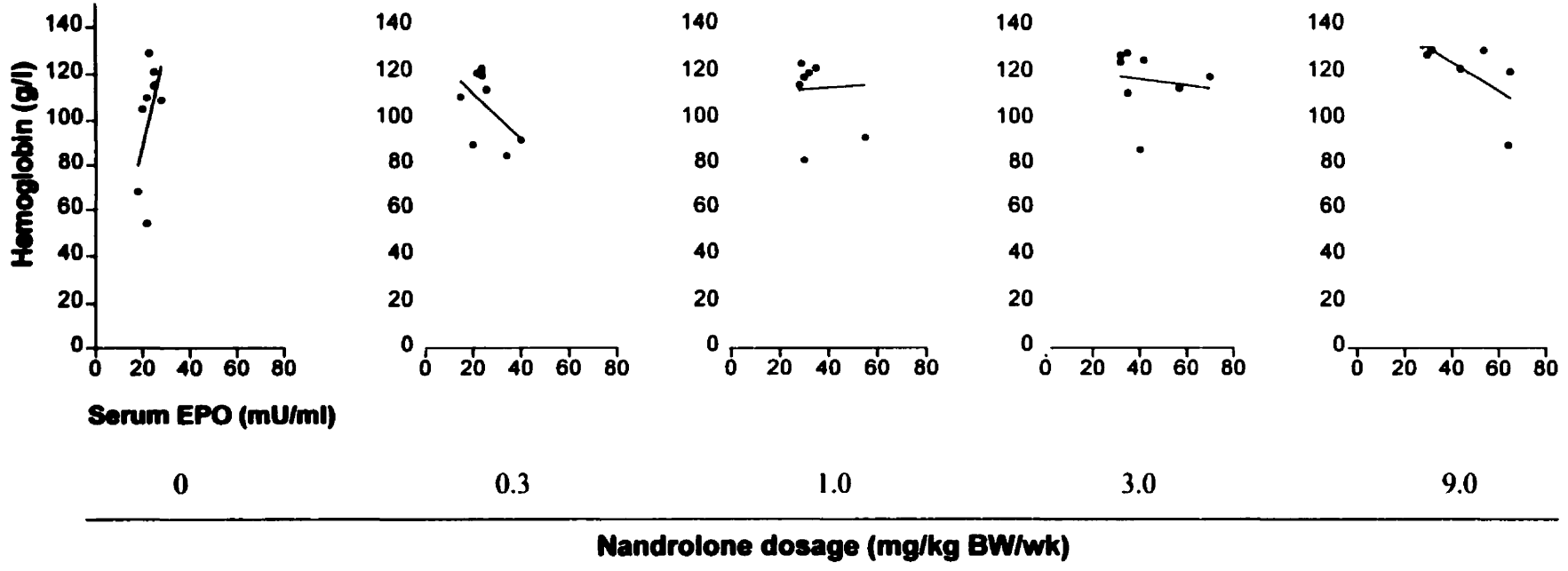



**Figure 15.** Correlation between serum EPO and hemoglobin concentrations in untreated and androgen-treated CRF mice, according to treatment dose.

The serum EPO level of 25 mU/ml and the hemoglobin concentration of 120 g/l are indicated by the dashed lines.

| Proportion of mice with serum EPO >25 mU/ml and hemoglobin >120 g/l | |
|---|---|
| CRF, untreated: | 0 / 8 |
| CRF, androgen-treated (0.3 mg/kg BW/wk): | 0 / 8 |
| CRF, androgen-treated (1.0 mg/kg BW/wk): | 4 / 8 |
| CRF, androgen-treated (3.0 mg/kg BW/wk): | 4 / 8 |
| CRF, androgen-treated (9.0 mg/kg BW/wk): | 6 / 7 |

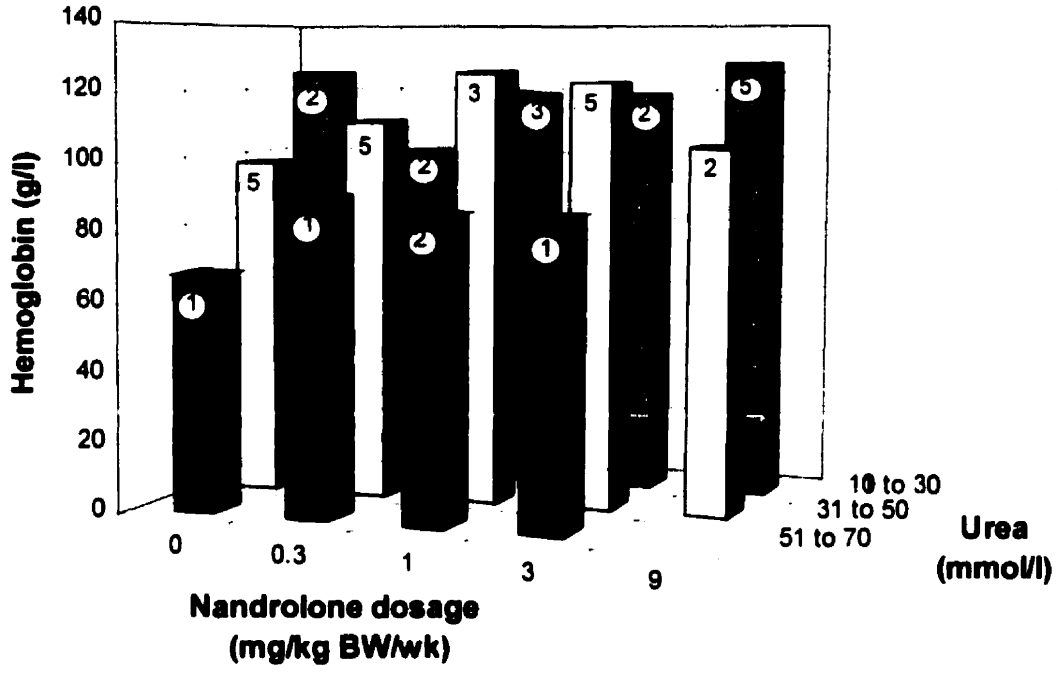



**Figure 16.** Influence of the severity of renal failure (as measured by urea levels) on the effect of androgen treatment on hemoglobin concentrations: Definitive experimental protocol (See Table 5).

Treatment was for 5 weeks starting one week after the onset of renal failure (left nephrectomy). Treatment groups received i.m. injections of 0.3, 1.0, 3.0, or 9.0 mg/kg BW once weekly. Untreated mice received no injections. Number of mice in each category is displayed in circles at the top of each bar. The Table below displays important information relevant to the Figure (serum EPO concentrations expressed as mean ± SD).

| Urea (mmol/l) | Serum EPO concentration (mU/ml) | | | | |
|---|---|---|---|---|---|
| | Nandrolone dosage (mg/kg BW/wk) | | | | |
| | 0 | 0.3 | 1 | 3 | 9 |
| 11 to 30 | 24 ± 1 | 23 ± 3 | 29 ± 1 | 39 ± 3 | 38 ± 6 |
| 31 to 50 | 23 ± 2 | 24 ± 4 | 41 ± 8 | 45 ± 12 | 65 ± 1 |
| 51 to 70 | 18 | 40 | 43 ± 10 | 40 | - |

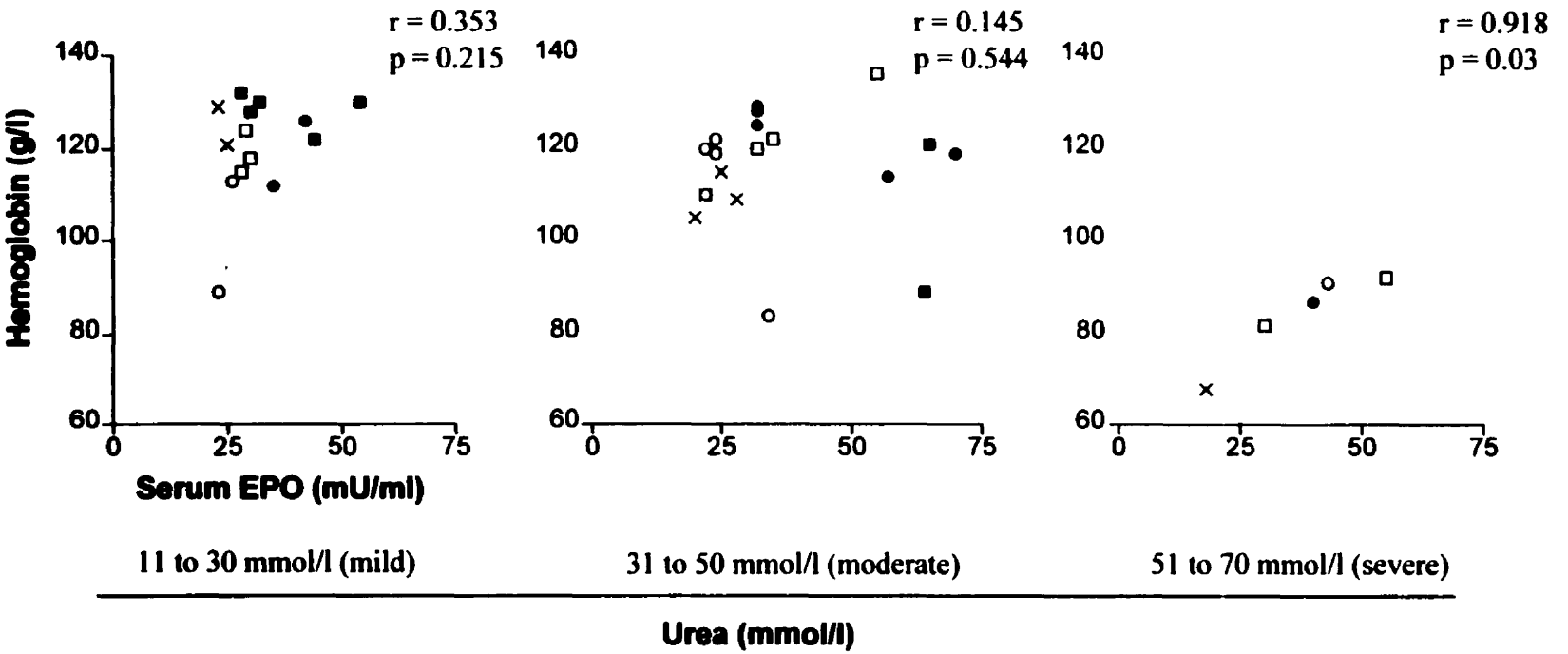



**Figure 17.** Correlation between serum EPO and hemoglobin concentrations in untreated and androgen-treated CRF mice, according to degree of renal failure.

