

# Distribution of Cyclosporin in Organ Transplant Recipients

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## Abstract

Cyclosporin is an immunosuppressive agent with a narrow therapeutic index. The total concentration of cyclosporin in blood is usually monitored to guide dosage adjustment and to compensate for substantial interindividual and intra-

individual variability in cyclosporin pharmacokinetics. Cyclosporin is a highly lipophilic molecule and widely distributes into blood, plasma and tissue components. It mainly accumulates in fat-rich organs, including adipose tissue and liver. In blood, it binds to erythrocytes in a saturable fashion that is dependent on haematocrit, temperature and the concentration of plasma proteins. In plasma, it binds primarily to lipoproteins, including high-density, low-density and very-low-density lipoprotein, and, to a lesser extent, albumin. The unbound fraction of cyclosporin in plasma ( $CsA_{fu}$ ) expressed as a percentage is approximately 2%.

It has been shown that both the pharmacokinetic and pharmacodynamic properties of cyclosporin are related to its binding characteristics in plasma. Furthermore, there is some evidence to indicate that the unbound concentration of cyclosporin ( $CsA_U$ ) has a closer association with both kidney and heart allograft rejection than the total (bound + unbound) concentration. However, the measurement of  $CsA_{fu}$  is inherently complex and cannot easily be performed in a clinical setting. Mathematical models that calculate  $CsA_{fu}$ , and hence  $CsA_U$ , from the concentration of plasma lipoproteins may be a more practical option, and should provide a more accurate correlate of effectiveness and toxicity of this drug in transplant recipients than do conventional monitoring procedures.

In conclusion, the distribution characteristics of cyclosporin in blood, plasma and various tissues are clinically important. Further investigations are needed to verify whether determination of  $CsA_U$  improves the clinical management of transplant recipients.

Transplantation is a unique method of treatment for a number of end-stage kidney, heart, liver, lung, pancreas, intestinal and bone marrow diseases and its success has been dependent upon the development of effective immunosuppression. The introduction of cyclosporin therapy in 1978, together with advances in surgical procedures, has made successful organ transplantation possible. The conventional maintenance immunosuppressive therapy administered by most transplant centres throughout the world is a triple regimen combining cyclosporin with azathioprine and corticosteroids. However, new immunosuppressive agents, including tacrolimus, sirolimus and mycophenolate mofetil, are now at various stages of clinical investigation in alternative regimens. The majority of immunosuppressive agents are highly bound to blood cells and plasma proteins. Most of these agents also distribute widely in various tissues and organs where they exert their therapeutic and often toxic effects.

Monitoring the trough blood concentration of cyclosporin is used to compensate for pharmaco-

kinetic variability and to individualise dosage. However, the total blood cyclosporin concentration, measured at trough, is an unreliable intermediate therapeutic endpoint as a guide to effectiveness of immunosuppressive agents.<sup>[1]</sup> This may be attributed to the fact that cyclosporin is extensively bound (approximately 98%) to blood and plasma components, and the measurement of total concentration in blood ( $CsA_T$ ) does not account for variability in  $CsA_{fu}$ .

For highly protein bound agents, the degree of protein binding may influence both the pharmacokinetic and pharmacodynamic characteristics of a drug. This includes the following points:

1. According to the principles of clinical pharmacology, the effect of drugs correlates better with the unbound concentrations in plasma than with the total concentration. Therefore the variability in the pharmacodynamics of immunosuppressive agents may also be at least partly attributable to the unbound concentration of the drugs in the central compartment.<sup>[2]</sup>

2. For drugs like cyclosporin, with a low to intermediate hepatic extraction ratio, the degree of hepatic clearance associates with the unbound fraction,<sup>[3]</sup> and therefore the total concentration should associate with the unbound fraction.

3. The small unbound concentration of a drug in plasma determines the extent of tissue distribution. Considering that the site of action of every pharmacological agent, with some exceptions like heparin, is situated within the intra- and intercellular space of various tissues, the concentration of a drug in the tissues, which is related to the unbound concentration in blood or plasma, should be a better indicator of drug concentration at the site of action.<sup>[4]</sup>

We have reviewed the literature describing the blood, plasma and tissue distribution of cyclosporin in order to illustrate the inter-relationships between these important pharmacokinetic characteristics and clinical outcomes related to cyclosporin therapy.

## 1. Physicochemical Properties

Cyclosporin is a neutral, highly lipophilic cyclic peptide containing 11 amino acids. Seven of these amino acids are *N*-methylated, but the amino groups in the remaining four amino acids undergo intramolecular hydrogen bonding with carbonyl groups to form a rigid cyclic polypeptide skeleton. The nine-carbon olefin-containing amino acid in position 1 has never been identified in nature before and is believed to be essential for the biological activity of the molecule.<sup>[5]</sup> The chemical structure of cyclosporin is shown in figure 1.

Cyclosporin is highly lipophilic, with an octanol to Ringer's solution partition coefficient of 991.<sup>[6]</sup> The solubility of cyclosporin at 25°C (expressed as mg/g) is 0.04 in water, 1.6 in *n*-hexane and greater than 500 in methanol, ethanol and acetonitrile.<sup>[7]</sup> In aqueous solution cyclosporin exhibits pH-independent, exothermic solubility behaviour characterised by an inverse proportionality with respect to temperature.<sup>[8]</sup> The solubility in water at 5°C is at least 10 times higher than that at

37°C, possibly as a result of stronger intramolecular hydrogen bonding at higher temperature.<sup>[8]</sup>

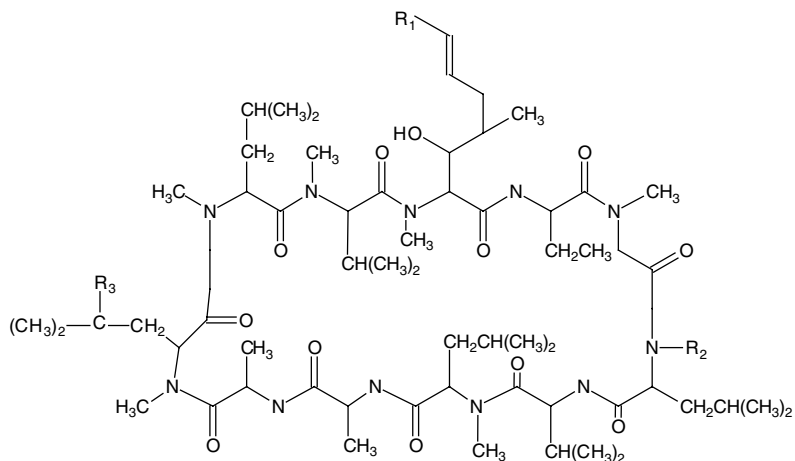
## 2. Mode of Action

The mechanism of immune activation, resulting in either allograft rejection or autoimmune diseases, is not fully understood, although *in vitro* models of the immune response, such as the mixed lymphocyte reaction, have provided some insight into the fundamental role of T-lymphocytes in this process.<sup>[9]</sup> Cyclosporin reversibly inhibits the T-lymphocyte mediated alloimmune response by inhibiting the production of lymphokines, including interleukin-2 (IL-2) and interferon- $\gamma$ , which are essential for the differentiation and proliferation of T helper lymphocytes and other immunocytes. Cyclosporin is not lymphotoxic, and its inhibitory activity is concentration-dependent and reversible.<sup>[10]</sup>

A cytosolic protein known as cyclophilin has been identified as the intracellular receptor for cyclosporin.<sup>[11]</sup> Upon binding to cyclophilin, cyclosporin undergoes a marked conformational change to produce a polar exterior surface. The cyclophilin-cyclosporin complex then competitively binds to and inhibits calcineurin, a heterodimeric calcium- and calmodulin-dependent serine/threonine phosphatase.<sup>[12]</sup> In so doing, the complex prevents the dephosphorylation and nuclear transcription of nuclear factors in activated T cells. The activity of this transcription factor is known to correlate with the level of IL-2 production after the T-cell receptor has been activated.

