REVIEW ARTICLE

Drug Saf 2011; 34 (2): 97-115
0114-5916/11/0002-0097/$49.95/0

Mammalian Target of Rapamycin (mTOR) Inhibitors

Potential Uses and a Review of Haematological Adverse Effects

Sofia Sofroniadou and *David Goldsmith*

Department of Renal Medicine and Transplantation, Guy's and St Thomas' NHS Foundation Trust, King's Health Partners Academic Health Science Centre (AHSC), London, UK

Contents

Abstract

Mammalian target of rapamycin (mTOR) inhibitors (mTORis) constitute a relatively new category of immunosuppressive and antineoplastic drugs. These share a unique mechanism of action that is focused on the inhibition of the mTOR. Their clinical applications have recently expanded significantly to cover a wide spectrum of immune and non-immune-mediated disorders, including, apart from solid organ transplantation, various solid organ and haematological malignancies, rheumatological and auto-immune diseases such as rheumatoid arthritis, systemic lupus erythematosus, fibrotic conditions, e.g. pulmonary and hepatic fibrosis, and even metabolic problems such as diabetes mellitus and obesity. The most challenging and frequent adverse effects of the mTORis are the haematological ones, especially anaemia, leukopenia and thrombocytopenia. A unique characteristic of mTORi-induced anaemia is concurrent marked microcytosis. Recently, mechanisms have been proposed to explain the microcytic appearance of this anaemia; these include globin production defect, erythropoietin resistance, chronic inflammation, dysregulation of cellular iron metabolism and hepcidin-mediated iron homeostasis interference. As the differential diagnosis of microcytic anaemia

includes pure iron deficiency, functional iron deficiency and haemoglobinopathies, characterization of the anaemia requires significant investigation, time and costs. Therefore, understanding of the likely interaction between mTORis and patients is valuable in clinical practice. Moreover, this could expand the drugs' therapeutic applications to other disorders, and suggest novel targets for further research.

Mammalian target of rapamycin (mTOR) inhibitors (mTORis) – rapamycin, also known as sirolimus, and its analogues everolimus, temsirolimus and deforolimus (ridaforilimus) – have received increasing attention recently because of their expanding roles, especially in solid organ transplantation and cancer. The mTORis share a unique mechanism of action inhibiting the mTOR. The mTOR, a 289 kDa serine/threonine kinase, was first identified in the yeast *Saccharomyces cerevisiae* and subsequently in mammalian cells. It is intracellular, with multiple subdomains that have been highly conserved throughout evolution from yeast to mammals.[1,2] It has key roles in cellular protein synthesis and energy balance, control of eukaryotic cell growth and proliferation, including cell-cycle progression, differentiation, protein degradation, apoptosis and angiogenesis.[3-5]

Two distinct mTOR complexes have been recognized: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). The former is composed of the proteins mTOR, regulatory-associated protein of mTOR (raptor), mLST8/GbL and proline-rich AKT substrate 40 kDa (PRAS40),[6-8] whereas the latter is composed of mTOR, rapamycin insensitive companion of mTOR (rictor), protor-1, mLST8/GbL and mammalian stress-activated protein kinase interacting protein 1 (mSIN1).[9,10] The main functions of mTORC1 include protein synthesis and cell-cycle progression, whereas mTORC2 plays an important role in actin cytoskeleton organization and cell survival.[11] Favourable concentrations of nutrients and growth factors stimulate mTORC1, whereas mTORC2 activation is limited to growth factor signalling.[12]

The purpose of this review is to examine the haematological adverse effect profile of this important class of drugs, as there are clear and predictable adverse effects that have been seen from the earliest period of mTORis use, and were commonly the reason for therapy discontinuation. Understanding how and why these haematological adverse effects occur will help clinicians in diverse clinical and research settings avoid unwanted drug toxicity.

1. Mammalian Target of Rapamycin (mTOR) Inhibitors (mTORis): Mechanisms of Action

All of the mTORis exhibit their action via the same mechanism. Following entry into the cytoplasm, the drug binds to the intracellular binding protein FK506-binding protein (FKBP12), an immunophilin, to form a complex. This complex then binds to mTOR at the FKBP12-rapamycin-binding domain, interfering with its ability to signal adequately to its downstream effectors. The inhibition of the mTOR signalling by the FKBP12-rapamycin complex is not fully understood but it may involve a destabilization of the interaction between mTOR and raptor.[4] Although it is known that only mTORC1 is inhibited by rapamycin, recent data suggest that prolonged treatment with rapamycin inhibits mTORC2 signalling by preventing the assembly of the mTOR-rictor complex.[13] The two most important effectors in the signalling pathway downstream of mTOR are S6K1 (ribosomal p70S6 kinase) protein and the initiation of translation factor 4E-BP1 (also known as PHAS-I), both of which control the translation of specific messenger RNAs (mRNAs) and the synthesis of particular proteins (figure 1). The inhibition of mTOR blocks the signal of the two downstream messengers, S6K1 and 4E-BP1, and prevents translation of key mRNAs required for cell-cycle progression from the G1 to S phase.[15,16]