The serum EPO level of 25 mU/ml and the hemoglobin concentration of 120 g/l are indicated by the dashed lines.
x = Untreated; O = 0.3 mg/kg BW/wk; □ = 1.0 mg/kg BW/wk; ● = 3.0 mg/kg BW/wk; ■ = 9.0 mg/kg BW/wk

| Proportion of mice with serum EPO >25 mU/ml and hemoglobin >120 g/l | |
|---|---|
| Mice with mild renal failure: | 7 / 14 |
| Mice with moderate renal failure: | 7 / 20 |
| Mice with severe renal failure | 0 / 5 |

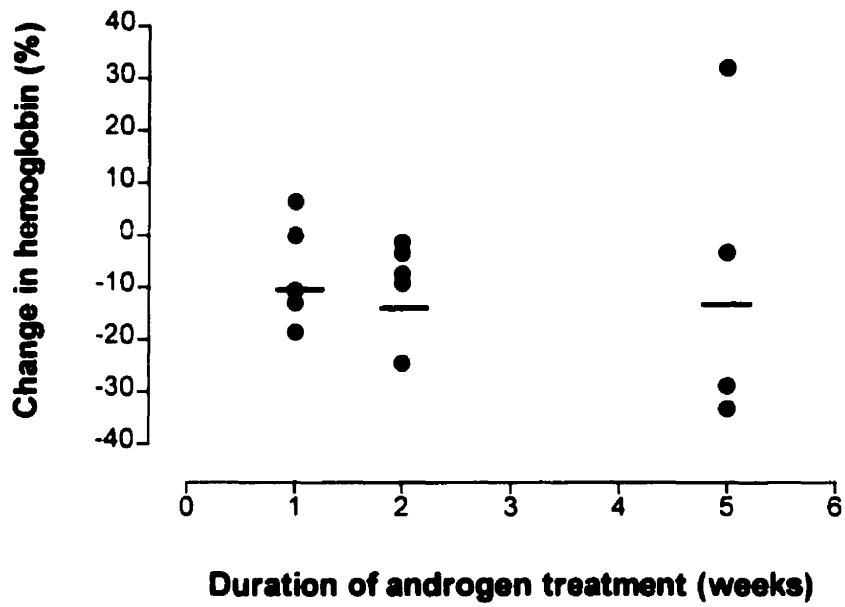



**Figure 18.** The effect of duration of androgen treatment (3.0 mg/kg BW/wk) on hemoglobin concentrations in mice with renal failure of different duration: Time-course study (See Table 6).

Mice with renal failure were treated with 3.0 mg/kg BW nandrolone decanoate once weekly for periods of 1, 2 and 5 weeks starting one week after the onset of renal failure (left nephrectomy). Baseline and final hemoglobin were not significantly different between the groups. Individual data shown with means indicated by horizontal bars. The Table below displays important information relevant to the Figure (hemoglobin concentrations expressed as mean ± SD).

| Duration of treatment | Hemoglobin (g/l) | | Proportion of mice with positive change in Hb |
|---|---|---|---|
| | Pre-treatment | Post-treatment | |
| 1 week (n=5) | 117.2 ± 9.9 | 109.0 ± 10.6 | 1 / 5 |
| 2 weeks (n=5) | 110.4 ± 11.5 | 99.2 ± 9.3 | 0 / 5 |
| 5 weeks (n=4) | 101.8 ± 13.9 | 90.5 ± 17.5 | 1 / 5 |

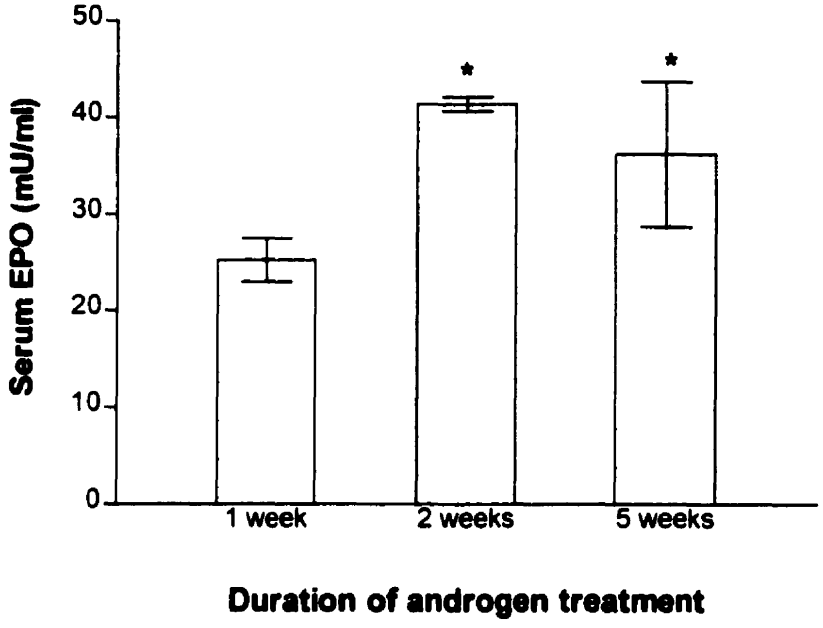



**Figure 19.** The effect of duration of androgen treatment (3.0 mg/kg BW/wk) on serum EPO concentrations in mice with renal failure of different duration: Time-course study (See Table 6).

Data expressed as mean ± SD.
Significant differences between mice treated for 2 and 5 weeks and mice treated for one week (*$p<0.05$).

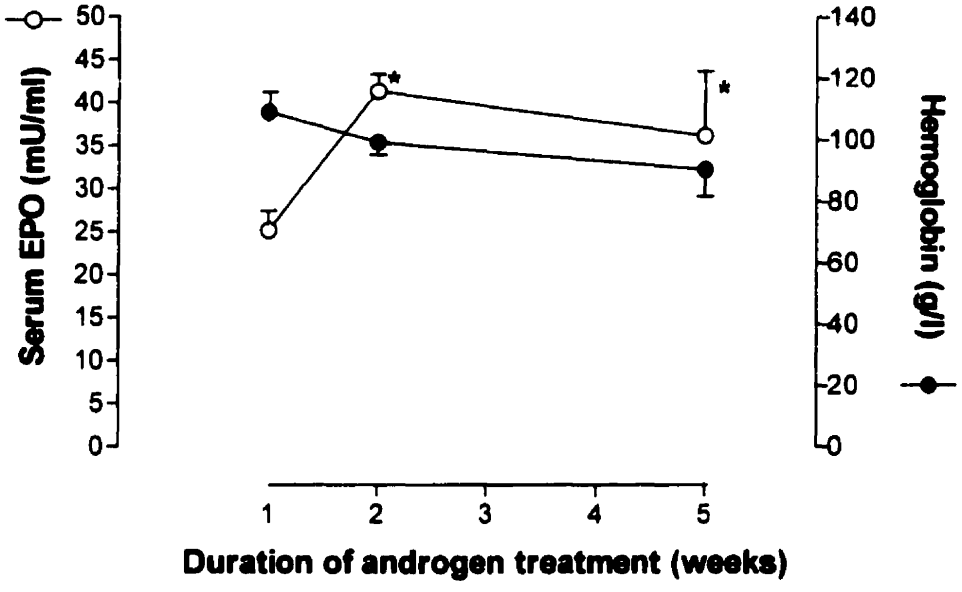



**Figure 20.** Relation between the changes in serum EPO and hemoglobin concentrations of mice with renal failure in response to different duration of androgen treatment (3 mg/kg BW/wk during 1, 2 and 5 weeks): Time-course study (See Table 6).

Data expressed as mean ± SD.
Significant differences in serum EPO concentrations between mice treated for 2 and 5 weeks and mice treated for one week (*$p<0.05$).

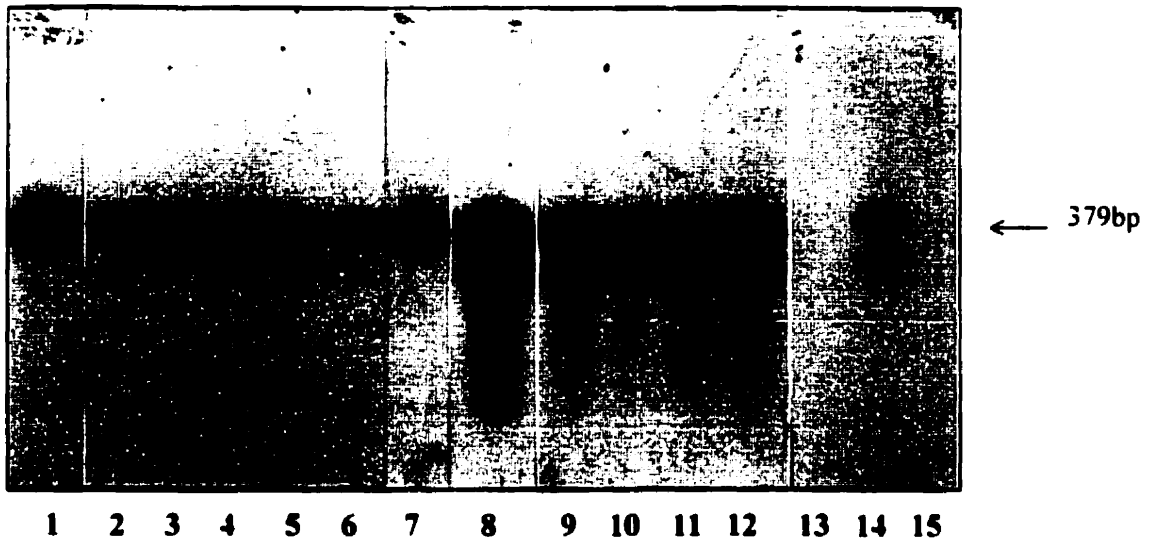



**Figure 21.** Expression of EPO mRNA in the kidney of normal. phlebotomized and CRF mice.

Southern blot analysis of cDNA amplified by RT-PCR with 4 days exposure:

Lanes 1-7: Normal mice
Lanes 8-12: Phlebotomized mice
Lanes 13-15: CRF mice (at 6 weeks)