## 3. Pharmacokinetics

Cyclosporin exhibits variable intra- and inter-individual pharmacokinetics which are influenced by several interrelated factors, including age, disease status, lipoprotein concentrations and concurrent medications.<sup>[13]</sup> Oral absorption of cyclosporin is slow and erratic. The absorption half-life ranges between 0.5 to 2 hours. The absolute bioavailability of cyclosporin has been estimated at 30% (range 10 to 60%) although may be as low as 5% in liver transplant recipients with cholestasis



	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
Cyclosporin	CH <sub>3</sub>	CH <sub>3</sub>	H
AM1	CH <sub>2</sub> OH	CH <sub>3</sub>	H
AM4N	CH <sub>3</sub>	H	H
AM9	CH <sub>3</sub>	CH <sub>3</sub>	OH

Fig. 1. Structure of cyclosporin and its major metabolites.

or gastrointestinal dysfunction.<sup>[13]</sup> A microemulsified formulation of cyclosporin (Neoral<sup>®</sup>) has been shown to reduce the variability in the gastrointestinal absorption of cyclosporin<sup>[14]</sup> with an average bioavailability 30% higher than that of the non-microemulsified formulation.<sup>[14]</sup> Cyclosporin is highly bound to blood and plasma components and widely distributes in various tissues. It is metabolised in the gastrointestinal tract and liver<sup>[15]</sup> by an isoenzyme of cytochrome P450 (CYP) belonging to the CYP3A gene family of monooxygenases.<sup>[16]</sup> The metabolism of the cyclosporin molecule involves mainly hydroxylation, demethylation and cyclisation of different amino acids while the cyclic structure of the cyclosporin mole-

cule remains intact. Biliary excretion is the major pathway for elimination of cyclosporin metabolites. More than 90% of a cyclosporin dose is excreted in bile, and only less than 1% is excreted as parent drug.<sup>[17]</sup> Less than 3% of a cyclosporin dose undergoes renal elimination, mainly as hydroxylated metabolites.<sup>[18]</sup>

In this article we have reviewed the pharmacokinetic studies that have described the distribution characteristics of cyclosporin. The other aspects of cyclosporin pharmacokinetics have been extensively reviewed by others.<sup>[13,19]</sup>

## 4. Distribution

### 4.1 Tissue Distribution

In the body, cyclosporin accumulates mainly in fat-rich organs such as the liver, adipose tissue and

1 Use of tradenames is for product identification only and does not imply endorsement.

lymph nodes.<sup>[20]</sup> Moreover, post-mortem studies in human have shown high concentrations of cyclosporin in large bowel, breast, stomach, adrenal glands, oesophagus, pancreas, spleen and kidney.<sup>[21,22]</sup>

More recently, a physiologically based pharmacokinetic model for tissue distribution of cyclosporin has been proposed.<sup>[23]</sup> Tissue distribution kinetics of intravenous cyclosporin in the rat were used in conjunction with cyclosporin concentration-time profiles from kidney transplant recipients to allow scaling up of the prediction of tissue concentrations to humans. Tissue to whole blood concentration ratios, calculated by the use of the area under the concentration-time curve from zero to 32 hours ( $AUC_{32}$ ) of parent cyclosporin in whole blood and tissues, were 1.7 in muscle, 2.8 in bone, 3.5 in skin, 3.7 in heart, 4.5 in gut, 5.4 in lung, 6.5 in thymus, 7.1 in kidney, 7.7 in spleen, 9.1 in fat and 11 in liver. Two modelling approaches were used, including a 'linear tissue distribution model' and a model that assumes a saturable intracellular binding pattern for cyclosporin. Considering the high degree of lipophilicity of cyclosporin, the authors originally assumed that the distribution of cyclosporin in various organs would only depend upon blood flow and not on membrane permeability. They therefore adapted a 'blood flow limited' distribution pattern while assuming a fixed value of 6% for  $CsA_{fu}$ , which is significantly higher than that observed by others for  $CsA_{fu}$  in plasma. Despite the fact that this modelling approach could predict the plasma concentrations reasonably well, it had limited success in predicting the pharmacokinetic parameters in humans. The authors attributed this to the failure to account for variability in the plasma protein binding of cyclosporin. They further hypothesised that a laboratory method that allows measurement of 'tissue membrane available' cyclosporin concentrations may provide a more accurate determination of local tissue distribution.<sup>[23]</sup>

In addition, placental transfer and tissue distribution of cyclosporin has been studied in pregnant New Zealand rabbits.<sup>[24]</sup> At delivery, the whole

foetus accumulated little cyclosporin and the drug was undetectable in either fetal brain or amniotic fluid. The mean cyclosporin concentration in fetal blood was 6% of the mother's blood concentration.<sup>[24]</sup>

#### 4.1.1 Tissue Concentration and Rejection

The concentration of cyclosporin within a transplanted organ has been shown to associate to a greater extent with the incidence of rejection than does  $CsA_T$ , although the unbound concentration in blood ( $CsA_U$ ) was not measured.<sup>[25,26]</sup> The concentration of cyclosporin in kidney biopsy samples was significantly lower in patients who had acute rejection than in those with stable graft function [mean  $\pm$  standard deviation (SD)  $20 \pm 8$  vs  $45 \pm 35$  ng/mg biopsy protein, respectively].  $CsA_T$ , however, was not significantly different between the two groups.<sup>[25]</sup> Others have also found that cyclosporin concentrations in liver biopsy samples were significantly lower ( $1879 \pm 998$  ng/g) in patients with a rejecting hepatic allograft than in those without rejection ( $3493 \pm 936$  ng/g), whereas  $CsA_T$  was not significantly different between the two groups.<sup>[26]</sup> The interrelationship between the concentration of cyclosporin in heart or lung allografts and the incidence of rejection in these organs has yet to be determined.

#### 4.2 Blood Distribution

In blood, cyclosporin is highly bound to blood cells and plasma components. The relative distribution of cyclosporin between blood and plasma is dependent on temperature, drug concentration, haematocrit and plasma lipoproteins. At a cyclosporin concentration of  $500 \mu\text{g/L}$  at  $20^\circ\text{C}$ , 58% of the drug is associated with erythrocytes, 4% with granulocytes, 5% with lymphocytes and the remaining 33% that resides in plasma is extensively bound to plasma lipoproteins.<sup>[27]</sup> The percentage of cyclosporin in plasma exhibits an inverse linear relationship with the haematocrit.<sup>[28,29]</sup> Red blood cell binding of cyclosporin is nonlinear, reaching saturation at  $4000 \mu\text{g/L}$  which is higher than the usual concentrations observed therapeutically. However, the amount of cyclosporin associated

with mononuclear cells and plasma proteins is unsaturable at concentrations even as high as 7000 µg/L.<sup>[30]</sup>

A study of the kinetic uptake of cyclosporin into erythrocytes showed that cyclosporin diffuses passively into red blood cells. Although the kinetics of uptake were temperature-dependent, the capacity of red blood cells for cyclosporin uptake was independent of temperature between 10 and 42°C.<sup>[31]</sup> Passage of cyclosporin into and out of erythrocytes was studied in the presence of a medium containing isotonic phosphate buffer, human serum albumin and plasma obtained from volunteers before and 90 minutes after ingestion of food.<sup>[32]</sup> It was found that the presence of both albumin and plasma in the medium retarded the uptake of cyclosporin into the red blood cells, but did not influence drug efflux out of the cells. The effects of albumin were proportional to the concentration of albumin present, whereas the plasma samples taken after ingestion of food led to a more pronounced decrease in cyclosporin uptake, possibly because of the presence of chylomicrons in plasma.<sup>[32]</sup>

The existence of an erythrocyte binding protein for cyclosporin with a binding constant of  $1.9 \times 10^7$  L/mol has been reported.<sup>[33]</sup> The protein was said to be distinct from haemoglobin, carbonic anhydrase, calmodulin and cytochrome b5 with a molecular weight of 15 000Da, similar to the molecular weight of cyclophilin. The binding constant between cyclosporin and this protein was also decreased with increasing temperature.<sup>[34]</sup>

#### **4.2.1 Temperature and Blood/Plasma Concentration Ratio**

The distribution of cyclosporin in blood is highly temperature dependent. The fraction of the total blood cyclosporin concentration present in plasma increases with temperature. The blood to plasma ratio has been shown to range from 5.1 at 20°C to 2.0 at 37°C.<sup>[35]</sup> At 37°C, 60% of the blood cyclosporin was localised in plasma compared with 46% at room temperature.<sup>[30]</sup> Temperature dependent binding to blood cells may be explained in part by a higher affinity of cyclosporin for plasma

proteins, including lipoproteins, at elevated temperatures<sup>[27]</sup> which, in turn, may be related to the higher degree of hydrophobicity of the cyclosporin molecule at 37°C than at 20°C.<sup>[8]</sup> Considering this temperature dependency, whole blood is presently the preferred matrix for the therapeutic monitoring of total cyclosporin because the total concentrations in blood are not altered by storing the blood at different temperatures. In addition plasma must be separated from whole blood at 37°C for any studies that aim to address the plasma protein binding of cyclosporin.

#### **4.2.2 Distribution of Metabolites in Blood**

The solubility characteristics of cyclosporin metabolites are different from those of cyclosporin. The metabolites are generally less lipophilic and their solubility is less temperature dependent than that of the parent compound (figure 1).<sup>[36]</sup> The tissue distribution of cyclosporin metabolites is also distinct from that of cyclosporin. Metabolites mainly reside in kidney, muscle, liver and spleen, unlike the parent compound, which resides predominantly in adipose tissue.<sup>[22]</sup>

The partitioning of cyclosporin metabolites in blood also differs from that of cyclosporin. The binding of cyclosporin metabolites to blood components is complex and varies with haematocrit and temperature as well as the structure and concentration of metabolites. Monohydroxylated metabolites AM1 and AM9 exhibit greater uptake into erythrocytes than the parent compound, whereas the concentration of the demethylated metabolite, AM4N, is higher in plasma than in erythrocytes.<sup>[36]</sup> In plasma, the affinity of metabolites for plasma proteins is lower than that of cyclosporin, but the binding of cyclosporin metabolites to lipoprotein has yet to be determined.<sup>[37]</sup>

#### **4.3 Plasma Distribution**

Cyclosporin is highly bound to plasma lipoproteins, but there is disagreement in the literature over the extent of association of cyclosporin with plasma proteins. This discordance is probably at least partly attributable to differences in the analytical methods used to determine binding. Lemaire

and Tillement<sup>[27]</sup> found that approximately two-thirds of cyclosporin bound in plasma is associated with plasma lipoproteins while the remaining third is associated with non-lipoprotein plasma proteins, including albumin and globulins. In contrast, another study<sup>[38]</sup> revealed that only between 10 and 15% of cyclosporin was associated with plasma proteins other than lipoproteins. Table I summarises reported percentages of cyclosporin associated with different classes of plasma proteins.

