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Erythrocyte amino acids in health and renal failure and their association to the IGF-I/IGFBP-1 axis

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LIST OF PUBLICATIONS

This thesis is based on the following studies, which will be referred to by their Roman numerals

I Divino Filho JC, Bergström J, Stehle P, Fürst P: **Simultaneous measurements of free amino acid patterns of plasma, muscle and erythrocytes in healthy human subjects**. Clinical Nutrition 16: 299-305, 1997

II Divino Filho JC, Bàràny P, Stehle P, Fürst P, Bergström J: **Free amino acid levels simultaneously collected in plasma, muscle and erythrocytes of uraemic patients**. Nephrology, Dialysis and Transplantation 12: 2339-2348, 1997

III Divino Filho JC, Hazel SJ, Fürst P, Bergström J, K.Hall: **Glutamate concentration in plasma, erythrocyte and muscle in relation to plasma levels of insulin-like growth factor (IGF-I), IGF binding protein-1 and insulin in patients on haemodialysis.** Journal of Endocrinology 156: 519-527, 1998

IV Divino Filho JC, Hazel SJ, Anderstam B, Bergström J, Lewitt M, Hall K: **Effect of protein intake on plasma and erythrocyte free amino acids, and serum IGF-I and IGFBP-1 concentrations in rats.**
Submitted to American Journal of Physiology

V Divino Filho JC, Hazel SJ, Anderstam B, Suliman ME, Bergström J, Hall K: **Erythrocyte glutamate as a marker of catabolism during moderate renal failure and protein restriction in rats**.
In manuscript form

ABSTRACT

Erythrocyte amino acids in health and renal failure, and their association to the IGF-I/IGFBP-1 axis

José Carolino Divino Filho

Abnormalities in amino acids (AA) metabolism in uraemia have mainly been reported to occur in plasma and muscle. No investigation has been undertaken to evaluate the AA levels in plasma, muscle and red blood cells (RBC) simultaneously, although the RBC pool of free AA constitutes a large proportion of the free AA in blood, and RBC are involved in the interorgan transport of AA. Moreover, in most of the earlier studies reporting RBC AA levels in different clinical conditions, including uraemia, AA were determined in whole blood, which also includes AA from white blood cells and platelets. In this thesis, muscle, plasma and RBC were sampled simultaneously and reversed-phase HPLC was used to determine free AA in these compartments. RBC were separated from plasma, white blood cells and platelets, and then hemolysed, deproteinised, filtered and analysed for AA. In study **I**, 27 healthy subjects were investigated in order to establish reference muscle, plasma and RBC free AA levels. These results may assist the clinical investigator when comparing the AA profiles in muscle, plasma and RBC in various disease conditions and also for evaluating the effect of various physiological stimuli on AA concentration changes in these compartments. In study **II,** muscle biopsy and blood samples (plasma and RBC) were obtained from 38 haemodialysis (HD), 22 continuous peritoneal dialysis (CPD) and 10 end-stage renal failure patients for determination of free AA and the results compared to data obtained from study **I**. Several AA abnormalities in all three compartments were observed in the uraemic patients End-stage renal failure is characterised by both disturbed protein metabolism and changes in the IGF-I/IGFBP-1 axis. Protein synthesis and degradation are regulated by a number of hormones, and hormonal regulation represents an important process to maintain coordination of nutrient flows among the various organs. In study **III,** a possible association between changes in AA levels and the IGF-I/IGFBP-1 axis in end-stage renal failure was investigated in 30 HD patients who had no clinical signs of malnutrition. RBC glutamate and plasma IGFBP-1 levels were elevated in the HD patients and they were positively correlated. Since high IGFBP-1 reduces the bioavailability of IGF-I, reduced bioavailability of IGF-I, due to elevated IGFBP-1 levels, may be linkedAA levels and the IGF-I/ IGFBP-1 axis in end-stage renal failure was investigated in 30 HD patients who had no clinical signs of malnutrition. RBC glutamate and plasma IGFBP-1 levels were elevated in the HD patients and they were positively correlated. Since high IGFBP-1 reduces the bioavailability of IGF-I, reduced bioavailability of IGF-I, due to elevated IGFBP-1 levels, may be linkedto the regulation of glutamate distribution in uraemia. Increased postabsorptive plasma glutamate levels have been linked to conditions with loss of body cell mass.

In studies **IV** and **V** investigated in a rodent model how the protein content in the diet (**IV** and **V**) and moderate renal failure (**V**) change the intra-and extracellular AA levels (particularly glutamine and glutamate), and whether these changes are associated with circulating IGF-I and IGFBP-1 levels (**IV** and **V**) and/or muscle ASP/DNA ratios (**V**). Elevated RBC glutamate levels found in rats fed a low protein diet (**IV**) may indicate alterations in glutamate flux and interorgan nitrogen transport. The RBC and muscle glutamate relationships to the IGF-I/IGFBP-1 axis and muscle ASP/ DNA ratio support the proposal that RBC glutamate or RBC glutamine/glutamate ratio may be used as markers of catabolism, and that changes in the bioavailability of IGFs are linked to the regulation of glutamate distribution.

In summary, this thesis has demonstrated that AA determination in RBC is a simple and sensitive method for detecting AA alterations, and that RBC have an important and yet not fully clarified role in AA and protein metabolism in catabolic conditions such as uraemia and protein restriction, in both man and rat.

Key words:Amino acid, ASP/DNA, catabolism, dialysis, erythrocytes, glutamate, glutamine, health, human, IGF-I, IGFBP-1, muscle, plasma,protein intake, rat, renal failure, uraemia.

CONTENTS

ABBREVIATIONS

AA	Amino acids
ALA	Alanine
AMC	Arm muscle circumference
APD	Automated peritoneal dialysis
ARG	Arginine
ASP	alkali-soluble protein
ASP	Asparagine
BCAA	Branched-chain amino acids
BP	Binding-protein
BW	body weight
CAPD	Continuous ambulatory peritoneal dialysis
CIT	Citrulline
CPD	Continuous peritoneal dialysis
CRF	Chronic renal failure
CSA	Cysteinesulfinic acid
CV	Coefficient of variation
DEXA	Dual energy X-ray absorptiometry
EAA	Essential amino acids
FFS	Fat-free solids
GH	Growth hormone
GLN	Glutamine
GLU	Glutamate
GLY	Glycine
GSH	Glutathione
HD	Hemodialysis
HIS	Histidine
HPLC	High performance liquid chromatography
ICW	Intracellular water
IGF	Insulin-like growth factor
ILE	Isoleucine
K	dialyser urea clearance
Kda	kilodaltons
LBM	lean body mass
LEU	Leucine
LYS	Lysine
MET	Methionine
NEAA	Non-essential amino acids
ORN	Ornithine
PD	Peritoneal dialysis

PHE	Phenylalanine
PNA	Protein nitrogen appearance
RBC	Erythrocytes
RIA	Radioimmunoassay
Rh	recombinant human
S	Sham-operated
SER	Serine
SSA	Sulphosalicylic acid
t	time
TAU	Taurine
THR	Threonine
TRP	Tryptophan
TYR	Tyrosine
U	Uraemic
V	urea distribution volume
VAL	Valine
∑	Sum

INTRODUCTION

URAEMIA

"Uraemia is a toxic syndrome caused by severe glomerular insufficiency, associated with disturbances in tubular and endocrine functions of the kidney. It is characterised by retention of toxic metabolites, associated with changes in volume and electrolyte composition of the body fluids and excess or deficiency of various hormones"[**19**] has been suggested as the definition of uraemia that comes closest to reality, emphasizing all aspects of loss of renal cell mass and functions. Uraemia, as a catabolic state, is associated with multiple metabolic and endocrine disturbances such as amino acid (AA) abnormalities, disturbances in protein and energy metabolism, hormonal derangement, alterations in intermediary metabolism, that contribute to protein-energy malnutrition and wasting [**21,148**]. Renal replacement therapy, in the form of haemodialysis (HD) and peritoneal dialysis (PD) has evolved considerably along the years and can promote long-term survival and rehabilitation. The worldwide dialysis population increases steadily and it is estimated to be over one million patients within a few years. When dialysis therapy starts, the clinical symptoms of uraemia diminish or disappear, and some of the metabolic and endocrine abnormalities are attenuated or disappear completely, provided that the patients are adequately dialysed and the protein intake is sufficient. However, many of the catabolic factors found at the onset of therapy remain abnormal and the dialytic procedure-induced catabolism [**100,101**] combined with other factors (such as, low energy intake, metabolic acidosis, loss of AA and proteins) may increase protein requirements above those for non-dialysed uraemic patients.

For decades it has been hypothesised that dietary protein restriction might be beneficial for patients with progressive renal disease. As early as 1918 Franz Vilhard wrote of using a low protein diet to ameliorate the signs and symptoms of uraemia [**233**]. In addition to symptomatic improvement, protein restriction has been demonstrated in several studies to prolong endogenous renal function and retard the commencement of dialysis [**5,,83**] although this effect seems to be of minor practical importance. The difficulty in implementing dietary protein restriction therapy is that an inadequate low protein and energy intake results in net degradation of endogenous protein stores, which contributes to the loss of lean body mass observed in severely uraemic subjects treated with protein restricted diets [**47**]. Dietary AA entering the free AA pool (in equilibrium with body protein because of protein turnover) are metabolised in a variety of pathways, resulting in either gains or losses of AA or protein by or from the organism. Oxidative losses of free AA occur during feeding because they are consumed at a rate which is usually in excess of the rate at which net protein synthesis can occur, so that oxidation occurs as part of the process of maintaining the small size of tissue-free AA pools. The principal changes in the major systems responsible for maintenance of AA homeostasis with reduced protein intakes are **a**) a reduced (altered rate) of AA oxidation, initially due to change in tissue AA concentrations, **b**) a decline in protein synthesis due to cytoplasmic mechanisms that are accompanied or followed by changes at the genomic level, and **3**) changes in protein degradation where in liver there is an initial increase and a later decline, whereas in muscle the rate of protein degradation decreases from the outset although it might not fall as markedly as does muscle protein synthesis [**241**].

In normal adults the average minimum require-

ments for protein are about 0.6 g/kg BW/day which, after correction for 25% variability to include 97.5% of the population of young adults raises the safe level of intake to 0.75 g/kg BW [**240**]. On the other hand, compensatory reduction in protein turnover and AA oxidation in response to protein restriction seems to be as efficient in chronic renal failure (CRF) patients as in normal individuals [**219**], provided that the patients are not acidotic or suffering from enhanced catabolism due to co-morbid conditions.

Evidence exists that a limited dietary intake of protein and energy occurs independent of dietary advice, with the development of uraemia associated with progressive renal impairment or inadequate dialysis [**120,131,174**]. In various studies, signs of malnutrition have been observed in 10% to 70% of HD patients and 18% to 51% of continuous ambulatory peritoneal dialysis (CAPD) patients [**26**].

In summary, low intake of protein and energy as well as catabolic effects of acidosis, energy depletion, losses of glucose, protein and amino acids, and inflammatory responses to dialysis may be involved in the prevalence of malnutrition in uraemia.

AMINO ACIDS

HISTORICAL BACKGROUND

In the beginning of this century, Van Slyke & Meyer [**226**] used their new nitrous acid method to show that the free AA were at much higher concentrations in tissues, specifically the liver, than in plasma of dogs, and that the injection of an AA mixture (hydrolysed casein) raised the tissue levels far more than the plasma levels. A quotation from these authors deserves mention: "The AA of the blood appear therefore to be in equilibrium with those of tissues, a condition which accounts for all the observed phenomena, and would also account for any transfer of AA which may occur from organ to organ, or from maternal organs to the fetus." Today one might replace the word *equilibrium* with a term more suitable for describing an energised transport system which is regulated.

Some of the earliest experiments suggesting that erythrocytes (RBC) could take up AA were performed by Constantino in 1913 [**48,49**]. He found that glycine and asparagine were taken up by RBC incubated in high concentrations of these AA. Much later, Ussing [**224**] established that leucine could be transported by RBC membranes, being the first to show that RBC were permeable to AA *in vitro*. Subsequently Christensen *et al* [**43**] demonstrated that RBC could concentrate both glycine and alanine from the external medium and that the presence of various AA could affect the distribution of other AA between cells and extracellular fluids. It was from the detailed study of these effects, and the application of enzyme kinetics, that the definition of distinct transport systems for AA types began to emerge [**164**].

McMenamy *et al* [**151**] reported in 1960 that some AA, measured by paper chromatography, were concentrated in the RBC relative to plasma *in vivo*. This investigation was followed by some other quantitative studies of RBC AA in normal individuals [**141,202**] and in patients with various metabolic and blood disorders [**2,15,141,150,161,197**]. The determinations of AA in RBC of healthy and diseased adults, and of foetal and maternal blood have also been reported [**28,29**]. Data presented by Hagenfeldt *et al*

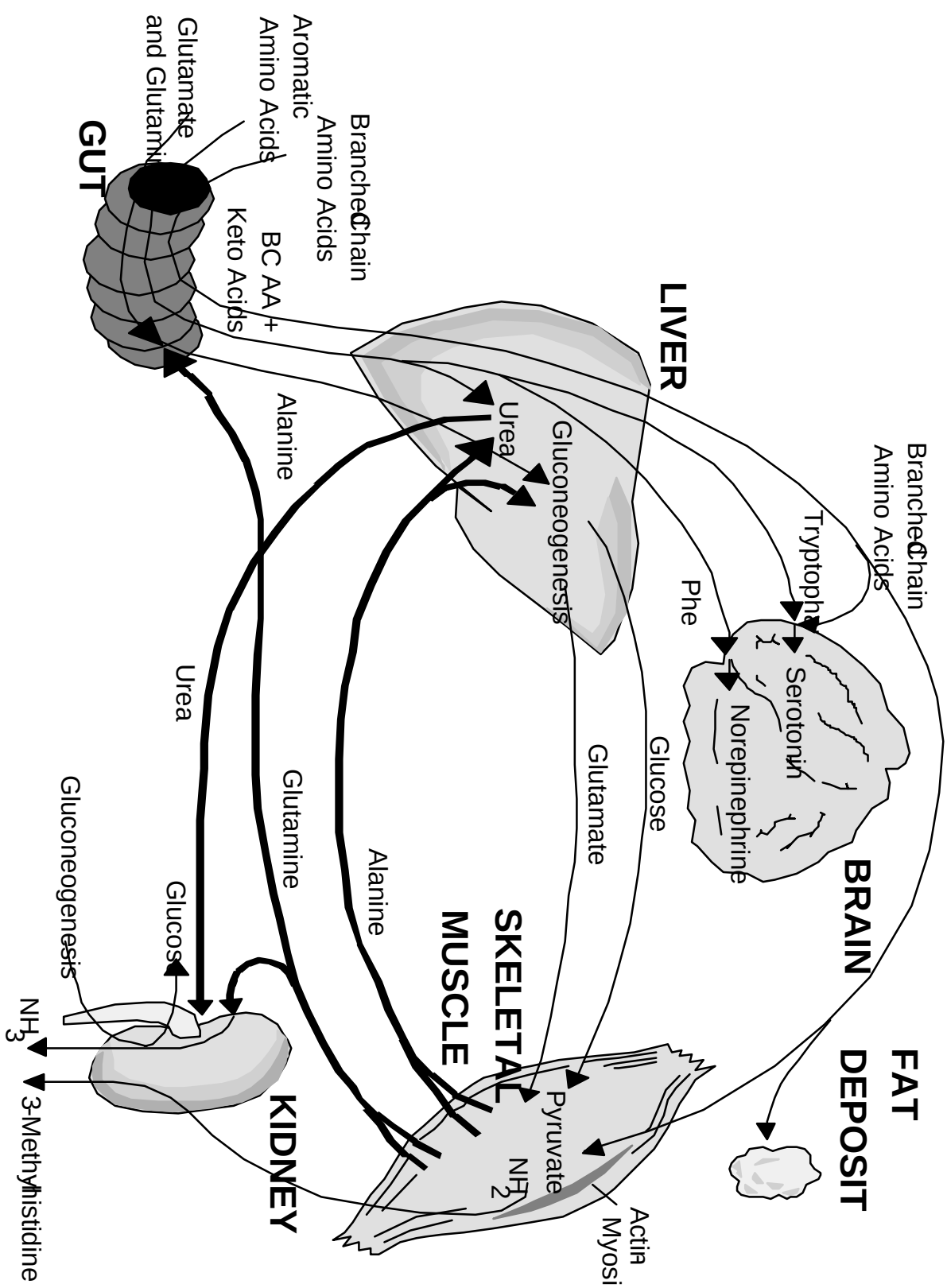


Fig.1. Major pathways of the transport and fate of various AA between organs. This is a slight modification from Munro [**160]**

[**103**] supported the presence of several AA transport systems in RBC which are operative *in*

vivo

and help to maintain the normal distribution of AA between plasma and RBC. AA derived from dietary protein or from catabolism of body proteins are distributed to tissues and cells by the circulation. In the last decades, animal and human studies have clearly suggested that the intestine and the liver also serve independent and important purposes in AA metabolism [**1,60,145,149,201,229**]. The active participation of various organs in AA metabolism emphasizes the importance of the interorgan circulatory transport of AA and the contributory role that RBC may have to this flow has to be taken into consideration (Fig.ure 1).

Earlier, it was generally believed that plasma rather than RBC was the vehicle of AA transport between tissues [**159**]. Nearly 35 years ago, Pitts reported that plasma measurements of AA transport rates reflected those occurring in whole blood [**171**]. In 1973 Felig *et al* found that blood cell elements contribute substantially to the net flux of AA from muscle and gut to liver in normal postabsorptive humans [**76**]. In 1982, O'Keefe *et al* [**163**] demonstrated by using L-[U-^{14}C], significant differences between blood cells and plasma AA indicating that the use of whole blood rather than plasma in protein turnover experiments will produce different results. Other studies with compartmentation of AA between plasma and RBC *in vivo* have been performed by using [^{14}C] alanine [**42**], ^{15}N-labeled glutamine [**52**]**,** and L-[1-^{13}C] leucine, [^{15}N] glycine, L-[^{15}N] alanine **[53]** in humans, confirming earlier findings obtained in animals [**112,113**] that one must be aware that plasma measurements underestimate true transport rates at least by as much as the packed cell volume.