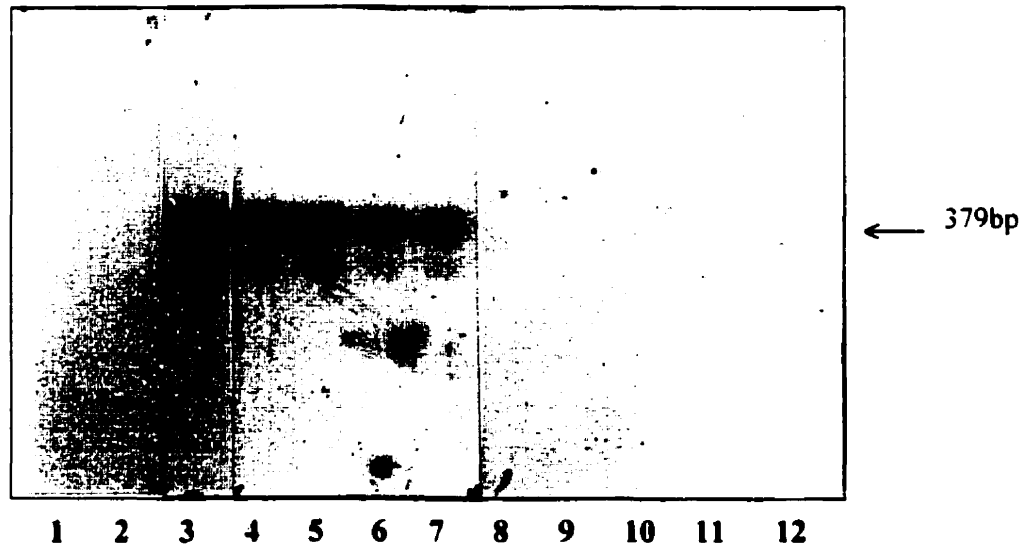



**Figure 22**. Expression of EPO mRNA in the liver of normal. phlebotomized and CRF mice.

Southern blot analysis of cDNA amplified by RT-PCR with 4 days exposure:

Lanes 1-2: Normal mice
Lanes 3-7: Phlebotomized mice
Lanes 8-12: CRF mice (at 6 weeks)

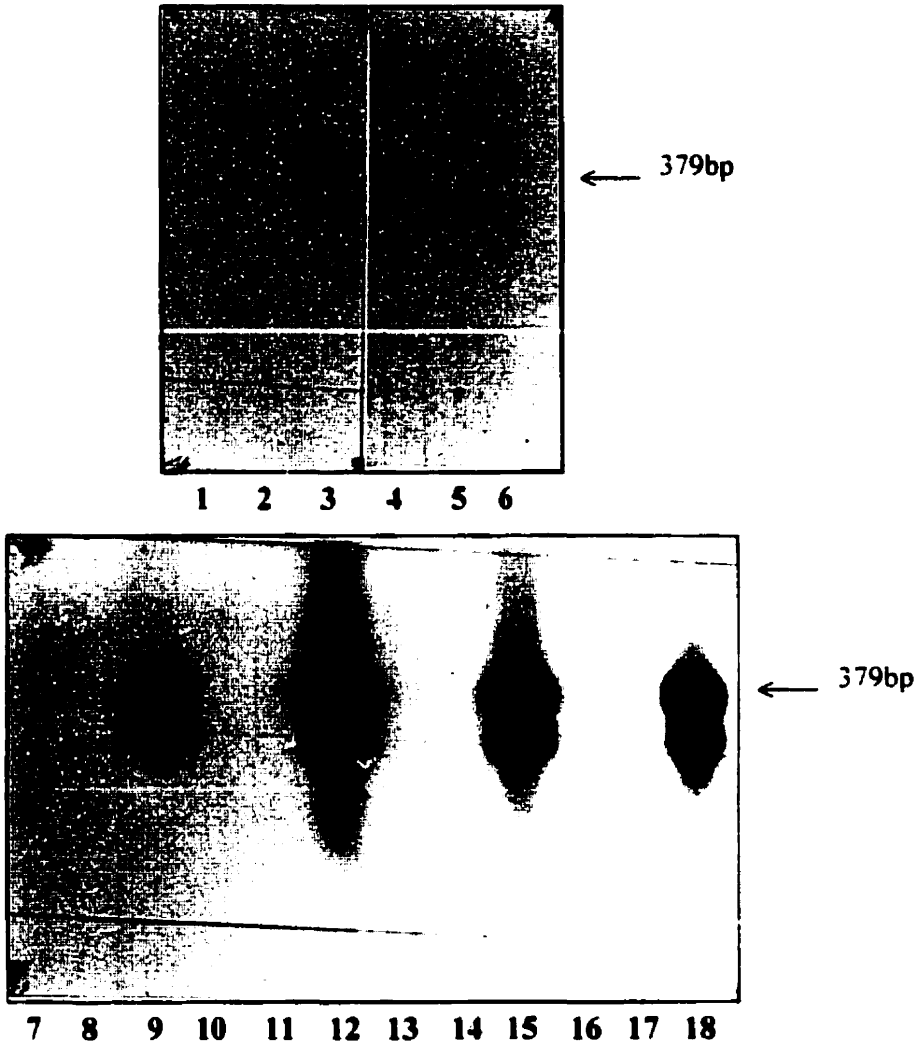



**Figure 23.** Expression of EPO mRNA in the remnant kidney of CRF mice treated with nandrolone decanoate.

Southern blot analysis of cDNA amplified by RT-PCR with 4 days exposure:

Lanes 1-6: Untreated CRF mice
Lanes 7-9: Androgen-treated CRF mice (0.3 mg/kg BW/wk)
Lanes 10-12: Androgen-treated CRF mice (1.0 mg/kg BW/wk)
Lanes 13-15: Androgen-treated CRF mice (3.0 mg/kg BW/wk)
Lanes 16-18: Androgen-treated CRF mice (9.0 mg/kg BW/wk)

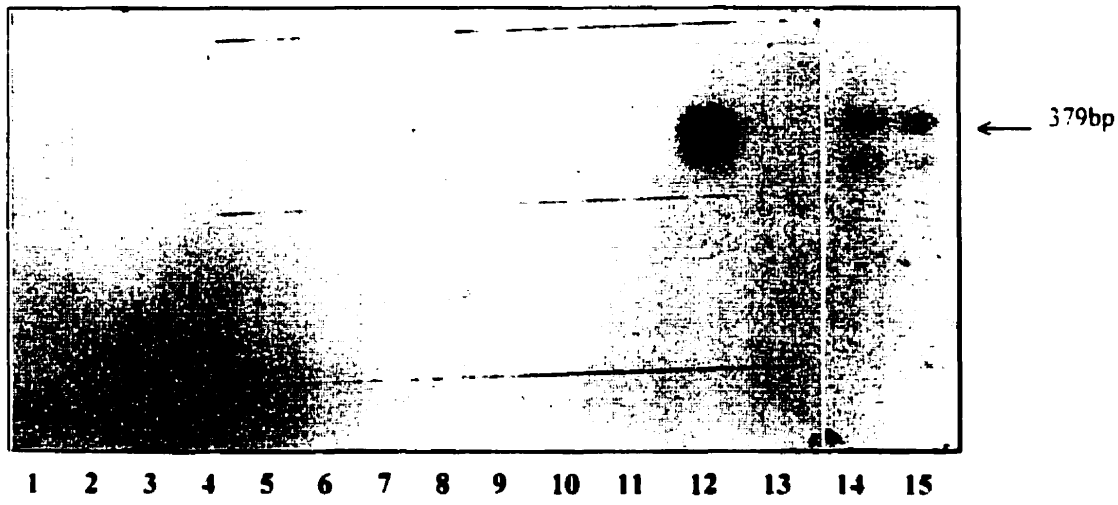



**Figure 24.** Expression of EPO mRNA in the liver and remnant kidney of mice with renal failure treated with a fixed dose of nandrolone (3.0 mg/kg BW/wk) for a duration of 1, 2, and 5 weeks.

Southern blot analysis of cDNA amplified by RT-PCR with 4 days exposure:

Lanes 1-2: Liver of androgen-treated CRF mice (1-week treatment)
Lanes 3-4: Liver of androgen-treated CRF mice (2-week treatment)
Lanes 5-7: Liver of androgen-treated CRF mice (5-week treatment)
Lane 8: Kidney of androgen-treated CRF mice (1-week treatment)
Lanes 9-10: Kidney of androgen-treated CRF mice (2-week treatment)
Lanes 11-15: Kidney of androgen-treated CRF mice (5-week treatment)

**Plate 2.** Comparison of bone and marrow histology between normal and CRF mice.

The tissue sections are all oriented in the same direction with the cartilaginous surface of the tibial plateau placed at the top of each panel (magnification x200). The top two panels (a, b) are from a normal mouse and the bottom two panels (c, d) are from a littermate, 14-week old mouse with renal failure of 6 weeks' duration.

a) *H&E staining of normal bone demonstrating*: sharp, linear interface between bone and marrow; large marrow spaces with easily identifiable hematopoietic progenitors and prominent adipocytes normal architecture of lamellar bone surrounding the marrow.

b) *Acid phosphatase staining of normal bone demonstrating*: few osteoclasts (bone resorbing cells indicated in red and observed only along subcortical bone); clear, smooth delineation between the large marrow spaces and the surrounding bone.

c) *H&E staining of CRF bone demonstrating considerable changes from normal bone*: extensive replacement fibrosis of the bone marrow; irregular interface between the lamellar bone and marrow with distinct scalloping in places due to excessive osteoclastic activity.

d) *Acid phosphatase staining of CRF bone demonstrating*: increased number and size of osteoclasts involving subcortical and cancellous bone. The bone shows prominent remodeling.