#### 4.3.1 Binding to Plasma Lipoproteins

Lipoproteins are macromolecular complexes of lipid and protein with the major function of transporting lipids through the vascular and extravascular compartments by dissolving compounds in their lipid core. Lipoproteins may also act as carriers for highly lipophilic drugs, vitamins and mediators such as platelet activating factor.<sup>[41]</sup> The general structure of lipoproteins is globular, consisting of a hydrophobic interior and an outer, more polar, region.<sup>[42]</sup> Within the core of the lipoprotein particle are located the more hydrophobic lipids, including esterified cholesterol and triglycerides, whereas the outer region consists of more hydrophilic molecules, including free cholesterol, phospholipid and protein. The protein components of lipoproteins are apolipoproteins, a structurally di-

verse group of proteins with different metabolic and immunological characteristics.<sup>[42]</sup> Based on their sedimentation properties, the lipoproteins can be classified into three major groups; the remaining fraction is regarded as the lipoprotein deficient fraction (LPD) and contains albumin and nonesterified fatty acid complexes. The lipoprotein fractions include very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). However, the existence of an intermediate density lipoprotein (IDL) fraction has also been reported.<sup>[42]</sup>

Cyclosporin widely distributes among different classes of lipoproteins (table I).<sup>[27,43,44]</sup> Distribution studies of cyclosporin in different classes of lipoproteins *in vitro* by ultracentrifugation of human plasma spiked with radiolabelled cyclosporin at 20°C showed that cyclosporin exhibits a higher affinity for HDL than for LDL and VLDL.<sup>[38]</sup> Examination of cyclosporin uptake by a phospholipid vehicle suggests that cyclosporin undergoes non-specific dissolution in the lipophilic core of the lipoprotein complex rather than binding to a specific binding site.<sup>[38]</sup> The proportion of cyclosporin distributed between different lipoprotein fractions is independent of cyclosporin concentration over a

**Table I.** Distribution of cyclosporin in various classes of lipoproteins as determined by density gradient ultracentrifugation

Subjects	Total cyclosporin concentration (µg/L)	Cyclosporin (%) <sup>a</sup>					Reference
		chylomicrons	VLDL	LDL	HDL	other proteins	
<b>Humans</b>							
Healthy							
fasted	100		8.4	31.0	45.7	14.7	38
nonfasted		8.6	6.7	28.4	38.9	11.2	
Hyperlipidaemic		13.0	5.9	30.3	36.9	12.3	
Kidney transplant	100-300		19.0 (7.0)	28.0 (8.0)	35.0 (6.0)	15.0 (5.0)	
Fasting volunteer	500-38000		7.0 (0.3)	35.0 (2.0)	42.0 (6.0)	12.0 (1.0)	39
Heart transplant			10.0 (4.0)	35.0 (9.0)	33.0 (7.0)	22.0 (5.0)	
Liver transplant			10.0 (2.0)	32.0 (3.0)	37.0 (4.0)	23.0 (6.0)	
<b>Rats</b>							
Bile duct ligated	809		12.0 (4.0)	32.0 (7.0)	68.0 (6.0)	6.0 (2.0)	40
Control	822		3.0 (0.4)	21.0 (5.0)	50.0 (7.0)	9.0 (2.0)	

a Percentage of cyclosporin associated with each lipoprotein fraction. Values are means, with standard deviation in parentheses.

**HDL** = high-density lipoprotein; **LDL** = low-density lipoprotein; **VLDL** = very-low-density lipoprotein.

range up to 38 000 µg/L, which is well above the therapeutic range.<sup>[39]</sup>

Based on the lipid content of each lipoprotein, triglyceride-rich lipoproteins (VLDL and IDL) associate with greater amounts of cyclosporin per mg of lipid than do HDL or LDL.<sup>[45]</sup> Cyclosporin also can transfer between lipoproteins *in vitro*. Lipoproteins containing cyclosporin were incubated with cyclosporin-free lipoproteins obtained from another individual [i.e. VLDL-cyclosporin was incubated with LDL, LDL-cyclosporin with VLDL, VLDL-cyclosporin with medium density HDL (HDL-M), and LDL-cyclosporin with HDL-M]. After 10 hours of incubation, under ultracentrifugation forces, they observed in the samples that cyclosporin had transferred from VLDL to LDL but not from LDL to VLDL. Furthermore, HDL-M was an acceptor of cyclosporin from either VLDL or LDL.<sup>[45]</sup>

#### 4.3.2 Pharmacokinetics in Different Lipoprotein Fractions

Pharmacokinetic parameters for cyclosporin in different lipoprotein fractions have been compared following single<sup>[46]</sup> or multiple doses<sup>[47]</sup> of cyclosporin. Cyclosporin 2 mg/kg was administered as a single intravenous infusion for 2 hours to nine candidates for bone marrow transplantation, and the concentration-time profiles of cyclosporin were evaluated in the different lipoprotein fractions.<sup>[46]</sup> After administration of cyclosporin for 1 month post transplant, the pharmacokinetic profiles of intravenously administered cyclosporin 2 mg/kg in lipoprotein fractions were reinvestigated in the same group of patients.<sup>[47]</sup> Results from the single-dose study demonstrated higher clearance and a shorter half-life for cyclosporin in the VLDL fraction than in whole blood, LDL or HDL fractions, possibly because of the lower affinity of cyclosporin for VLDL. After 1 month of cyclosporin therapy, the percentage of cyclosporin in the HDL fraction declined significantly but in the LDL fraction was increased by 68%. After taking into consideration the increases in the concentration of total triglyceride and HDL and LDL cholesterol that occur following transplantation,<sup>[47]</sup> it was con-

cluded that after multiple doses the affinity of cyclosporin for any particular lipoprotein probably remains unchanged. An increase in the relative proportion of different lipoproteins however may be responsible for the observed changes in cyclosporin pharmacokinetics in the lipoprotein fractions.

#### 4.3.3 Effects of Hyperlipidaemia

Hyperlipidaemia alters the distribution of cyclosporin among different lipoprotein fractions. Cyclosporin distribution among different lipoprotein fractions was investigated in bile-duct-ligated rats, an experimental model of cholestasis and hyperlipidaemia.<sup>[40]</sup> Non-ligated rats served as controls. It was found that the percentage of cyclosporin associated with VLDL and LDL was higher in the hyperlipidaemic rats than in the controls (table I).

The percentage of cyclosporin associated with lipoprotein fractions was studied in 12 patients awaiting heart transplantation.<sup>[48]</sup> The patients were divided into three groups having low, intermediate or high total cholesterol concentration. The percentages of cyclosporin associated with VLDL, IDL and LDL correlated positively with the serum concentrations of cholesterol, triglyceride and phospholipid. In contrast, the percentage of cyclosporin associated with the HDL fraction correlated negatively with serum lipid concentrations. The investigators concluded that higher concentrations of plasma lipids result in an increased binding of cyclosporin to the VLDL, IDL and LDL fractions and decreased binding to the HDL fraction.<sup>[48]</sup>

In another study, the influence of dyslipidaemia on distribution of cyclosporin among lipoprotein fractions was examined.<sup>[49]</sup> It was found that the percentage of cyclosporin in VLDL and LDL fractions combined was  $32 \pm 4\%$  in normolipidaemic patients as compared with  $46 \pm 8\%$  in hypercholesterolaemic patients,  $54 \pm 13\%$  in hypertriglyceridaemic patients and  $6 \pm 9\%$  in patients who had elevated levels of both cholesterol and triglyceride. This increase was associated with a significant decrease in the HDL fraction from ap-

proximately 40 to 20% in all the dyslipidaemic patients studied. The proportion of cyclosporin associated with lipoprotein-deficient plasma was similar (approximately 20%) in all groups, but it was significantly reduced to  $13 \pm 5\%$  in patients who had a combination of hypercholesterolaemia and hypertriglyceridaemia.<sup>[49]</sup>

## 5. Methods for Measurement of the Unbound Fraction of Cyclosporin

The hydrophobic nature of the cyclosporin molecule can cause nonspecific binding to surfaces of standard equipment used for this type of analysis. For example, attempts to use standard ultrafiltration devices (Amicon) have yielded nonreproducible results owing to variable levels of nonspecific binding.<sup>[50]</sup> Expensive, purpose-built, precision-engineered apparatus is therefore necessary for determination of  $CsA_{fu}$ . Hence, relatively few published articles have reported measurements of  $CsA_{fu}$  or  $CsA_U$  (table II). A number of methods have been applied in the determination of  $CsA_{fu}$ , including ultracentrifugation, erythrocyte parti-

tioning, equilibrium dialysis and microdialysis methods.

The protein binding of cyclosporin in plasma behaves as a nonsaturable linear process.  $CsA_{fu}$  in plasma is constant over the therapeutic concentration range,<sup>[55]</sup> and  $CsA_U$  may be calculated by multiplying the total plasma concentration ( $CsA_p$ ) by  $CsA_{fu}$  (equation 1):

$$CsA_u = CsA_p \times CsA_{fu}$$

Therefore, for any given total concentration in plasma, the fraction unbound will provide a direct estimation of the unbound concentration.