INTRA-AND EXTRACELLULAR AMINO ACIDS

Growth and differentiation, protein synthesis, osmoregulation, neurotransmission, gluconeogenesis, glutathione (GSH) biosynthesis, and other metabolic pathways depend on AA requirements availability in cells and tissues. Moreover, the ways in which these needs might modify transport of AA into and out of cells are complex and still not fully understood. The factors involved in AA intra-extracellular gradient maintenance are: **a**) dietary protein, **b**) the rate of delivery to tissues, **c**) the kinetic properties of the systems that transport the individual AA into and out of tissues, **d**) the intracellular fate of AA which, in turn, depends on activity and kinetic properties of key enzymes and hormonal stimuli to the cells, **e**) the relative rates of protein synthesis and degradation and the meta-

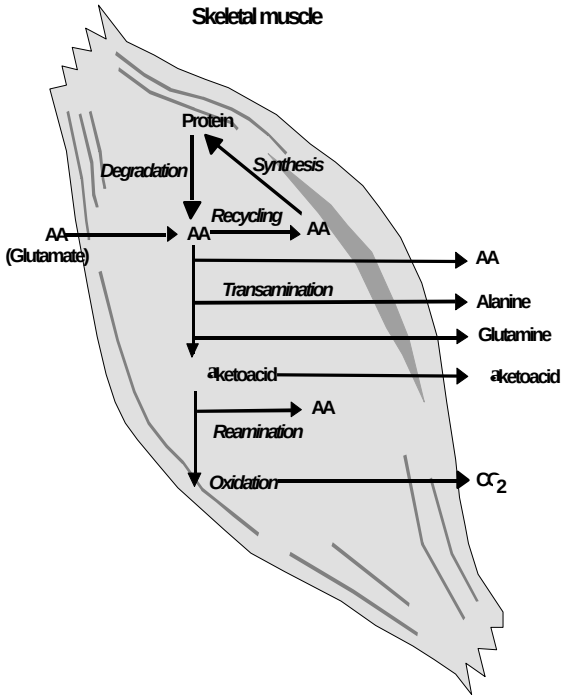


Fig.2. Amino acid (AA) metabolism in skeletal muscle.

bolic interconversion of AA.

Skeletal muscle is the major protein and free AA reserve of the body (Figure 2) and the alkali-soluble protein (ASP) in relation to DNA (ASP/DNA ratio) in muscle has been used as a quantitative index for assessing the muscle protein content, since it reflects the amount of protein per cell nucleus [**82**]. From a biochemical point of view, measurement of the muscle ASP/DNA ratio is comparatively simple and such measurements can be regarded as reliable and reproducible. Reduction in the muscle ASP/DNA ratio has been reported during catabolism in both man and rats, being associated with abnormalities in AA composition in skeletal muscle and impaired body growth [**51,168**]. The intracellular free AA pool is one of the major factors involved in protein synthesis regulation and by measuring the AA concentration important information on protein metabolism can be obtained.

Plasma AA are commonly determined for evaluation of nutritional status and for detecting specific abnormalities in protein and AA metabolism. The concentrations of the different AA may differ considerably, but each AA seems to vary within relatively narrow limits in healthy individuals [**235**] which is surprising in view of the intermittent influx of AA derived from dietary proteins, short-term changes in rates of protein synthesis and degradation, and the large inter-tissue fluxes of certain AA. However, the largest store of intracellular free AA is confined to to the skeletal muscle tissue [**25**], which also contains the largest pool of body protein whereas the plasma pool represents a small fraction of the body total free AA content. Moreover, the plasma concentration of most AA are much lower and bear little relation to the intracellular concentrations in muscle and other tissues, and they do not reflect the composition of tissue or dietary proteins [**25,159**].

RBC AA

The RBC contain all the AA present in plasma; and as in other tissues, certain of them (particularly some of the NEAA) are maintained at higher concentrations than in plasma. In contrast to typical nucleated cells from other tissues, mature mammalian RBC have no nuclei, no mitochondria, no ribosomes or other organelles which exist in muscle and other cells, and therefore they are incapable of protein synthesis. This absence of an obvious AA requirement may have contributed to the reluctance of investigators to study AA transport and concentrations in RBC . On the other hand, RBC provide a unique experimental preparation in that they are easily obtainable as a homogenous isolated cell preparation and the RBC is one of the classical cell systems being used for membrane transport studies. Numerous AA transport systems for RBC and muscle cell membranes have been characterised **[13,32,61,66,67,68,69,95,107,222, 228,232,238,]**.

Several reasons have been proposed [**66,238**] for the existence of AA transport systems in RBC: **a**) they might be functional relics of the large requirements for AA during reticulocyte development; **b**) the RBC could play a role in interorgan transport as proposed by Elwyn [**71**] and discussed further by Christensen [**44**], **c**) AA might be “accidental” substrates of systems designed to transport other substances [**70**]; **d**) RBC have a requirement for AA for GSH biosynthesis, a vital part

of RBC metabolism. The integrity of the RBC is maintained by the presence of millimolar concentrations of GSH, which protect the cell from injury by free radicals. It has been estimated that the intracellular half-life of GSH in human RBC is 4-6 days [**97**]. Thus there is a continual need to synthesise new GSH peptide from its constituent AA (glutamate, cysteine and glycine) which takes place in two ATP-dependent steps catalysed by gamma-glutamyl cysteine synthetase and GSH synthetase [**146,153**] and to remove GSH from the cell by a gluthatione disulfide (GSSG) transport system. It is still unclear how glutamate enters the RBC as their membranes are virtually impermeable to glutamate whereas glycine and cysteine enter the RBC by their transport systems; **e**) another finding is the definition of a peptide transporter in the RBC membrane [**132,133**] which suggests a role of RBC AA transport in exporting the products of digested peptides. King and Kuchel have demonstrated significant uptake and hydrolysis of dipeptides by RBC [**132,133**]. This suggests glutamyl dipeptides as the source of glutamate for GSH biosynthesis, and provides a new digestive role for RBC in terms of scavenging peptides and hydrolysing them. The AA transport systems could then conceivably even be acting in reverse, i.e. exporting AA derived from peptide hydrolysis from the cell. This is a major new role for RBC and much work needs to be done to confirm and establish the principles of peptide uptake by RBC.

Incubating human RBC with various ^{14}C-labeled AA *in vitro*, Winter and Christensen [**232**] determined the time course of their distribution ratios across the RBC membrane. They concluded that if the equilibration times (>30 minutes) documented *in vitro* were indeed valid *in vivo*, essentially no exchange could occur between the tissues and RBC for any AA except leucine, because it takes only a few seconds for blood to transverse a capillary bed [**76**]. In that case, RBC would only function as passive reservoirs rather than as active interorgan AA carriers. Some *in vivo* studies, however, using multiorgan arteriovenous catheterisation, provided evidence for an active participation of RBC in AA exchange between blood and tissues, implying that AA uptake or delivery by/to the RBC was faster *in vivo* than expected from the *in vitro* data [**8,71,72,73,76,112,113**]. Elwyn *et al* measured arteriovenous amino acid concentration gradients in both plasma and whole blood by using multiorgan catheterisation in dogs [**71, 72,73**]. Across several organs, such as liver, changes in the whole blood levels of several AA could not be accounted for solely by changes occurring in plasma, implying that the intracellular RBC content changed much faster *in vivo* than expected from the *in vitro* data. Heitman and Bergman [**112**] found that plasma measurements underestimated whole blood AA flux rates across most tissues of the sheep. Although RBC are impermeable to L-glutamate and L-aspartate *in vitro* [**238**], participation of RBC in AA exchange between circulating blood and tissues was documented by Aoki *et al* [**8**] for glutamate during insulin infusion, and Felig *et al* [**76**] proposed that RBC fulfilled a major role in alanine, leucine and glutamine interorgan transport.

RBC contain most AA at concentrations as high or higher than plasma [**93,103,180**]. In addition, studies by Elwyn *et al* [**72**] have suggested that an exchange of AA between plasma and tissue cells and an exchange between RBC and tissue cells might take place independently. Several other studies have also implicated the RBC as important carriers in

the net flux of various AA between peripheral tissues, gut and liver in both human individuals [**10,76,77**] and rats [**169,200]**.

Although the RBC contain a large proportion of the free AA in blood and are actively involved in the interorgan transport of AA, only a few studies have been performed to evaluate the RBC AA profile in comparison to plasma AA, in metabolic disorders such as homocystinuria and haemolytic anemia [**2,15,141,150,161,197**].

INTRA-AND EXTRACELLULAR AA IN MALNUTRITION

It is well known that in protein-energy malnutrition of the kwashiorkor type and in experimental protein deficiency, plasma EAA tend to be decreased, while some NEAA are increased. The usefulness of the EAA/NEAA ratios as an index of protein nutritional status has been demonstrated in children [**199**], growing rats [**208**] and young men [**86,,87**].

Among the NEAA, glutamine plays a pivotal role in overall nitrogen metabolism, acts as a "nitrogen shuttle" among various organs and has been proposed as a conditionally essential amino acid [**135**]. The concentration of glutamine is remarkably high in human muscle (20 mM), equivalent to > 15 g of nitrogen in an adult [**25,186**], being an important determinant of intracellular osmolarity and, possibly, of protein turnover [**108**]. This pool is depleted in response to a variety of insults including surgery [**231**], fasting and diabetes; but no abnormalities in intracellular glutamine levels have been observed in uraemia in spite of the altered nitrogen metabolism present in this catabolic condition. Glutamine is synthesized from glutamate and ammonia in a wide variety of tissues containing glutamine synthetase; and it is also widely hydrolyzed by glutaminases to yield back glutamate and ammonia [**230**]. Glutamate is one of the most important NEAA, playing numerous essential and multifactorial roles: it is central to all transamination reactions in the body, whereas its transamination product, -ketoglutarate, is central to the tricarboxylic acid cycle providing a critical link between carbohydrate and AA metabolism (**Figure 3**). Glutamate is also a highly concentrated intracellular AA, but is on the other hand, one of the least abundant of the plasma AA [**25,75**]. Thus, an extremely high concentration gradient across a variety of cell membranes exists for glutamate [**8,12,25,103,188**]. Although the principal role of glutamine may be interorgan transport and glutamate action seems to be primarily intracellular as the focal point for transamination; it is not apparent, at this point, whether the body needs glutamine as glutamine or as glutamate. Impairment of glutamate uptake by the skeletal muscle tissue is a common and relatively early event in the development of cachetic conditions [**102**], and abnormally high postabsorptive venous plasma glutamate levels have been reported for several diseases that are associated with a loss of body cell mass including cancer [**64,170**], human/simian immunodeficiency virus infection [**65**], and amyotrophic lateral sclerosis [**173**]. A linkage between postabsorptive AA release and glutamate uptake in skeletal muscle tissue of healthy young subjects, cancer patients and the elderly has been postulated by Holm *et al* **[117]** (Figure 4).

INTRA-AND EXTRACELLULAR AA IN URAEMIA

The kidney plays a major role in the regulation of body pools of many AA through synthesis, degradation and/or urinary excretion. The renal handling of AA has been investigated by measuring renal clearance, arteriovenous differences, micropuncture, perfusion of isolated kidneys or tubules, studies of renal cortical or medullary slices, and cell culture. In a normal adult about 70 grams of non-protein bound AA are filtered by the kidney

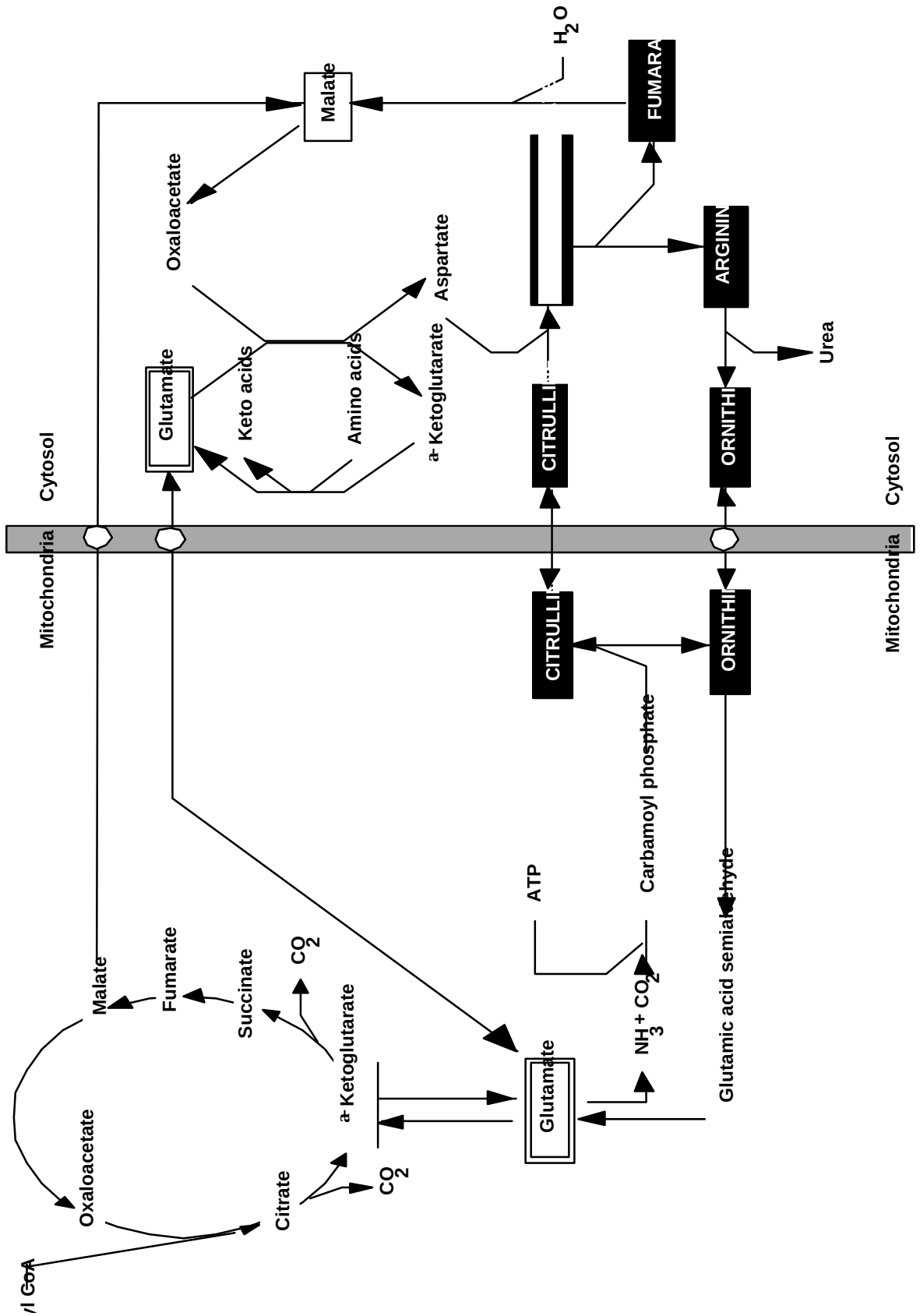


Fig.3. AA metabolism shown as part of the central pathways of energy metabolism.

each day, 97% of which are actively reabsorbed in the proximal tubules [**134**]. The distribution of

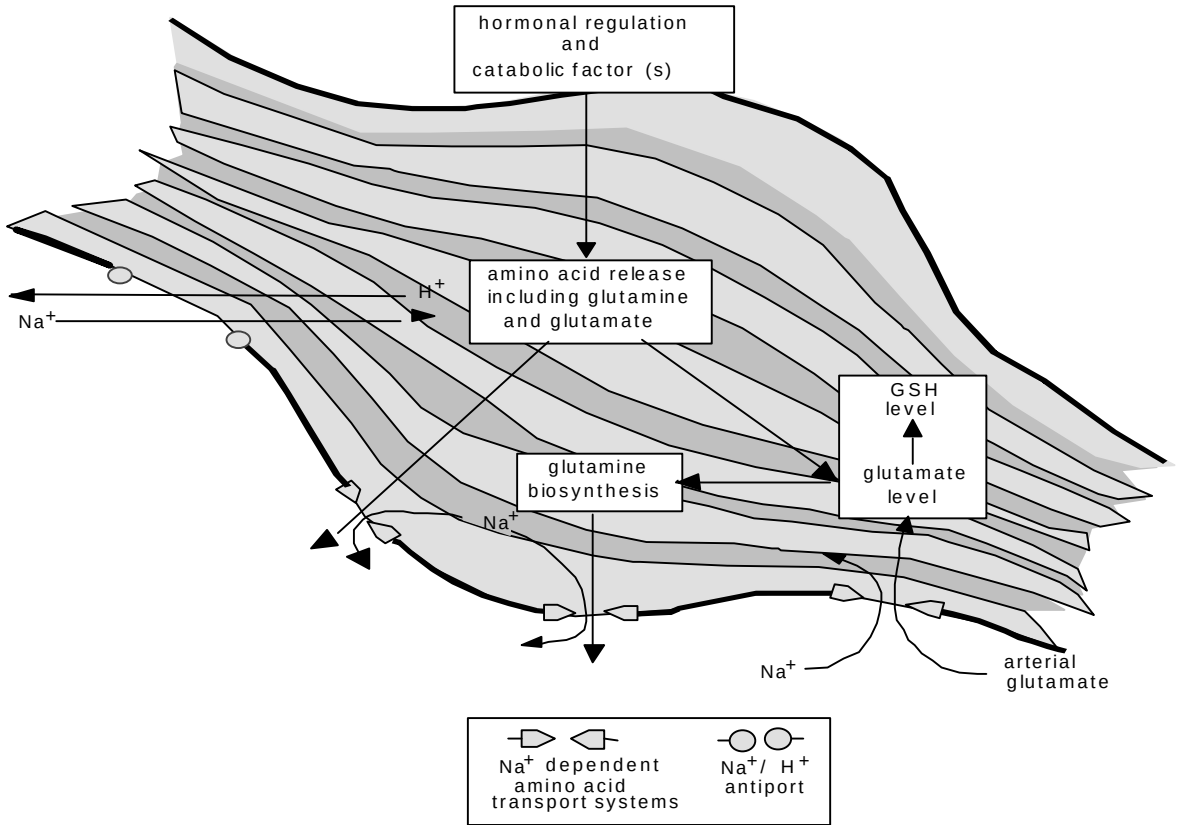


Figure.4. Schematic illustration of skeletal muscle catabolism and the resulting release of AA, and glutamate uptake. Modified from Holm *et al* **[117]**

AA to the different pools may be altered by impairment of either excretion (e.g. 3-methylhistidine), renal metabolism (e.g. citrulline and glycine), or synthesis (e.g. serine and tyrosine). Increased plasma citrulline concentrations may result from decreased conversion to arginine by the decreased kidney function [**41**]. Low plasma serine and high glycine in chronic renal failure may be due to reduced renal conversion of glycine to serine [**216**], the kidney being the major endogenous source of serine under normal conditions [**172**].

Low plasma tyrosine and low ratio of tyrosine to phenylalanine may be due to inhibition in the uraemic state of phenylalanine hydroxylase, which converts phenylalanine to tyrosine [**236**], and to a reduction in the renal cell mass, where a large part of the conversion of phenylalanine to tyrosine normally takes place [**216**]. Other abnormalities in AA metabolism may be related to several features of chronic uraemia, such as disturbances in protein and energy metabolism, hormonal derangement, and alterations in the intermediary metabolism and, in dialysis patients, loss of protein and AA by the dialytic procedure.