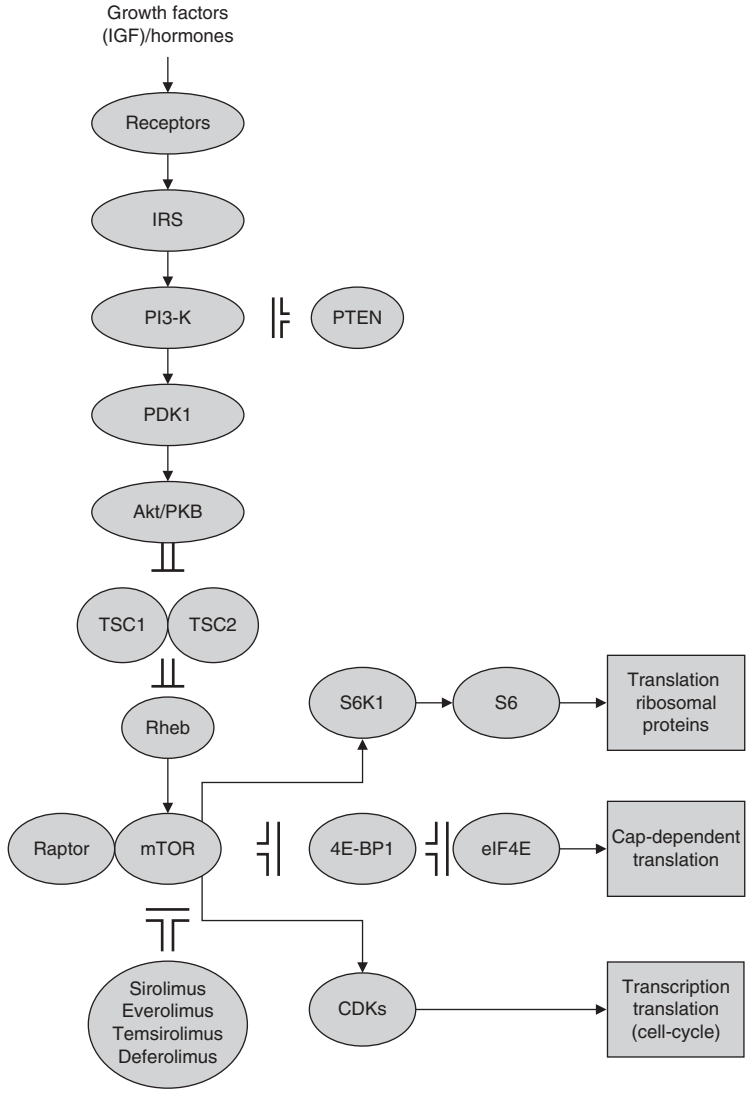

Fig. 1. The mammalian target of rapamycin (mTOR) signalling pathway. Receptors on the cell surface are activated by growth factors such as the insulin-like growth factor (IGF). The receptors signal to phosphatidylinositol 3-kinase (PI3-K), through insulin-receptor substrates (IRS). The PI3-K can be inhibited by PTEN, which is the phosphatase and tensin homologue tumour suppressor. PI3-K, in turn, activates phosphoinositide-dependent protein kinase 1 (PDK1) and the protein kinase B (Akt/PKB). Akt phosphorylates the tuberous sclerosis proteins TSC1–TSC2 complex, which is thought to act as a negative regulator of the small guanosine-5′-triphosphate (GTP)-binding protein Rheb, which may be the direct activator of mTOR. The activated mTOR complex 1 (mTORC1) phosphorylates and activates S6K1, which subsequently phosphorylates the 40s protein S6 of ribosome, resulting in initiation of messenger RNA (mRNA) translation with a 50-terminal oligopyrimidine and encoding of ribosomal proteins and/or elongation factors that lead to stimulation of protein synthesis. mTOR directly phosphorylates and inactivates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), the suppressor of eIF4E. The release of eIF4E from phosphorylated 4E-BP1 is required for cap-dependent translation of mRNAs, including those that are needed for cell-cycle progression and are involved in cell-cycle regulation.[14] **CDKs** = cyclin-dependent kinases; **raptor** = regulatory-associated protein of mTOR.

As a consequence, the mTOR inhibition leads to G1 arrest.[15] It also blocks the interleukin (IL)-2 receptor-dependent and the CD28-dependent signalling pathways, resulting in inhibition of growth factor driven proliferation of haematopoietic and non-haematopoietic cells. Thus, the

mTORis inhibit T- and B-lymphocyte activation, proliferation and differentiation in response to antigenic and cytokine stimulation, and block antibody production, effects that constitute the cornerstone of their immunosuppressive activity.

2. Clinical Applications

The clinical applications of the mTORis cover a wide spectrum of immune-mediated and increasing numbers of non-immune-mediated disorders, while their preclinical development involves other diseases such as diabetes mellitus or obesity (table I). This places this class of drug at the centre of many different disease pathways and clinical and laboratory research.

The first of the mTORis to be discovered was sirolimus (Rapamune®; Wyeth-Ayerst, Collegeville, PA, USA) as part of a screening programme for new antifungal agents, and was named rapamycin because it was isolated from a strain of the fungus *Streptomyces hygroscopicus* in a soil sample from the Rapa Nui, or Easter Island.[68] The inhibitory effect of sirolimus on the immune system was recognized in rats not long after its discovery in 1977.[69] However, the initial studies

Table I. Clinical applications of mammalian target of rapamycin inhibitors (mTORis)

Clinical field	Disease/disorder	mTORis	References (clinical and preclinical data)
Transplantation	Kidney transplantation	Sirolimus, everolimus	17-20
	Pancreas transplantation	Sirolimus, everolimus	21,22
	Liver transplantation	Sirolimus, everolimus	23,24
	Heart/lung transplantation	Sirolimus, everolimus	25-28
	Graft-versus-host disease	Sirolimus	29
Cancer	Kaposi's sarcoma, glioma, advanced solid malignancies, leukaemia, non-Hodgkin's lymphoma	Sirolimus	30-35
	Non-small-cell lung cancer, renal cell carcinoma, colorectal, neuroendocrine tumours, post-transplant lymphoproliferative disorder, haematological malignancies	Everolimus	36-41
	Renal cell carcinoma, metastatic breast, advanced solid tumours, endometrial, myeloid cell leukaemia	Temsirolimus	42-45
	Advanced malignancies, advanced sarcomas,	Deforolimus	46-48
Hamartomas syndromes	TSC1/TSC2, neurofibromatosis, Peutz-Jeghers, Cowden disease	Sirolimus	32,49
Autoimmune disorders	Rheumatoid arthritis	Sirolimus, everolimus	50,51
	Systemic lupus erythematosus	Sirolimus	52
	Autoimmune lymphoproliferative syndrome	Sirolimus	53
	Autoimmune myocarditis	Sirolimus	54
	Autoimmune hepatitis	Sirolimus	55
	Membranous nephropathy	Sirolimus	56
Infectious diseases	HIV	Sirolimus	57
Neurological disorders	Huntington's disease	Sirolimus	58
Metabolic disorders	Type II diabetes mellitus, obesity	Sirolimus	59-61
Proliferative disorders	Autosomal dominant polycystic kidney disease	Sirolimus	62
	Pulmonary fibrosis	Everolimus	63
	Hepatic fibrosis	Sirolimus	64
	Cardiac hypertrophy	Sirolimus	65
	Drug-eluting coronary stents	Sirolimus, everolimus, zotarolimus	66,67

in animal models that demonstrated *in vivo* the immunosuppressive efficacy of sirolimus were conducted a decade later.[70,71] The drug was approved in 1999 by the US FDA as an immunosuppressant for the prevention of acute rejection episodes in combination with ciclosporin (cyclosporine) and corticosteroids in renal transplantation. Later, in 2000 in Europe and 2003 in the US, sirolimus was approved as a maintenance immunosuppressant instead of ciclosporin 3 months following transplantation in low-risk kidney transplant recipients. In pancreas transplantation, sirolimus use, both as *de novo* and as rescue therapy, was successful.[21,22] Although the initial experience of its use in liver transplantation was not encouraging,[72] two studies demonstrated excellent results and suggested the use of sirolimus-based immunosuppression in liver transplantation.[23,24] Sirolimus has also been used with success in heart transplantation,[25,26] while some small studies also showed good outcomes in lung transplant recipients.[27,28]

Despite demonstrating significant antitumour activity,[73,74] sirolimus was not developed as an anticancer drug in the early days due to solubility and stability problems that were later eclipsed by the development of appropriate formulations. Sirolimus was used both as an immunosupppresant and an anticancer drug in renal transplant recipients with Kaposi's sarcoma.[30] It was also evaluated in renal transplant patients as a prophylactic agent for cutaneous and non-cutaneous malignancies.[31] Because of its antiproliferative and anticancer properties, sirolimus was also used in tuberous sclerosis complex patients with angiomyolipomas and lung lymphangioleiomyomatosis,[32] as well as in various malignancies (table I).