### 5.1 Ultracentrifugation

Legg and Rowland<sup>[50]</sup> used the ultracentrifugation technique to estimate  $CsA_{fu}$ . Estimates of  $CsA_{fu}$  in kidney transplant patients ranged from 3 to 12%. Their method involved ultracentrifugation of plasma containing [<sup>3</sup>H]cyclosporin at 37°C for 12 to 18 hours under conditions chosen to distribute LDL, HDL and albumin to the bottom of ultracentrifugation tube while VLDL and chylomicrons were distributed to the top section, leaving the in-

**Table II.** Published values of the unbound fraction of cyclosporin in plasma. Values are expressed as means, means  $\pm$  standard deviation or range

Method	Sample	Temperature (°C)	$CsA_{fu}$ (%)	Reference
Ultracentrifugation	Normal plasma + cyclosporin	20	5.0	27
		4	30.0	
Ultracentrifugation	Normal plasma + cyclosporin	37	3.1-4.5	50
		20	6.0-11.2	
		5	16.2-23.2	
Erythrocyte partitioning	Kidney transplant (n = 5)	37	4.2-12.2	51
	Healthy volunteers	37	$17.0 \pm 3.0$	
	Kidney transplant	37	$9.0 \pm 5.0$	
	Liver transplant	37	$8.0 \pm 1.0$	
Equilibrium dialysis	Healthy volunteers (n = 7)	37	1.6	52
			1.0-0-2.4	
Equilibrium dialysis	Kidney transplant (n = 66)	37	1.4	52
			0.5-4.2	
			1.5 $\pm$ 0.4	
Equilibrium dialysis	Heart transplant (n = 64)	37	1.5 $\pm$ 0.4	53
	Heart-lung transplant (n = 8)		$1.47 \pm 0.40$	53
	Lung transplant (n = 13)		$1.38 \pm 0.44$	53
Microdialysis	Healthy volunteers	37	$1.5 \pm 0.3$	54

**$CsA_{fu}$**  = unbound fraction of cyclosporin in plasma (expressed here as percentage).

intermediate levels free from the binding species. The plastic tubes were frozen by slow immersion in liquid nitrogen and cut into sections. The content of each section was placed in liquid scintillant and allowed to thaw before removal of the tube remains. The  $CsA_{fu}$  was calculated from the ratio of the radioisotope activity in the protein-free section to that in the unfractionated plasma.

There are a number of possible artifacts associated with this method that may influence the accuracy of estimates of the fraction unbound. First, binding of cyclosporin to lipoproteins is highly temperature-dependent.<sup>[27]</sup> It is therefore possible that cyclosporin may re-equilibrate between the lipoprotein and protein-free partitions during the freezing process.<sup>[50]</sup> Secondly, although protein contamination in the lipoprotein-free partition was small (less than 300 mg/L), if the contaminant were albumin or lipoproteins, these could bind strongly to cyclosporin. Finally, because of the extensive nonspecific uptake of cyclosporin by plastics, cyclosporin could have penetrated into the polyallomer ultracentrifuge tube surface before or during the 18-hour ultracentrifugation period. Moreover, it may have been partially released into the liquid scintillant later during thawing. Each of these potential artifacts would contribute to an overestimation of  $CsA_{fu}$ .

### 5.2 Erythrocyte Partitioning

The use of erythrocyte partitioning techniques to determine  $CsA_{fu}$ <sup>[51,56]</sup> may have similar limitations to the ultracentrifugation method. In this method, [<sup>3</sup>H]cyclosporin was added to whole blood samples obtained from transplant recipients. After centrifugation at 37°C to isolate plasma, the red blood cells were resuspended and washed three times with phosphate buffer. Finally the amount of radioactivity associated with whole blood, red blood cells, plasma and buffer compartments were measured by the use of liquid scintillation counting. The  $CsA_{fu}$  in plasma was then calculated according to equation 2:

$$\frac{WB}{P} = \frac{RBC}{B} \times \frac{B}{P} [H + (1-H)]$$

where RBC is red blood cell, WB is whole blood, P is plasma, B is buffer, H is the haematocrit and B/P is the  $CsA_{fu}$  in plasma. This method may however overestimate  $CsA_{fu}$  (table II).

### 5.3 Equilibrium Dialysis

Equilibrium dialysis is the established method for estimation of the unbound fraction of highly protein-bound drugs. However, the use of this method for determination of  $CsA_{fu}$  may be subject to artifacts arising from the hydrophobic nature of cyclosporin and its affinity for plastics. The material most commonly used in the manufacturing of the dialysis chambers, polytetrafluoroethylene (Teflon®), is unacceptable because of extensive adsorption of the drug to surfaces of the dialysis chambers.<sup>[57]</sup> Henricsson<sup>[58]</sup> minimised this problem by equilibrating in dialysis chambers made from stainless steel. The method was used to evaluate  $CsA_{fu}$  in healthy volunteers and kidney transplant recipients.<sup>[52]</sup> The mean of estimated values was approximately 1.5%, substantially lower than values determined by ultracentrifugation or erythrocyte partitioning (table II). We were able to reproduce this method and measure  $CsA_{fu}$  in a cohort of 89 heart and lung transplant recipients, and found an average value of 1.5% for  $CsA_{fu}$  in these patients.<sup>[53,59]</sup>

### 5.4 Microdialysis

An *in vitro* microdialysis method for measurement of  $CsA_{fu}$  has been reported.<sup>[54]</sup> This method involves the use of a flow-through loop-type probe made of silica tubing and a probe made of regenerated cellulose tubing with a molecular weight cutoff of 13 kDa. The probe was placed in plasma containing cyclosporin (including radiolabelled material) and perfused with phosphate buffer. The amount of radioactivity associated with the perfusate was then measured. Estimates of  $CsA_{fu}$  by this technique were similar to those obtained using equilibrium dialysis chambers made of stainless steel.<sup>[54]</sup>

### 5.5 Mathematical Models

Owing to the complexity of the analytical methods required for determination of  $CsA_{fu}$ , a number of researchers have attempted to develop mathematical methods to estimate this fraction from other known parameters. In general, the fraction unbound ( $f_u$ ) of a drug is the ratio of its unbound concentration to its total concentration. The fraction unbound can be shown to be dependent upon the association constant for the drug-protein interaction and the concentration of binding proteins according to equation 3:<sup>[60]</sup>

$$f_u = \frac{1}{1 + K \cdot P}$$

where  $K$  is the association constant for the drug-protein interaction and  $P$  is the concentration of binding proteins. Thus, it may be possible to estimate the unbound fraction of drugs when  $K$  and  $P$  are known.

A number of authors have attempted to develop mathematical models for derivation of  $CsA_{fu}$ , with varying degrees of success. These methods are listed in table III. The most common modelling approach was to derive  $CsA_{fu}$  from the concentration of lipoproteins.<sup>[61-63]</sup>

Legg and colleagues<sup>[61]</sup> developed a model to estimate  $CsA_{fu}$  from the concentrations of total cholesterol and triglyceride. An ultracentrifugation method as described in section 5.1 was used

to determine  $CsA_{fu}$  in five kidney transplant recipients in the first 10 days post-transplant. The estimated  $CsA_{fu}$  ranged between 4 and 13%. The proposed model for calculation of  $CsA_{fu}$  was based on binding constants which described binding to two matrices. One matrix contained the triglyceride-rich components of plasma floating at the top of the ultracentrifuge tube and the other the cholesterol-rich section in the bottom section of the tube. This model accounted for only 23% of the variability in  $CsA_{fu}$ . The limited success of this model might be related to the failure to differentiate between LDL-cholesterol and HDL-cholesterol as binding species with different affinities for the drug. Technical difficulties associated with the ultracentrifugation method may also have contributed to the error.

In an attempt to develop models for the estimation of  $CsA_{fu}$ , Urien and colleagues<sup>[62]</sup> studied the interaction of cyclosporin and lipoproteins both *in vitro* and *in vivo* in rats. The binding constants were determined from partitioning between erythrocytes and plasma in the presence of different concentrations of the various lipoproteins. The effects of cyclosporin binding to lipoproteins on brain uptake was investigated by the intracarotid injection technique of Oldendorf,<sup>[62]</sup> in which [<sup>3</sup>H]cyclosporin in solution containing various concentrations of lipoproteins was injected into the rat brain. The amount of cyclosporin extracted

**Table III.** Proposed models for the determination of the unbound fraction of cyclosporin in plasma

Methodology (prediction element)	Subjects	Model or model requirements	Reference
Ultracentrifugation ( $CsA_{fu}$ )	5 kidney transplant recipients	$CsA_{fu} = 1/(1.346 \times TG + 2.815 \times C + 1)$	61
Erythrocyte partitioning method ( $CsA_u$ )	5 kidney transplant recipients	Haematocrit, ratio of cyclosporin concentrations in whole blood to plasma	56
Erythrocyte partitioning followed by rat brain study ( $CsA_{fu}$ )	Rats and plasma from healthy volunteers	$CsA_{fu} = 1/1 + 13 \times HDL-C + 2.7 \times (C - HDL-C)$	62
Physiological modelling of renal clearance ( $CsA_{fu}$ )	Rabbits and heart transplant recipients	Cyclosporin plasma and urine concentrations, urinary flow rate	64
Equilibrium dialysis ( $CsA_{fu}$ )	Plasma from healthy volunteers	$C$ , HDL-C, TG	65
Equilibrium dialysis ( $CsA_{fu}$ )	89 heart or lung transplant recipients	Equation 4	63

**C** = total cholesterol concentration (mmol/L);  **$CsA_{fu}$**  = unbound fraction of cyclosporin in plasma;  **$CsA_u$**  = unbound concentration of cyclosporin in plasma; **HDL-C** = concentration of cholesterol in high-density lipoprotein fraction (mmol/L); **TG** = triglyceride concentration (mmol/L).

from brain was related inversely to the lipoprotein concentration in the injected solution and showed that brain uptake occurred from the free drug pool and possibly from a small part of the originally LDL-bound pool of cyclosporin. The results allowed the calculation of  $CsA_{fu}$  (table III). The predicted range of  $CsA_{fu}$  over the normal range of plasma lipoprotein concentrations was between 5 and 11%.