By far the largest pool of free AA is within the skeletal musculature [**25**], which in an adult man represents 40% of the body weight. For most AA the muscle intracellular concentration is higher than the plasma concentration and the skeletal muscle tissue also contains the largest pool of body protein [**25**]. The intracellular free AA pool is one of the major factors involved in the regulation of protein synthesis, and by measuring the muscle AA concentrations important

information on protein metabolism can be obtained. However, muscle biopsy is an invasive and sometimes uncomfortable procedure, which precludes its utilisation in large groups of patients.

In chronic renal failure, a specific pattern with high concentrations of several non-essential AA (NEAA) and low concentrations of essential AA (EAA), including branched-chain AA (BCAA), has been reported both in plasma and muscle [**3,23,37,99,130,143**] and the distribution of some AA between the extra- and intracellular compartments is altered [**6,23,24,143**]. This AA profile is in many respects similar to that observed in individuals suffering from protein malnutrition [**105**].

Typical changes in muscle AA of patients with untreated chronic uraemia are low concentrations of valine (but not leucine and isoleucine), threonine, lysine, histidine, and tyrosine [**6**]. Some of the AA abnormalities of uraemia are corrected in patients treated with maintenance HD, whereas other abnormalities are not restored to normal. Intracellular valine depletion occurs in muscle even in apparently well-nourished HD patients [**23**].

Reduced muscle valine concentrations in HD patients have been shown to correlate with the degree of metabolic acidosis in these patients [**23**]. It has been demonstrated that acidosis appears to enhance muscle protein catabolism in rats with chronic renal failure [**106**]. This effect is mediated by stimulation of the ATP dependent ubiquitin-proteasome catabolic pathway [**154**] and by activation of skeletal muscle branched-chain keto-acid dehydrogenase, which increases the catabolism of the BCAA that are mainly metabolised in muscle tissue [**147**]. Low intracellular concentrations of tyrosine and a reduced ratio of tyrosine to phenylalanine also persist in patients on HD [**23**].

Besides alterations in AA concentration gradients between the extra- and intracellular compartments [**3**], renal failure is also associated with alterations in the postprandial and post-absorptive interorgan transport of AA [**55,217**]. It has been suggested that these abnormalities may contribute to the increased catabolism associated with renal failure, and to the rate of progression of renal disease [**55,217,218**]. The cellular basis for the phenomenon is poorly understood, but altered membrane transport of AA could contribute to the changes seen.

As previously mentioned, RBC contain a large proportion of the free AA in blood, the intra-erythrocyte pool of free AA being actively involved in the interorgan transport of AA [**44,71,76**]. There is considerable evidence for the presence of altered membrane ion transport in renal failure [**78,128**]. Fervenza *et al* [**79,80**] have demonstrated specific changes of selected membrane transport systems for AA in uraemia: increased lysine and glycine uptake in RBC and reduced RBC transport capacity for serine.

In a few previous studies performed in patients with chronic renal failure RBC AA pattern was not identical to that in plasma (taken simultaneously) or in muscle (as compared to the other reports) [**81,90,125**]. Two of these studies [**90,125**] were based on blood cells (whole blood minus plasma) AA levels whereas one [**81**] was based on RBC AA levels. These studies indicated that the altered RBC AA pattern is not identical to that in plasma (taken simultaneously) or in muscle (as compared with values reported in the literature).

HORMONAL REGULATION OF AA METABOLISM

Whole body proteins are subjected to continuous turnover, and the overall rates of protein synthesis and breakdown are precisely regulated. Because

skeletal muscle constitutes the major protein reservoir in the body, the hydrolysis of muscle protein to generate AA is also an important first step in gluconeogenesis. Consequently, negative protein balance in muscle, leading to a net loss of soluble and myofibrillar protein, is characteristic of catabolic states where gluconeogenesis from body protein stores rises. The overall rates of protein synthesis and degradation in muscle are regulated by a number of hormones; and hormonal regulation of nutrient flows among the various organs represents an important process in order to maintain this coordination. The RBC pool of AA is known to be involved in the interorgan transport of AA, whereas the effect of hormones on the kinetics of AA uptake has been reported for a large number of tissues and cell types [**98,198**].

The whole body growth and anabolism in healthy subjects is dependent on GH throughout postnatal life and protein turnover in muscle requires insulin. The growth promoting and anabolic actions of GH are assumed to be mediated via the insulin-like growth factor I (IGF-I) (Figure 5). IGF-I and its close homologue IGF-II belong to the same peptide family as insulin, having approximately 50% percent of their AA in common. [**54,194**]. Insulin circulates at picomolar concentrations and has a half-life of minutes. The IGFs, on the other hand, circulate at much higher (nanomolar) concentrations and are mainly bound to IGF-binding proteins (IGFBPs) (Figure 6) that modulate IGF activity [**124**]. The IGFs in contrast to insulin are expressed in nearly all tissues and function as both endocrine and paracrine hormones [**54**] (Figure 5). The liver is assumed to be the source of the IGFs reaching the circulation. The growth promoting effect and anabolic effects of IGFs are mediated via a membrane receptor (IGF-I

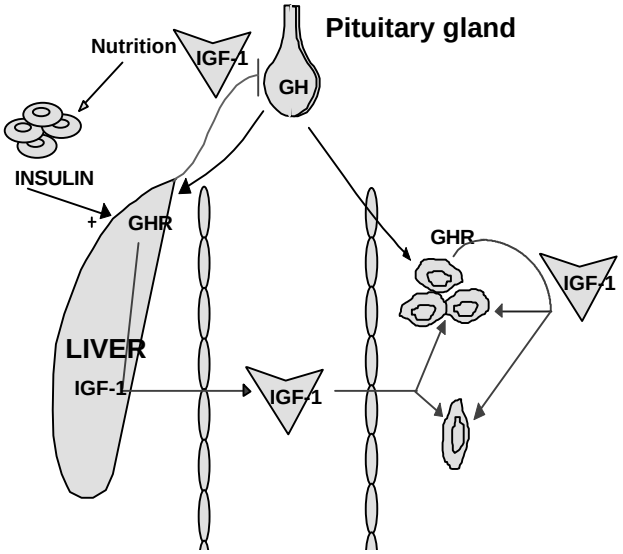


Fig.5. GH regulation of IGF-I as endocrine and paracrine hormone.

receptor), which is structurally closely related to the insulin receptor, and contains tyrosine kinase activity. IGF-I receptors are present in all cells apart from differentiated hepatocytes and adipocytes, which are target cells for insulin [**127**]. IGFs in

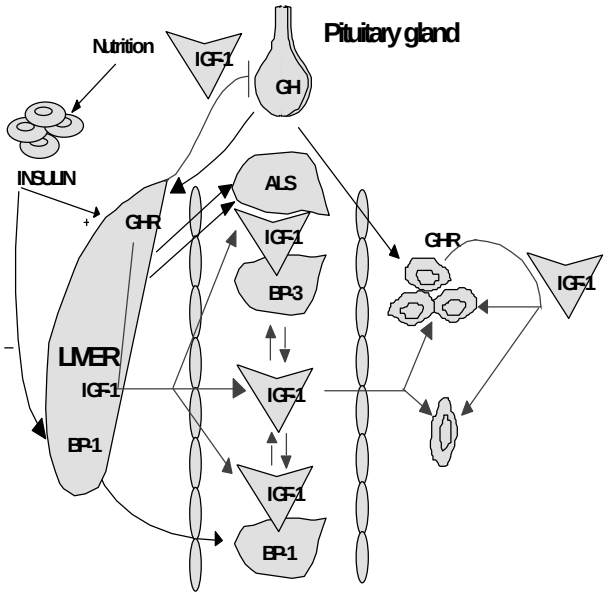


Fig.6. IGF-I in the circulation, its regulation and dependency on IGFBP-1 and IGFBP-3.

high concentration can crossreact with the

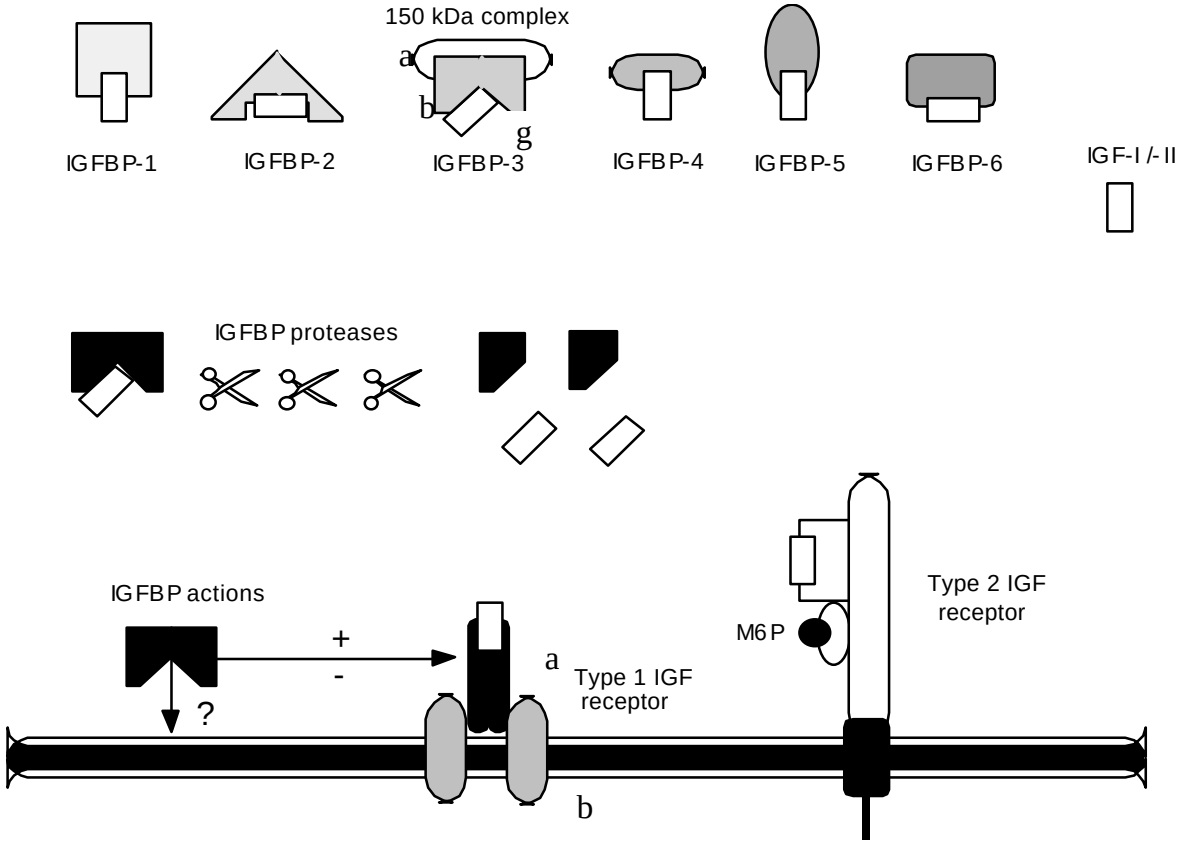
insulin receptor and conversely insulin in high concentration can act via IGF-I receptors. In muscle, where both insulin and IGF-I receptors are present, IGF-I and insulin stimulate glucose uptake via their own receptors. After birth GH starts to regulate the IGF-I expression and the GH-induced rise in IGF-I is nutrition dependent.

[**194,346**] The factors regulating IGF-II expression are still unknown. The serum IGF-II concentrations in healthy adults are about fourfold higher than IGF-I concentration, whereas serum IGF-II levels in the rat decline after birth. Activation of either the insulin receptor or the IGF-I receptor evokes similar initial responses within the cell [**139**]. However, since insulin regulates mainly metabolic functions and the IGFs regulate mainly growth and differentiation, the final pathways these hormones activate within the cell must be separate and distinct.

INSULIN

Numerous studies have established that insulin plays a role in the regulation of protein turnover and AA metabolism in man, and this has been the subject of several reviews [**56**] [**75**]. *In vitro* studies using incubated muscle and the perfused hindquarter from rats indicate that insulin suppresses protein degradation and stimulates protein synthesis [**45,123**]. In contrast, insulin's anabolic effect in humans *in vivo* appears to be limited to a suppression of protein degradation and leucine oxidation; no change or a reduction in protein synthesis has been observed [**88,92,209**]. The lack of stimulation of whole-body protein synthesis has been attributed to the reduction in plasma [**40**] and intracellular **[7]** amino acid concentra-

Fig.7. IGF binding proteins (IGFBP-1 to IGFBP-6) and IGF receptors..



tions induced by raising plasma insulin. When plasma AA were maintained at basal levels during an insulin infusion (by simultaneously infusing AA), the reduction in protein synthesis was blunted; and raising the plasma AA above basal levels stimulated protein synthesis [**40**]. Thus, hyperaminoacidemia, not hyperinsulinemia, was the stimulus to increase whole-body protein synthesis. Barret *et al* have demonstrated that insulin infusion inhibited the rate of phenylalanine appearance (i.e., proteolysis) across the forearm, whereas phenylalanine rate of disappearance (i.e., protein synthesis) did not change [**16**]. When hyperaminoacidemia was induced by simultaneously infusing AA, protein synthesis increased when protein turnover was measured across the leg [**17**].

GH/IGF SYSTEM

The interaction of growth hormone (GH) with its receptor stimulates the expression of the IGF-I gene and the release of the IGF-I peptide, which has a molecular weight of 7.6 Kda. IGF-I mediates many of the anabolic effects of GH and inhibits the secretion of pituitary GH via feedback system **[138]**. It stimulates bone formation, protein synthesis, glucose uptake in muscle, AA transport, neuronal survival and myelin synthesis. IGF-I also reverses negative nitrogen balance during underfeeding and inhibits protein degradation in muscle. For these reasons, IGF-I has been proposed as a therapy for osteoporosis, various catabolic conditions, diabetes, obesity, neuromuscular disorders, GH resistance, and insulin resistance [**124,138**].

In contrast to insulin the IGFs in the circulation are bound to binding proteins and less than 1 % is present in free form. Six IGF binding proteins (IGFBPs) with high affinity for IGFs have been characterised [**124**] and they were termed IGFBP-1 to IGFBP-6 in the order they were sequenced (**Fig**.**7**). IGFBP-2 and IGFBP- 6 bind preferentially IGF-II, whereas the other IGFBPs bind IGF-I and IGF-II with approximately equal affinity (figure 7). The IGFBPs modulate the action of IGFs but the physiological role of these IGFBPs are not fully understand. They function as storage and transport proteins, and at the target tissues they compete with the receptors for the binding of IGFs. IGFBP-1, IGFBP-2 and IGFBP-4 have been shown to block the effect of IGFs, whereas IGFBP-3 and IGFBP-5 can enhance the IGF effects. The percentage of IGFs bound to the IGFPBs in binary complexes depend on the equilibrium between IGFs and IGFBPs. In healthy adults the majority of IGF-I circulates bound to IGFBP-3 together with another GH regulated protein (acid labile subunit, ALS) in a ternary complex. The ternary complex is considered to be a storage form and in this form IGFs cannot leave the circulation (Figure 6). Proteases, which cleave IGFBP-3, release IGFs. Even if it is generally assumed that the concentration of free IGF-I in serum reflects the bioavailability of endocrine IGFs for the target tissue, there are difficulties involved in the determination of free IGF-1. Therefore, determinations of total IGF-I levels are determined in clinical studies. The serum levels of total IGF-1 are age dependent, with increasing levels during childhood, reaching their peak during puberty, and followed by a decline to low levels in old age. Accordingly, the IGF-1 levels have to be expressed in SD score of age-matched healthy subjects, when evaluating whether an IGF-I value is elevated or decreased.

IGFBP-1

A 30 Kda IGFBP was purified from amniotic fluid and medium conditioned by the human hepatoma cell-line HepG2 [**175,176**]. IGFBP-1 was later purified by others [**36**].

IGFBP-1 is mainly derived from the liver and counteracts the effects of IGFs *in vitro*. The high hepatic IGFBP-1 production rate and the rapid turnover rate in the circulation [**33**] could give IGFBP-1 a role as a regulator of IGFs bioavailability at the target tissue, in spite of the low serum concentration in relation to IGFBP-3. The mean IGFBP-1 serum level in adult human is around 35 ug/l [**34**]. IGFBP-1 binds IGF-1 with an affinity constant in the same range as the type 1 IGF receptor. IGFBP-1 has been shown to be mainly regulated by nutrition [**35,36,104**] and insulin [**33,34,205**]. In obese subjects the free IGF-I, which is considered to be the biological active fraction, was inversely correlated to IGFBP-1 [**85**]. Furthermore, IGFBP-1 administered to hypophysectomised rats inhibits the anabolic effects of both IGF-I and GH [**50**] and transgenic IGFBP-1 mice have retarded growth [**183**]. In healthy subjects the IGFBP-1 levels are inversely correlated to insulin levels [**115**]. While insulin appears to be the major inhibitor of IGFBP-1 [**33, 136**], the IGFBP-1 expression is stimulated by other regulators of metabolism such as glucagon [**114**].

There are several potential physiological roles for IGFBP-1 including: **a**) acting as a "shuttle", transporting the IGFs from the large reserves held in the circulation to the tissues, **b**) acting as an "address" molecule, directing the IGFs to appropriate sites of action or possibly to sites for clearance. **c**) alternatively, acting to regulate IGF-activity in a tissue-specific manner, either damping or enhancing activity in different tissues according to the binding protein interacting with other factors within any specific tissue, **d**) acting as a "scavenger", mopping up excess IGFs in the tissues and preventing a hypoglycemic response, or more specifically acting as a counter-regulator, removing insulin-like activity when fuel supplies are low, **e**) acting as a modulator of mitogenic activity, regulating tissue growth according to metabolic status. There is evidence to support each of these postulated roles and, of course, they are not mutually exclusive as under varying conditions and in different tissues IGFBP-1 may serve a number of roles [**116**].

GH/IGF SYSTEM IN URAEMIA

Patients with end stage renal disease, whether they are treated with HD or CPD, may suffer from protein-energy malnutrition, which is associated with increased morbidity and mortality. Besides low protein and energy intake, acidosis and accumulation of uraemic toxins in uraemia, changes in circulating hormone levels are proposed to enhance protein breakdown and/or decrease protein synthesis. A disturbed balance between pro-inflammatory cytokines and anabolic polypeptide hormones, such as GH, IGFs and insulin, are supposed to play a role in enhancing protein breakdown and attenuating protein synthesis.

Insulin resistance and impaired pancreatic insulin secretion leading to glucose intolerance in uraemic patients are well-established features of chronic renal failure [**58,4,57**]. Maintenance HD commonly leads to a partial correction of insulin

resistance. However, the stimulatory effect of insulin with respect to the cellular uptake of AA appears to be intact in chronic renal failure patients [**7**].