The initial solubility and stability issues with sirolimus led to the development of rapamycin analogues with superior pharmacokinetic and biological properties. Three rapamycin analogues or rapalogues [everolimus (RAD001; 40-O-[2-hydroxyethyl]-rapamycin), Novartis Pharma, temsirolimus (cell-cycle inhibitor-779), Wyeth; and deforolimus (AP23573), Ariad Pharmaceuticals] have been synthesized and introduced to clinical trials in different, mainly malignant, disorders. Everolimus is an oral derivative of sirolimus (not a prodrug) that has been approved in Europe as an immunosuppressant for the prevention of renal and cardiac allograft rejection[20] Moreover, it has also demonstrated antitumour activity in phase I studies in solid organ tumours,[36-38] and in phase II studies in low-grade neuroendocrine tumours.[39] Additionally, it has shown effectiveness in post-transplant lymphoproliferative disorders caused by Epstein-Barr virus (EBV) transformation, as well as in advanced haematological malignancies.[40,41]

Temsirolimus is a dihydroxylmethyl propionic acid ester prodrug of sirolimus that is available in both oral and intravenous formulations. It has mainly been developed as an anticancer agent as it showed potent antitumour activity, and has been extensively used in clinical trials in oncology. The drug was approved by the FDA for the treatment of advanced renal cell carcinoma in 2007. Encouraging results have been demonstrated by its utilization in advanced solid tumours[42-44] and in relapsed and refractory mantle cell lymphoma (MCL).[45]

One rapamycin analogue, deforolimus or AP23573, has recently entered phase I and II trials (for advanced malignancies[46,47] and advanced sarcomas[48]), whereas other analogues, such as AP23841 (an analogue that was designed to treat primary bone malignancies or metastatic bone lesions), are in advanced preclinical development.

Apart from the above major clinical applications, the mTORis have also been studied clinically in graft-versus-host disease,[29] in neurodegenerative disorders,[58] as well as in infectious diseases such as HIV.[57] Recently, sirolimus was studied in autoimmune disorders, such as experimental autoimmune myocarditis,[54] post-transplant autoimmune hepatitis,[55] patients with autoimmune lymphoproliferative syndrome[53] and experimental membranous nephropathy.[56] The results from the clinical trials were encouraging in rheumatoid arthritis,[50,51] where sirolimus as well as everolimus were administered, and in systemic lupus erythematosus (SLE) where sirolimus was shown to be a safe and effective treatment for SLE refractory to traditional medications.[52] Further new promising applications of the mTORis

highlighting its antifibrotic effects include autosomal dominant polycystic kidney disease (ADPKD),[62] pulmonary[63] and hepatic fibrosis[64] and cardiac hypertrophy.[65] Additionally, sirolimus-eluting cardiovascular stents have been approved for clinical use since 2003[66,67] and, more recently, two more mTORis (everolimus, zotarolimus) have been used in drug-eluting stents following coronary angioplasty.[75,76]

3. Haematological Adverse Effect Profile

Since the early clinical trials with sirolimus in transplantation in 1996, it emerged that anaemia, leukopenia and thrombocytopenia were amongst the most common adverse effects of the mTORis introduction. These adverse effects were the most likely reason for the intervention with mTORis to be terminated.[17,18,77] Although initially these haematological adverse effects seemed to result from dose- or concentration-limited toxicities in the bone marrow (myelosuppression), the underlying mechanisms leading to anaemia development are likely to be more complex and are still poorly understood.

3.1 Thrombocytopenia and Leukopenia

In one of the early, randomized, double-blinded, phase I studies with sirolimus, the safety and tolerance of multiple doses of the drug were assessed in renal transplant patients under simultaneous ciclosporin and corticosteroid immunosuppression.[77] The study cohort was partitioned into four sirolimus dosage groups: placebo, low dose (1–3 mg/m^2/day), medium dose (5–6 mg/m^2/day) and high dose (7–13 mg/m^2/day). Platelet count demonstrated the clearest evidence of a dose-dependent toxicity during sirolimus treatment, with patients receiving medium- and high-doses of sirolimus exhibiting the greatest decrease in platelet count. The mean baseline value of all patients in the sirolimus group decreased from $[238.7 \pm 62.9] \times 10^3$ cells/mm^3 to 169.1 ± 74.9 cells/mm^3 ($p = 0.0003$) at day 15. A statistically significant, but not convincingly dose related, decrease in the mean white blood cell (WBC) count after sirolimus administration was also recorded.

The same centre subsequently conducted an open-label, single-centre, phase I/II, dose-escalation trial to examine the safety and efficacy of this drug combination[17] Forty mismatched living-donor renal transplant recipients were sequentially assigned to receive escalating initial dosages of sirolimus (0.5–7.0 mg/m^2/day) in addition to courses of prednisone and a concentration-controlled regimen of ciclosporin. The above cohort was compared with a historical cohort of 65 demographically similar recipients treated with the same concentration-controlled regimen of ciclosporin and tapering doses of prednisone. The addition of sirolimus produced haematological effects manifested as decreased WBC ($p < 0.0001$) and platelet ($p = 0.02$–0.0004) counts.

One year later a randomized multicentre study also demonstrated thrombocytopenia and leukopenia under sirolimus-based immunosuppression.[18] Eighty-four first cadaveric renal allograft recipients were randomized to ciclosporin ($n = 42$) or sirolimus ($n = 41$), while they all received corticosteroids and azathioprine. Thrombocytopenia was a striking adverse effect of sirolimus (37% vs 0% mean of 161 vs 261 g/L at month 1; $p < 0.001$), followed by leukopenia (39% vs 14%) These abnormalities improved 2 months after transplantation when the sirolimus target trough level was lowered from 30 to 15 ng/mL.

In another study from 14 European centres, 78 first cadaveric renal allograft recipients were randomized to receive sirolimus ($n = 40$) or ciclosporin ($n = 38$) in an open-label design.[19] All patients received corticosteroids and mycophenolate mofetil 2 g/day. Sirolimus doses were adjusted to achieve steady-state trough levels of approximately 30 ng/mL for 2 months, and 15 ng/mL thereafter. Sirolimus was associated with a higher incidence of thrombocytopenia, with all but one of the cases appearing in the first 3 months of the study, while WBC counts were also significantly lower in the sirolimus group in the same study period.

Moreover, the incidence of sirolimus-induced thrombocytopenia and leukopenia was assessed in a study that compared two cohorts of renal transplant recipients over 1 year: 119 patients received sirolimus in addition to ciclosporin and

prednisolone, and 65 demographically similar, concurrent patients received only ciclosporin and prednisolone.[78] Thrombocytopenia (platelet count $<150 \times 10^3$ cell/mm^3) was usually observed during the first 4 weeks of treatment ($p = 0.004$). Compared with the ciclosporin-prednisolone group, the sirolimus-ciclosporin-prednisolone treatment group showed a 2.2-fold greater overall relative risk of thrombocytopenia. The incidence of leukopenia (WBC count <5000/mm^3) was also highest during the first 4 weeks. The occurrence, but not the severity or the persistence, of both thrombocytopenia and leukopenia correlated significantly with sirolimus trough concentrations ≥16 ng/mL ($p = 0.001$ and 0.0001, respectively). A significant correlation was demonstrated between the two adverse effects ($p = 0.001$). The authors proposed two hypotheses to explain the above adverse effects. The one they favoured less was based on *in vitro* findings that sirolimus potentiates platelet destruction in a time- and dose-dependent manner via agonist-induced aggregation,[79] although the phase III trials failed to show an increased rate of venous thrombosis among patients in the sirolimus arms.[80,81] Their second hypothesis was the inhibition by sirolimus of the signal transduction via the glycoprotein 130 (β). This receptor is shared by a variety of cytokines, including IL-11,[82] granulocyte colony-stimulating factor[83] and erythropoietin, which stimulate the production of platelets, leukocytes and erythrocytes, respectively.