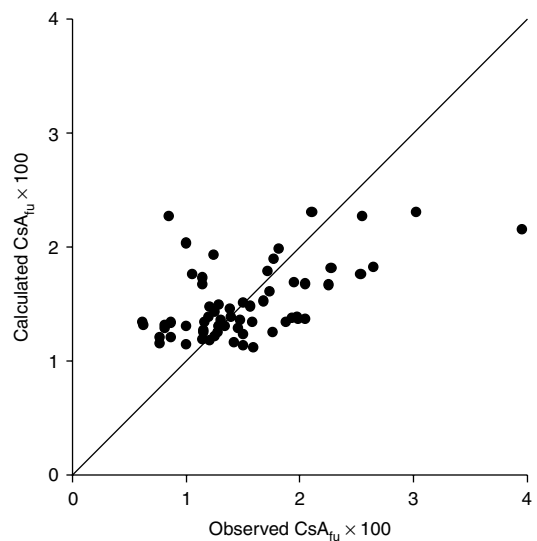
Fois<sup>[65]</sup> used equilibrium dialysis to estimate binding constants between cyclosporin and lipoproteins. Lipoprotein fractions containing HDL, LDL, VLDL and lipoprotein-free plasma were separated from the pooled plasma of normolipidaemic volunteers by using a preparative sequential floating ultracentrifugation technique. The lipoprotein fractions were dialysed against isotonic phosphate buffer.  $CsA_{fu}$  was measured in each lipoprotein fraction and in lipoprotein-deficient plasma by equilibrium dialysis using cells made of chromium plated brass to minimise nonspecific binding. Lipoprotein fractions at various concentrations were spiked with chromatographically purified [<sup>3</sup>H]-cyclosporin and dialysed against phosphate buffer until equilibrium was reached. The binding constants for each class of lipoprotein were obtained by regressing  $CsA_{fu}$  against the concentration of lipoproteins. The model was then re-fitted against the concentration of total cholesterol, HDL-cholesterol and LDL-cholesterol and total triglyceride to allow use of these commonly measured parameters in the model.<sup>[65]</sup>

We have developed another mathematical method for predicting  $CsA_{fu}$  from known parameters<sup>[63]</sup> and, to our knowledge, it is the only method that is derived using information obtained from a large number of transplant recipients. Values of  $CsA_{fu}$  were determined *ex vivo* by equilibrium dialysis of 126 plasma samples obtained from 58 heart and lung transplant recipients. The concentrations of serum lipids and albumin, measured by means of standard enzymatic techniques, were used as concentration markers for lipoprotein and albumin fractions. We randomly divided the patients into two equal sized groups, used the infor-

mation from the first group to derive the model, and from the second group to investigate the predictive performance of the model. A model incorporating the serum concentrations of HDL-cholesterol, LDL cholesterol and albumin, and time after transplantation, gave the best fit. The final model is shown in equation 4 below:

$$CsA_{fu} = \frac{1}{3.39 \times C_{LDL-C} + 6.20 \times C_{HDL-C} + 1.52 \times C_{Alb} + (-0.099 \times t_{TPX}) + 1}$$

where  $C_{LDL-C}$  is the concentration of LDL-cholesterol in mmol/L,  $C_{HDL-C}$  is the concentration of HDL-cholesterol in mmol/L,  $C_{Alb}$  is the concentration of serum albumin in g/L and  $t_{TPX}$  is time post-transplantation in days. The model was tested using criteria suggested for evaluating the predictive performance of models using the second dataset (figure 2). Mean prediction error (ME) was calculated as a measure of bias, and root mean squared error (RMSE) and mean absolute error (MAE) were calculated as measures of precision.<sup>[66]</sup> The ME (95% confidence interval) for percentage unbound ( $CsA_{fu} \times 100$ ) was 0.07% (-0.065, 0.020%),



**Fig. 2.** Relationship between predicted and observed unbound fraction of cyclosporin ( $CsA_{fu}$ ) [reproduced from Akhlaghi et al.,<sup>[63]</sup> with permission].

indicating that the model provided an unbiased estimate of  $CsA_{fit}$ . The values of RMSE and MAE were 0.536% (0.398, 0.645%) and 0.270% (0.266, 0.409%), respectively. Prediction error was normally distributed. Approximately 30% of the prediction errors were less than 10% and less than 5% of the prediction errors were greater than 50%.

## 6. Protein Binding and Cyclosporin Pharmacokinetics

The impact of protein binding on the pharmacokinetics of cyclosporin is not fully understood. There are, however, a large number of mainly anecdotal studies that describe the inter-relationship between the degree of protein binding of cyclosporin and its pharmacokinetics. These studies can be classified into three major groups: (i) investigations that describe the influence of lipoproteins on cyclosporin uptake into various tissues; (ii) investigations that indirectly relate variability in cyclosporin pharmacokinetics to its protein binding; and (iii) reports on the influence of hyperlipidaemia on cyclosporin pharmacokinetics.

### 6.1 Tissue Uptake

Perfusion, or the rate of blood flow to the organ, and permeability, the ability of a drug to pass through capillary membranes, govern uptake of a drug by an organ. In general, capillary membranes in liver and kidney are more permeable to drug movement, whereas passage of drugs into brain is restricted by the blood-brain barrier.<sup>[67]</sup> The proportion of intravenously administered [<sup>3</sup>H]cyclosporin extracted by rat brain has been shown to be 4% compared with 75% hepatic extraction.<sup>[6,68]</sup> Addition of human plasma to the injection solution significantly diminished cyclosporin extraction by rat brain, indicating that increased binding reduces cyclosporin penetration into the brain tissue. This addition did not, however, alter hepatic extraction.<sup>[6]</sup> Addition of whole blood into the injection solution decreased liver extraction as well as brain extraction.<sup>[68]</sup> It was proposed that binding of cyclosporin to blood and plasma components may alter the uptake of cyclosporin into different tis-

ues including brain and kidney, whereas hepatic uptake of cyclosporin is only influenced by red blood cell binding and not plasma protein binding.<sup>[68]</sup> This finding is somewhat inconsistent with other *in vitro* and *ex vivo* studies<sup>[69]</sup> and must be treated with caution.

Lipoprotein binding may decrease the uptake of cyclosporin by hepatocytes.<sup>[69-71]</sup> In the absence of lipoproteins, cyclosporin is rapidly taken up by isolated rat hepatocytes and is highly associated (>80%) with the cells. Addition of lipoproteins (LDL and HDL) to the culture medium significantly reduced the uptake and metabolism of cyclosporin. The decrease in uptake was related to the concentration of LDL present.<sup>[70]</sup> A similar inhibitory effect on the uptake and metabolism of cyclosporin was observed when an isolated perfused rat liver system was used.<sup>[71]</sup> Rifai and co-workers<sup>[69]</sup> investigated the role of LDL on cyclosporin uptake into human hepatoma cell lines (HepG2 and Jurkat). Approximately 80% of [<sup>3</sup>H]-cyclosporin entered the cells within the first hour of incubation. Addition of various concentrations of LDL to the cell culture medium decreased the uptake of cyclosporin in a concentration-dependent manner by up to 60%.<sup>[69]</sup>

Furthermore, by means of an isolated perfused rat kidney model, Strong and Ueda<sup>[72]</sup> have demonstrated that addition of lipoproteins to the perfusion medium inhibits the renal tissue distribution and renal elimination of cyclosporin. The tissue concentration of cyclosporin was  $33.9 \pm 23.4 \mu\text{g/g}$  in the absence of lipoproteins in the perfusion medium, decreasing to  $9.5 \pm 6.1 \mu\text{g/g}$  and  $5.3 \pm 1.8 \mu\text{g/g}$  when 200 mg/dl of LDL or HDL, respectively, was added to the medium.<sup>[72]</sup>