During malnutrition and GH deficiency, the low IGF-I levels may explain the growth retardation. In children and adults with renal failure, growth retardation and loss of body weight occur in spite of elevated fasting GH levels [**184**] and normal serum IGF-I levels [**30**].

However, the bioavailability of IGFs is reduced in uraemic patients due to the high concentrations of IGFBPs [**30**]. Elevated serum levels of IGFBP-1, IGFBP-2 , IGFBP-3 and recently IGFBP-6 have been reported in patients with renal failure [**119,126,142,177,178,220**]. Of these IGFBP-2 and IGFBP-6 bind preferentially IGF-II, whereas high concentration of IGFBP-1 and IGFBP-3 are supposed to reduce the bioavailability of IGF-I. The elevated levels of immunoreactive IGFBP-3 is not due to an increased production but have been attributed to an accumulation of IGFBP-3 fragments not able to form the ternary complex [**30,178**]. Moreover, accumulation of immunoreactive IGFBP-3 fragments has been attributed to reduced clearance since no increase in IGFBP-3 protease activity is found in sera from patients with renal failure [**182**]. During therapy with pharmacological doses of GH the percentage increase in IGF-I is considerable larger than the increase in IGFBP-3 and therefore the bioavailability of IGFs for tissue is proposed to increase. However, the question has recently been raised whether the excess IGFBP-3 fragments present in chronic renal failure serum can bind IGF-I with high affinity, and attenuate the bioavailability of IGF-I [**63**]. Hence the elevated levels of IGFBP-1 have come into greater focus as an inhibitor of IGFs action, in spite of the low IGFBP-1 concentration in relation to IGFBP-3.

Malnourished patients with chronic renal failure have been given rhIGF-I to improve their protein balance [**166**]. Moreover, the decline in plasma AA levels after a single injection of recombinant human (rh) IGF-I has been observed, by Fouquet *et al*, to be less in dialysis (HD and CAPD) patients than in controls [**84**]. The altered response of plasma AA to rhIGF-I in dialysis patients might be part of a more general resistance to the effects of rhIGF-I in advanced renal failure [**62**]. However, the effects of IGFs on the AA concentrations in different compartments and how IGFBP-1 may modify IGF-I induced actions have not been clarified.

AIMS OF THE THESIS

The present research project was undertaken to study the role of the RBC AA pool in relation to muscle and plasma in amino acid metabolism in uraemia. The specific aims of the studies were :

1. To establish reference RBC, muscle and plasma (simultaneously collected) AA levels in healthy subjects

2. To investigate the RBC, muscle and plasma (simultaneously collected) AA profile in uraemic patients and compare the results to the reference levels established

3. To evaluate if RBC AA determination can give important additional information to that obtained from plasma and muscle aminograms

4. To investigate the possibility of an association between changes in AA levels and the IGF-I/ IGFBP-1 axis in end-stage renal failure

5. To examine in a rodent model how the protein content in the diet changes the plasma and RBC AA levels; and whether these changes are associated with circulating IGF-I and IGFBP-1 levels

6. To study in a rodent model how the protein content in the diet and moderate renal failure change the intra-and extracellular AA levels; and whether these changes are associated with circulating IGF-I and IGFBP-1 levels and /or muscle ASP/DNA ratios

METHODS

Sampling procedures

Studies I-III

All subjects were studied on the morning after an overnight fast. HD patients were studied in the morning of a non-dialysis midweek day whereas controls and CPD patients were studied on any weekday. Venous blood (first) and muscle samples were obtained after the subject had been resting in a supine position for 30 minutes. Needle biopsy muscle samples were obtained from the lateral portion of the quadriceps femoris muscle [**22**].

Studies IV-V

Fifty-two days following the start of the experiment, the rats received an intraperitoneal injection of fluanisonum and fentalynium (Hypnorm®, 10 mg/ml and 0.2 mg/ml, respectively), followed by exsanguination using cardiac puncture. Rats were killed in the period 1000 to 1730 hours. For practical reasons the rats could not be killed simultaneously, but the groups were uniformly distributed to ensure that there was no biased effect of the time of the day. Gastrocnemius muscle was dissected, weighed, frozen on dry ice and stored at -70°C until further processing.

Analytical Procedures

Serum biochemistry (Studies I-V)

Serum biochemistry measurements for urea, creatinine, bicarbonate, cholesterol and glucose were evaluated by routine methods. Serum albumin was determined by the bromcresol purple method and serum total protein by the biuret method.

Plasma free amino acids separation (Studies I-V)

Heparinised blood sample was centrifuged for 10 minutes at 4°C in order to obtain plasma, which was then deproteinised with sulphosalicylic acid (30 mg/ml plasma) and centrifuged. The supernatant was stored at -70°C until analysis of AA.

RBC amino acids separation (Studies I-V)

For measurement of RBC AA, white cells and platelets were carefully removed and 1 g of packed red cells was rapidly haemolysed by adding 1.0 ml of 1% Saponin (Sigma, St. Louis, MO, U.S.A.). The sample was then extracted with 0.3 ml 50% SSA, mixed and centrifuged at 1700 x g for 20 minutes at 4°C. The supernatant was filtered using 0.45 um HA filter (Millipore) and frozen at -70°C until analysed [**81**]. Norvaline was used as the internal standard. For calculation of intracellular AA concentrations in RBC, the water content was taken as 66% of RBC weight in all samples, as described by Flügel-Link *et al* [**81**]. We also performed a pilot study with six RBC samples from healthy subjects and six from HD patients where we weighed each sample and then dried it and reweighed the sample. Our results showed a mean RBC water content of 67% (64-69%).

Muscle free amino acids, DNA, alkali-soluble protein and chloride (Studies I-III)

Muscle samples from controls and patients, obtained by needle biopsy [**22**], were dissected free from blood and visible connective tissue, weighed repeatedly for

extrapolation of the wet weight to time zero, frozen in liquid nitrogen and freeze-dried**.** The freeze-dried samples were weighed, fat was extracted in petroleum ether during 60 minutes, dried at room temperature and reweighed. The weight is referred to as fat-free solids (FFS). The sample was powdered in an agat mortar and rinsed from flakes of visible connective tissue. The powder was divided into two portions: about 2.5 mg of it was used for the analyses of electrolytes and total creatine and about 3 mg for the determinations of DNA, RNA and ASP or DNA, AA and ASP. The electrolyte sample was dried at 80°C for 30 minutes. This procedure reduced the water content by 4% to 6%. The true dry weight of the other portion of the powder was therefore calculated as 95% of the observed weight after powdering at room temperature and humidity. All glassware and utensils used in contact with the muscle biopsy were rinsed in nitric acid (1 mol/L) to remove traces of sodium and other electrolytes. Chloride was determined by electrometric titration, as described earlier [**22**]. ASP and DNA were determined after extraction with 0.35 ml 4% SSA in an ice bath for 1 hour of the 3 mg freeze-dried powder and the supernatant was used for AA analysis. The precipitate was incubated for one hour in 0.3 M KOH (0.3 mol/L) and ASP was determined in an aliquot by the Lowry method [**144**]. DNA was extracted by a procedure described by Schmidt and Tannhauser [**195**] and determined by the diphenylamine reaction [**94**]. The calculation of extra- and intracellular water content and the intracellular amino acid concentrations in muscle based on the chloride method has been described previously [**25,82**].

Muscle free amino acids, DNA, alkali-soluble protein and chloride (Studies IV-V)

The same method was used as for Studies **I-III**, excepting that for Studies **IV-V** the gastrocnemius muscle was dissected, weighed, frozen on dry-ice and stored at -70°C until further processing.

High performance liquid chromatography (HPLC) and free AA determination

Free amino acids in RBC, muscle and plasma were determined using an automated on-line HPLC system with pre-column derivatization (ortho-phthaldialdehyde/3-mercaptopropionic acid, OPA/3-MPA) and norvaline as the internal standard. The reproducibility of the method was assessed on the basis of 25 standard analysis and yielded values between 0.4 and 2.2% (coefficient of variations, CV). The error of the method was determined from 180 duplicate analyses of human plasma samples, ranging between 1.0 and 4.7% (CV) [**96**]. Tyrosine, considered as an indispensable AA under special conditions such as uraemia and infancy, was listed as an EAA.

IGF-I determination

In Study **III**, samples for determination of IGF-I were acid-ethanol extracted and cryoprecipitated prior to the radioimmunoassay (RIA) and des(1-3) IGF-I was used as the ligand to eliminate interaction of remaining IGFBPs in the samples [**14**]. The intra and interassay CV were 4% and 11% respectively. This assay has been validated in uraemic sera, using separation by gel chromatography at low pH. Since the IGF-I levels are age dependent they

were also expressed as SD scores calculated from the regression equation based on serum samples from a reference material consisting of 247 healthy men and women aged 20-70 years [**115**].
In papers **IV-V**, serum rat IGF-I was measured using the same RIA as above [**14**].

IGFBP-I determination
In Study **III**, IGFBP-1 was determined using the RIA of Povoa *et al*. [**177**] in which both phosphorylated and unphosphorylated IGFBP-1 are equipotent. Crossreaction was less than 0.05 for IGFBP-3 and less than 0.5 % for IGFBP-2,-4,-5 and -6. The intra and interassay CV were 3% and 10% respectively. The relation of IGFBP-1 to insulin levels was judged comparing the values with those obtained in a middle-aged and elderly twin population consisting of 360 subjects aged 40-80 years [**118**].
In Studies **IV-V**, serum IGFBP-1 was measured by RIA using a rat IGFBP-1 standard [**140**].

Insulin determination
In Studies **I-III**, insulin was measured with a commercial assay kit (Pharmacia Insulin RIA 100, Pharmacia AB, Stockholm, Sweden). In Studies **IV-V**, immunoreactive insulin was measured by RIA using guinea pig antibodies raised against porcine insulin and ^{125} I labeled porcine insulin as a tracer. Rat insulin (Novo, Denmark) was used as a standard. Dextran coated charcoal was used to separate bound from free insulin. The sensitivity of the assay is approximately 0.2 µU per tube and intraassay coefficient of variation 5.1%.

Protein nitrogen appearance (PNA) and Kt/V (Studies II-III)
The predialysis patients had a median creatinine clearance of 9 ml/min (5-31 ml/min). Their daily protein intake was estimated on the basis of their daily urinary urea excretion using an equation presented by Borah *et al* [**31**].
The PNA, which in stable patients gives an estimate of the protein intake, was calculated from urea appearance. The dialysis dose, expressed as Kt/V urea, was calculated from the dialyser urea clearance (K), dialysis time (t) and the calculated urea distribution volume (V, ml); both according to the urea kinetic modelling method of Farrell and Gotch [**74**]. PNA and dialysis index in CPD patients were calculated from total daily (dialysate + urine) urea excretion and the total loss of protein in the dialysate was added, according to Bergström *et al* [**18**].

Anthropometric parameters (Studies I-III)
Body mass index (BMI= BW in kg/height in m^2) and a relative weight index (weight index= BW x 100/reference BW) were calculated using actuarial tables from the Metropolitan Life Insurance Company as reference [**234**]. In the HD patients, postdialysis body weight was assessed. Arm muscle circumference (AMC) was calculated as mid-arm circumference (cm) -0.1 (x triceps skinfold (mm)). Skinfold thickness was measured with a Harpenden skinfold caliper (British Indicators Ltd., St Albans, Herts, UK).

Statistics
Data are reported as mean ± SE and a p-value < 0.05 was considered significant if not otherwise stated. Values on IGF-I, insulin, IGFBP-1 and urea were log transformed before analysis in Studies **III**, **IV** and **V** because the

transformed values more closely approximated the Gaussian distribution. Unpaired or paired Studentís t-test was used to assess significant differences. Analysis of variance, followed by Studentís t-test with the Bonferroni procedure was used for comparison of multiple groups (Studies **II**, **IV** and **V**). Linear regression analysis (Pearson's correlation) or non-parametric analysis (Spearman rank correlation) was used to assess relationships between two variables, as appropriate (Studies **I**, **III**, **IV** and **V**). Multiple regression analysis was used in Studies **I**, **III**, **IV** and **V.**

The nature, purpose and potential risks of the human studies were carefully ex-

MATERIAL, RESULTS AND DISCUSSION

plained to all patients before they consented to participate. The human studies protocols were approved by the Ethics Committee of the Karolinska Institute at Huddinge University Hospital. The rodent model experimental design was approved by the Animal Ethics Committee of the Karolinska Hospital.

Study I

Simultaneous measurements of free amino acid patterns of plasma, muscle and erythrocytes in healthy human subjects
JC.Divino Filho, J.Bergström, P.Stehle, P.Fürst
Clinical Nutrition 16: 299-305, 1997

Twenty-seven healthy volunteers (16 male and 11 female) with a mean age of 38.5 years (21-64 years) took part in the investigation. Weight, height and BMI are shown in Table 1(Study **I**). All the subjects were in normal physical condition and had not been on a controlled diet. The free AA concentrations in plasma, muscle and RBC for the healthy subjects (mean $\pm$ SE) are presented in Table 2 (Study **I**). Tyrosine and histidine which under special conditions (uraemia, infancy), appear to be indispensable AA, were listed as EAA.

Plasma, RBC and muscle free AA and intra-extracellular AA gradients

In plasma, glutamine had the highest concentration whereas methionine showed the lowest level. The sum ($\sum$) of concentrations of the NEAA was almost twice as high as that of the EAA.

In muscle, most of the EAA were present in lower concentrations than the NEAA. Exceptions were histidine, threonine and lysine which also had high muscle/plasma gradients (Table 2, Study **I**). Of the NEAA, glutamine and taurine had by far the highest muscle intracellular concentrations (20050 and 19200 umol/l, respectively), and the highest gradient between muscle and plasma (29:1 and 385:1, respectively) together with glutamic acid (123:1).

The EAA concentrations in RBC were similar to or only slightly higher than the plasma concentrations. The NEAA concentrations in RBCs were generally higher than in plasma, but most of them were considerably lower than in muscle, especially glutamine, taurine and alanine. The muscle/RBC ratio for valine, leucine, isoleucine, phenylalanine and tyrosine was around 1.0-1.1 (Table 2). All other AA showed a higher gradient (1.8-184).

In the present evaluation we could confirm earlier reported results [**25,156**] that the gradient between muscle ICW and plasma were around 1.0 (Table 2, Study **I**) for some AA (valine, leucine, isoleucine, phenylalanine and tyrosine). Indeed, the mean concentrations for each of these AA were also similar in muscle and RBC with little, if any, concentration gradients.

These observations indicate that in healthy subjects, under basal conditions, the concentrations of these AA are in equilibrium among all three compartments which is also corroborated by the significant correlations between individual AA concentrations in these compartments (Tables 5 and Fig 1, study **I**). In accordance with previ-

ous studies [**25,156**], some of the EAA (histidine, lysine, threonine) exhibited relatively high gradients between muscle and plasma. In contrast, the RBC/plasma gradient was about one, which demonstrates a difference between muscle and RBC regarding the maintenance of IC/EC gradients for these AA.

Most of the NEAA, especially taurine, glutamate and glutamine, had much higher muscle/plasma gradients than the EAA. On the other hand, the RBC/plasma gradients for most NEAA were much lower than the muscle/plasma gradients, although RBC accumulation of glutamate, glycine, arginine, asparagine, ornithine and taurine (range from 2.2 to 14.0) was observed.

These results suggest that there are differences in the membrane transport characteristics of several AA between RBC and muscle and that the concentrations of these AA in RBC do not have any relationship to those in muscle.

Influence of age and sex on plasma, RBC and muscle free AA

Muscle arginine and lysine as well as RBC citrulline showed higher concentrations in younger females when compared to males whereas all other AA (in RBC, muscle and plasma compartments) were present in higher concentrations in the male subjects. Muscle valine and asparagine as well as RBC serine, ornithine, methionine and BCAA levels were lower in the elderly than in the younger subjects whereas muscle arginine and plasma histidine levels were higher in the elderly subjects.

In previous studies only minor differences in muscle and plasma AA were observed in elderly as compared to younger healthy subjects [**156**,**157,158**]. This difference might be due to the larger age range in the present study (21-64 years) in comparison to the previous one (52-77 years).

Regarding the sex-dependent AA differences, our results are in keeping with earlier observations.[**11,89,129] [152,162,196**]. However, a novel finding was that not only plasma BCAA [**152**], but also muscle and RBC BCAA are lower in females than in males.

Correlations

A positive correlation was observed between individual as well as BCAA concentrations in plasma, muscle and erythrocytes (Fig.1, study **I**). For several other AA, correlations were also found between the concentrations in the different compartments (Table 6, study **I**).

Skeletal muscle is the major protein and free AA reserve of the body and probably the primary site in mammals for the catabolism of BCAA. The ASP/DNA ratio in muscle is probably the best quantitative index for assessing the muscle protein content, since it reflects the amount of protein per cell nucleus [**82**]. The observed correlations between individual BCAA and BCAA in the three compartments and the observations that ASP/DNA and BCAA concentration in these compartments (Fig 3, paper **I**) were all interrelated suggests that the levels of BCAA may be useful for assessing protein nutritional status.

Study II

Free amino acid levels simultaneously collected in plasma, muscle and erythrocytes of uraemic patients

JC.Divino Filho, P.Bàràny, P.Stehle, P.Fürst, J. Bergström:
Nephrology, Dialysis and Transplantation 12: 2339-2348, 1997

The free AA concentrations in plasma, muscle and RBC for the uraemic patients in tables 2, 3, 4 (study **II**) (figures 9 & 10). shows the plasma, muscle and RBC free AA concentrations for the uraemic groups, given as percentages of the mean concentrations in the twenty-seven healthy subjects group (controls) comprised in paper **I**. Tyrosine and histidine which under special conditions (uraemia, infancy), appear to be indispensable AA, were listed as EAA. The patients characteristics are shown in Table 1 (Study **II**) and Figure 8.

The HD patients were dialysed thrice weekly with hollow-fibre dialysers, using a glucose-free dialysate with acetate or bicarbonate as the buffer, blood flow between 200 and 350 ml/min and dialysate flow 500 ml/min. The CPD patients were treated either with CAPD (four to five daily 2-litre exchanges) or automated peritoneal dialysis (APD) (10-25 l of dialysis fluid per night with standard PD solution). The patients used different glucose concentrations according to their need to remove excess fluid. The CPD patients had no episodes of peritonitis in the 30 days prior to the investigation. The predialysis patients had a median creatinine clearance of 9 ml/min (5-31 ml/min) and were not given AA supplementation. Four of the HD patients had residual renal function (creatinine clearance between 1.0 and 1.5 ml/min). Three of the CPD patients had residual renal function (creatinine clearance between 0.5 and 1.8 ml/min). Routine medication included vitamin B and C supplementation, sodium bicarbonate, phosphate binders, and diuretics.