The use of sirolimus or other mTORis in oncology was also accompanied by haematological adverse effects. Sirolimus was assessed in a phase I study of patients with recurrent glioma who received sirolimus combined with gefitinib.[33] Sirolimus was administered either at 10 mg in patients receiving anticonvulsant drugs that are known to induce cytochrome P450 3A or at 5 mg in patients not receiving any anticonvulsant drugs. The mean trough sirolimus level for patients treated with 5 mg/day (i.e. 7.0) was significantly lower than that of patients treated with 10 mg/day (16.7; $p < 0.0001$); however, trough sirolimus levels did not differ based on stratum ($p = 0.136$). Thrombocytopenia was the most common dose-limiting toxicity.

A phase I study with everolimus in 33 patients with advanced solid tumours reported neutropenia as grade III dose-limiting toxicity.[84] Twelve patients (22%) presented grade 1–3 neutropenia (only one patient presented grade 3 that was reported as drug-related toxicity) and 17 (31%) patients presented grade 1–2 thrombocytopenia. Patients were treated with either 20, 50 and 70 mg weekly doses (trough everolimus concentrations = 0.7 ng/mL, 1.0 ng/mL and 4.2 ng/mL, respectively) or 5 and 10 mg daily (trough everolimus concentrations = 8.5 ng/mL and 17.0 ng/mL, respectively).

The use of temsirolimus in a phase II clinical trial in 35 patients with relapsed and refractory MCL in a weekly dose of 250 mg was also accompanied by dose-limiting thrombocytopenia.[45] Thrombocytopenia accompanied by anaemia were also the main drug-related adverse effects of deforolimus in a phase II study of 25 patients with advanced sarcomas.[48]

3.2 Anaemia

The anaemia induced by sirolimus and other mTORis has been the subject of recent interest due to its ubiquity, its characteristics and the proposed mechanisms for its development. The development of anaemia in transplantation (post-transplantation anaemia [PTA]) has started to receive attention over the last decade since the improvement in patient and graft survival highlighted the importance of chronic attention to anaemia and of cardiovascular risk factors control. The WHO definition of anaemia is a haemoglobin level <13 g/dL in adult males and <12 g/dL in adult females. The overall picture from the available evidence is that PTA is common (12–76%). Two large European surveys have shown that 38.6% and 45.6% of the patients, respectively, were anaemic at enrolment.[85,86]

The mTORis are amongst the immunosuppressants associated with PTA; others are mycophenolate mofetil and azathioprine.[87] The first study to demonstrate this correlation was a phase I and II study of 40 mismatched living-donor renal transplant recipients who were assigned to receive escalating initial dosages of sirolimus

(0.5–7.0 mg/m^2/day), in addition to courses of prednisone and ciclosporin.[17] The above cohort was compared with a historical cohort of 65 patients, and a slower recovery of the haemoglobin, particularly after the first month, ($p = 0.05$ to <0.0001) was shown for the sirolimus-treated patients.

One year later, a randomized, open-label, parallel-group, multicentre study demonstrated a 37% incidence of anaemia early post-transplantation in sirolimus-treated patients versus 24% in a ciclosporin-treated group. The anaemia was found to be sirolimus concentration-dependent and was improved or reversed when the target trough concentration was lowered from 30 to 15 ng/mL.[18]

A worldwide phase III study of a sirolimus/ciclosporin regimen in combination with corticosteroids in primary mismatched renal allograft recipients revealed a higher incidence of anaemia compared with the placebo/ciclosporin/corticosteroid group.[88] The anaemia appeared to be dose-dependent, with 27% incidence in the 5 mg/day sirolimus-treated cohort compared with 16% incidence in the sirolimus 2 mg/day cohort, and no statistically significant difference between the last cohort and the placebo group.

A phase III study of 719 primary human leukocyte antigen-mismatched renal allograft recipients who were randomized to receive either sirolimus (2 or 5 mg/day) or azathioprine in combination with ciclosporin and prednisone also revealed a dose-dependent effect of sirolimus on the occurrence of anaemia.[89] The mean haemoglobin values were significantly lower for the 5 mg/day sirolimus group compared with the sirolimus 2 mg/day group and the azathioprine group, although the rates of graft failure and acute rejection were lower at both doses of sirolimus.

The contribution of sirolimus to PTA was also demonstrated in a study where the combination of sirolimus/calcineurin inhibitors/corticosteroids was compared with mycophenolate mofetil/calcineurin inhibitors/corticosteroids in kidney and kidney-pancreas allograft recipients.[90] Sirolimus-treated patients had an almost 2-fold increase in the prevalence of anaemia (57% vs 31%; $p = 0.024$) at 12 months post-transplantation. Sirolimus use remained an independent determinant of anaemia even after multivariate analysis.

In a prospective study in renal transplantation, 165 patients were randomized to receive either sirolimus or tacrolimus as maintenance immunosuppression in combination with mycophenolate mofetil and corticosteroids and antithymocyte globulin as induction therapy.[91] Calcineurin inhibitors were completely avoided. The sirolimus-treated group exhibited a higher incidence of anaemia towards the tacrolimus-treated group (haemoglobin 10.6 ± 1.2 vs 9.9 ± 1.2 g/dL; $p < 0.001$), at 1 month post-transplantation, a difference that disappeared at 1-year post-transplantation.

In a multicentre study from 15 countries that recruited 1645 renal allograft recipients,[92] four treatment groups were compared: mycophenolate mofetil with standard-dose ciclosporin and corticosteroids; mycophenolate mofetil with low-dose ciclosporin, daclizumab and corticosteroids; mycophenolate mofetil with low-dose tacrolimus, daclizumab and corticosteroids; or mycophenolate mofetil with low-dose sirolimus (9 mg/day for 3 days, and 3 mg/day thereafter, adjusted to achieve trough levels of 4–8 ng/mL), daclizumab and corticosteroids. Anaemia was 25% in the low-dose sirolimus group compared with 18.5% in the standard-dose ciclosporin group, 17.4% in the low-dose ciclosporin group and 17.1% in the low-dose tacrolimus group ($p > 0.05$).

Apart from the renal and renal/pancreas transplantation, the use of sirolimus in liver transplantation was also accompanied by anaemia. A study of 70 patients with hepatocellular carcinoma (HCC) who received *de novo* immunosuppression with sirolimus showed anaemia and leukopenia in 56% of patients.[93]

Sirolimus has also been used as an alternative immunosuppressant strategy to allow either dose minimization or complete withdrawal of calcineurin inhibitors in 131 heart and 55 lung transplant patients where a significant drop in haemoglobin levels was recorded after sirolimus introduction.[94]

As anaemia appears to be an adverse effect of mTORis, a randomized, multicentre 36-month trial in *de novo* renal allograft recipients ($n = 588$)

compared everolimus 1.5 or 3 mg/day with mycophenolate mofetil 2 g/day in combination with ciclosporin and corticosteroids.[95] The mean haemoglobin levels were significantly lower in both everolimus groups.