# Plate 2

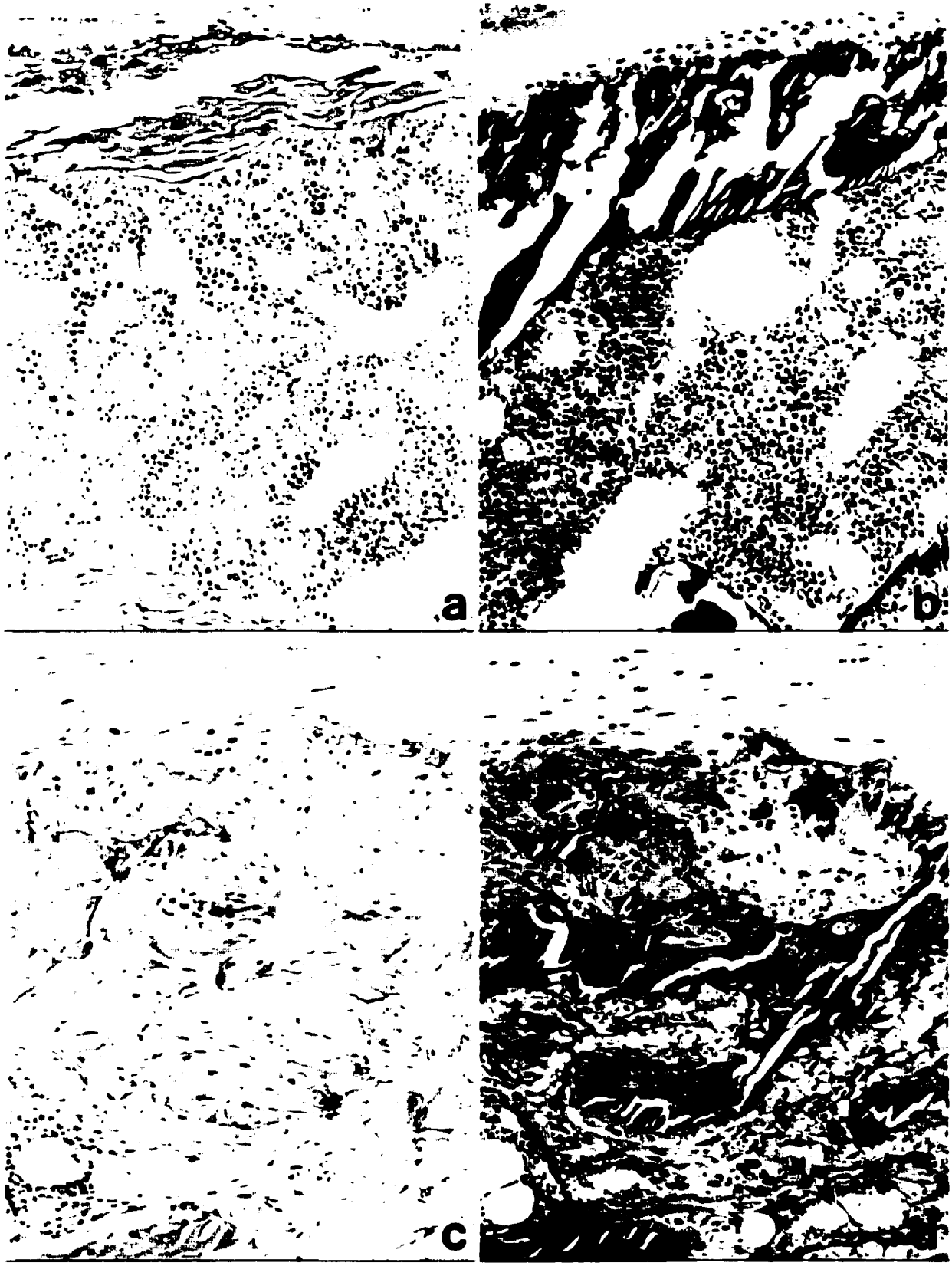


**Plate 3.** Comparison of bone and marrow histology between mice with renal failure of different duration: 1, 3 and 6 weeks.

The tissue sections are all oriented in the same direction with the cartilaginous surface of the tibial plateau placed on the left of each panel. The top two panels (a, b) are from a mouse with 1 week of renal failure, the middle two panels (c, d) are from a mouse with 3 weeks of renal failure and the bottom two panels (e, f) are from a mouse with 6 weeks of renal failure.

a) *H&E staining of bone after 1 week of renal failure*: Changes in bone and marrow morphology are already evident. Marrow fibrosis is extensive in addition to the loss of normal architecture of the surrounding bone. Adipocytes are less common than in normal marrow and scalloping of the bone edges has already begun in some areas.

b) *Acid phosphatase staining of bone after 1 week of renal failure*: Again, there is evident morphological changes in the architecture of the marrow and the surrounding bone. The number and size of osteoclasts (indicated in red) is already significantly increased from normal. Note the minimal activity between cancellous bone and fibrous tissue.

c) *H&E staining of bone after 3 weeks of renal failure*: With further progression of the renal failure, more severe morphological changes occur in the bone and marrow. The fibrosis is more extensive while scalloping of the bone edges is well developed.

d) *Acid phosphatase staining of bone after 3 weeks of renal failure*: Although adipocytes are seen in this panel, the cells are much smaller and less common than normal. The number and size of osteoclasts, however, has increased significantly from one week of renal failure. Osteoclasts extend along cancellous bone but remain predominantly in the fibrous tissue of the marrow.

e) *H&E staining of bone after 6 weeks of renal failure*: In this panel, severe fibrosis and damage is evident throughout the whole section. Large marrow spaces have been replaced by fibrosis. Scalloping of the bone edges is widespread.

f) *Acid phosphatase staining of bone after 6 weeks of renal failure*: It is clear in this panel that the number and size of osteoclasts is greatly increased from normal and one week of renal failure. Also evident is the increased osteoclastic activity in relation to all bony lamellae.

# Plate 3

# DISCUSSION

The mouse model utilized in this study presents the main characteristics of severe chronic renal failure in man with the expected retention of nitrogenous compounds together with related biochemical abnormalities such as hyperkalemia and acidosis, growth retardation, immunological deficits with increased susceptibility to infection, lipid changes with atherosclerosis, florid bone disease and anemia. The present study confirmed some of the observations previously made in this model of chronic severe failure of a major organ (43, 44, 45, 46, 47, 116). Clinical cases of CRF represent a heterogeneous mixture of underlying renal disease, age, gender, ethnicity, co-morbid factors, and other characteristics complicated by variations in dietary and therapeutic regimens that may affect the study parameters. In addition, repetitive hemodialysis sessions causing hemodynamic stress and cytokine activation were obviously lacking from this model. Therefore, the selected mouse model of CRF derives its strength from its ability to accurately reproduce the human condition without the above confounding factors. This model remains stable compared to other animal models, especially in the rat, showing undesirable hypertrophy of the remnant kidney and subsequent improvement in renal function. After six weeks of renal failure, the mouse model displays the characteristic features of severe stable renal failure observed in the human condition.

The key advantages of ease of induction and maintenance of the mouse model of CRF were demonstrated in the present investigation. The surgical procedures involved in the induction of renal failure were technically straightforward, of rapid execution

thereby limiting exposure to anesthesia, associated with minimal blood loss, and free of immediate as well as long-term infectious and inflammatory complications. Following the onset of renal failure, the mice were maintained for periods up to six weeks using standard procedures of animal husbandry. No restrictions in dietary intake (solid food and water) were required and the mice enjoyed unlimited activity. The androgen-treated CRF mice were housed in an all-purpose animal facility together with the selected control groups of untreated CRF mice as well as normal and acutely phlebotomized mice. The availability of this model of CRF enabled the present investigation to be conducted in the mouse, a species which has been previously investigated extensively with regards to erythropoiesis and its regulation (16, 73, 138).

A technical improvement of the first surgical step to induce renal failure, i.e. electrocoagulation of the surface of the right kidney, was introduced during this study. As shown in Plate 1, the metallic plate upon which the mouse rests during the electrocoagulation procedure was reduced in size in order to limit the contact of the plate only to the body of the mice. This modification reduced the likelihood of causing electrical burn to the more fragile extremities, namely, the ears, limbs and tail.

The mode of induction of renal failure by electrocoagulation of the renal surface may have direct repercussions on the EPO- sensing and producing sites located in the kidney. Both these sites have been identified with cells of the cortical interstitium lying in close proximity to the capillaries of convoluted tubular segments. All these sites can potentially be damaged by the electrocoagulation procedure which is aimed at destroying the entire superficial cortical area. The destruction of the renal cortex would eliminate the superficial cortical nephrons which constitute the majority (85%) of

nephrons while sparing the minority population of deeply-seated juxtamedullary nephrons. As a consequence, this mode of induction of renal failure has the potential to destroy, either partially or completely, the EPO- sensing and producing sites. However, the electrocoagulation is not applied to the surface of the kidney close to the hilum and the inner part of the lower pole in order to prevent injury to the delicate hilar structures (renal vessels, nerves and lymphatics) and the ureter, respectively. Additionally, since the electrocoagulation procedure is applied solely on the basis of observation by the naked eye, there may be other areas of the cortex unintentionally spared from the burning. As a consequence, it is therefore possible that for the same degree of renal failure, there might be differential destruction or preservation of EPO- sensing and producing sites, thereby explaining the intra-group variability of the results..

The blood urea level as determined by autoanalyzer served as the measure of renal function. Usually, the serum creatinine concentration is used as an index of renal function because it is not influenced by changes in fluid balance, thus directly reflecting renal function. However, the normal creatinine levels in the blood of mice are significantly lower than in man (45), and therefore require a special method of measurement. Alternatively, blood urea levels, which are similar in normal mice and man, can be used to monitor renal function in the euvolemic state. Blood urea levels can be affected by fluid status independently of renal function e.g. urea levels increase with fluid depletion whereas they decrease during fluid overload. By the usual criteria of evaluation, the CRF mice appeared to be in relatively normal fluid balance: they were edema-free therefore excluding marked fluid excess and they were active with free access to water suggesting the absence of significant fluid depletion.

The degree of renal failure, as assessed by serum urea levels, showed fluctuations with time. This parameter was increased on average 7- to 8-fold, indicating a reciprocal fall in renal function. A significant increase in serum urea was observed at one and three weeks following the surgical induction of renal failure. Subsequently, with the duration of renal failure, the serum levels of urea decreased. Several factors may be responsible for this downward trend. The increased urea levels early after the onset of renal failure are a reflection not only of definitive destruction of renal parenchyma by the electrical burn but also of reversible functional impairment of residual intact renal tissue caused by interstitial inflammation secondary to the thermal injury. These combined lesions account for the large losses of mice from severe renal failure during the first week following the left nephrectomy when the only functional renal parenchyma consists of the deep tissues of the right kidney which escaped the thermal injury. Only the mice with milder renal impairment will survive this early period, thus explaining the lower serum urea values observed after six weeks of renal failure.

Evidence for severe bone disease was found in CRF mice. Studies of bone histology in CRF mice revealed extensive changes affecting bone and marrow morphology (44). To further evaluate these profound changes, histological sections were made of the tibial plateau of normal mice and mice with renal failure of different duration. Evidence of prominent bone remodeling was observed as early as one week after the onset of renal failure. After six weeks of renal failure, widespread remodeling was evident showing the typical changes of osteitis fibrosa cystica: replacement fibrosis of the marrow, irregular bone / marrow interface, and increased size and number of

osteoclasts together with evidence of increased osteoclastic activity such as numerous bone lacunae with frequent scalloping.

The rapidity with which the bone changes develop during renal failure in this animal model deserves comment. The structural changes in the bone were associated with significant serum biochemical abnormalities, all supporting marked secondary byperparathyroidism. In fact, hypercalcemia was observed as further evidence of severe secondary hyperparathyroidism giving rise to tertiary hyperparathyroidism. Possible explanations for the rapid development of severe hyperparathyroidism may include the strain and sex of young animals in a rapid growth phase. It is also possible that the secondary hyperparathyroidism was initiated even before the onset of the renal failure as a direct result of the electrocoagulation of the surface of the right kidney. It is well known that unilateral or bilateral renal diseases associated with calcium and/or magnesium wasting, with secondary hypocalcemia and/or hypomagnesemia, are potent stimuli of parathyroid function. It is possible that electrocoagulation of the right kidney may induce such renal leakage with stimulation of parathyroid activity causing secondary hyperparathyroidism even before the onset of renal failure induced by the left nephrectomy. In this instance, the onset of renal failure would further aggravate the existing hyperparathyroidism, readily causing hypercalcemia as seen in the model. Experimental support for this hypothesis could be readily obtained from correlations of blood, urine and bone measurements made between the first and second surgical procedures to induce renal failure.