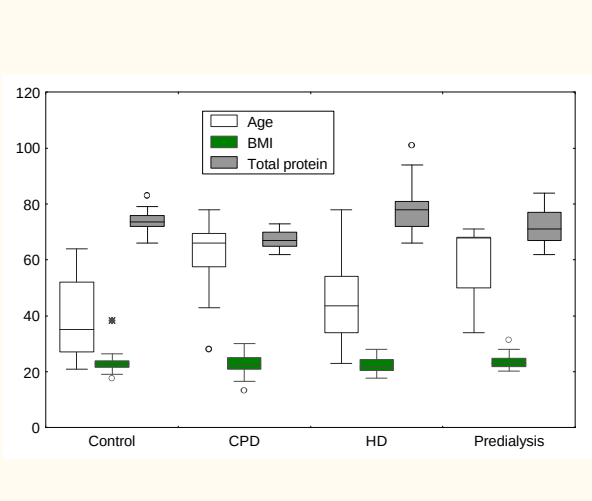


Fig.8. Box plots showing age, BMI and total proteins in controls, CPD, HD and predialysis patients. The box plots display the 10th, 25th, 50th, 75th and 90th percentiles. The individual values above the 90th and below the 10th percentiles (outliers) are plotted as small circles, and the extreme values as stars..

Plasma, RBC and muscle free AA

Low concentrations of EAA (leucine, valine, tyrosine) were observed in RBC and plasma, in the presence of normal concentrations in muscle. On the other hand, elevated concentrations of several NEAA (alanine, glycine, asparigine, arginine) and lysine were present simultaneously in RBC and muscle, but not in plasma. In contrast, RBC taurine concentrations were elevated in both HD and CPD patients. The HD and CPD patients showed evidence of taurine depletion with significant reduced muscle levels and in the HD patients the plasma levels were also reduced, when compared to the controls. RBC histidine levels were elevated whereas plasma levels were low, when compared to controls. Moreover, muscle and RBC serine levels in the patients were not different from controls whereas plasma levels were decreased and normal, respectively.

Low levels of EAA and elevated levels of NEAA have been described in different compartments in uraemia and other catabolic conditions [**6,23,24,38,**

39,143,**199,208**,**237**]. For example, reduced muscle valine concentrations in HD patients have been shown to correlate with the degree of metabolic acidosis [**23**], and the low plasma tyrosine levels are attributed to a reduced synthesis [**236**] and/or to increased metabolism [**193]**. The AA patterns in plasma and muscle of the present study were less abnormal than those earlier reported which may possibly be attributed to the more efficient dialysis treatment with full correction of acidosis and to the better nutrition.

Flügel et *al* were the first to report AA concentrations determined in RBC instead of blood cells or washed RBC in a study performed on patients with renal failure

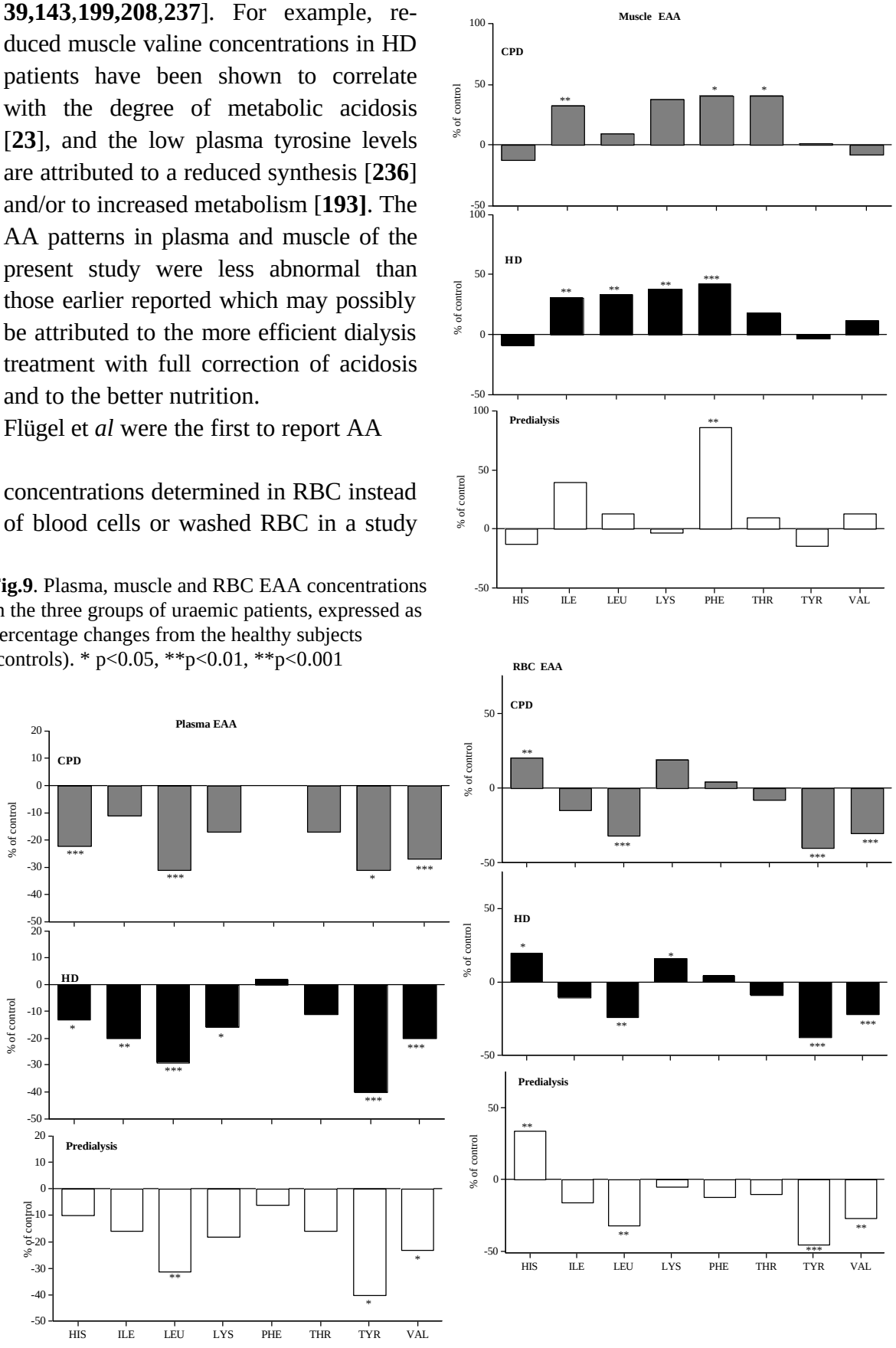


Fig.9. Plasma, muscle and RBC EAA concentrations in the three groups of uraemic patients, expressed as percentage changes from the healthy subjects (controls). * p<0.05, **p<0.01, **p<0.001

and compared to healthy subjects [**81**]. They observed several abnormal RBC AA levels (increased histidine, glutamate, glycine, ornithine, taurine, citrulline) similarly to the findings of Ganda at al [**90**] who measured plasma and blood cell AA before and after a HD treatment in six anephric patients; and to the findings of Jontofsohn *et al* [**125**] who measured blood cell AA in chronically uraemic and HD patients and observed increased cystine, ornithine and citrulline as well as decreased tyrosine. However, there were also many AA differences which might be due to the fact that blood cells include the values from white blood cells and platelets which have high intracellular concentrations of certain AA [**151**]. Our results on RBC AA concentrations in renal failure

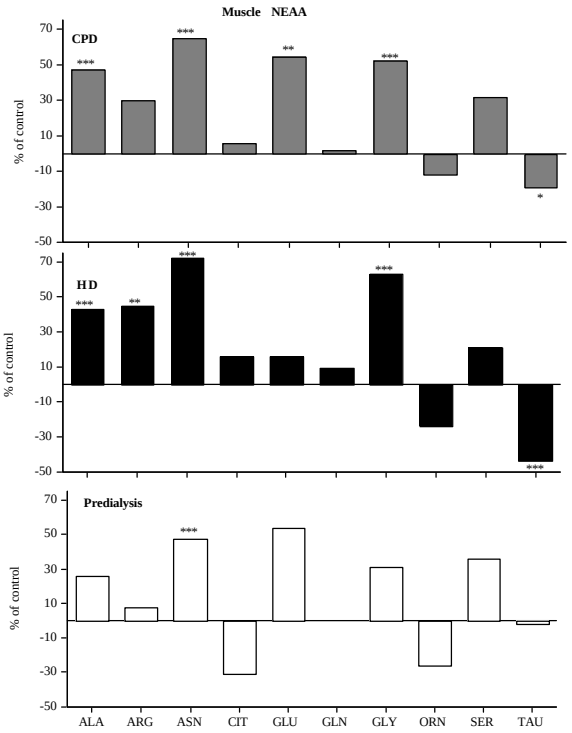
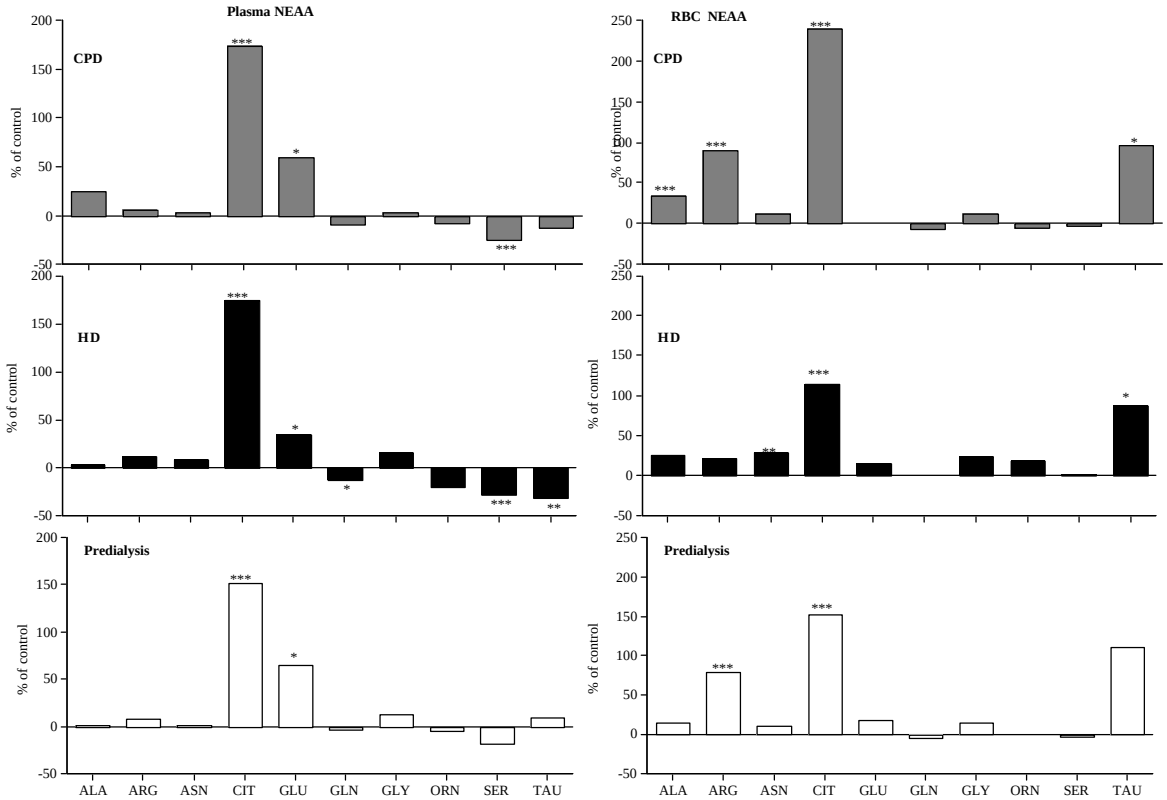


Fig.10. Plasma, muscle and RBC NEAA concentrations in the three groups of uraemic patients, expressed as percentage changes from the healthy subjects (controls). * p<0.05, **p<0.01, **p<0.001



are to a large extent in agreement with the data presented by Flugel *et al* [**81**].

On the other hand, this study reports the first data on AA levels obtained simultaneously from three different compartments in uraemic patients. The low concentration of some EAA in RBC (but not in muscle) reflecting the concentration in plasma, and the elevated concentrations of some NEAA (and lysine) present simultaneously in RBC and muscle (but not plasma) may suggest that the determination of AA in RBC is a more sensitive method for detecting AA abnormality than AA analysis in muscle tissue, an exception being taurine.

The findings of low levels of taurine in muscle and plasma and elevated levels in RBC are in keeping with earlier results [**20,81,206**]. The observation that low plasma taurine is associated with high concentrations of cysteinesulfinic acid (CSA) [**206**] suggests that the metabolic conversion of CSA to taurine is impaired in uraemic patients and this metabolic abnormality may cause taurine depletion. The increased RBC taurine levels in uraemia may indicate an active accumulation due to altered membrane transport. We have recently reported that one month vitamin (pyridoxine and folic acid) supplementation,in a group of HD patients, led to a reduction in RBC taurine concentration, despite unchanged plasma taurine levels [**207**].

Intra-extracellular AA gradients

Most of the NEAA, especially taurine and glutamine, had much higher muscle/plasma gradients than RBC/plasma gradients, although RBC accumulation of glycine, serine, arginine, asparagine, ornithine, glutamate and taurine was also observed. All EAA (except histidine) showed higher muscle/plasma gradients in the uraemic patients when compared to the controls whereas the only EAA RBC/plasma gradients significantly elevated were histidine and lysine.

These results indicate a difference between muscle and RBC regarding the maintenance of intra/extracellular gradients for certain AA in uraemia. Several AA transport systems have been demonstrated in human RBC [**238,66**] as well as specific changes in some of these AA transport systems (lysine, serine, glycine) have been described (*in vitro)* in HD patients [**79,80**]. Our *in vivo* findings of elevated lysine and glycine levels, both in muscle and RBC, in the presence of normal serine levels give clinical support to the *in vitro* studies earlier reported [**79,80**]

Study III

Glutamate concentration in plasma, erythrocyte and muscle in relation to plasma levels of insulin-like growth factor (IGF-I), IGF binding protein-1 and insulin in patients on haemodialysis.
JC.Divino Filho, SJ.Hazel, P.Fürst, J.Bergström, K.Hall
Journal of Endocrinology 156: 519-527, 1998

Thirty patients (24 men, 6 women) with a mean age of 50 years (range 24-70 years) on maintenance haemodialysis were investigated (Table 1, study **III**) (Figure 11). Thirteen of these subjects, in which muscle biopsies were taken, were included in study **II**. The causes of renal failure included chronic glomerulonephritis in 18 patients, chronic pyelonephritis in 3 patients, polycystic kidney disease in 5 patients and in 4 patients the aetiology was

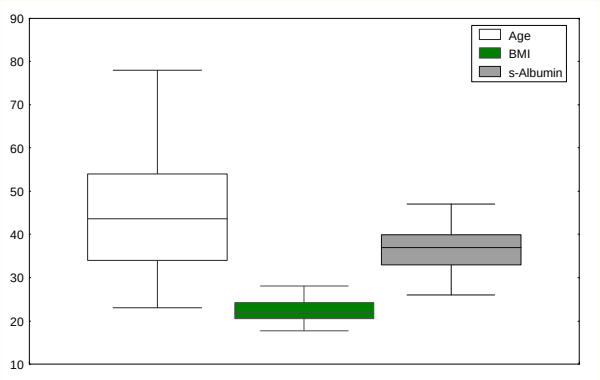


Fig.11. Box plots showing age, BMI and total proteins in 30 HD patients. The box plots display the 10th, 25th, 50th, 75th and 90th percentiles.

unknown. The patients had been on regular (thrice weekly) haemodialysis for a mean of 35 months (range 3-283 months). They were all dialysed with hollow-fiber dialysers, using a glucose-free dialysate with bicarbonate as the buffer, blood flow between 200 and 350 ml/min and dialysate flow 500 ml/min. No patient was on insulin or GH therapy. All patients were on an *ad libitum* diet and in stable clinical conditions, without clinical signs of malnutrition. BMI value below 20 was only present in one patient (BMI=17); and his IGF-I (183 ug/l) and IGBPP-1 (95 ug/l) levels were not different from the whole group. In Figure 11, age, BMI and total proteins are displayed as box plots.

IGF-I, IGFBP-1 and insulin levels

The IGF-I levels in the patients on haemodialysis (geometric mean of 211 µg/l) were slightly elevated in comparison with age matched controls. The mean IGF-I SD score was + 0.74±0.30 SD and no patient had a value below -2 SD. The plasma insulin levels, with a geometric mean of 10.9 U/ml, were within normal range. However, the geometric mean level of IGFBP-1 (89 g/l) was significantly higher than that of age matched controls (36g/l)

The normal or slighted elevated IGF-I levels and the threefold elevated IGFBP-1 levels found in these HD patients are comparable to previous reports in patients with renal disease [**104,119,192**]. In addition we found that the IGFBP-1 levels remained inversely related to insulin levels as in healthy subjects (Fig 1, paper III).

The absence of hyperinsulinemia and the identical slope of the regression line of IGFBP-1 on insulin levels in comparison to controls indicate that insulin resistance is not the cause of elevated IGFBP-1 levels.Tönshoff *et al* have demonstrated an increased hepatic expression of IGFBP-1 in uraemic rats [**220**], suggesting an increased IGFBP-1 production in renal failure.

Intra-extracellular AA levels

Most of the NEAA in both RBC and plasma displayed a pattern with higher concentration in the patient group than in the controls and this increase was more pronounced in RBC than in plasma (Fig 2, study III).

Several of the elevated RBC and plasma NEAA concentrations displaying a tendency to elevation in the HD patients of the previous study (paper II), reached significance in the HD patients of the present study (Figure 2, study III).

Intra-extracellular glutamate levels

Plasma and RBC glutamate were signifi-

cantly higher in the patient group than in controls ($p< 0.05$), although no difference between the groups was observed in muscle glutamate (Table 2, study **III**). Mean glutamate concentrations were ten fold higher in RBC (524 ±26 mol/l) than in plasma (45 ± 4 mol /l) and glutamate was the only AA, which displayed an inverse correlation between the concentrations in RBC and muscle ($r = -0.65$. $p< 0.02$, $n=12$) (Fig 3, study III).

High postabsorptive venous plasma glutamate levels have been reported for several diseases associated with loss of body cell mass such as cancer and immunodeficiency virus infection **[64,170]** , human/simian immunodeficiency virus infection [**65**]. Studies on exchange rates in well-nourished cancer patients have shown that high venous plasma glutamate levels may serve as an indicator for a decreased uptake of glutamate by the peripheral muscle tissue in the postabsorptive period and may be indicative for a precachectic state [**102**].

The muscle glutamate levels were inversely correlated to the IGFBP-1 levels ($r = -0.73$, $p<0.01$, $n=12$) and positively correlated to IGF-I ($r = 0.64$, $p<0.02$) (Figure .4, paper III). The relationship between RBC glutamate and IGFBP-1 and/ or IGF-I displayed a pattern which was opposite that for muscle glutamate.