### 6.2 Clearance and Volume of Distribution

The hepatic extraction ratio of cyclosporin in a 4-year old liver transplant recipient with impaired liver function was 0.16. This value was estimated by measuring the concentration of cyclosporin in the portal and hepatic veins during a liver transplant procedure. This extraction ratio places cyclo-

sporin in the rank of low to intermediately cleared drugs.<sup>[73]</sup>

Clearance of cyclosporin varies with age, type of allograft and disease status. In 69 nonobese bone marrow transplant recipients, a significant negative correlation was found between age and both cyclosporin clearance and volume of distribution following an intravenous cyclosporin dose of 2.6 to 3.5 mg/kg.<sup>[74]</sup> In this study, patients were grouped into those who were younger than 10 years, those between 11 and 40 years, and those older than 40 years. Cyclosporin clearance and volume of distribution were compared among the three groups. The mean cyclosporin clearance in the three groups was 4.93, 2.58 and 1.21 L/h/kg (82.2, 43.0 and 20.2 ml/min/kg), respectively, and the volumes of distribution at steady state were 34.4, 20.6 and 4.7 L/kg, respectively. Thus, both cyclosporin clearance and volume of distribution at steady state decreased with age.<sup>[74]</sup> Since cyclosporin is not a drug with high hepatic extraction and its metabolism does not depend on hepatic blood flow (which is reduced in the elderly), the authors speculated that increased cyclosporin binding to the elevated levels of serum lipoproteins found in the elderly might be responsible for this observation.<sup>[74]</sup>

Significant negative correlations have been observed between cyclosporin clearance and the concentrations of VLDL-triglyceride, VLDL-cholesterol and LDL-triglyceride in patients with uraemia prior to kidney transplantation.<sup>[75]</sup> Moreover, the concentration of HDL-cholesterol was highly correlated ( $r > 0.9$ ) with the plasma half-life and volume of distribution of cyclosporin after a single 2 mg/kg intravenous infusion dose of the drug.<sup>[46]</sup> In contrast, Gardier and colleagues<sup>[48]</sup> did not observe a significant correlation between cyclosporin clearance in plasma or whole blood and lipoprotein concentrations. They observed that the volume of distribution at steady state, based on plasma concentration data, was negatively correlated with the concentration of total cholesterol, phospholipid and LDL-cholesterol.

### 6.3 Hyperlipidaemia

Both cyclosporin clearance and volume of distribution at steady state were significantly lower in hyperlipidaemic obese Zucker rats when compared with nonobese Zucker or Sprague-Dawley rats.<sup>[76]</sup> Pharmacokinetic parameters of cyclosporin were also different between normolipidaemic and hyperlipidaemic kidney transplant recipients.<sup>[77]</sup> One hundred and sixty patients were divided into two groups according to their cholesterol levels, with a cut-off value of 5.2 mmol/L. A 24-hour pharmacokinetic study was then carried out in each patient following oral administration of cyclosporin. High performance liquid chromatography (HPLC) was used to measure parent cyclosporin and metabolites in blood and urine. Cyclosporin clearance (total body, apparent and renal) was significantly lower in the hyperlipidaemic patients. In addition, the area under the concentration-time curve, maximum concentration in the dosage interval, dose-adjusted 12-hour trough concentration and half-life of cyclosporin were all greater in the hyperlipidaemic group. The authors concluded that greater binding of cyclosporin in the hyperlipidaemic patients was probably responsible for the decrease in its clearance.<sup>[77]</sup> Consistent with this finding, a significantly lower trough concentration of total cyclosporin has been found in hypocholesterolaemic than in hypercholesterolaemic kidney transplant recipients.<sup>[78]</sup> The total cyclosporin concentrations were 103 and 188 µg/L respectively, despite comparable cyclosporin dosages in the two groups.<sup>[78]</sup> Similarly, the mean cyclosporin clearance was significantly lower in paediatric patients with nephrotic syndrome than in those who received a transplanted kidney (0.43 vs 0.65 L/h/kg). The mean concentration of cholesterol was also significantly higher in these children (11.8 mmol/L) than that in the transplant recipients (5 mmol/L).<sup>[79]</sup>

The total concentration of cyclosporin at steady state is unusually high in patients with type V hyperlipoproteinaemia (familial mixed hypertriglyceridaemia) without signs of cyclosporin toxicity.<sup>[80,81]</sup> In these patients, the concentrations of

VLDL-triglyceride and chylomicrons are also markedly increased. Anecdotally, it has also been reported that a bone marrow transplant recipient who had an extremely high serum triglyceride level (15 mmol/L), despite having a very high trough cyclosporin concentration (1000 µg/L) for the given dose, showed no signs of nephrotoxicity.<sup>[80]</sup> Further investigation revealed that the patient had type V hyperlipoproteinaemia, and that the increase in total cyclosporin concentration paralleled the increase in concentration of triglyceride. In another case report<sup>[81]</sup> of a kidney transplant recipient with type V hyperlipoproteinaemia with very high whole blood and plasma cyclosporin concentrations, a large proportion of cyclosporin in plasma was associated with chylomicrons. The investigators also showed that the uptake of cyclosporin into isolated peripheral blood lymphocytes and kidney tissue *in vitro* occurred to a much lesser extent when plasma from that patient was used in the incubation medium compared with plasma from normolipidaemic individuals. This may also suggest that chylomicron-associated cyclosporin is not biologically available. The investigators proposed that in patients with type V hyperlipoproteinaemia, the cyclosporin dosage should be adjusted with reference to the concentration of cyclosporin in chylomicron-cleared plasma.<sup>[81]</sup>

During the coadministration of the lipid-lowering agent fenofibrate to long-term survivors of heart transplantation, the apparent clearance of cyclosporin was increased from  $1.40 \pm 0.25$  to  $1.52 \pm 0.25$  L/h/kg ( $23.4 \pm 4.0$  to  $25.4 \pm 4.1$  ml/min/kg) before and during administration of fenofibrate, respectively, although the magnitude of this increase was not clinically significant.<sup>[82]</sup> We have also found that administration of the HMG-CoA reductase inhibitor simvastatin to heart transplant recipients resulted in a 29% increase in  $CsA_{fu}$ . The whole blood trough concentration of cyclosporin was also significantly lower after administration of simvastatin (334 vs 235 µg/L before and after simvastatin administration, respectively) although cyclosporin doses were comparable.<sup>[83]</sup> The increase in clearance observed as a result of fenofibr-

ate or simvastatin might have been due to the increase in  $CsA_{fu}$  caused by lower lipoprotein levels. Such findings support the view that cyclosporin binding influences its clearance.

## 7. Protein Binding and Cyclosporin Pharmacodynamics

A number of studies have addressed the role of unbound or lipoprotein-bound cyclosporin for its pharmacological activity. These studies can be catalogued into four major groups: (i) studies that measured  $CsA_{fu}$  and  $CsA_U$  and related these values to clinical outcomes post-transplantation; (ii) *in vitro* investigations that evaluated the effects of protein binding on cyclosporin pharmacodynamic properties; (iii) studies that have related hyperlipidaemia to outcome after transplantation (including graft survival); and (iv) studies that have attempted to establish a correlation between protein binding and cyclosporin toxicity.

### 7.1 Possible Association Between Unbound Cyclosporin and Clinical Outcome

#### 7.1.1 Kidney Transplantation

The only comprehensive study that measured  $CsA_U$  and attempted to relate it to clinical outcome was performed by Lindholm and colleagues in 66 kidney transplant recipients.<sup>[52,55,84]</sup>  $CsA_{fu}$  and  $CsA_T$  in 1806 samples of plasma (median 30.5 samples per patient) were measured by means of equilibrium dialysis and HPLC, respectively, for a period of 6 months after transplantation. The mean  $\pm$  SD of  $CsA_{fu}$  was  $1.37 \pm 0.42\%$ , ranging from 0.50 to 4.20%.  $CsA_{fu}$  decreased significantly during the week prior to acute rejection. At 1 week before acute rejection,  $CsA_{fu}$  was  $1.59 \pm 0.57\%$ , decreasing to  $1.34 \pm 0.30\%$  by the day of acute rejection ( $p < 0.01$ ). Changes in  $CsA_{fu}$  observed during episodes of nephrotoxicity and infection were not statistically significant.  $CsA_T$  in plasma, as measured by HPLC, decreased from  $100 \pm 131$  µg/L to  $58 \pm 59$  µg/L in the period between 1 week before acute rejection to the day of acute rejection, and  $CsA_U$  decreased from  $1.79 \pm 3.23$  µg/L to  $0.73 \pm 0.76$  µg/L over the same period of time.

Lindholm<sup>[55]</sup> concluded that measurement of  $CsA_U$  did not provide sufficient additional information to warrant recommending it as part of routine cyclosporin therapeutic monitoring when taking into consideration the laborious and complicated methodology involved. However, Lindholm proposed that the measurement of  $CsA_{fu}$  needs further investigation in patients with abnormal lipoproteins, such as liver and heart transplant recipients.