Many biochemical changes were associated with the bone disease. Serum calcium, phosphate and alkaline phosphatase levels were significantly elevated from

normal. Elevations in serum calcium levels indicated prominent bone resorption; in addition to increases in osteoclastic activity, increased alkaline phosphatase levels indicated increased osteoblastic activity. Phosphate retention was secondary to reduced renal excretion in mice with a relatively high phosphate content in their diet in captivity, explaining the high phosphate concentrations of normal rodents compared to man. Unfortunately, parathyroid hormone (PTH) serum levels were undetectable when measured with the human PTH kit, probably due to lack of cross-reactivity of the human anti-PTH antibody, of the kit with the mouse PTH.

The mice with renal failure demonstrated the salient hematological features observed in man with severely impaired renal function (3, 80, 83). Anemia usually progresses as renal failure worsens and, as expected, a statistically significant correlation was found between the urea and the degree of anemia (as measured by hemoglobin) in the CRF mice. An evaluation of selected parameters in normal and CRF mice (at six weeks) demonstrated the significant changes that occur in CRF: hemoglobin, hematocrit, and red blood cell count all were decreased significantly from normal. These hematological tests were also performed in groups of mice 1, 3, and 6 weeks after the onset of renal failure in order to determine the influence of the duration of the renal failure on hematological parameters.

This study confirmed that serum EPO levels are unchanged from normal in CRF mice of six weeks' duration (19, 100). The serum EPO levels were measured by a commercial radioimmunoassay originally developed for determinations in man using an anti-human EPO antibody known to cross-react with rabbit EPO. This study confirmed that this antibody also cross-reacts with mouse EPO. The serum EPO levels in the

normal mouse were comparable to that of man and to published observations (32). The serum EPO levels in mice were also unchanged from normal at 1, 3 and 6 weeks of renal failure.

The unchanged serum EPO levels in CRF mice were inappropriately low for the degree of anemia. It remains unclear why these serum EPO levels, within the normal range, were unable to prevent the anemia from occurring. A possible explanation may lie in one of the known disadvantages of all radioimmunoassays used in the measurement of EPO in that they detect immunoreactive but not necessarily bioactive hormone. Thus, in renal failure, when serum EPO levels are low or undetectable, the radioimmunoassay detects higher levels than those detected by the traditional polycythemia mouse assay. Immunologically reactive EPO fragments that are biologically inactive may account for this difference.

As in man with CRF, a relative deficiency of endogenous EPO production therefore seemed to be the most important of the anemia-causing factors in these mice. The importance of this mechanism is further supported by the previous observation of anemia correction with administration of exogenous r-HuEPO at doses equivalent, on a body weight basis, to those used in dialysis patients (138). This study did not investigate the possible contribution to the anemia of other mechanisms, particularly hemolysis. Circulating red blood cells of CRF mice are smaller than normal which may increase their sensitivity to lysis thus leading to a more severe anemia (83). The MCV of mouse erythrocytes is close to half of that in man and their spherocytic morphology may promote osmotic fragility in conditions such as renal failure.

Brox et al. have previously demonstrated a role for IGF-I in the regulation of erythropoiesis (15, 16, 24). In order to examine the second part of the hypothesis of this study, serum levels of free IGF-I were measured in normal control mice as well as in untreated and androgen-treated CRF mice. Serum IGF-I levels in normal mice were measured to be higher than those in man whereas there were no significant differences in the levels between normal mice and untreated CRF mice. Treatment with androgens had no effect on the serum IGF-I levels of CRF mice. The elevated serum levels of IGF-I in all mouse groups, compared to normal control human volunteers, may be due to the fact that the mice were not fasting at the time of blood sampling while all normal human subjects were fasting overnight. Since IGF-I binding proteins which have a major influence on bioavailable IGF-I were not measured, further studies on the IGF-I/BP profile are required. However, for the purpose of this study, this part of the hypothesis was not pursued further.

Testosterone and various androgen preparations were first used in the treatment of anemia in the 1940s (87). While serious side-effects such as masculinization and jaundice were observed with high doses of testosterone, increased RBC counts were the higher priority. Subsequent studies showed that androgens are potentially effective agents for the treatment of anemia associated with other disease states, including CRF (106).

With the clinical introduction of r-HuEPO, the use of androgens in CRF has diminished greatly. However, lately, the elevated cost of r-HuEPO and occasional cases of hyporesponsiveness to r-HuEPO (79) have prompted renewed interest in the use of androgens. More recent studies utilize novel androgen preparations which have fewer

side-effects. Symptomatic individuals with a hematocrit less than 25% are good candidates for androgen therapy. In utilizing androgens to treat the anemia of CRF, the hematocrit unfortunately rarely rises above 36% (17). Parenteral administration of androgens may be more effective than administration by other routes and intra-muscular injections are, clinically, the usual route. Agents belonging to several classes (testosterone esters, 17 α-alkylated compounds, and norsteroids) are commonly used. It has been suggested that androgens having an angular configuration (5-β-epimers) stimulate stem cell proliferation to a greater degree than those whose configuration is planar (5-α-epimers), which may act primarily by enhancing EPO production (35). Nandrolone decanoate (Deca-Durabolin®), whose configuration is planar, is a popular choice for both nephrectomized and non-nephrectomized individuals (17). Patients are unlikely to respond to androgens if, after 6-9 months of therapy at different dosages, a favorable clinical outcome has not been achieved (35).

The effect of androgen treatment on the anemia of CRF in the mouse was investigated. The androgen doses selected for this study were based on doses used in patients with end-stage renal disease treated with androgens. Clinically, the doses of androgens used usually range from 100 to 200 mg/wk which correspond, in a 70 kg man, to approximately 1.5 to 3.0 mg/kg BW/wk. Doses of 0.3, 1.0, 3.0, and 9.0 mg/kg BW/wk were therefore selected in this study and administered intra-muscularly as used clinically. Initially, treatments began three weeks after the onset of renal failure (time of left nephrectomy) and continued for three weeks. Using this treatment regimen, the mice did not show any increase in hemoglobin concentration at the time of sacrifice. According to the literature, androgens need to be administered for longer periods to be

effective (35). Therefore, in the following set of experiments, androgen treatment began one week after the onset of renal failure and continued for five weeks. Following this modified experimental protocol, the mice showed a consistently increasing trend in hemoglobin commensurate to the androgen dose, although the increases never reached statistical significance. A potential limitation of this modified experimental protocol is the difficulty to randomize mice on the basis of hemoglobin concentration at the start of the treatment period at one week of renal failure. At that point time, the anemia has not yet reached its nadir, shown in previous experiments to occur after 3 weeks of renal failure (43). Despite this limitation, this experimental protocol was nevertheless adopted because it was considered more important to terminate the experiment after 6 weeks of renal failure rather than extend experiments beyond that period in order to compare the results with the bulk of previous experimental findings obtained after 6 weeks of renal failure. Lastly, it was surmised that the above limitation was comparable in all animal groups.

Serum EPO levels in androgen-treated CRF mice showed significant changes commensurate with the treatment dose. Serum EPO levels increased with increasing doses of nandrolone, with significant increases compared to untreated animals observed at the doses of 3.0 and 9.0 mg/kg BW/wk. It is important to note, however, that these significant increases in serum EPO levels were not accompanied by significant increases in hemoglobin.

Several explanations can be offered for the discrepancy between the response of serum EPO and hemoglobin concentrations to androgen treatment. The half-life of red blood cells, of 90 days, in mice (58) may be too long to observe a change in hemoglobin

in response to the increased serum EPO concentrations during the 5-week experimental period. In addition, uremic inhibitors to erythropoiesis, such as spermine and spermidine, found in CRF may inhibit the maturation of hematopoietic progenitors into viable red blood cells. Thirdly, the serum EPO concentration attained with androgen treatment may not have been sufficiently elevated for a change in hemoglobin concentration to occur. In a separate experiment, following treatment with 10U r-HuEPO, CRF mice exhibited a serum EPO level of 249 ± 180 mU/ml. In comparison, the highest EPO level attained by androgen-treated mice, i.e. those treated with 9.0 mg/kg BW/wk, was only 45.3 ± 15.9 mU/ml (Table 5). This discrepancy in measured serum EPO levels suggests that the lack of significant increase in hemoglobin levels with increasing androgen doses is due to the failure of the selected androgen to raise serum EPO levels sufficiently o produce a hemopoietic effect. Lastly, this model of renal failure using young female mice may not have been the optimal model to test the initial hypothesis. In clinical studies, a favorable response to androgen treatment was often observed in older, male hemodialysis patients (120).

Mice with renal failure treated with a fixed dose of 3.0 mg/kg BW/wk for periods of 1, 2, and 5 weeks did not show a significant change in hemoglobin between 1 and 5 weeks of treatment. However, they did show a significant increase in serum EPO levels at 2 and 5 weeks of treatment compared to one week of treatment. These results indicated that the increased EPO production in response to androgens occurs early but is not readily translated into an increased hemoglobin concentration.

A prime objective of this investigation was to correlate the circulating EPO levels in normal mice and in untreated and androgen-treated mice with renal failure with

the mRNA expression of the hormone in relevant tissues. This goal was achieved successfully by the systematic examination of EPO mRNA expression in the kidney and liver, the two main EPO-producing sites in the body. According to current concepts of EPO production, the kidney is the main organ of production in the normal physiological state. In non-renal anemias, the liver provides 10-15% of total EPO production (31, 41). Several studies have postulated that androgens act by increasing EPO production from the kidney (7, 20, 28, 40, 111, 120), while others maintain that androgens increase EPO production from, as yet unknown, extra-renal site(s) (26, 35, 112).

As anticipated, EPO mRNA message in normal mice was present in moderate intensity in the kidney, however no message was found in the liver. Phlebotomized mice had a marked increase in EPO mRNA message in both the kidney and liver, with the more intense message detected in the kidney. These results are consistent with those observed in the normal rat after phlebotomy, where a much higher EPO mRNA expression was detected in the kidney by Northern blot and ten- to hundred-fold increases of serum EPO levels were measured by RIA (72). Similarly increased serum EPO levels were previously observed in the normal mouse post-phlebotomy (138). Increased EPO mRNA expression in the liver in response to phlebotomy has also been previously described (38).

Untreated CRF mice had reduced EPO mRNA expression in the remnant (burnt) right kidney. These results are comparable with many similar observations in animals with surgically reduced renal mass (119). This reduced EPO expression could explain the low or normal serum EPO levels observed in the CRF mice with anemia, a finding corroborated by numerous previous reports in humans and other animal species (3, 11).