In the extended group of patients (with and without muscle biopsy) a positive correlation was found between RBC glutamate and IGFBP-1 levels ($r = 0.51$ $p<0.01$) and an inverse correlation to IGF-I ($r = -0.46$ $p<0.01$) (Figure 5, study **III**), whereas no correlation was observed between RBC glutamate and insulin levels. None of the individual plasma AA was correlated to IGF-I, IGFBP-1 or insulin levels.

The finding that glutamate concentration in RBC but not plasma was inversely correlated to muscle glutamate levels in haemodialysis patients indicates that glutamate accumulates in RBC when glutamate uptake in muscle is reduced. Aoki *et al* [**8**] have demonstrated that RBC are important in the transport of glutamate into muscle; and IGF-I could be expected to increase the glutamate uptake in muscle, since IGF-I stimulates glucose uptake in vitro as well as protein synthesis in vivo and reduces protein degradation [**189**]. IGFBP-I has been shown to block the IGF induced glucose transport into muscle *in vitro* [**242**].

Increased concentrations of IGFBPs may be responsible for the reduced bioactivity of IGF-I in uraemic serum [**30**]. Because of its rapid turnover, IGFBP-1 is assumed to modulate the free fraction of IGF-I [**137**]; a ready supply of nutrients which normally decreases IGFBP-1 levels enables IGF-I to bind to its specific receptor and, during a shortage of nutrients, high IGFBP-1 level binds IGF-I and inhibit its bioactivity. The majority of circulating IGF-I is bound to IGFBP-3 which has been observed to be elevated in uraemic serum [**30**]. Moreover, it has been recently raised the question whether the IGFBP-3 fragments present in excess in chronic renal failure serum really bind IGF-I with high affinity and reduce the concentration of bioavailable IGF-I [**63**]; therefore the elevated levels of IGFBP-1 have come into focus as inhibitors of IGFs action, in spite of its low concentration in relation to IGFBP-3.

The findings of a significant positive correlation between RBC glutamate and IGFBP-1 levels as well as an inverse correlation between RBC glutamate and IGF-I levels are interesting considering the relation between lean body mass (LBM) and these two variables in GH-deficient adult patients [**210**]. LBM determined by Dexa was shown to be inversely related to IGFBP-1 levels, but not to IGFBP-3 levels, both before and during GH therapy whereas a positive correlation was found between LBM and IGF-I levels only during GH therapy. The LBM dependency of IGFBP-1 levels was attributed to its reduction of the free fraction of IGFs. These observations raised the question whether RBC glutamate could be used as an indicator of enhanced protein degradation.

Study IV

Effect of protein intake on plasma and erythrocyte free amino acids, and serum IGF-I and IGFBP-1 concentrations in rats

. JC.Divino Filho, SJ.Hazel, B.Anderstam, J. Bergström, M.Lewitt, K.Hall:

Submitted to American Journal of Physiology

Female Sprague Dawley rats were purchased from B&K Universal, Sollentuna, Sweden. A total of 29 rats were used in the experiments and divided randomly into groups fed 6%, 21% and 35% protein diets ad libitum (n=9, 10 and 10 respectively). Five days following the sham operation the rats were changed from the ordinary rat chow to the specific protein diets, and fed these diets for forty-seven days. The BW of the groups of animals at the beginning of the experiment were 159±1, 162±1 and 163±1 (mean ± SEM) for the 6%, 21% and 35% protein groups. The diets were calculated to contain: raw protein 6.1%, 21.0% and 35.0%, and non-fat energy 67.9%, 53.0% and 39.0% respectively, with 7.0% raw fat, 4.0% fibre, 5.0% ash, 10.0% water and 13.3 MJ/kg in each diet (AnalyCen Nordic, Special Diets, Lidköping, Sweden). The raw materials were casein, corn starch, sucrose, dextrose, cellulose powder, mineral premix and soy bean oil. All rats were maintained on a 12 hour day/night rhythm with free access to water. In this study we evaluated the effect of protein restriction on RBC and plasma AA and their correlation to the IGF-I/IGFBP-1 axis in normal rats.

Growth and IGF-I, IGFBP-1 and insulin concentrations

The growth of rats fed 6% protein was significantly retarded in comparison to the other two groups ($p<0.0001$) (Table 1, study IV).

There was an inverse correlation between IGFBP-1 and insulin ($r= - 0.68$) as well as between IGFBP-1 and IGF-I ($r= - 0.84$) when the three groups were combined. Box plots of weight gain, serum urea, IGF-I, insulin and IGFBP-I are displayed in figure 12.

Dietary protein restriction caused growth retardation associated with low serum concentrations of IGF-1 and high serum concentrations of IGFBP-1 as expected Decreased IGF-1 is not the only mechanism involved in the growth retardation as the anabolic effects of IGF-1 are also impaired by protein restriction [**167,211,212, 213,214**]. Moreover, IGFBP-1 inhibits the IGF-1 effect in the tissues and the high levels observed in the low protein intake group might suggest decreased IGF-1 bio-

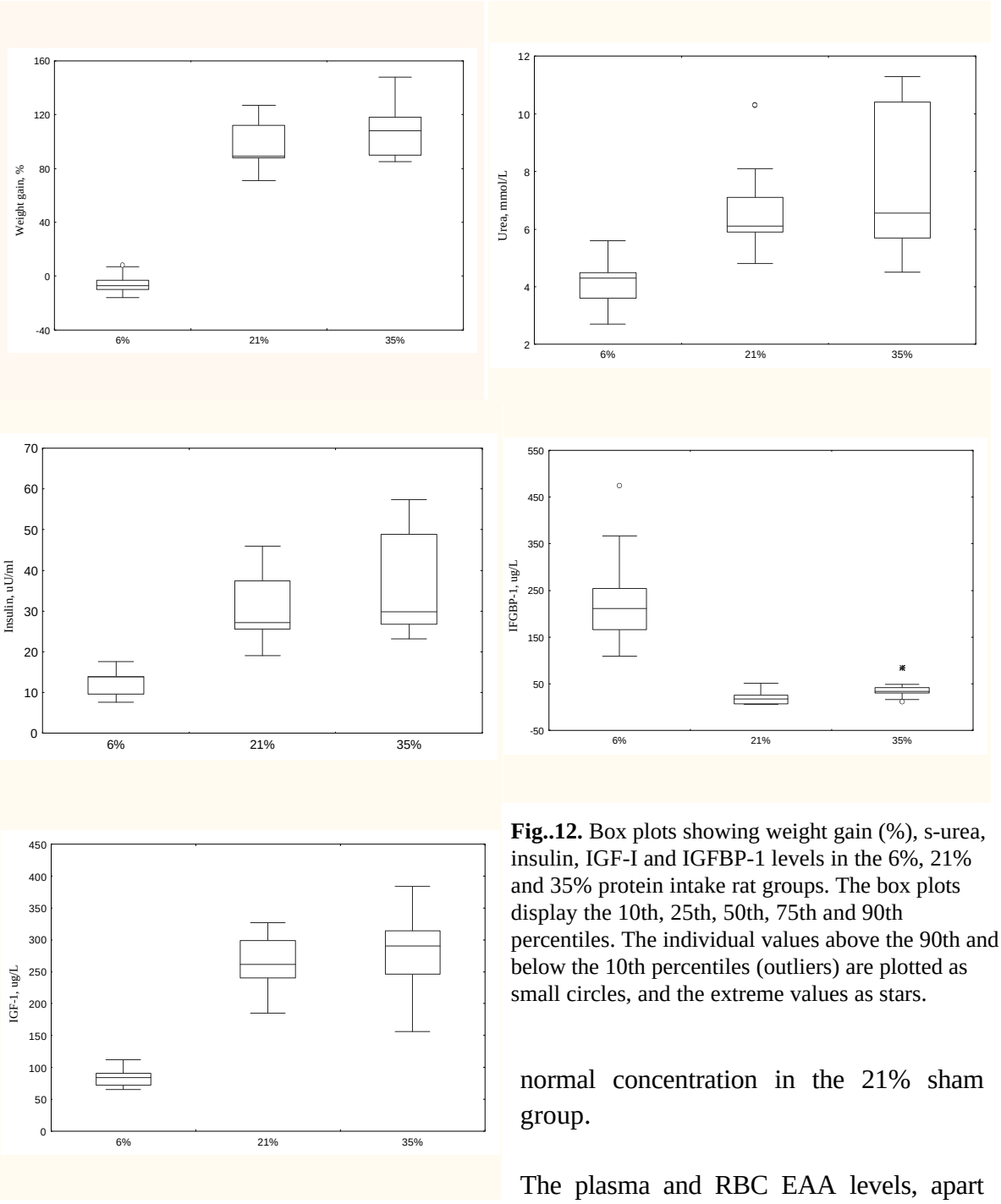


Fig..12. Box plots showing weight gain (%), s-urea, insulin, IGF-I and IGFBP-1 levels in the 6%, 21% and 35% protein intake rat groups. The box plots display the 10th, 25th, 50th, 75th and 90th percentiles. The individual values above the 90th and below the 10th percentiles (outliers) are plotted as small circles, and the extreme values as stars.

availability.

AA concentrations in plasma and RBC

The AA concentrations in plasma, RBC and the gradients RBC/plasma are shown in tables 2, 3 and 4 of paper IV, respectively**.** Figure 13 shows the plasma and RBC free AA concentrations for the uraemic groups, given as percentages of the normal concentration in the 21% sham group.

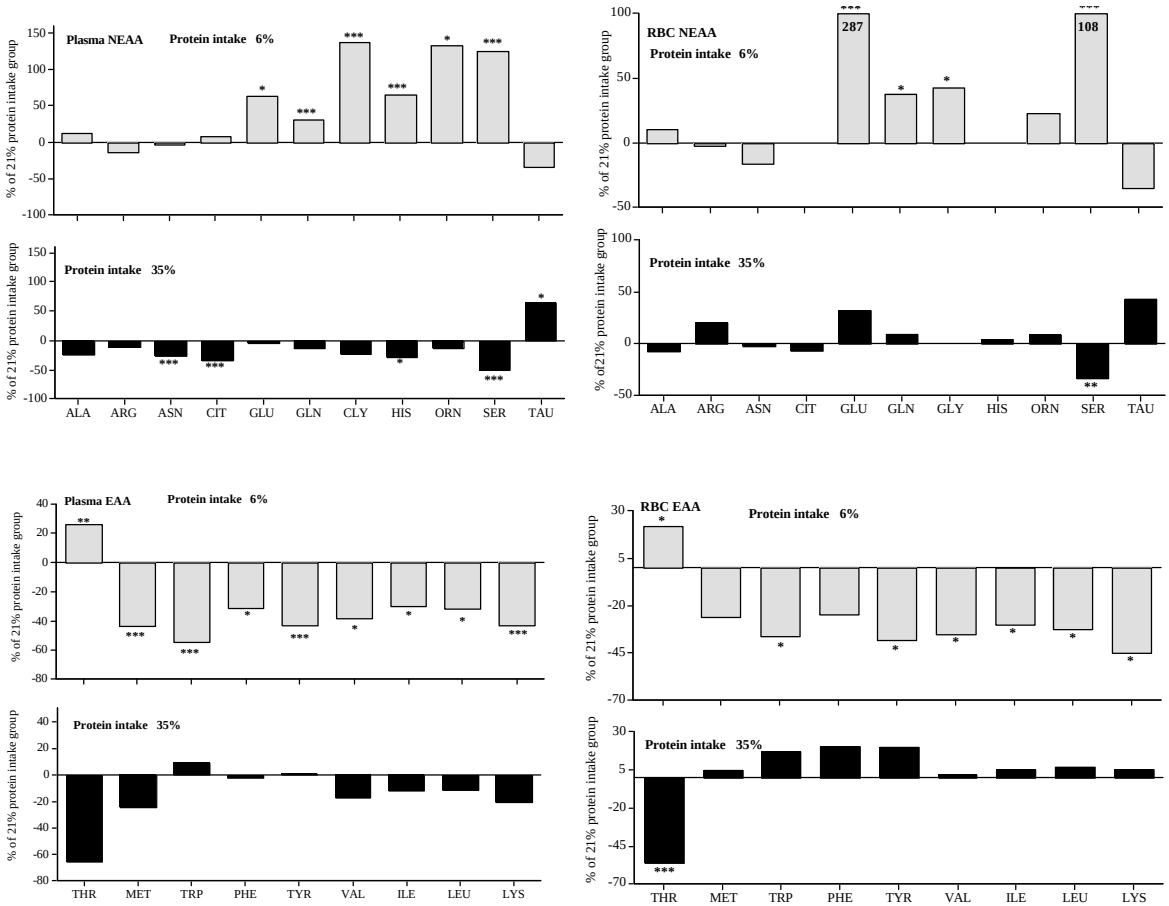
The plasma and RBC EAA levels, apart from threonine, were significantly lower in the 6% protein group than in the two other groups. In contrast, most of the NEAA were elevated in plasma and RBC in the 6% group.

Threonine levels were higher and taurine lower in both compartments in the 6% protein group, compared to the other

groups. The decreased availability of threonine by excess dietary protein constitutes a dietary paradox. Threonine shares a common metabolic pathways (threonine-serine dehydratase) with serine, and high-protein diets consistently induce a fall in their concentrations in liver, plasma and muscle [**155**]. Our results demonstrate that the variations in threonine and serine concentrations related to protein intake observed in plasma **[155]** are also present in RBC. Low taurine levels in plasma and RBC in the 6% group presumably reflect taurine depletion due to insufficient dietary supply of taurine and/or the precursor AA, considering that the methionine levels are also low in this group. Taurine is known to be actively absorbed by the various tissues of the rat [**203**] and its plasma and RBC decrease in the low protein diet group may reflect a mechanism of preventing the loss of taurine via urine.

The EAA/NEAA ratios in both plasma and RBC were significantly lower in the 6% protein intake group when compared to the 21% and 35% groups (Tables 2 and 3, study IV). The EAA/NEAA ratio is considered to be an index of protein nutritional status [**225**], and the lowered EAA/NEAA ratio with the 6% protein diet as well as the impaired weight gain provide evidence for the nutritional inadequacy of this diet in rats.

Fig.13. Plasma and RBC AA concentrations in the 6% and 35% protein intake rat groups, expressed as percentage changes from the 21% group (controls). * p<0.05, **p<0.01, **p<0.001



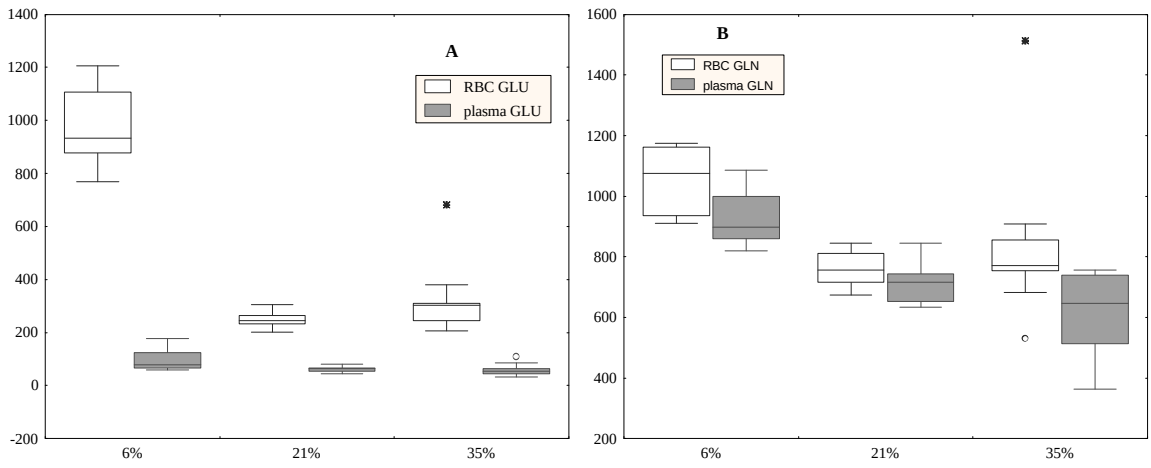
The individual plasma and RBC AA concentrations in the different protein diet groups were similar; therefore the RBC/plasma gradients for most of the AA did not differ between rats on different diets. Glutamate was the only AA in which the RBC/plasma gradient increased significantly as the protein intake decreased (4.3 to 12.7, $p<0.0001$). This increase in RBC glutamate is due to either a decreased capacity of the peripheral muscle tissue to extract glutamate from the circulating blood and/or an increased hepatic glutamate release. Aoki *et al* have demonstrated in humans that insulin selectively increases muscle glutamate uptake from whole blood [**8**] and that changes occur in whole blood fluxes with changing states of nutrition [**9**]. Therefore, the reduced insulin levels in the low protein group may have resulted in reduced glutamate uptake by the muscle, and accumulation of glutamate in the RBC.

RBC glutamate levels, which were weakly related to plasma glutamate, were strongly correlated to RBC and plasma glutamine. The observed relationship of RBC-glutamate to plasma and RBC glutamine but not plasma glutamate reinforces the importance of the RBC in AA transport and also suggests a role for RBC in the interconvertibility of glutamine and glutamate. Glutamate and glutamine are known to be linked to gluconeogenesis, renal ammoniagenesis and cycling of nitrogen among various organs of the body and therefore are two of the most important NEAA. Figure 14 displays box plots of plasma and RBC glutamate and glutamine in three groups of rats.

Correlations between AA and/or Urea, and Insulin, IGF-I and IGFBP-1 levels

When the results from the three groups were combined, the RBC EAA/NEAA ratio showed a positive correlation to IGF-I (Fig.1A, study IV) and insulin (r= 0.76 and r= 0.58, respectively) whereas an inverse correlation was found to IGFBP-1 (r= -0.67)(Fig.1B, study IV). Similarly, the plasma EAA/NEAA ratio showed a positive correlation to IGF-I (r= 0.80) insulin (r=0.54), and an inverse correlation to IGFBP-1 (r= - 0.78).

Fig.14. Box plots showing plasma and RBC glutamate(A) and glutamine (B) levels in rats fed 6%, 21% and 35% protein intake. The box plots display the 10th, 25th, 50th, 75th and 90th percentiles. The individual values above the 90th and below the 10th percentiles (outliers) are plotted as small circles, and the extreme values as stars.