### 7.1.2 Heart Transplantation

We have measured  $CsA_{fu}$  in plasma obtained from 89 recipients of heart ( $n = 76$ ), heart-lung ( $n = 5$ ) and lung ( $n = 8$ ) transplant recipients by means of an equilibrium dialysis method. In this study, however,  $CsA_T$  in plasma was not available, and therefore the values of  $CsA_U$  could not be calculated.  $CsA_{fu}$  ranged from 0.52 to 3.94%, very similar to values found in kidney transplant recipients.  $CsA_{fu}$  did not vary according to the type of transplant or the primary diagnosis.  $CsA_{fu}$  was correlated with the serum concentration of total cholesterol and triglyceride and it was significantly lower in hypercholesterolaemic (cholesterol  $>6.5$  mmol/L) than in normocholesterolaemic patients (mean  $\pm$  SD  $1.37 \pm 0.52\%$  versus  $1.60 \pm 0.63\%$ , respectively).  $CsA_{fu}$  was also positively correlated with serum bilirubin and  $\gamma$ -glutamyl transpeptidase concentrations in the first post-operative month, but was not correlated with either serum creatinine or creatinine clearance.<sup>[53]</sup>

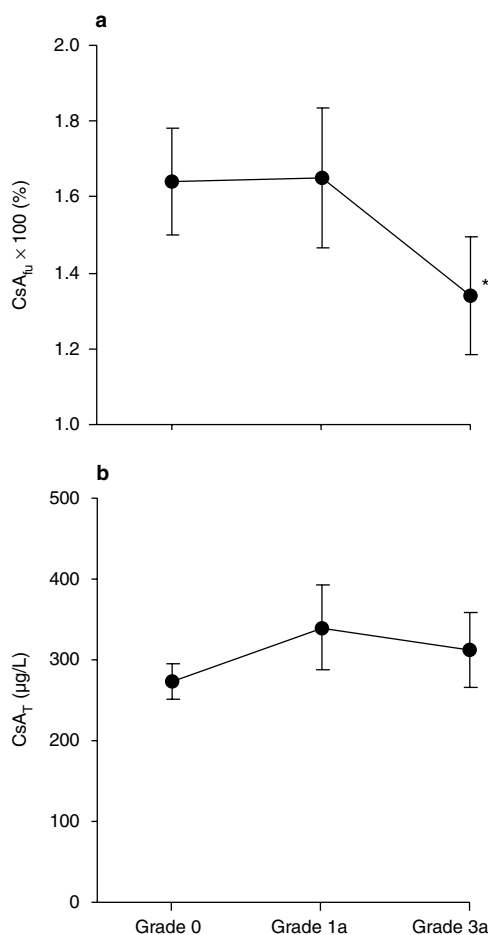
The association between  $CsA_{fu}$  and the incidence of heart allograft rejection was investigated. The diagnosis of heart allograft rejection is based upon the histological examination of endomyocardial biopsies carried out at routine intervals post-transplantation. The International Society for Heart and Lung Transplantation (ISHLT) guidelines for the diagnosis of heart allograft rejection classify the histological findings according to the intensity of leucocyte infiltration and/or myocyte damage.<sup>[85]</sup> The mean  $CsA_{fu}$  was significantly lower at the time of a clinically significant rejection episode (ISHLT grade 3a) than at times when there was either no rejection or when mild rejection (grade 1a) was diagnosed (figure 3). To further

evaluate the association between  $CsA_{fu}$  and rejection, patients were divided into three groups according to whether the mean ( $\pm$  SD)  $CsA_{fu}$  was low ( $1.33 \pm 0.10\%$ ,  $n = 15$ ), intermediate ( $1.60 \pm 0.07\%$ ,  $n = 16$ ) or high ( $1.99 \pm 0.30\%$ ,  $n = 15$ ) during the first post-operative year. In a review of biopsies taken within the first post-operative months, we found that 40.9% of those taken from patients with a low  $CsA_{fu}$  indicated a rejection warranting treatment. This was significantly greater than the proportions of 28.5% in the intermediate  $CsA_{fu}$  group and 32.1% in the high  $CsA_{fu}$  group. In the first month after transplantation, the linearised rate of treated rejection in the low  $CsA_{fu}$  group was  $6.5 \pm 1.7$  episodes of rejections/100 patient-days, and this was significantly higher ( $p < 0.05$ ) than  $3.5 \pm 0.8$  for the intermediate  $CsA_{fu}$  group and  $4.3 \pm 0.9$  for the high  $CsA_{fu}$  group. Moreover, the rejection episodes in the group with low  $CsA_{fu}$  tended to be less responsive to treatment with intravenous methylprednisolone, and a significantly larger proportion of these patients required second-line treatment with total lymphoid irradiation and/or monoclonal antibody therapy.<sup>[59]</sup>

In summary, it can be concluded that variability in  $CsA_{fu}$ , which is related to the concentration of lipoproteins in plasma, is likely to be of importance in relation to the immunosuppressive activity of cyclosporin in heart transplant recipients. However, further clinical investigations are warranted to characterise fully the inter-relationship between  $CsA_T$ ,  $CsA_U$ ,  $CsA_{fu}$  and organ rejection, and to evaluate whether monitoring of  $CsA_U$  would provide a more meaningful index for the immunosuppressive activity of cyclosporin.

### 7.2 Effects of Lipoproteins on Pharmacological Activity *In Vitro*

Phytohaemagglutinin (PHA) is a plant lectin with mitogenic activity that induces T lymphocytes to proliferate. Proliferative response of lymphocytes to PHA or other mitogenic agents *in vitro* is commonly used to evaluate the immunosuppressive activity of cyclosporin. To establish the influence of lipoproteins on the inhibition of PHA-



**Fig. 3.** Association between grade of endomyocardial biopsy and (a) unbound fraction of cyclosporin (CsA<sub>fu</sub>) or (b) total blood cyclosporin concentration (CsA<sub>T</sub>).<sup>[53]</sup> Data are expressed as means with 95% confidence intervals. Different grades of endomyocardial biopsy are defined according to International Society of Heart and Lung Transplantation guidelines;<sup>[85]</sup> **0** = no rejection; **1a** = focal (perivascular or interstitial) infiltrate without necrosis; **3a** = multifocal aggressive infiltrates and/or myocyte damage. \* indicates significantly different,  $p < 0.05$ .

induced mitogenicity, T lymphocytes were cultured in a medium containing cyclosporin (0 to 1000 µg/L) in the presence of either lipoprotein-deficient serum or varying concentrations of VLDL, LDL and HDL.<sup>[86]</sup> The cyclosporin concentration required to produce 50% inhibition in pro-

liferative activity of T lymphocytes (IC<sub>50</sub>) was higher for cyclosporin alone than for cyclosporin in the presence of VLDL or LDL (300 µg/L for cyclosporin alone, 180 and 80 µg/L in the presence of VLDL and LDL, respectively). In further experiments, lipoproteins and cyclosporin were added to the cell culture medium either separately or as a complex of cyclosporin and lipoprotein. The LDL-cyclosporin complex was shown to potentiate the immunosuppressive activity of cyclosporin when compared with the activity following addition of cyclosporin and LDL to the culture medium separately. This activity was significantly less, however, for the VLDL-cyclosporin complex than for VLDL and cyclosporin added separately. As a result of these findings, the authors<sup>[86]</sup> speculated that binding to LDL might potentiate the immunosuppressive activity through an increase in cyclosporin uptake via LDL receptors, whereas binding to VLDL inhibits this process. This *in vitro* observation, however, may be difficult to extrapolate to the *in vivo* situation where, in the presence of physiological concentrations of plasma lipoproteins, the LDL receptors on the surface of lymphocytes are downregulated.<sup>[87]</sup>

The effect of hyperlipidaemia on cyclosporin pharmacodynamics was studied in the obese Zucker rat as a model of morbid obesity and hyperlipidaemia.<sup>[88]</sup> It was found that trough cyclosporin concentrations in whole blood, serum and HDL and LDL fractions were significantly higher in obese rats than in lean animals. To investigate cyclosporin pharmacodynamics, rats were administered different doses of cyclosporin for 9 consecutive days, and the proliferation of T lymphocytes in the presence of PHA was determined for each rat. Although dose-related immunosuppression in the different groups of rats was observed, no significant difference in the suppression of T lymphocyte proliferation was found between obese and nonobese rats.

### 7.3 Effects of Lipoproteins on Clinical Activity

Transplants recipients are prone to hyperlipidaemia.<sup>[89]</sup> These patients develop coronary artery

disease (CAD) at a much faster rate than the normal population, and CAD remains the major cause of death in long-term survivors of transplantation.<sup>[90]</sup> Immunosuppressive therapy with corticosteroids and cyclosporin is considered to be an important contributing factor to the hyperlipidaemia after transplantation.<sup>[88,89,91]</sup> It has, therefore, been speculated that hyperlipidaemia may reduce the biological activity of cyclosporin and so reduce cyclosporin responsiveness.<sup>[92-95]</sup>

In paediatric patients with nephrotic syndrome, the degree of cyclosporin nonresponsiveness was correlated with hypercholesterolaemia.<sup>[92]</sup> To treat these patients, higher doses of cyclosporin were required to achieve a higher total concentration. Despite this high concentration, the concentration of serum creatinine was not increased, indicating that the higher doses did not cause nephrotoxicity in this group of patients.<sup>[92]</sup>

The lipoprotein concentration in 155 kidney transplant recipients was investigated by the use of an ultracentrifugation method.<sup>[93]</sup> Concentrations of cholesterol and triglyceride were measured in each fraction before and at 6 months after transplantation. On both occasions, serum lipid concentrations, including serum total cholesterol and triglyceride, as well as the cholesterol and triglyceride content of VLDL and LDL fractions, were significantly higher in the group of patients who had a greater incidence of acute rejection. The serum creatinine was also higher in those patients, as a result of the increased frequency of rejection episodes. In a more recent study, it was observed that chronic failure of kidney allografts correlated better with hypertriglyceridaemia than with hypercholesterolaemia.<sup>[96]</sup> These investigators subdivided 706 renal transplant recipients with long-term follow-up according to their serum concentrations of triglyceride or cholesterol. The period of graft survival was significantly shorter in hypertriglyceridaemic patients than in those with normal triglyceride. Conversely, cholesterol concentration had no effect on allograft survival.