Moreover, a meta-analysis of randomized trials with sirolimus or everolimus in kidney transplantation demonstrated a dose-dependent increase in the risk of anaemia compared with a regimen of calcineurin inhibitor (ciclosporin or tacrolimus) without mTORis (relative risk [RR] 1.67; 95% CI 1.27, 2.20), a finding that was not noted when sirolimus was juxtaposed to the antimetabolites mycophenolate mofetil and azathioprine (RR 0.94; 95% CI 0.72, 0.99).[96]

The use of mTORis in oncology and haematology is also characterized by anaemia as an adverse effect. In a phase I study, the pharmacokinetics and activity of a once-weekly oral sirolimus formulation were tested and the 24 patients with advanced solid tumours recruited received doses of sirolimus tablets 10, 20, 30 or 60 mg once weekly. Anaemia presented in 46% of patients.[34]

Temsirolimus was tested in patients with advanced renal cell carcinoma and no prior systemic therapy who were randomly assigned to one of three groups: (i) interferon subcutaneously up to 18 mega units (MU) three times weekly; (ii) temsirolimus intravenously 25 mg weekly; or (iii) temsirolimus intravenously 15 mg weekly plus interferon subcutaneously 6 MU three times weekly.[97] Among 208 patients, the most common temsirolimus-related grade III–IV toxicity was anaemia in 13% of patients.

3.3 Mechanisms of mTORis-Induced Anaemia and Microcytosis

The anaemia associated with sirolimus and other mTORis is characterized by marked red blood cell (RBC) mean corpuscular volume (MCV) decline and microcytosis. This is unique amongst immunosuppressive drugs.

The above phenomenon was first described in 2003 in lung transplant recipients with chronic allograft nephropathy.[98] The patients were switched from mycophenolate mofetil or azathioprine to sirolimus in combination with ciclosporin. After institution of sirolimus, the MCV declined progressively in all patients ($p = 0.001$), with a mean drop of 10.4 fl (range 2.7–19.9 fl). No correlation between MCV decline and deterioration of renal function was demonstrated.

A further study demonstrating erythrocyte microcytosis following use of sirolimus compared the haematological effects of sirolimus-ciclosporin-corticosteroids versus sirolimus-corticosteroids.[99] The nadir in MCV occurred at 12 months for both regimens, at which time 25.7% of patients receiving sirolimus-ciclosporin-corticosteroids and 43.6% of patients receiving sirolimus-corticosteroids had microcytic RBC indices The sirolimus-induced microcytosis was persistent and to some extent independent of anaemia as it was shown that at 24 months only 8.5% of the patients receiving sirolimus-corticosteroids had severe anaemia, while 29.8%, 27.3%, 35.7% and 58.3% of the patients with normal haemoglobin, mild anaemia, moderate anaemia and severe anaemia, respectively, presented with microcytosis.

Another trial, CONVERT (Conversion from calcineurin inhibitors to sirolimus maintenance therapy in renal allograft recipients), also reported a higher incidence of anaemia in patients who had been converted to sirolimus (37.4% vs 18.7% for the patients who continued on calcineurin inhibitors) and a large decrease in MCV from 90 fl to 82 fl over weeks 2–24 after conversion to sirolimus.[100] The microcytosis was progressive over time, although anaemia stabilized after a period of time.

Sirolimus-induced microcytosis was also demonstrated in ADPKD patients during a randomized controlled clinical trial that assessed the effect of sirolimus on disease progression.[62] A total of 25 patients were randomized to sirolimus 2 mg/day and 25 patients were randomized to no treatment except standard care. The haematological parameters were similar in both groups, except for a mild reduction in the MCV of erythrocytes in patients receiving sirolimus.

It seems that microcytosis is not only related to sirolimus treatment but is an mTORis class adverse effect. Recently, a study assessing anaemia caused by everolimus in 43 renal transplant patients after late introduction of everolimus

revealed a significant decline in MCV, serum iron and transferrin saturation, while haemoglobin and ferritin levels remained stable and C-reactive protein (CRP) levels were elevated.[101]

The effects of everolimus monotherapy on haematological parameters was also assessed in *de novo* liver transplant recipients comparing everolimus versus a ciclosporin immunosuppressive regimen. Anaemia in everolimus patients showed a trend toward the features of a microcytic and hypochromic anaemia, with mean MCV of 79.6 ± 7.5 and 76.4 ± 7.5 at 3 and 6 months, respectively.[102]

Several possible mechanisms have been proposed to explain the mTORis-induced anaemia and microcytosis.

3.3.1 Suppression of Bone Marrow Cells

Although the suppressive effect of mTORis on bone marrow cells seems to be a convincing explanation for the adverse effects of leukopenia and thrombocytopenia, it is probably only a weak pathophysiological parameter for the induced anaemia. Sirolimus and the other mTORis are more likely to interrupt the production of bone marrow elements by inhibiting cytokine signal transduction. Sirolimus blocks cytokine-driven *in vitro* proliferation of various haematopoietic cell lines, possibly via signal transduction inhibition of the glycoprotein 130β chain, which is shared by the IL-2, IL-6 and IL-11 receptor, leading to depletion of erythrocytes, leukocytes and platelets. Moreover, as S6K1 (ribosomal p70S6 kinase) is a downstream form of phosphatidylinositol 3-kinase (PI3-K), which is produced by the binding of erythropoietin to its cytoplasmic receptor, and since S6K1 modifies cellular translation in response to erythropoietin, thrombopoietin and IL-3,[103,104] it seems logical that the inhibition of the mTOR, which controls the S6K1, may lead to diminished replication of the erythroid cell population and anaemia. However, the above hypothesis is supported by limited data.[105]

3.3.2 Globin Production Defect

The development of a marked erythrocyte microcytosis within 6 months post-transplant was analysed in a prospective study that compared sirolimus + mycophenolate mofetil + prednisone with ciclosporin + mycophenolate mofetil + prednisone.[106] Mean haemoglobin levels were similar in the two groups at 6 months (11.2 ± 1.0 vs 11.7 ± 1.4 g/dL, respectively), whereas MCV was significantly lower with the sirolimus-containing regimen (78.5 ± 3.8 vs 88.4 ± 3.4, respectively) [table II]. The study demonstrated marked erythrocyte microcytosis without significant anaemia (most of the patients in the sirolimus group showed mild anaemia with haemoglobin concentration between 10 and 12 g/dL). According to the above study, microcytosis was unlikely to be related to sirolimus trough concentrations that were assessed as mean values at only three study timepoints. Instead, it has been proposed that the microcytosis could be caused by a globin production defect as in thalassaemia, mainly due to high haemoglobin being out of proportion to low MCV. In accordance with the above mechanism, it has recently been suggested that the clinical use of sirolimus may result in early differentiation of erythroid precursors and diminished globin synthesis, contributing to microcytic anaemia.[110]

However, the above hypothesis was not supported by the findings of three separate studies. A study in ten patients with β-thalassaemia assessed the effects of rapamycin on cultures of erythroid progenitors from the peripheral blood of the patients.[111] The results demonstrated that rapamycin induced an increase of fetal haemoglobin (HbF) in cultures from all the β-thalassaemia patients studied and an increase in their overall haemoglobin content/cell. The inducing effect of rapamycin was restricted to c-globin mRNA accumulation, being only minor for b-globin and none for a-globin mRNAs. According to the results, a strong correlation between the increase in HbF and c-globin mRNA content was demonstrated.