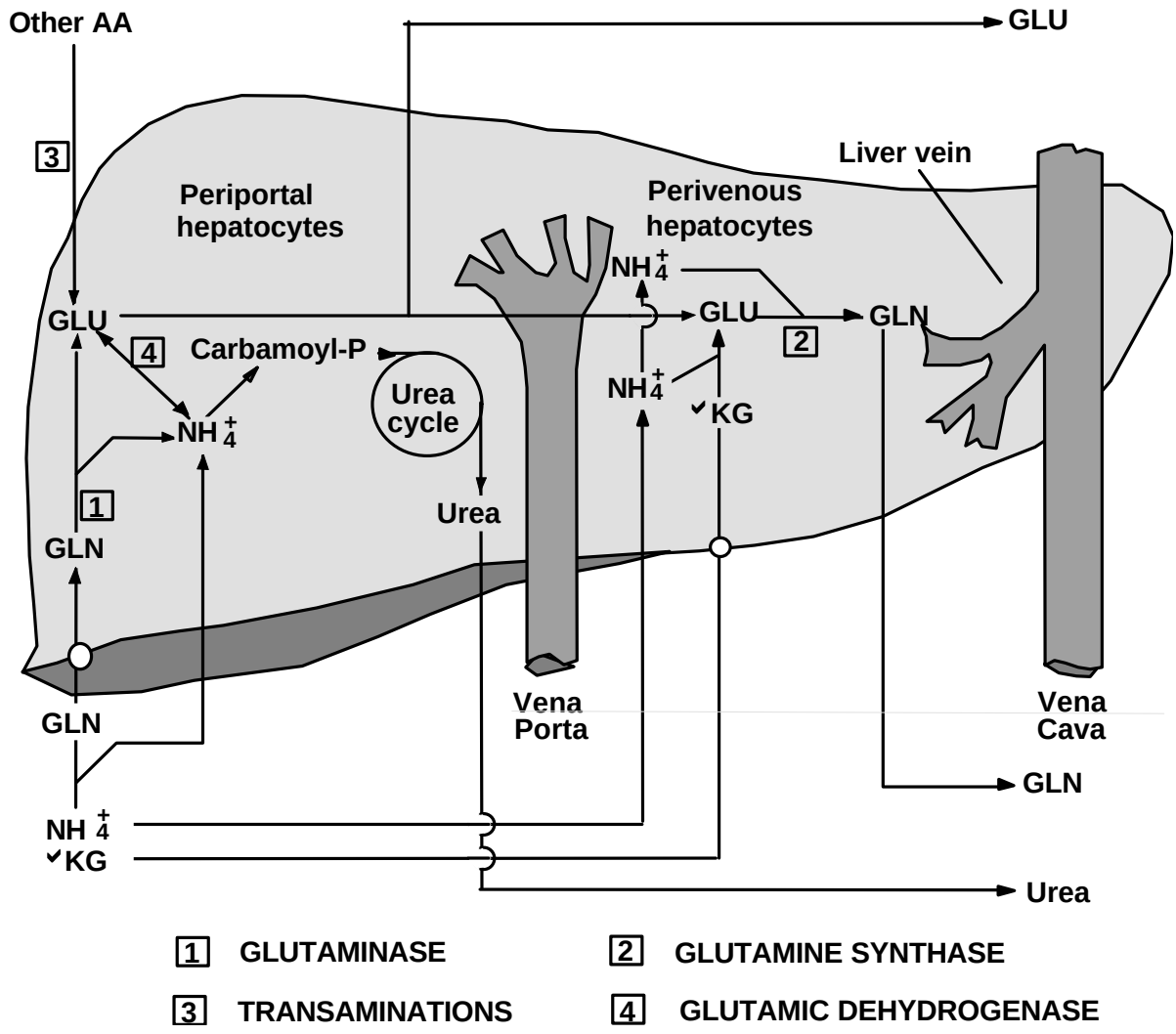


Fig.15. Schematic model for respective role of periportal and perivenous hepatocytes in liver nitrogen cycling in rats.

The highly significant inverse correlation of plasma and RBC EAA/NEAA ratio to IGFBP-1 as well as the positive correlation to IGF-1 support the use of EAA/NEAA ratio as an index of protein nutritional status. IGF-I and IGFBP-1 are presently used as indexes for malnutrition in humans [**46,124**].

Several plasma AA correlated to IGF-I and IGFBP-1 (Table 5, study IV). The plasma NEAA were inversely related to IGF-I and positively related to IGFBP-1, excepting for glutamate and taurine. Conversely, plasma EAA displayed an opposite pattern, with positive correlations to IGF-I and negative to IGFBP-1.

Among RBC AA, the most significant correlations to IGF-I and IGFBP-1 were found with RBC glutamate and serine. A highly significant inverse correlation existed between RBC glutamate and IGF-I (r= - 0.85, Fig. 1C, study IV) and insulin (r= - 0.72) levels whereas a positive correlation was found between RBC glutamate levels and IGFBP-1 (r= 0.78, Fig. 1D,

study IV).

Serum urea was inversely correlated to RBC glutamate (r= - 0.62) (Figure 2 A, paper IV), plasma glutamine(r= - 0.57) and IGFBP-1 (r= - 0.46); and positively correlated to EAA/NEAA plasma ratio (r= 0.69) and IGF-I (r= 0.72)(Figure 2 B, study IV).
In catabolic states, nitrogen flows from muscle largely in the form of glutamine [**227**] to the gut and liver, where it ultimately supports accelerated ureagenesis. In the low protein diet group the urea levels were significantly lower than in the other groups suggesting depressed ureagenesis. Rèmèsy *et al* have recently reported that in rats fed a protein-deficient diet (11%) it appears that nitrogen is extensively recycled (64%) as a result of glutamate and glutamine release by the liver. This cycling is much lower (15%) when the dietary protein level is sufficient (22%) to fulfill the nitrogen requirements [**185**].

The enzymes of the urea cycle and glutaminase are present in periportal hepatocytes, whereas glutamine synthetase is found only in perivenous hepatocytes (Figure 15). Whereas most AA are taken up by periportal cells capable of urea synthesis, more than 70% of total hepatic glutamate uptake must be ascribed to the perivenous hepatocytes [**110**]. Hazel *et al* have demonstrated [**110**] that the levels of mRNA for IGFBP-1 and IGF-I in hepatocytes can vary according to their position within the liver acinus. In rats fed a low protein diet higher levels of IGF-I mRNA were found in the periportal versus the perivenous whereas IGFBP-1 mRNA levels were higher in the perivenous area of the liver acinus.

When rat liver cells are cultured in the absence of specific AA or rats are fed a protein restricted diet, the expression of IGFBP-1 mRNA is increased [**204**]. It has earlier been shown, in primary rat hepatocyte culture [**211**], that AA deprivation decreased IGF-1 mRNA (-56% after 24 h) and increased IGFBP-1 mRNA abundance (+69%).

Our results with high RBC glutamate and low urea levels suggest that low protein diet results in shunting of extracted glutamine nitrogen from urea to hepatic glutamate release in the periportal hepatocytes. The hormonal signals regulating such a switch are still unknown. In this context it is of interest that the high RBC glutamate was related to low insulin levels, low IGF-1 levels and high IGFBP-1 levels. This raises the suspicion that a switch in glutamine towards RBC glutamate in the liver can be attributed to insulin as the regulator, since IGF-I and IGFBP-1 are not expected to have any direct effect on the amino acid metabolism in the liver due to lack of IGF-I receptors in hepatocytes.

The significant correlations between RBC glutamate to IGF-I/IGFBP-1 axis found in a group of non-malnourished HD patients who had normal IGF-I but three-fold increased IGFBP-1 levels were also observed in the present study in rats fed varying protein dietary levels.

Study V

Erythrocyte glutamate as a marker of catabolism during moderate renal failure and protein restriction in rats.
JC.Divino Filho, SJ.Hazel, B.Anderstam, ME Suliman, J.Bergström, K.Hall
In manuscript form

Female Sprague Dawley rats (BK Universal, Sollentuna, Sweden) were used in the experiments and divided randomly into groups fed 6 % and 21 % protein diets *ad libitum*. Surgery was performed with 2/3 of the right kidney resected on the first day, and the left kidney removed two days later, resulting in removal of approximately 5/6 of the functional renal mass in 21 rats. Some results from the control group have previously been published in paper IV and in another publication [**110**]. In the control rats (n=19) the kidney was exteriorised, and then replaced without removing any tissue. At five days following the surgery, rats were changed from the ordinary rat chow to the specific protein diets, and then monitored with regular measurement of the body weight changes until seven weeks following the onset of renal failure in the nephrectomised animals. The body weights of the four groups of animals at the beginning of the experiment were 159±1 and 162±1 for the 6% and 21% protein diet, respectively, sham-operated rats, and 150±1 and 148±1 (mean ± SEM) for the 6% and 21% protein diet, respectively, 5/6 nephrectomized rats. The diets were the same as stated above in study **IV**. All rats were maintained on a 12 hour day/night rhythm with free access to water. The rats were fed these diets for forty-seven days.

In this rat study we investigated how both renal failure and protein diet changed the plasma, muscle and RBC AA levels (especially glutamine and glutamate); and whether these changes were associated with the IGF-I/IGFBP-1 axis and/or muscle ASP/DNA ratios.

Body weight and serum levels of IGF-I, IGFBP-1, insulin urea and creatinine in the different groups of rats (Table 1, study **V)** (figure 16).
The BW of rats fed 6% protein were significantly retarded in comparison to the other groups. Uraemia resulted in significantly lower BW gains, with a significant difference between the 21% S and U groups but no significant difference between the 6% groups. Muscle ASP/DNA ratio was significantly lower in the 6% groups compared with the 21% groups, whereas no significant differences were observed between the U and S groups. Serum levels of urea were lower and creatinine higher in the 6% than in the 21% groups. In the U-groups both urea and creatinine were significantly elevated compared to the S-groups (Table 1, study V).

The similar weight gain in both the U and S-rats suggests that growth failure is due to protein deficiency as the 6 % protein diet was insufficient to promote a normal growth in normal rats. In a previous study, children who were uraemic or on maintenance haemodialysis were reported to show marked protein depletion, as assessed by muscle ASP levels, that were low in relation to FFS, potassium, phosphate and low serum transferrin levels [**59**]. The authors suggested that the protein depletion resulted chiefly from an inadequate protein intake. In a more recent study, Canepa *et al* reported a group of end-stage renal failure

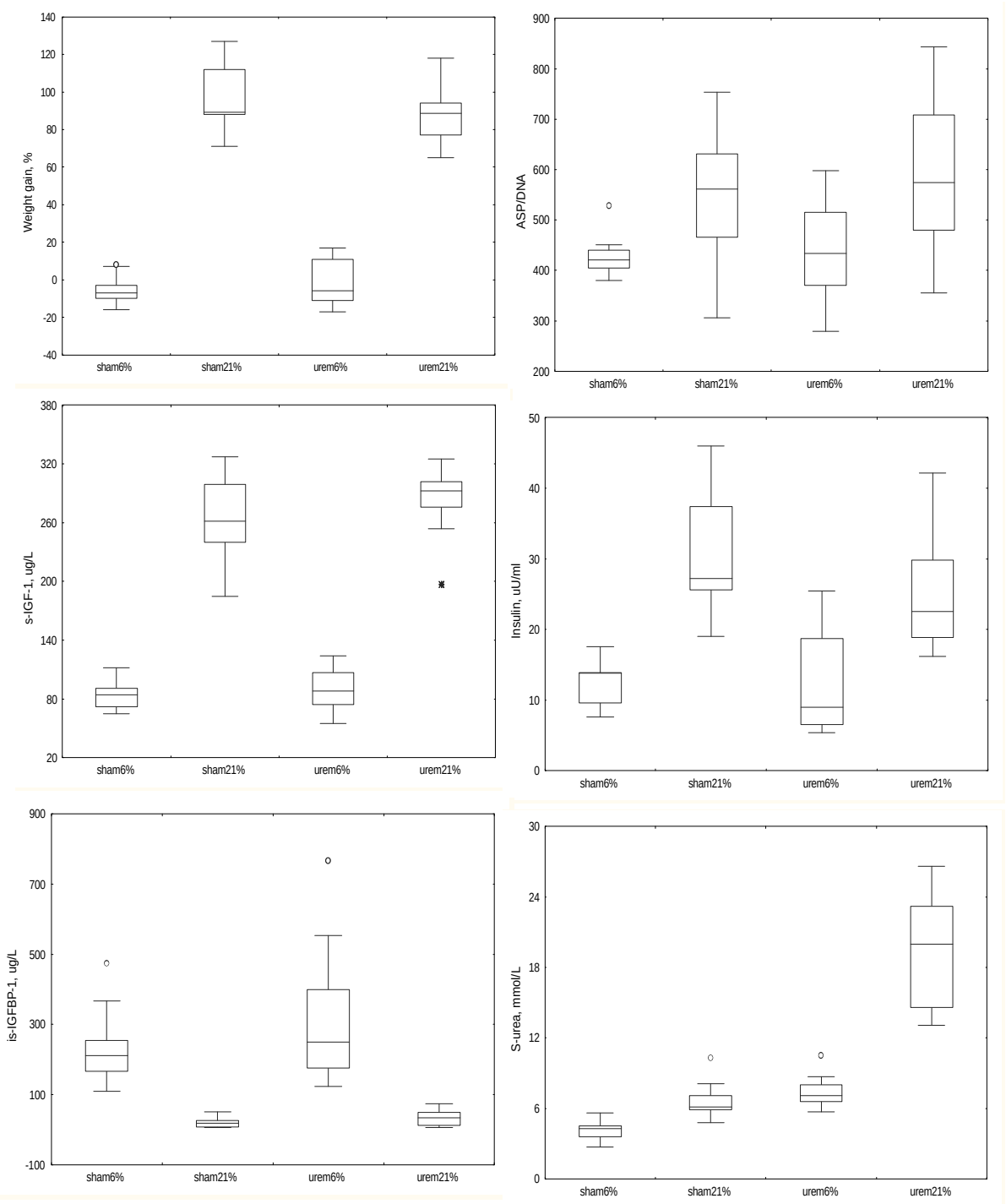
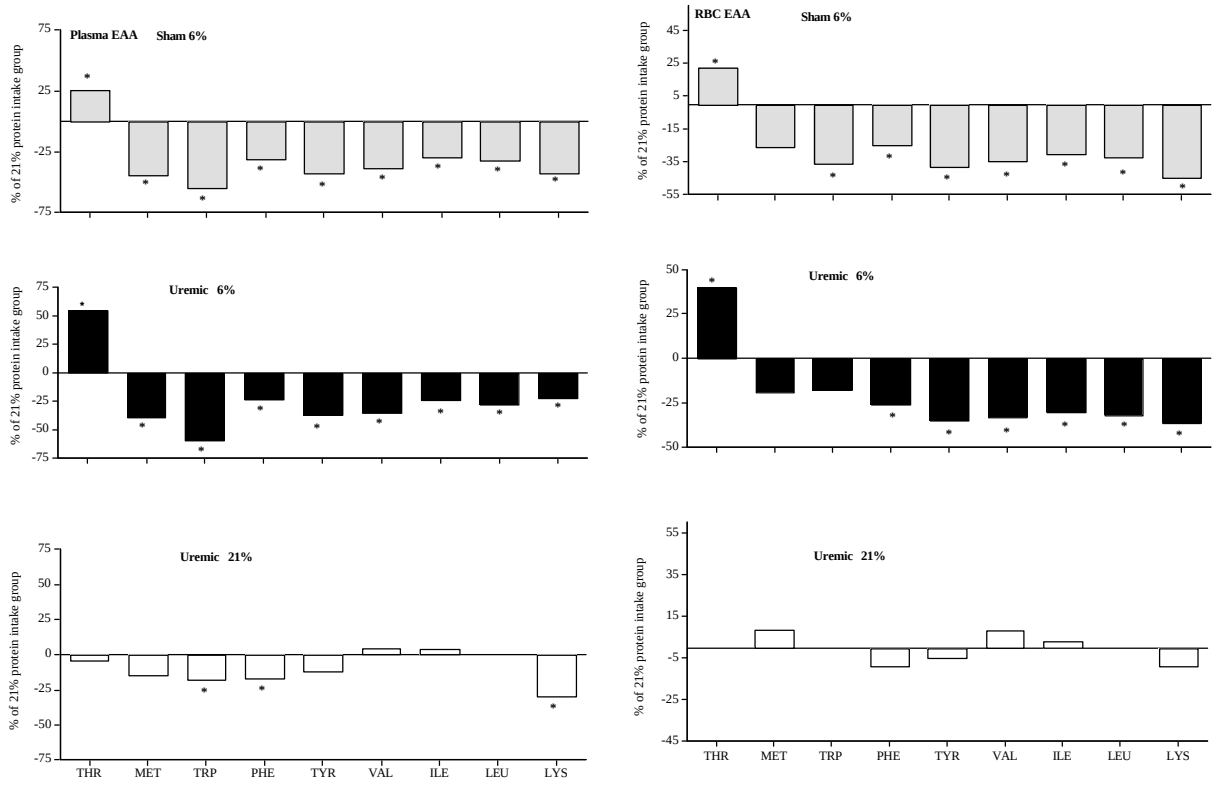


Fig.16. Box plots showing weight gain (%), muscle ASP/DNA ratio, IGF-I, insulin, IGFBP-1 and serum urea levels in the 6% and 21% protein intake S– and U- rat groups. The box plots display the 10th, 25th, 50th, 75th and 90th percentiles. The individual values above the 90th and below the 10th percentiles (outliers) are plotted as small circles, and the extreme values as stars.

children whose muscle ASP/DNA ratio did not differ from a control group suggesting this as evidence that they did not suffer from protein malnutrition [**37**]. On the other hand these children had AA abnormalities such as increased citrulline and glycine levels, reflecting alterations in their protein metabolism.



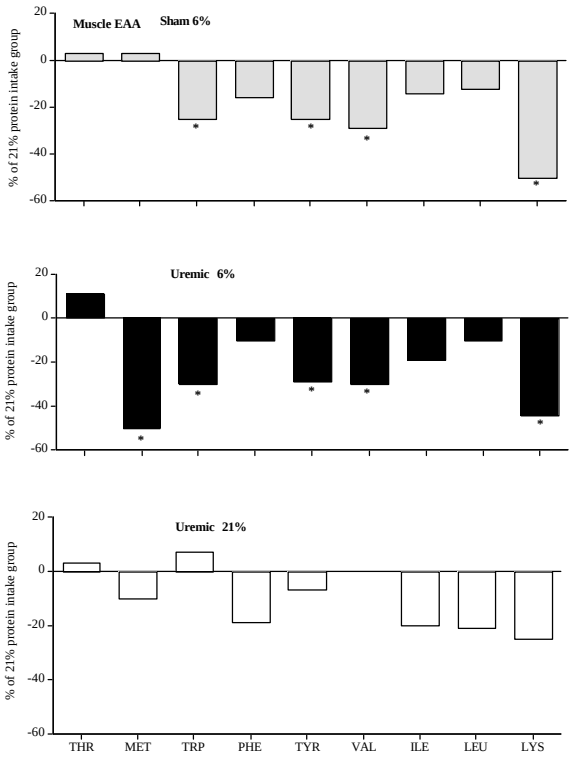


Fig.17. Plasma, muscle and RBC EAA concentrations in the S-6%, U-21% and U-6% protein intake rat groups, expressed as percentage changes from the S-21% group (controls). * p<0.05, **p<0.01, **p<0.00.