The effect of the fat content of food on cyclosporin pharmacodynamics was investigated in a

crossover trial of 16 stable kidney transplant recipients after 1-week consumption of a low-fat or high-fat diet.<sup>[97]</sup> No significant differences in cyclosporin pharmacodynamics were found. However, the total cholesterol, triglyceride and LDL-cholesterol concentrations were similar in both arms of the study despite the different diets.

An efficient lipid-lowering therapy is essential to prevent hyperlipidaemia after transplantation.<sup>[98,99]</sup> Administration of HMG-CoA reductase inhibitors (statins), including simvastatin,<sup>[100-102]</sup> pravastatin<sup>[100,103,104]</sup> and fluvastatin,<sup>[101,102]</sup> is effective in counteracting this complication of immunosuppressive drug therapy.<sup>[105]</sup> Statins significantly reduce total cholesterol and LDL-cholesterol concentrations. Considering the high affinity of cyclosporin for lipoproteins, a reduction in plasma lipoprotein concentrations could increase  $CsA_{fu}$ . Indeed, we have already shown that simvastatin can increase  $CsA_{fu}$  by 30% in heart transplant recipients.<sup>[83]</sup>

Administration of pravastatin to transplant recipients may not only control hypercholesterolaemia but may also increase the likelihood of successful organ transplantation.<sup>[100,104,106]</sup> In a randomised placebo-controlled clinical trial involving 97 patients (47 pravastatin vs 50 controls), Kobashigawa and colleagues demonstrated that the administration of pravastatin to heart transplant recipients resulted in less frequent haemodynamically compromising acute rejection episodes (3 vs 14 patients) and improved survival (94% vs 78%).<sup>[104]</sup> Furthermore, administration of pravastatin to kidney transplant recipients resulted in a reduction in the incidence of biopsy-proven rejection episodes (25% vs 58%).<sup>[100]</sup> Indeed, the administration of pravastatin to cyclosporin-treated patients has been associated with a significant reduction in natural killer cell cytotoxicity, suggesting that such drugs may have additive immunosuppressive activity.<sup>[100,104]</sup>

The mechanism of the additive immunosuppressive activity of the HMG-CoA reductase inhibitors is not clear. It has been speculated that pravastatin may either exhibit independent immu-

nosuppressive activity or it may indirectly increase the immunosuppressive activity of cyclosporin through alteration in cyclosporin pharmacokinetics, possibly by reducing lipoprotein binding.<sup>[107,108]</sup> To date, neither of these hypotheses has been verified, although the second mechanism seems more credible as pravastatin does not exhibit immunosuppressive activity in non-transplant patients.<sup>[108]</sup>

#### 7.4 Cholesterol Level and Cyclosporin Toxicity

de Groen and colleagues<sup>[109]</sup> observed that three liver transplant patients who developed severe cyclosporin-induced CNS toxicity after transplantation had very low serum cholesterol concentrations. Further, analysis of retrospective records of 54 liver transplant patients demonstrated that 13 patients who exhibited evidence of CNS toxicity had significantly lower cholesterol levels in the first week after transplantation than did patients without symptoms ( $2.44 \pm 0.10$  vs  $3.43 \pm 0.16$  mmol/L, respectively). They also showed that serum creatinine and blood pressure were higher in the low-cholesterol group.<sup>[110]</sup> These investigators speculated that low cholesterol concentrations in the patients with signs of neurotoxicity may have been associated with higher  $CsA_{fu}$ , and suggested that measurement of  $CsA_{fu}$  could be a better predictor of cyclosporin toxicity.<sup>[110]</sup> Moreover, in 19 hypercholesterolaemic kidney transplant recipients, it was shown that signs of nephrotoxicity,<sup>[78]</sup> including high blood pressure and raised serum creatinine concentration, were absent despite a significantly higher  $CsA_T$  compared with patients with normal plasma cholesterol concentrations.

### 8. Conclusions

Therapeutic monitoring of  $CsA_T$  is widely practised for dosage individualisation in transplant recipients. The main purpose for implementing this practice clinically is to account for interindividual and intra-individual variability in the pharmacokinetics of cyclosporin, and to protect individual transplant recipients against cyclosporin-related adverse effects, including nephrotoxicity and

neurotoxicity. Although the consensus report on cyclosporin monitoring recommends measurement of trough cyclosporin in whole blood using the most specific method available,<sup>[111]</sup> the value of monitoring  $CsA_T$  is subject to considerable debate.<sup>[112]</sup>  $CsA_T$  appears to be a particularly poor guide to the immunosuppressive effects of cyclosporin and for the prediction of rejection or infection.<sup>[113-117]</sup> However, a number of more recent studies suggest that monitoring of cyclosporin concentrations at 2 hours post-dose may provide a better index of its immunosuppressive activity than the trough concentration of cyclosporin.<sup>[118-120]</sup>

Cyclosporin is an exceedingly lipophilic molecule with unusual solubility characteristics in aqueous media.<sup>[6,8]</sup> It is transported throughout the body bound to plasma lipoproteins and other proteins. Lipoproteins are, therefore, serving as a biological vehicle for cyclosporin. To implement its immunosuppressive effects, cyclosporin has to cross the membrane barrier of its target cells, activated T lymphocytes. It has been speculated that the cyclosporin-lipoprotein complex may be taken up into the cells by means of LDL receptors.<sup>[110]</sup> However, this hypothesis does not seem to be valid because, in the presence of physiological concentrations of lipoproteins, the LDL receptors of human lymphocytes are downregulated.<sup>[87]</sup>

It is generally accepted that only unbound drug is able to penetrate through the cell membrane and diffuse into the cytosol. The amount of cyclosporin passing through the cell membrane and reaching cyclophilin would therefore be proportional to the  $CsA_U$  in plasma. The evidence from the clinical and experimental studies summarised in this review corroborates the importance of the protein binding of cyclosporin with respect to its pharmacokinetic and pharmacodynamic characteristics. Studies that have measured cyclosporin concentrations in allograft tissue<sup>[25,26]</sup> or unbound cyclosporin concentrations in plasma<sup>[55,59]</sup> have revealed a closer association between tissue or unbound cyclosporin concentrations and allograft rejection than between total blood cyclosporin

concentrations and rejection. In addition, the reports on the association between hyperlipidaemia and cyclosporin nonresponsiveness<sup>[89,96]</sup> and the evidence that administration of HMG-CoA reductase inhibitors reduces the likelihood of allograft rejection<sup>[100,104]</sup> indirectly add weight to the paradigm that unbound cyclosporin is the pharmacologically active form of the drug.

The reason why  $CsA_U$  correlates more closely with immunosuppressive effects than does  $CsA_T$  is probably related to both the distribution characteristics of cyclosporin and its mode of action in activated T cells. Allorecognition of donor antigens induces a cascade of events that results in the differentiation of null cells into activated T lymphocytes within the lymph nodes and spleen. The activated T lymphocytes are then released into the lymphatic system and, via the blood stream, infiltrate the allograft where they elicit their cytotoxic effects.<sup>[121]</sup> The proportion of cyclosporin available to the lymphocytes residing in the lymphatic organs, peripheral blood and tissues (including the allograft) should, therefore, provide a better indicator of the immunosuppressive effects of cyclosporin. Assuming that only unbound cyclosporin can cross the capillaries, reaching the interstitial fluids of either lymphatic organs or allograft, the amount of cyclosporin associated with the activated lymphocytes would be proportional to  $CsA_U$ , which is a function of  $CsA_{fu}$  and  $CsA_T$ . Indeed, attempts to deliver cyclosporin directly to the lymphatic system have resulted in a significant prolongation of allograft survival in murine skin and rat heart allograft experimental models.<sup>[122,123]</sup>

In clinically stable and pharmacologically unchallenged transplant recipients, measurement of  $CsA_T$  probably does provide a proportionate representation of biologically active  $CsA_U$ . However, there may be many occasions when this relationship breaks down or becomes inconsistent – particularly in clinically unstable patients with dyslipidaemia or impaired metabolic function, or in those receiving polytherapy. Such circumstances prevail in the early postoperative period, at a time when the risk of rejection is greatest. Under such

circumstances, measurement of unbound cyclosporin may provide a closer index of cyclosporin immunosuppressive activity than the total concentration of cyclosporin.

The complexity of the analytical techniques required for the accurate measurement of  $CsA_U$  is the major drawback in recommending the monitoring of this parameter in clinical practice. In the meantime, interpretation of  $CsA_T$  in guiding adjustment of cyclosporin dosage should involve careful consideration of the patient's lipid status. The use of mathematical models to estimate  $CsA_{fu}$ , and hence  $CsA_U$ , can provide supplementary, objectively derived, information to help guide dosage selection in patients with dyslipidaemia.

In summary, the large body of evidence outlined in this review suggests that binding of cyclosporin in blood and plasma is of importance in relation to the immunosuppressive activity of this drug after organ transplantation. Further investigations are needed to verify whether determination of unbound concentrations of cyclosporin improves the clinical management of transplant recipients.

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