A second study in human leukaemia cell line and culture of human erythroid progenitors isolated from healthy donors and patients with β-thalassaemia also showed that rapamycin is a powerful inducer of erythroid differentiation and c-globin mRNA accumulation, in leukaemia

Table II. Studies of sirolimus (SRL)-induced anaemia with reference to mean corpuscular volume (MCV) change

Study	Design	N	Early/ late use	Hb change	MCV change	Ferritin change	Fe change	TSAT change	Mean trough SRL levels (ng/mL)
Kim et al.[106]	Prospective, single-centre SRL + MMF + PDS vs CsA + MMF + PDS	59	Early	Only on day 91	21/26 MCV <79 5/26 MCV <85	19/21 microcytic patients ferritin >10 ng/mL	NE	19/21 microcytic patients TSAT >15%	17.1 ± 7.8 day 7, 20.2 ± 5.8 day 91
Maiorano et al.[107]	Prospective, randomized, single-centre SRL + MMF + PDS vs CsA + MMF + PDS	42	Late (CAN)	Significant reduction	Significant reduction	No change	Reduction	Reduction	Target trough 6–10, mean not mentioned
Thaunat et al.[108]	Retrospective single-centre, switch from CNI to SRL for CAN	46	Late (CAN)	Decline in 40/46 patients	Decline	High levels	Low levels	NE	Target trough 12–20
Augustine et al.[109]	Prospective, single-centre switch from SRL to eMPS	25	Late	Raised post-conversion	Raised post-conversion	No alteration	No alteration	No alteration	9.5 ± 2.3
Friend et al.[99]	Prospective, randomized, multicentre SRL-CsA - corticosteroids vs SRL-corticosteroids	430	Late	Lower with SRL-corticosteroids at 6 mo, dropped more with SRL-CsA-corticosteroids at >2 y	Low in both groups, lower in SRL-corticosteroids	NE	NE	NE	10.6 for the SRL-CsA-corticosteroids group vs 25.1 for the SRL-corticosteroids group month 12
Sanchez Fructuoso et al.[101]	Prospective, single-centre, late introduction of everolimus	43	Late	No change	Significant reduction	Stable	Reduced	Reduced	8.6 ± 5.0 at day 4

CAN = chronic allograft nephropathy; **CNI** = calcineurin inhibitors; **CsA** = ciclosporin; **eMPS** = enteric-coated mycophenolate sodium; **Hb** = haemoglobin; **MMF** = mycophenolate mofetil; **NE** = not estimated; **PDS** = prednisone; **TSAT** = transferrin saturation.

K562 cells as well as in normal human erythroid precursors and erythroid precursor cells from patients with β-thalassaemia.[112] Erythroid differentiation was found to be associated with a sharp increase in the production of c-globin mRNA, whereas these effects were not associated with inhibition of cell growth.

The third study assessed the effects of everolimus on the erythroid differentiation of human leukaemic cells and on cultures of erythroid progenitors from the peripheral blood of patients with β-thalassaemia.[113] Everolimus was demonstrated to be a potent inducer of erythroid differentiation increasing the production of α- and γ-globin mRNAs in the leukaemic cells and of γ-globin mRNA in the patients with β-thalassaemia.

3.3.3 Erythropoietin Resistance Hypothesis

In a study by Augustine et al.,[109] 25 stable renal transplant patients receiving maintenance tacrolimus and sirolimus therapy were enrolled in a prospective trial with conversion from sirolimus to enteric-coated mycophenolate sodium. It was shown that conversion from sirolimus to enteric-coated mycophenolate sodium was accompanied by significant increase in haemoglobin and decrease in erythropoietin resistance, as assessed by the endogenous erythropoietin : haemoglobin ratio (table II). Mean MCV levels rose from 84.5 ± 3.6 fl pre-conversion to 89.5 ± 4.9 fl at 6 months ($p = 0.001$). No effect of sirolimus on ferritin levels or renal function was demonstrated.

3.3.4 Chronic Inflammatory State Hypothesis

In another study, by Thaunat et al.,[108] 48 renal transplant patients who had been switched from calcineurin inhibitors to sirolimus for biopsy-proven chronic allograft nephropathy were retrospectively analysed. A clinical pattern of microcytic anaemia, together with low serum iron, high ferritinaemia and high fibrinogen and CRP levels, were described. IL-6 and tumour necrosis factor (TNF)-α levels at the nadir of anaemia were significantly higher than before sirolimus introduction and after its withdrawal, whereas an absence of any increase in IL-10 was recorded. It was therefore postulated that this was the effect of sirolimus inhibition of monocyte IL-10 production via the p70 S6-kinase pathway, leading to a defective IL-10-dependent inflammatory autoregulation and a chronic inflammatory state.

3.3.5 Dysregulation of Cellular Iron Metabolism Hypothesis

A prospective study by Maiorano et al.[107] compared a group of 14 patients with CAN randomized to 40% ciclosporin reduction with a second group of 28 patients randomized to immediate ciclosporin withdrawal and sirolimus commencement. While ferritin levels remained stable after sirolimus introduction, iron serum levels, together with haemoglobin, transferrin saturation, MCV and mean corpuscular haemoglobin, were markedly reduced in the patients assigned to sirolimus switch, whereas no significant change was observed regarding the above parameters in the patients who continued receiving ciclosporin (table II). Serum hepcidin levels were similar in the two groups after randomization, whereas serum levels of hepcidin are usually markedly increased with chronic inflammation-related anaemia. The hypothesis that sirolimus affects a downstream mechanism directly influencing ferroportin expression or function, and consequently cellular iron metabolism, was therefore made.

3.3.6 Hepcidin-Mediated Iron Homeostasis Interference: Reversal of Microcytosis with Intravenous Iron

In a retrospective analysis of 93 renal and renal/pancreas transplant patients who were treated with sirolimus for >3 months, we showed that sirolimus induced marked erythrocyte microcytosis from the first month of introduction and throughout the whole study period of 24 months.[114] The microcytosis was not accompanied by anaemia as haemoglobin improved, probably due to an increase in erythropoiesis stimulating agents and to estimated glomerular filtration rate improvement. An association between dMCV (MCV change on sirolimus) and sirolimus levels was shown at 3, 6, 12 and 24 months post-sirolimus ($p = 0.015$, $p = 0.037$, $p = 0.002$ and

p = 0.001, respectively). The multiple linear regression analysis demonstrated that intravenous iron administration was an independent predictor of dMCV at 12 and 24 months on sirolimus (p = 0.031 and p = 0.048). All six patients who were treated with intravenous iron only for putative iron deficiency presented an RBC MCV increase and restoration of microcytosis, an event that did not occur with the three patients who took oral iron only. We support a functional iron-deficient hypothesis on the basis of microcytosis accompanied by increased red cell volume distribution width (RDW), unchanged and almost normal ferritin levels, stable levels of CRP and the lack of response to oral iron. We also provided an indirect clue for a key role of hepcidin based on the marked impact of intravenous iron supplementation on MCV, and the marked 'rebound' in plasma ferritin levels in those patients in whom sirolimus was discontinued.[114]

Although the above proposed mechanisms are presented separately, some of them are related and probably have to be analysed under the spectrum of their interaction (figure 2).