Amino acid concentrations in muscle, RBC and plasma

The majority of EAA in muscle, RBC and plasma were significantly lower in rats on 6% protein than in those fed the 21% protein, apart from threonine, which was significantly higher in RBC and plasma in the 6% groups (Figures 17,18) (table 2, study **V**).

In general the effect of protein restriction on NEAA was opposite to that on EAA. Muscle taurine, RBC glutamate and glutamine, and plasma glutamate, glutamine and citrulline were higher in the 6% groups (Table 3, study **V**). The lack of difference in muscle glutamate in spite of 50% lower mean levels in the 6% groups

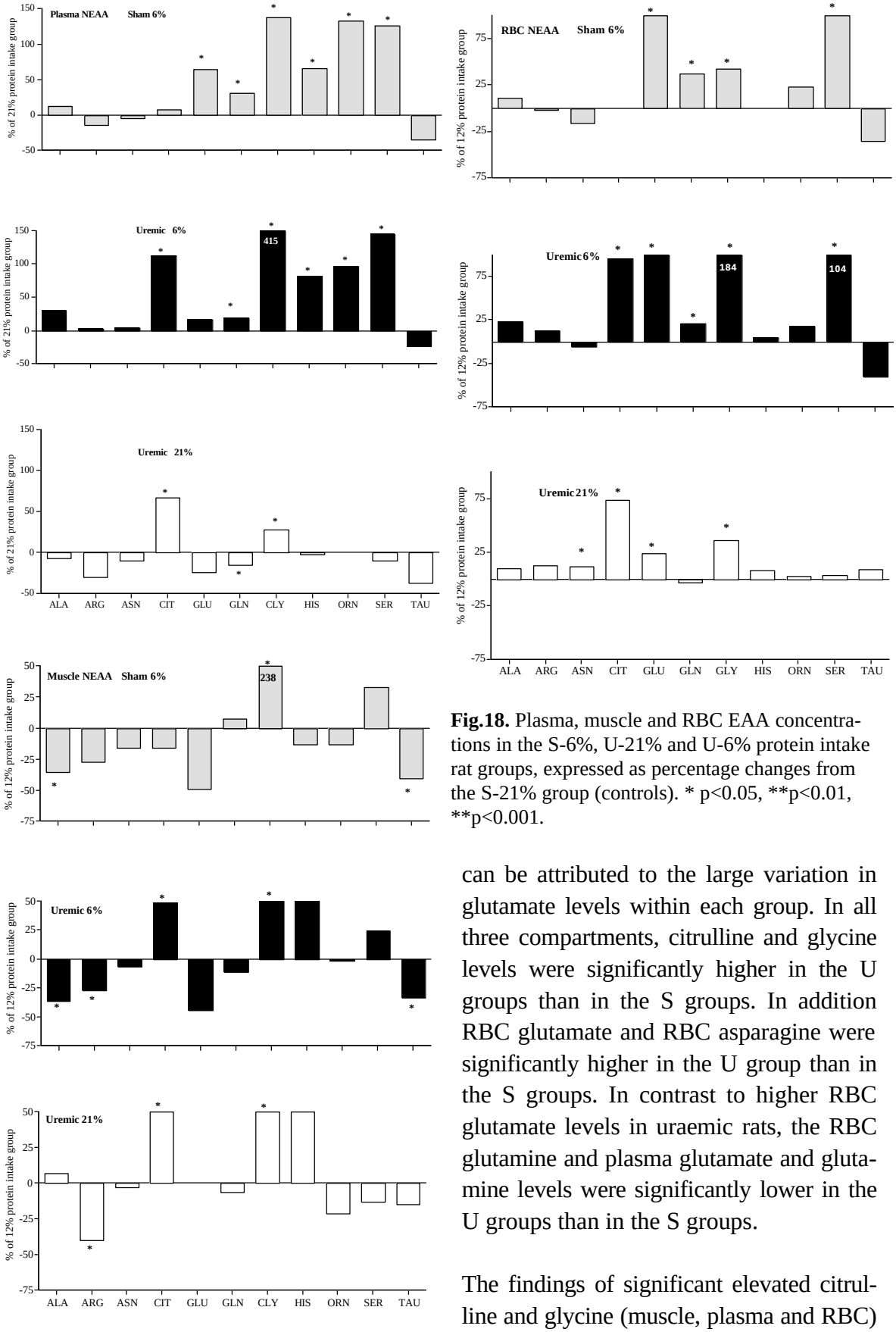


Fig.18. Plasma, muscle and RBC EAA concentrations in the S-6%, U-21% and U-6% protein intake rat groups, expressed as percentage changes from the S-21% group (controls). * p<0.05, **p<0.01, **p<0.001.

can be attributed to the large variation in glutamate levels within each group. In all three compartments, citrulline and glycine levels were significantly higher in the U groups than in the S groups. In addition RBC glutamate and RBC asparagine were significantly higher in the U group than in the S groups. In contrast to higher RBC glutamate levels in uraemic rats, the RBC glutamine and plasma glutamate and glutamine levels were significantly lower in the U groups than in the S groups.

The findings of significant elevated citrulline and glycine (muscle, plasma and RBC)

levels in the uraemic rats when compared to the sham groups confirm the presence of the uraemic state in these rats. AA abnormalities, which were observed in all three compartments, are among the first signs of protein metabolism derangement in the progression of renal failure.

In the present study we have found that low protein diet caused a threefold increase in RBC glutamate levels, not only in the sham-operated rats as reported in study **IV**, but also in uraemic rats; and that uraemia *per se* results in a further increase in RBC glutamate levels.

In spite of the lack of difference in muscle glutamate and muscle glutamine levels between the four groups of rats, the muscle glutamine/glutamate ratios were significantly higher in the 6% groups than in the 21% groups (Table 4, study **V**). Conversely the RBC glutamine/glutamate ratio was significantly lower in the 6% groups when compared to the 21% groups. In addition, the RBC glutamine/ glutamate ratios were significantly lower in the U groups compared with the S groups. Data published by Jasper *et al* [**122**] have suggested that glutamine/glutamate ratio is an useful indicator of the capacity for glutamine output; thus, the lower RBC glutamine/glutamate ratio in the low protein and uraemic groups may be an indication of decreased capacity of the peripheral muscle tissue to extract glutamate from the circulating blood and/or increased hepatic glutamate output during catabolism [**215**].

Relation between glutamate, glutamine or urea, and IGF-I, insulin, or IGFBP-1 levels

Muscle glutamate was positively correlated to IGF-I (r= 0.38) and inversely correlated to IGFBP-1 (r= -0.37), and the muscle glutamine/glutamate ratio correlated inversely to IGF-I (r= -0.56) and positively to IGFBP-1 (r= 0.40). Both RBC glutamate and RBC glutamine displayed inverse and highly significant correlations to IGF-I (r= -0.84 and r= -0.79) and insulin (r= -0.75 and r= -0.64) and positive correlations to IGFBP-1 (r= 0.84 and r= 0.70) (Fig 2, study V). With the use of RBC glutamine/glutamate ratio as the dependent variable the relations became positive to IGF-I (r= 0.83) and insulin (r= 0.73), and inverse to IGFBP-1 with a linear regression line (r= -0.83). In multiple regression analyses with RBC glutamate or RBC glutamine/glutamate ratio as the dependent variables, both IGF-I and IGFBP-1 remained as significant independent variables resulting in an adjusted r^2 of 0.74 and 0.72, respectively. Thus, the variation in RBC glutamate or RBC glutamine/ glutamate ratio can be attributed, by approximately 70%, to the bioavailabilty of IGF-I.

Glutamine accounts for over 50% of the intracellular AA content [**75**] and its efflux from muscle increases in catabolic disease states by **a**) increased protein breakdown with direct production of glutamine and liberation of AA precursors of glutamine (i.e., glutamate, aspartate, isoleucine, and valine) **b**) by inducing the activity of glutamine synthetase. It is known that glucocorticoid administration enhances the rate of release of glutamine from skeletal muscle of rats and this may be due to changes in efflux and/or increased intracellular formation of glutamine [**191**]. The higher muscle glutamine/glutamate ratio found in the low protein groups and its significant inverse correlation to IGF-I and positive to IGFBP-1 confirm earlier findings of high muscle glutamine output during protein

catabolism.

An opposite effect on urea by protein deficiency and uraemia resulted in no difference being observed between urea levels in the uraemic rats on the 6% diet and sham-operated rats on the 21% diet. When combining all four groups, urea was positively correlated to IGF-I (r=0.62) and inversely to IGFBP-1 (r= -0.46), but no significant correlation was found to insulin.

Serum urea was inversely related to RBC glutamine (r=-0.68) and RBC glutamate (r=-0.50).

IGF-I and its binding proteins appear to be important links between nutrient intake and cellular anabolic responses. Elevated IGFBP-1 levels decrease IGF-I bioavailability which may lead to a glutamate shift from muscle to RBC; or common factors stimulate both RBC glutamate uptake and IGFBP-1 liver expression. In vitro studies have shown that IGF-I decreases the rate of release of glutamine from skeletal muscle in rat [**165**]. Growth hormone administration to severely traumatised patients has been reported to reduce protein losses from skeletal muscle and also to reduce muscle glutamine efflux; this effect completely accounted for by a suppression of glutamine *de novo* synthesis in muscle tissue [**27**]. Garibotto *et al* have demonstrated that GH treatment of malnourished haemodialysis patients result in an increase in whole blood arterial glutamate levels, despite a tendency for its muscle balance to become more positive [**91**]. In rats, GH has been shown to markedly reduce liver glutamate uptake and to increase glutamate levels, thus reducing ureagenesis [**227**]. These results altogether may indicate that GH treatment (with increasing IGF-I levels and decreasing IGFBP-1 levels) promotes increased glutamate output from the liver and improves muscle uptake of glutamate for protein synthesis in uraemia. One may assume that when the metabolic balance is reestablished the glutamate levels will normalise.

ASP/DNA ratio in muscle and its relation to other variables

Muscle ASP/DNA ratio was significantly lower in the 6 % than in the 21% groups, whereas no significant differences were found between the U and the S groups. ASP/DNA ratio correlated significantly to IGF-I (r= 0.49) and insulin (r= 0.56), whereas a negative correlation was observed between ASP/DNA ratio and IGFBP-1 (r= -0.45).

ASP/DNA ratio showed significant inverse correlations to muscle glutamate (r= -0.42), muscle glutamine (r= -0.59), RBC glutamate (r= -0.55), RBC glutamine (r= -0.46) (FIGURE 4) and plasma glutamine (r= -0.37), but not to plasma glutamate.

In multiple regression analyses with ASP/DNA ratio as the independent variable, both muscle glutamate and IGF-I remained as significant independent variables and resulted in an adjusted r^2 of 0.63 as compared to r^2 = 0.30 with muscle glutamate alone. Further addition of insulin as independent variable increased the r^2 to 0.74.

Changes in the ASP/DNA ratio have been reported in different catabolic conditions in both man and animals [**51,168,**]. In both low protein intake rat groups (S and U), the ASP/DNA ratio was reduced in comparison with the respective normal intake groups (S and U), suggesting that skeletal

muscle cells in the S-6% and U-6% rats failed to grow, most likely due to an increase in protein catabolism linked to protein depletion. The experimental rat model of renal failure applied in this study has been reproduced earlier by one of the authors [**111**]. Interestingly neither ASP/DNA ratio nor weight gain, IGF-I, IGFBP-1, insulin, blood glucose were significantly different between S and U groups with the same protein intake although serum levels of urea and creatinine were higher in the uraemic groups when compared to their respective sham groups.

The AA changes attributed to protein deficiency in the present study are consistent with the non-uremic related AA changes observed in non-malnourished haemodialysis patients in study **III**. However, the renal failure in the rats was less pronounced than in haemodialysis patients and their IGFBP-1 levels were not significantly elevated as in the patients.

SUMMARY

Previous studies have demonstrated that RBC are involved in AA transport between different tissues of the body [**9,73,76**]. Plasma carries free AA from gut or peripheral tissues to liver whereas AA are carried from liver to peripheral tissues as free AA in RBC or as protein in plasma [**73**]. The need to carry out simultaneous determinations of AA concentrations, both in RBC and plasma, in order to obtain a more complete picture of the inter-organ AA relationship under different physiological situations, has been suggested [**112,200**].

The measurement of AA in RBC can give important additional information to that obtained from plasma aminograms, especially as it does not require extra blood samples or laborious procedures for preparing the samples. Furthermore, muscle biopsy is an invasive method and muscle sample analysis is a laborious and expensive procedure which precludes its utilisation in large groups of patients.

In this thesis reversed-phase HPLC was used for determination of AA concentrations in plasma, muscle and RBC whereas in most earlier studies the analyses were performed by ion exchange chromatography [**25,81,89,99,129,151,156,158,196**]. The concentrations reported in the present study are in good agreement with earlier data [**25,156,158**]; yet, standard deviations with HPLC analytics are much lower for most of the AA as compared with earlier reported values measured with ion-exchange chromatography. Hence, the appropriate use of reversed-phase HPLC constitutes a refinement of the results by improving precision and reliability of the AA analysis [**96**]. It should be remembered that measurements of tissue free AA are usually made of a single point in time, and it is upon such a measurement that one decides whether a subject manifests "normal" or pathological AA metabolism [**196**]. Therefore, reliable references of AA concentrations preferentially obtained simultaneously in various tissue compartments are of importance. This thesis presents the first data on AA levels obtained simultaneously from three different compartments in healthy subjects and uraemic patients.

In most earlier studies, RBC free AA were calculated as the difference between whole blood and plasma values, and therefore included AA from white blood cells and platelets [**8,71,77,90,125**]. Furthermore, direct measurement in washed RBC [**103**] led to underestimation of several of the AA (especially methionine, valine, isoleucine, tyrosine and phenylalanine). Our RBC free AA results are in keeping with the data published by Flugel *et al* [**81**].

Our study is the first to report on elevated RBC and plasma glutamate levels and their possible relation to catabolism in non-malnourished HD patients. In a recently published study on protein-energy malnutrition in HD patients, it could be observed from the plasma AA tables that glutamate was the only NEAA to be significantly elevated in the patients identified as mildly, moderately or severely (classified by subjective global nutritional assessment) malnourished, but RBC free AA were not determined in this study [**181**].

End-stage renal failure is characterised by both disturbed protein metabolism and

changes in the IGF-I/IGFBP-1 axis. The presence of high IGFBP-1 accompanied by low IGF-I levels is a common finding during protein and caloric restriction [**124,192,223**] and elevated IGFBP-1 levels in uraemia were already reported in 1984 [**177**]. In contrast to the protein-restricted rats in study **IV** and **V**, the HD patients in study **III** had normal or slightly elevated IGF-I levels in the presence of threefold elevated IGFBP-1 levels. On the other hand, both protein-restricted rats as well as non-malnourished HD patients presented similar patterns of elevated RBC NEAA and an association between RBC glutamate and the levels of IGF-I and IGFBP-1.

The correlations between RBC or muscle glutamate and the IGF-I / IGFBP-1 axis found in study **III**, **IV** and **V** do not clarify the cause. It is most likely that these variables share some common regulators although a causal relationship is not excluded. A direct stimulatory effect of glutamate or any other AA on IGFBP-1 expression in hepatocytes is unlikely, since it has been reported that withdrawal of AA from medium increased IGFBP-1 release from hepatocytes *in vitro* [**204**]. Hormones with hepatic receptors such as glucagon are tentative factors. An increased hepatic extraction of glutamine and an increased release of glutamate from the liver have been reported in a patient with glucagonoma, who displayed several AA abnormalities and weight loss [**188**].

The elevated IGFBP-1 levels with a low protein diet can be attributed to an increased expression of IGFBP-1 mRNA **[110,211]** and it has previously been shown that hepatic IGFBP-1 mRNA expression is increased in rats fed a protein restricted diet **[110]** . Glucagon and lymphokines have been considered as stimulators of IGFBP-1 expression during starvation, but their effect on glutamine and glutamate turnover in the liver is unknown. Whether the change in hepatic glutamine/ glutamate metabolism shares a common regulator with hepatic IGFBP-1 expression and decreased IGF-I expression during reduction of protein intake has to be considered [**204**].

Significant relationships between several AA, and IGF-1 and IGFBP-1 levels with the protein intake in sham-operated and 5/6 nephrectomized rats were found in studies IV and V. These findings raise the possibility that the AA availability to the hepatocytes in rats fed low protein intake might be related to the changes in IGF-1 and IGFBP-1 expression. Moreover, the increase in RBC glutamate levels as the protein intake decreases may indicate alterations in the interorgan nitrogen transport involving glutamate flux.

In summary, this thesis has demonstrated that AA determination in RBC is a simple and sensitive method for detecting AA alterations, and that RBC have an important and yet not fully clarified role in AA and protein metabolism in catabolic conditions such as uraemia and protein restriction, in both man and rat. The determination of RBC AA should be considered when undertaking metabolic and clinical studies of AA and protein disturbances occurring in uraemia and other disease conditions. The relationships found between the RBC glutamate and glutamine, and the IGF-I/ IGFBP-1 axis enable hypotheses to be made and studies to be designed to address the mechanisms involved.

CONCLUSIONS

1. The RBC, muscle and plasma AA results from the healthy subjects have been established as reference data for comparing AA profiles in various disease conditions; and also for evaluating the effect of various physiological stimuli on AA concentration changes in these compartments.

2. Elevated concentrations of some NEAA in RBC and muscle (but not plasma) and lower concentration of some EAA in RBC and plasma (but not muscle) of uraemic patients in comparison to healthy subjects have been demonstrated. Therefore, the determination of AA in RBC may be a more sensitive method for detecting a deficiency in these AA than AA analysis in the muscle.

3.In patients on haemodialysis, the high RBC glutamate levels positively correlated to the elevated serum IGFBP-1 levels leading to the proposal that changes in the bioavailability of IGFs, induced partly by IGFBP-1, are linked to the regulation of glutamate distribution in uraemia.

4. Elevated RBC NEAA and EAA levels found in rats fed low protein diet are similar to RBC AA findings observed in non-malnourished haemodialysis patients. The increase in RBC glutamate levels with a low protein diet in rats may indicate alterations in glutamate flux and interorgan nitrogen transport. Furthemore, the findings of a positive correlation between RBC glutamate and IGFBP-1 and inverse correlation to IGF-I, reinforces the possible role of RBC glutamate as a marker of catabolism in nutrition.

5. The effects of protein restriction and renal failure on AA in an experimental rodent model demonstrate some opposite effects on RBC glutamate and glutamine and serum urea levels. The RBC and muscle glutamate relationships to the IGF-I/IGFBP-1 axis and muscle ASP/DNA ratio support the proposal that RBC glutamate or RBC glutamine/ glutamate ratio should be evaluated as a marker of catabolism in different clinical situations; and that changes in the bioavailability of IGFs are linked to the regulation of glutamate distribution.

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Oscar Wilde