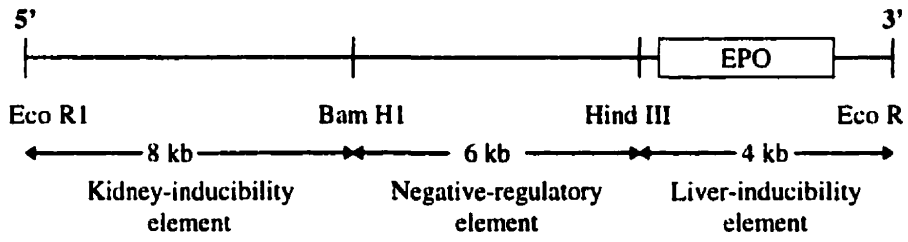
Surprisingly, no mRNA expression was detected in the liver of CRF mice despite the presence of significant anemia, thus corroborating previous findings in the same animal model (138). Based on the observations made in phlebotomized mice, one would expect the liver to increase its production of EPO in the anemia associated with CRF, however that, surprisingly, was not the case. The reasons for this differential response of the liver to two anemic states remain obscure.

A central part of this study was to examine the effect of androgen treatment of CRF mice with graded doses of nandrolone on EPO mRNA expression in the kidney and liver. CRF mice treated with androgens demonstrated a significant increase in the intensity of message in the remnant kidney compared to untreated CRF mice. This finding, however, was only observed in 1 of the 3 mice in each treatment group. Surprisingly, there was no EPO mRNA expression found in the liver of these same mice, thus indicating that androgens do not modify the absent EPO mRNA expression observed in the liver of untreated CRF mice. These observations support the hypothesis that androgens alleviate the anemia of CRF by increasing EPO production from the remnant kidney.

The fact that not all mice treated with androgens responded with an increased mRNA expression in the remnant kidney deserves consideration. Several factors could explain this response to androgens. Firstly, female mice were utilized in this study. The female gender was chosen because of its generally docile character and its heightened immune response compared to the male counterpart. Previous studies utilizing androgens in the treatment of the anemia of CRF have involved mostly male patients. The main disadvantage of using females in the study of the effect of androgens is the

inhibitory effect of estrogens on EPO production and erythropoiesis (82), although the administration of androgens may have, in some animals, overcome this negative effect. Secondly, and perhaps more importantly, is the possibility of a different degree of electrocoagulation of the surface of the right kidney between the mice with an accompanying different level of destruction of EPO- sensing and producing renal sites. As explained earlier, for the same degree of renal failure produced by the electrocoagulation procedure, the EPO- sensing and producing sites may have been left intact or destroyed to various degrees. For this reason, animals with a similar degree of renal failure (as measured by serum urea levels) may respond differently to androgen treatment.

Additionally, androgens were not able to stimulate the absent EPO mRNA expression in the liver of untreated CRF mice. Failure of the CRF mouse to express EPO mRNA in the liver corroborated the unexpected finding of a previous study (138) and the lack of change in liver EPO mRNA expression after androgen treatment in the present study was also unexpected. In order to better understand the issue of EPO gene regulation in the kidney and liver, Semenza et al. (110) generated a transgenic mouse for the human EPO gene to localize the *cis*-acting DNA sequences required for EPO gene expression in the kidney and liver. This may be pertinent to the mechanism of androgen effect on the kidney and liver. The expression patterns in these constructs allowed the localization of three distinct elements namely, kidney-inducibility (KIE). negative regulatory (NRE) and liver inducibility elements (LIE) (See Figure below).




**Localization of *cis*-acting DNA sequences regulating human EPO gene expression in transgenic mice (Semenza, 1993). Human EPO gene represented in box.**

In this particular construct, the KIE lies 5' to the EPO gene and is within the 8 kb fragment shown, while the NRE also lies 5' to the EPO gene but 3' to the KIE, and is within the 6 kb fragment. The LIE is located within a 4 kb fragment. Binding of the promoter to the KIE or the LIE allows for transcription of the gene in the appropriate organ. In normal states of EPO production, occurring mostly in the kidney, the promoter will bind to the KIE and will subsequently drive transcription of the EPO gene. Since we have shown absent EPO mRNA expression in the liver of CRF mice, it is possible then that the EPO promoter is prevented from binding to the LIE, thus preventing transcription of the EPO gene and causing eventual inhibition of EPO production from the liver. Another possibility is that, in the context of CRF, promoters of the EPO gene may bind to the NRE thereby inducing an inhibitory effect on transcription of the EPO gene. Additionally, the presence of low molecular weight peptides, retained in renal failure, that inhibit the LIE is yet another explanation of the absence of EPO mRNA expression in the liver of CRF mice.

Androgen treatment does not reverse this apparent inhibition on LIE thus explaining the lack of expression of EPO mRNA in the liver. However, increased EPO mRNA expression in the remnant kidney of androgen-treated CRF animals was observed compared to untreated CRF animals. This novel observation suggests that androgens likely act positively on the KIE to increase transcription of the EPO gene. Since the expression of EPO mRNA is more intense in androgen-treated mice, it also likely that androgens may inhibit the previously active NRE and thus increase transcription of the EPO gene in the kidney. No such mechanism appears probable in the liver of these same mice.

During the course of this project, several difficulties were encountered. For example, in order to study animals with severe CRF the initial surgical procedure (electrocoagulation of the right kidney) was required to be extensive. This approach entailed large animal losses following the second procedure (left nephrectomy) when the electrocoagulation had left too few intact nephrons to sustain life. Another difficulty inherent to mouse work in general was the small blood volume of the animals which limited repeated samplings without inducing significant anemia. Furthermore, the small volume of samples limited the number and type of tests capable of being performed.

Despite the difficulties encountered, the aims of the research project were effectively met:

1. Further characterization of the mouse model of renal failure was achieved by conducting a time-course study examining selected blood parameters and observing the morphological changes occurring in the bone with advancing renal failure. In this animal model, the development of osteitis fibrosa cystica occurred early after

the onset of renal failure. Further, osteoclastic activity was demonstrated to originate in the bone marrow before localizing on the bone surface.

2. The effects of CRF on circulating hematological parameters were delineated and found to corroborate previous data.
3. A dose-response curve to nandrolone decanoate was established.
4. Nandrolone treatment was effective in significantly increasing serum EPO concentrations but did not increase hemoglobin concentrations significantly although a consistent increasing trend in hemoglobin (commensurate with androgen dose) was observed.
5. The effects of CRF on the gene expression of EPO were determined in the kidney and liver using RT-PCR. In CRF mice, the mRNA expression of EPO was reduced in the remnant (burnt) right kidney and absent in the liver thus confirming earlier observations in this animal model (138).
6. Upon treatment with androgens, EPO mRNA expression in CRF mice was increased in the kidney compared to untreated controls but remained surprisingly absent in the liver.
7. These results led to the conclusion that the mechanism of action of androgens on the anemia of CRF is by increasing EPO production from the remnant kidney.

# REFERENCES


1. Akahane K, Hosoi T, Urabe A. Effects of recombinant human tumor necrosis factor (rhTNF) on normal human and mouse hematopoietic progenitor cells. Int J Cell Cloning 16: 16-26, 1987.

2. Akahane K, Tojo A, Urabe A, Takaku F. Pure erythropoietic colony and burst formations in serum-free culture and their enhancement by insulin-like growth factor I. Exp Hematol 15: 797-802, 1987.

3. Anagnostou A, Kurtzman NA. Hematologic consequences of renal failure. In: The Kidney, Brenner BM and Rector FC (eds.). Saunders Publishing, Philadelphia, 1991, pp 2019-2035.

4. Andress DL, Sherrard DJ. The osteodystrophy of chronic renal failure. In: Diseases of the Kidney, Schrier RW and Gottschalk CW (eds.). Boston, Brown and Co., 1996, pp. 2597-2624.

5. Aron DC. Insulin-like growth factor-I and erythropoiesis. Biofactors 3: 211-6, 1992.

6. Bagatell C, Bremner W. Androgens in men - uses and abuses. Review. N Eng J Med 334: 707-14, 1996.

7. Ballal S, Domoto D, Polack D, Marciulonis P, Martin K. Androgens potentiate the effects of erythropoietin in the treatment of anemia of end-stage renal disease. Am J Kidney Dis 17: 29-33, 1991.

8. Baxter RC, De Mellow JS. Measurement of insulin-like growth factor-II by radioreceptor assay using ovine placental membranes. Clin Endocrinol 24:267-78, 1986.

9. Beckman B, Belegu RD, Belegu M, Katsuoka Y, Fisher JW. Hypoxic enhancement of murine erythroid colony formation. In: Experimental Hematology Today, Baum SJ, Ledney GD, Thierfelder S (eds). New York, NY, Karger, 1982, pp 53-62.

10. Berns JS, Rudnick MR, Cohen RM. A controlled trial of recombinant human erythropoietin and nandrolone decanoate in the treatment of anemia in patients on chronic hemodialysis. Clin Nephrol 37: 264-7, 1992.

11. Besarab A, Girone JF, Erslev A, Caro J. Recent developments in the anemia of chronic renal failure. Semin Dial 2: 87-97, 1989.

12. Besarab A. Anemia in renal disease. In: Diseases of the Kidney, Schrier RW and Gottschalk CW (eds.): Boston, Brown and Co., 1996, pp. 2581-2596.

13. Blum WF, Jenne EW, Reppin F, Kietzmann K, Ranke MB, Bierich JR. Insulin-like growth factor I (IGF-I)-binding protein complex is a better mitogen than free IGF-I. Endocrinology, 125:766-72, 1989.

14. Boyer SH, Bishop TR, Rogers OC, Noyes AN, Frelin LP, Hobbs S. Roles of erythropoietin, insulin-like growth factor I, and unidentified serum factors in promoting maturation of purified murine erythroid colony-forming units. Blood 80: 2503-12, 1992.

15. Brox AG, Congote LF, Fafard J, Fauser AA. Identification and characterization of an 8-kd peptide stimulating late erythropoiesis. Exp Hematol 17: 769-73, 1989.

16. Brox AG, Zhang F, Guyda H, Gagnon RF. Subtherapeutic erythropoietin and insulin-like growth factor-1 correct the anemia of chronic renal failure in the mouse. Kidney Int 50: 937-43, 1996.

17. Buchwald D, Argyres S, Easterling R, Oelshlegel FJ, Brewer GJ, Schoomaker EB, Abbrecht PH, Williams GW, Weller JM. Effect of nandrolone decanoate on the anemia of chronic hemodialysis patients. Nephron 18: 232-8, 1977.

18. Camitta BM, Thomas ED, Nathan DG. A prospective study of androgens and bone marrow transplantation for treatment of severe aplastic anemia. Blood 53: 504-14, 1979.

19. Caro J, Brown S, Miller O, Murray T, Erslev AJ. Erythropoietin levels in uremic nephric and anephric patients. J Lab Clin Med 93: 449-58, 1979.