Hepcidin, a 25-amino acid peptide converted from prohepcidin, which is produced by the hepatocytes, is a key regulator of the extracellular iron homeostasis.[115,116] It acts by binding to ferroportin, the sole known cellular iron efflux channel,[117] inducing its internalization and degradation in the cytoplasm.[118] Thus, hepcidin regulates iron efflux into plasma, either from enterocytes that absorb dietary iron, macrophages that recycle iron, or hepatocytes that store iron. The upregulation of hepcidin results in inhibition of intestinal iron absorption and iron release from macrophages and hepatocytes.[119] Hepcidin expression is upregulated by inflammation and elevated iron concentration and downregulated by erythropoietic activity and hypoxia.[120-123] As inflammation increases, cytokine stimulation of hepcidin production and high hepcidin levels limit iron availability for erythropoiesis (as a result of hepcidin-induced loss of ferroportin from macrophages). It thus seems that hepcidin plays a crucial role in the anaemia of inflammation and erythropoietin resistance as well as in the iron-resistant, iron deficiency anaemia. Apart from the above interaction, inflammation may also impact on erythropoiesis through a cytokine (IL-1, TNF, IL-6, interferon)-dependent, hepcidin-independent pathway.[124,125]

Although the detection of hepcidin has been hampered by technical difficulties, a few methods for hepcidin quantification in serum and urine have been proposed. While the major form of

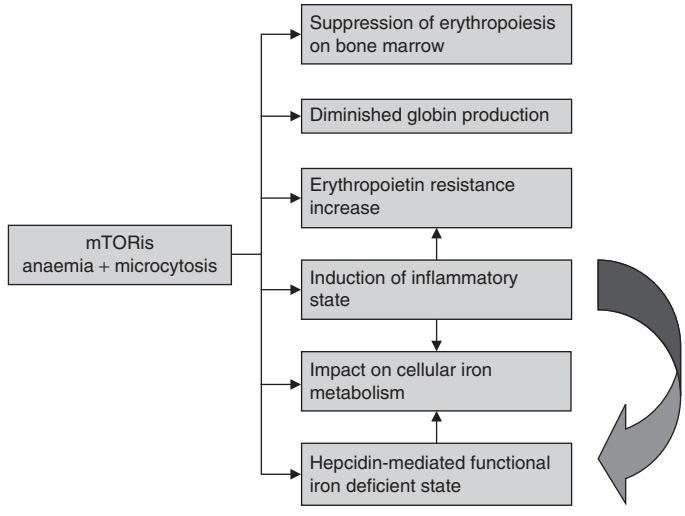

Fig. 2. Possible mechanisms of mammalian target of rapamycin inhibitor (mTORi)-induced anaemia and microcytosis and their interactions.

hepcidin that can be detected in urine and serum is hepcidin-25 and hepcidin-20,[126] prohepcidin (a 60 amino acid peptide) is easily detectable in serum. However, the diagnostic utility of serum prohepcidin determination is disputed since prohepcidin is a processing intermediate lacking a significant biological role in iron metabolism.[127,128] This was exactly the main disagreement in the study published by Maiorano et al.[107] where the enzyme-linked immunosorbent assay (ELISA) that was used did not measure mature hepcidin but prohepcidin, which demonstrates a poor correlation with body iron stores.[129]

In a study by Park et al.,[126] human urinary hepcidin-25 and hepcidin-20 was measured using cation exchange chromatography and reverse-phase high-performance liquid chromatography (HPLC). Subsequently, other assays for urinary hepcidin determination were reported.[130,131] More recently, studies using methods for serum hepcidin detection and quantification-like, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS)[132] liquid chromatography (liquid chromatography-tandem mass spectrometry method [LC-MS/MS])[133] or isotope dilution micro-HPLC-tandem MS[134,135] were published. Furthermore, in the last 2 years, ELISA was used in studies reporting promising results regarding accuracy.[136-138] However, specific studies designed to evaluate the reliability and reproducibility of all the assays for hepcidin determination, either in serum or urine, are warranted in order to establish universal standards and widely, commercially available assays.

4. Conclusions

The mTORis are novel immunomodulatory agents used increasingly frequently in organ transplantation and cancer. Their clinical applications have expanded recently to cover a very wide range of immunological and non-immunological disorders. Haematological adverse effects from this class of drug are important and frequent, and are seen across all of the patient groups and in all the clinical contexts, and can constitute an important reason for treatment withdrawal. Of course, patients with chronic kidney disease are themselves more prone to anaemia, and this is a factor in this particular context. The haematological adverse effects that are demonstrated by the mTORis are quite distinctive from many of the other immunosuppressants. There are some particularly interesting issues in relation to the actions of these agents on bone marrow and on the separate cell lines therefrom. Thrombocytopenia and leukopenia seem mainly to occur on the basis of a direct effect on the bone marrow cells. On the other hand, anaemia, which is common, cannot be explained solely on the above basis as potentially several other proposed mechanisms seem also to be involved. Studying and understanding the pathophysiology and underlying mechanisms of mTORis-induced anaemia could be important in discriminating this anaemia from anaemia of pure iron deficiency or anaemia of chronic disease, and could also reveal useful clues about how these complex systems interact in health and in disease.

Anaemia postrenal transplantation is complex and multifactorial, but is often microcytic. This low MCV is most commonly due to iron deficiency, usually absolute, more rarely, relative. As the clinically important differential in a transplant patient with microcytosis would be iron deficiency due to chronic blood loss (requiring extensive and expensive investigations), haemoglobinopathy, or functional iron deficiency, understanding the characteristics of sirolimus-induced RBC microcytosis/anaemia could thus spare patients unnecessary investigation and intervention. It could also help to generate novel hypotheses for the mechanisms of action of these drugs in humans, their potential uses (e.g. sickle-cell disease and β-thalassaemia) and possibly even lead to novel targets for future drug design.

Acknowledgements

No sources of funding were used to prepare this manuscript. David Goldsmith has received consultancy fees from Wyeth and Novartis, and honoraria from Novartis. Sofia Sofroniadou has no conflicts of interest to declare.