20. Cattran DC, Fenton SSA, Wilson DR, Oreopoulos D, Shimizu A, Richardson RM. A controlled trial of nandrolone decanoate in the treatment of uremic anemia. Kidney Int 12: 430-7, 1977.

21. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-9, 1987.

22. Claustres M, Chatelain P and Sultan C. Insulin-like growth factor 1 stimulates human erythroid colony formation in vitro. J Clin Endocrinol Metab 65: 78-82, 1987.

23. Clemmons DR. Competition for binding to insulin-like growth factor (IGF) binding protein-2, 3, 4, and 5 by the IGFs and IGF analogs. Endocrinology, 131: 890-5, 1992.

24. Congote LF, Brox A, Lin FK, Lu HS, Fauser AA. The N-terminal sequence of the major erythropoietic factor of an anephric patient is identical to insulin-like growth factor 1. J Clin Endocrinol Metab 72: 727-9, 1991.

25. Correa PN, Axelrad A. Production of erythropoietic bursts by progenitor cells from adult human peripheral blood in an improved serum-free medium: Role of IGF-I. Blood 78: 2823-9, 1991.

26. Dainiak N. The role of androgens in the treatment of anemia of chronic renal failure. Semin Nephrol 5: 147-53, 1985.

27. Davies M, Muckle TJ, Cassels-Smith A, Webster D, Kerr DNS. Oxymetholone in the treatment of anemia in chronic renal failure. Br J Urol 44: 387-94, 1972.

28. DeGowin R, Lavender AR, Forland M, Charleston A. Erythropoiesis and erythropoietin in patients with chronic renal failure treated with hemodialysis and testosterone. Ann Intern Med 72: 913-8, 1970.

29. Drop SL, L. Schuller AG, Lindenbergh-Kortleve DJ, Groffen C, Brinkman A, Zwarthoff EC. Structural aspects of the IGFBP family. Growth Regulation 2: 69-79. 1992.

30. Ekblom B, Berglund B. Effect of erythropoietin administration on maximal aerobic power. Scand J Med Sci Sports 1: 88-93, 1991.

31. Erslev AJ, Caro J, Kansu E, Silver R. Renal and extrarenal erythropoietin production in anaemic rats. Br J Hematol 45: 65-72, 1980.

32. Erslev AJ, Wilson J, Caro J. Erythropoietin titers in anemic, non-uremic patients. J Lab Clin Med 109: 429-33, 1987.

33. Erslev AJ. Erythropoietin and testosterone in vitro. Proceedings of the IX European Cong Haemat, Lisbon. 1963, p 143-8.

34. Erslev AJ. Management of the anemia of chronic renal failure. Clin Nephrol 2: 174-8, 1974.

35. Eschbach J, Adamson J. Improvement in the anemia of chronic renal failure with fluoxymesterone. Ann Intern Med 78: 527-32, 1973.

36. Eschbach JW, Adamson J. Anemia in renal disease. In: Diseases of the Kidney, Schrier RW, Gottschalk CW (eds). Little Brown and Co., Boston, 1988, pp 3019-3034.

37. Eschbach JW. Erythropoietin 1991--An overview. Am J Kidney Dis 18: 3-9, 1991.

38. Fandrey J, Bunn HF. In vivo and in vitro regulation of erythropoietin mRNA: Measurement by competitive polymerase chain reaction. Blood 81: 617-23, 1993.

39. Fisher J, Samuels AI, Malgor LA. Androgens and erythropoiesis. Isr J Med Sci 7: 892-900, 1971.

40. Fried W, Jonasson O, Lang G. The hematologic effect of androgen in uremic patients. Ann Intern Med 79: 823-7, 1973.

41. Fried W. The liver as a source of extrarenal erythropoietin. Blood 40: 671-8, 1972.

42. Fried W. Anemia in patients with chronic renal failure. Int J Artif Organs 3: 62-4, 1980.

43. Gagnon RF, Ansari M. Development and progression of uremic changes in the mouse with surgically induced renal failure. Nephron 54: 70-6, 1990.

44. Gagnon RF, Duguid WP. A reproducible model of chronic renal failure in the mouse. Urol Res 11: 11-4, 1983.

45. Gagnon RF, Gallimore B. Characterization of a mouse model of chronic uremia. Urol Res 16: 119-26, 1988.

46. Gagnon RF, Gold J, Gerstein W. A mouse model for delayed-type hypersensitivity skin changes in chronic renal failure. Uremia Invest 8: 121-5, 1984-85.

47. Gallimore B, Gagnon RF, Richards GK. Response to intraperitoneal Staphylococcus epidermidis challenge in renal failure mice. Kidney Int 32: 678-83, 1987.

48. Garcia JF, Ebbe SN, Hollander L. Radioimmunoassay of erythropoietin: Circulating levels in normal and polycythemic human beings. J Lab Clin Med 99: 624-35, 1982.

49. Gardner FH, Pringle JC. Androgens and erythropoiesis. I. Preliminary clinical observations. Arch Intern Med 107: 846-62, 1961.

50. Gardner FH. Introduction. In: Medullary Aplasia, Najean Y (ed). New York, Masson, 1980, pp 1-8.

51. Gimenez L, Scheel P. Clinical application of recombinant erythropoietin in renal dialysis patients. Hematol/Oncol Clin NA 8: 913-26, 1994.

52. Gold AP, Michael AF. Congenital adrenal hyperplasia associated with polycythemia. Pediatrics 23: 727-30, 1959.

53. Goldberg AC, Trivedi B, Delmez. Uremia reduces serum insulin-like growth factor 1, increases insulin-like growth factor 2, and modifies their serum protein binding. J Clin Endocrinol Med 55: 1040-5, 1982.

54. Gonzalez E, Martin K. Bone cell response in uremia. Semin Dial 9: 339-46, 1996.

55. Goodman WG, Coburn JW, Slatopolsky E, Salusky IB. Renal osteodystrophy in adults and children. In: Primer on the metabolic bone diseases and disorders of mineral metabolism, Favus MJ et al. (eds.). Philadelphia, Lippincott-Raven, pp 341-360.

56. Goodnough L, Monk T, Andriole G. Erythropoietin therapy. Current Concepts, N Eng J Med 336: 933-8, 1997.

57. Guler HP, Zapf J, Schmid C, Froesch ER. Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. Acta Endocrinol 121: 753-8, 1989

58. Harris JW, Kellerman RW. Production, Metabolism, Destruction: Normal and Abnormal. In: The Red Cell, Harris JW, Kellerman RW (eds.). Cambridge, Mass, Harvard University Press, 1972, pp 281-333.

59. Hermine O, Beru N, Pech N, Goldwasser E. An autocrine role for erythropoietin in mouse hematopoietic cell differentiation. Blood 78: 2253-60, 1991.

60. Hintz RL, Liu F, Rosenfeld RG, Kemp SF. Plasma somatomedin-binding proteins in hypopituitarism: Changes during growth hormone therapy. J Clin Endocrinol Metab 53:100-4, 1981.

61. Hruska K, Teitelbaum S. Renal osteodystrophy. Review, N Eng J Med 333: 166-74, 1995.

62. Humphries JE. Anemia of renal failure: Use of erythropoietin. Med Clin North Am 76: 711-25, 1992.

63. Isley W, Underwood L, Clemmons D: Dietary components that regulate serum somatomedin-C concentrations in humans. J Clin Invest 71: 175-82, 1983.

64. Izumi T, Kasuga M, Kadowaki T, Hizuka N, Takaka F, Akanuma Y: Characterization of human erythrocyte insulin-like growth factor I receptors. J Clin Endocrinol Metab 62: 1206-12, 1986.

65. Jacobs S, Kull FC, Earp HS, Svoboda M, Van Wyk JJ, Cuatrecasas P. Somatomedin-C stimulates the phosphorylation of the β subunit of its own receptor. J Biol Chem 258: 9581-4, 1983.

66. Jansen M, Van Schaik M, Ricker A, Bullock B, Woods D, Gabbay K, Bussbaum A, Sussenbach J, Van den Brande J. Sequence of CDNA encoding human insulin-like growth factor I precursor. Nature 306: 609-11, 1983.

67. Jepson J, Gardner FH, Gorshein D, Hait WM. Current concepts of the action of androgenic steroids on erythropoiesis. J Pediat 83: 703-8, 1973.

68. Kanamura A, Okamoto T, Hara H, Nagai K. Developmental changes in erythropoietin responsiveness of late erythroid precursors in mouse hemopoietic organs. Developmental Biol 92: 221-8, 1982.

69. Kenney BJ, Gilberstein AS. Increased erythropoiesis induced by androgenic hormone therapy. N Eng J Med 256: 719-26, 1957.

70. Koch KM, Frei U. Treatment of renal anemia, 1960-1990. Adv Nephrol Necker Hosp 20: 19-30, 1991.

71. Koch KM, Patyna WD, Shaldon S, Werner E. Anemia of the regular hemodialysis patient and its treatment. Nephron 12: 405-19, 1974.

72. Koury ST, Bondurant MJ, Koury MC. Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood 71: 524-7, 1988.

73. Krantz SB. Erythropoietin. Blood 77: 419-34, 1991.

74. Kurtz A, Hart W, Jelkmann W, Zapf J, Nauer C. Activity in fetal bovine serum that stimulates erythroid colony formation in fetal mouse livers is insulin-like growth factor I. J Clin Invest 76: 1643-52, 1985.

75. Kurtz A, Jelkmann W, Bauer C. A new candidate for the regulation of erythropoiesis insulin like growth factor I. FEBS Lett 149: 105-11, 1982.

76. Kurtz A, Matter R, Eckardt KU, Zapf J. Erythropoiesis, serum erythropoietin, and serum IGF-I in rats during accelerated growth. Acta Endocrinol 122: 323-8, 1990.

77. Kurtz A, Zapf J, Eckardt KU, Clemons G, Froesch ER, Bauer C. Insulin-like growth factor-I stimulates erythropoiesis in hypophysectomized rats. Proc Natl Acad Sci USA 85: 7825-9, 1988.

78. Lacombe C, Casadevall N, Varet B. Polycythemia vera: In vitro studies of circulating erythroid progenitors. Br J Haematol 44: 189-92, 1980.

79. Lacombe, C. Resistance to erythropoietin. Review. N Eng J Med 334: 660-1, 1996.

80. Lazarus JM, Hakim RM. Medical aspects of hemodialysis. In: The Kidney, Brenner BM and Rector FC (eds.). Saunders Publishing, Philadelphia, 1991, pp 2019-2035.

81. Le Roith, D. Insulin-like growth factors. N Eng J Med 336: 633-40, 1997.

82. Lee RG. Nutritional factors in the production and function of erythrocytes. In: Wintrobe's Clinical Hematology. Ninth ed. Lee RG, Bithell TC, Foerster J, Athens JW, Lukens JN (eds.). Lea and Febiger, Philadelphia, 1993, pp 158-185.

83. Lee RG. The normocytic normochromic anemias. In: Wintrobe's Clinical Hematology. Ninth ed. Lee RG, Bithell TC, Foerster J, Athens JW, Lukens JN (eds.). Lea and Febiger, Philadelphia, 1993, pp 885-991.

84. Litwack G. Biochemistry of Hormones II: Steroid hormones. In: Textbook of biochemistry with clinical correlations, Devlin TE (ed.). New York, John Wiley and Sons, 1992, pp. 901-924.

85. Manolagas S, Jilka R. Bone marrow, cytokines, and bone remodelling: Emerging insights into the pathophysiology of osteoporosis. Review, N Eng J Med 332: 305-11, 1995.

86. Martin JL. Insulin-like growth factor binding protein-3: Biochemistry and physiology. Growth Regulation 2:88-99, 1992.

87. McCullagh EP. Jones R. Effect of androgens on the blood count of men. J Clin Endocrinol Metab 2: 243-51, 1942.

88. McGonigle RJS, Wallin JD, Shadduck RK. Erythropoietin deficiency and inhibition of erythropoiesis in renal insufficiency. Kidney Int 25: 437-44, 1984.

89. Merchav S, Silvian-Drachsler I, Tatarsky I, Lake M and Skottner A. Comparative studies of the erythroid-potentiating effects of biosynthetic human insulin-like growth factors-I and -II. J Clin Endocrinol Metab 74: 447-52, 1992.

90. Mirahmadi MK, Vaziri ND, Gorman JT. Controlled evaluation of hemodialysis patients on nandrolone decanoate vs testosterone enanthate. Trans Am Soc Artif Organs 25: 449-54, 1979.

91. Mirza A, Correa P, Axelrad A. Increased basal and induced tyrosine phosphorylation of the insulin-like growth factor I receptor β subunit in circulating mononuclear cells of patients with polycythemia vera. Blood 86: 877-86, 1995.

92. Moriyama Y, Fisher JW. Effects of testosterone and erythropoietin on erythroid colony formation in human bone marrow cultures. Blood 45: 665-70, 1975.

93. Naets JP, Wittek M. The mechanism of action of androgens on erythropoiesis. Ann NY Acad Sci 149: 366-72, 1968.

94. Necheles TF. Studies on the control of hemoglobin synthesis. The in vitro effects of androgens and erythropoietin. Proc Symp Erythropoieticum, Prague. 1970, p 40-45.

95. Parker L. Adrenal androgens. In: Endocrinology, deGroot L (ed.). Philadelphia, W.B. Saunders Co., 1995, pp 1836-1849.

96. Pascual J, Teruel JL, Marcen R, Liano F, Ortuno J. Blood pressure after three different forms of correction of anemia in hemodialysis. Intl J Artif Organs 15: 393-6, 1992.

97. Phillips L, Fusco A, Unterman T, del Greco F: Somatomedin inhibitor in uremia. J Clin Endocrinol Metab 59: 764-72, 1984.

98. Prewitt T, D'Ercole A, Switzer B, Van Wyk J: The relationship of serum immunoreactive somatomedin-C to dietary protein and energy in the growing rat. J Nutrition 112: 144-50, 1982.

99. Raich PC, Korst DR. Plasma erythropoietin levels in patients undergoing long-term hemodialysis. Arch Pathol Lab Med 102: 73-5, 1978.

100. Rege AB, Brookins J, Fisher JW. A radioimmunoassay for erythropoietin: Serum levels in normal human subjects and in patients with hemopoietic disorders. J Lab Clin Med 100: 829-43, 1982.

101. Reichlin S: Neuroendocrinology. In: Williams, Textbook of Endocrinology. Saunders Publishing, Philadelphia, 1985, pp 492-567.

102. Reisner EH Jr. Tissue culture of bone marrow. Effect of steroid hormones on hematopoiesis in vitro. Blood 27: 460-9, 1966.

103. Richardson JR, Weinstein MB. Erythropoietic response of dialyzed patients to testosterone administration. Ann Intern Med 73: 403-7, 1970.

104. Rishpon-Meyerstein N, Kilbridge T, Simone J, Fried W. The effect of testosterone on erythropoietin levels in anemic patients. Blood 31: 453-60, 1968.

105. Rotwein P. Two insulin-like growth factor I messenger RNAs are expressed in human liver. Biochemistry 83: 77-81, 1986.

106. Sanchez-Medal L. The hemopoietic action of androstanes. Prog Hematol 7: 111-36. 1971.

107. Sanengen T and Halvorsen S. Regulation of erythropoiesis during rapid growth. Br J Haematol 61: 273-9, 1985.

108. Sawada K, Krantz SB, Dessypris EN, Koury ST and Swayer ST. Human colony-forming units-erythroid do not require accessory cells, but do require direct interaction with insulin-like growth factor-I and/or insulin for erythroid development. J Clin Invest 83: 1701-9, 1989.

109. Segal GM, Eschbach JW, Egrie JC. The anemia of end-stage renal disease: Progenitor cell response. Kidney Int 33: 983-88, 1988.

110. Semenza G. Regulation of human erythropoietin gene expression in transgenic mice and human hepatoma cell. In: Erythropoietin, molecular physiology and clinical applications. Bauer C, Koch KM, Scigalla P, Wieczorek L (eds.). Marcel Dekker, Inc, New York, 1993, pp 21-31.

111. Shahidi N. Androgens and erythropoiesis N Eng J Med 289: 72-80, 1973.

112.Shaldon S, Koch KM, Oppermann F, Patyna WD, Schoeppe W. Testosterone therapy for anemia in maintenance dialysis. Br Med J 3: 212-4, 1971.

113.Sherard D, Hercz G, Pei Y, Maloney N, Greenwood C, Manuel A, Saiphoo C, Fenton S, Segre G. The spectrum of bone disease in end-stage renal failure - an evolving disorder. Kidney Int 43: 436-42, 1993.

114.Staats J. Standardized nomenclature for inbred strains of mice: Eighth listing. Cancer Res. 45: 945-77, 1985.

115.Steinglass P, Gordon AS, Chariopper HA. Effect of castration and sex hormones on the blood of the rat. Proc Soc Exp Biol Med 48: 169-77, 1941.

116.Stewart-Phillips JL, Gagnon RF, Lough J. Atherosclerosis in a mouse model of chronic renal failure. Clin Ter Cardiovasc 8: 241-4, 1989.

117.Stivelman JC. Resistance to recombinant human erythropoietin therapy: A real clinical entity? Semin Nephrol 9: 8-11, 1989.

118.Sundraram K, Kumar N, Monder C, Bardin CW. Different patterns of metabolism determine the relative anabolic activity of 19-norandrogens. J Steroid Biochem Molec Biol 53: 253-7, 1995.

119.Tan CC, Eckardt K, Ratcliffe PJ. Organ distribution of messenger RNA in normal and uremic rats. Kidney Int 40: 69-76, 1991.

120.Teruel JL, Marcen R, Navarro-Antolin J, Aguilera A, Fernandez-Juarez G, Ortuno J. Androgen versus erythropoietin for the treatment of anemia in hemodialyzed patients: A prospective study. J Am Soc Nephrol 7: 140-4, 1996.

121.Thomopoulos P, Postel-Vinay MC, Testa U, Guyda HJ and Posner BI. Receptors for insulin-like peptides (ILAS) in rat reticulocytes and erythrocytes. Endocrinology 108: 1087-90, 1981.

122.Thorosson AV and Hintz RL. Specific I-125 somatomedin receptors on circulating human mononuclear cells. Biochem Biophys Res Comm 74: 1566-71, 1977.

123.Tonshoff B, Owell D, Zhao D, Durham S, Coleman M. Decreased hepatic insulin-like growth factor-1 and increased IGD binding protein-1 and -2 gene expression in experimental uremia. Endocrinology 138: 938-46, 1997.

124.Tregor A, Shaw DB, Grover RF. Secondary polycythemia in adolescents at high altitude. J Lab Clin Med 66: 304-14, 1965.

125.Trippel S, Van Wyk J, Foster M, Svoboda M. Characterization of a specific somatomedin-C receptor on isolated bovine growth plate chondrocytes. Endocrinology 112: 2128-36, 1983.

126.Underwood L, Van Wyk J: Normal and aberrant growth. In: Williams, Textbook of Endocrinology. Saunders, Philadelphia, 1985, pp 155-205.

127.Ureña P, Bonnardeaux A, Eckardt KU, Kurtz A and Drueke TB. Insulin-like growth factor I: A modulator of erythropoiesis in uraemic patients? Nephrol Dial Transpl 7: 40-4, 1992.

128.Vahlquist B. The cause of sexual differences in erythrocyte hemoglobin and serum iron levels in human adults. Blood 5: 874-5, 1950.

129.Valderrabano F. Erythropoietin in chronic renal failure. Kidney Int 50: 1373-91, 1996.

130.Vollmer LP, Gordon AS. Effect of sex and gonadotropic hormones upon the blood picture of the rat. Endocrinology 29: 828-37, 1941.

131.von Hartitzsch B, Kerr DNS, Morley G, Marks B. Androgens in the anemia of chronic renal failure. Nephron 18: 13-20, 1977.

132.Walls J. Hemoglobin - is more better? Neprol Dial Transpl 10 (suppl 2): 56-61. 1995.

133.Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. Reference range data base for serum chemistry and hematology values in laboratory animals. J Toxicol Environ Health 18: 161-88, 1986.

134.Yarden Y, Ullrich A. Growth factor receptor tyrosine kinases. Annu Rev Biochem 57: 443-8, 1988.

135.Zapf J, Schoenle E, Jagars G, Sand I, Grunwald J, Froesch ER. Inhibition of the action of nonsuppressible insulin-like activity on isolated rat fat cells by binding to its carrier protein. J Clin Invest 63:1077-84, 1979.

136.Zevin D, Turani H, Cohen A, Levi J. Androgen-associated hepatoma in a hemodialysis patient. Nephron 29: 274-6, 1981.

137.Zhang F, Gagnon RF, Richards GK, Brox AG. Anemia of chronic renal failure: Characterization in the mouse and correction with human recombinant erythropoietin. Nephron 72: 654-61, 1996.

138.Zhang F, Laneuville P, Gagnon RF, Morin B, Brox AG. Effect of chronic renal failure on the expression of erythropoietin message in a murine model. Exp Hem 24: 1469-74, 1996.

139.Zhou X-J, Pandian D, Wang X, Vaziri N. Erythropoietin-induced hypertension in rat is not mediated by alterations of plasma endothelin, vasopressin, or atrial natriuretic peptide levels. J Am Soc Nephrol 8: 901-5, 1996.