References

1. Gingras AC, Raught B, Sonenberg N. Regulation of translation initiation by FRAP/mTOR. Genes Dev 2001; 15: 807-26
2. Jacinto E, Hall MN. Tor signalling in bugs, brain and brawn. Nat Rev Mol Cell Biol 2003; 4: 117-26
3. Avruch JK, Hara Y, Lin X, et al. Insulin and amino-acid regulation of mTOR signaling and kinase activity through the Rheb GTPase. Oncogene 2006; 25 (48): 6361-72
4. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell 2006; 124: 471-84
5. Yang Q, Guan K-L. Expanding mTOR signaling. Cell Res 2007; 17: 666-81
6. Kim DH, Sarbassov DD, Ali SM, et al. MTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 2002; 110: 163-75
7. Loewith R, Jacinto E, Wullschleger S, et al. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell 2002; 10: 457-68
8. Sancak Y, Thoreen CC, Peterson TR, et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell 2007; 25: 903-15
9. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 2004; 14: 1296-302
10. Pearce LR, Huang X, Boudeau J, et al. Identification of Protor as a novel Rictor-binding component of mTOR complex-2. Biochem J 2007; 405: 513-22
11. Sarbassov DD, Ali SM, Sabatini DM. Growing roles for the mTOR pathway. Curr Opin Cell Biol 2005; 17: 596-603
12. Morelon E, Mamzer-Breneel MF, Peraldi MN, et al. Sirolimus: a new promising immunosuppressive drug. Towards a rationale for its use in renal transplantation. Nephrol Dial Transplant 2001; 16: 18-20
13. Sarbassov DD, Ali SM, Sengupta S, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell 2006; 22: 159-68
14. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov 2006; 5: 671-88
15. Hidalgo M, Rowinsky EK. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene 2000; 27: 6680-6
16. Rowinsky EK. Targeting the molecular target of rapamycin (mTOR). Curr Opin Oncol 2004; 16: 564-75
17. Kahan BD, Podbielski J, Napoli KL, et al. Immunosuppressive effects and safety of a sirolimus/cyclosporine combination regimen for renal transplantation. Transplantation 1998; 66: 1040-6
18. Groth CG, Bäckman L, Morales JM, et al. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. Transplantation 1999; 67: 1036-42
19. Kreis H, Cisterne JM, Land W, et al. Sirolimus in association with mycophenolate mofetil induction for the prevention of acute graft rejection in renal allograft recipients. Transplantation 2000; 69: 1252-60
20. Chapman TM, Perry CM. Everolimus. Drugs 2004; 64: 861-73
21. Vincenti F, Stock P. De novo use of sirolimus in immunosuppression regimens in kidney and kidney-pancreas transplantation at the University of California, San Francisco. Transplant Proc 2003; 35 (3 Suppl.): 18-6S
22. Rogers J, Ashcraft EE, Emovon OE, et al. Long-term outcome of sirolimus rescue in kidney-pancreas transplantation. Transplantation 2004; 78: 619-22
23. McAlister VC, Peltekian KM, Malatjalian DA, et al. Orthotopic liver transplantation using low-dose tacrolimus and sirolimus. Liver Transpl 2001; 7: 701-8
24. Trotter JF, Wachs M, Bak T, et al. Liver transplantation using sirolimus and minimal corticosteroids (3-day taper). Liver Transpl 2001; 7: 343-51
25. Keogh AM, the Sirolimus Cardiac Transplant Trial Group. Sirolimus immunotherapy reduces the rates of cardiac allograft rejection: 6-month results from a phase 2, open-label study [abstract]. Am J Transplant 2002; 2 Suppl. 3: S246
26. Groetzner J, Meiser B, Landehr P, et al. Mycophenolate mofetil and sirolimus as calcineurin inhibitor-free immunosuppression for late cardiac-transplant recipients with chronic renal failure. Transplantation 2004; 77: 568-74
27. Villanueva J, Boukhamseen A, Bhorade SM. Successful use in lung transplantation of an immunosuppressive regimen aimed at reducing target blood levels of sirolimus and tacrolimus. Heart Lung Transplant 2005; 24: 421-5
28. Venuta F, De Giacomo T, Rendina EA, et al. Recovery of renal impairment with sirolimus after lung transplantation. Ann Thorac Surg 2004; 78: 1940-3
29. Benito AI, Furlong T, Martin PJ, et al. Sirolimus (rapamycin) for the treatment of steroid-refractory acute graft-versus-host disease. Transplantation 2001; 72: 1924-9
30. Stallone G, Schena A, Infante B, et al. Sirolimus for Kaposi's sarcoma in renal transplant recipients. N Engl J Med 2005; 352: 1317-23
31. Campistol JM, Eris J, Oberbauer R, et al. Sirolimus therapy after early cyclosporine withdrawal reduces the risk for cancer in adult renal transplantation. J Am Soc Nephrol 2006; 17: 581-9
32. Bissler JJ, McCormack FX, Young LR, et al. Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. N Engl J Med 2008; 358: 140-51
33. Reardon DA, Quinn JA, Vredenburgh JJ, et al. Phase 1 trial of gefitinib plus sirolimus in adults with recurrent malignant glioma. Clin Cancer Res 2006; 12: 860-8
34. Ratain MJ, Napoli KL, Moshier KK, et al. A phase 1b study of oral rapamycin (sirolimus) in patients with advanced malignancies. ASCO Annu Meet Proc 2007; 25 Suppl. 18 (Pt I): 3510
35. Chen W, Grammatikakis I, Li J, et al. Inhibition of Akt/mTOR signaling pathway induces cell cycle arrest and apoptosis in acute myelogenous leukaemia [abstract]. Blood (ASH Annual Meeting Abstracts) 2005; 106: 2355
36. Milton DT, Riely GJ, Azzoli CG, et al. Phase 1 trial of everolimus and gefitinib in patients with advanced non-small-cell lung cancer. Cancer 2007; 110: 599-605
37. Bendell JC, George D, Nixon A, et al. Results of a phase I study of bevacizumab (BV), everolimus (EV), and erlotinib

(E) in patients with advanced solid tumors. ASCO Annu Meet Proc 2007; 25 Suppl. 18 (Pt 1): 3548

38. Speca JC, Mears AL, Creel PA, et al. Phase I study of PTK787/ZK222584 (PTK/ZK) and RAD001 for patients with advanced solid tumors and dose expansion in renal cell carcinoma patients. ASCO Annu Meet Proc 2007; 25 Suppl. 18 (Pt I): 5039
39. Yao JC, Phan A, Chang DZ, et al. Phase II study of RAD001 (everolimus) and depot octreotide (sandostatin LAR) in advanced low grade neuroendocrine carcinoma (LGNET). ASCO Ann Meet Proc 2007; 25 Suppl. 18 (Pt 1): 4503
40. Majewski M, Korecka M, Kossev P, et al. The immunosuppressive macrolide RAD inhibits growth of human Epstein-Barr virus-transformed B lymphocytes in vitro and in vivo: a potential approach to prevention and treatment of posttransplant lymphoproliferative disorders. Proc Natl Acad Sci U S A 2000; 97: 4285-90
41. Yee KWL, Wierda W, O'Brien S, et al. A phase I/II study of the oral mTOR inhibitor RAD001 in patients with advanced hematologic malignancies [abstract]. Blood (ASH Annual Meeting Abstracts) 2004; 104: 4818
42. Hidalgo M, Buckner JC, Erlichman C, et al. A phase I and pharmacokinetic study of temsirolimus (CCI-779) administered intravenously daily for 5 days every 2 weeks to patients with advanced cancer. Clin Cancer Res 2006; 12: 5755-63
43. Carpenter JT, Roche H, Campone M, et al. Randomized 3-arm, phase 2 study of temsirolimus (CCI-779) in combination with letrozole in postmenopausal women with locally advanced or metastatic breast cancer. ASCO Annu Meet Proc 2005; 23 Suppl. 16 (Pt 1 of II): 564
44. Oza AM, Elit L, Biagi J, et al. Molecular correlates associated with a phase II study of temsirolimus (CCI-779) in patients with metastatic or recurrent endometrial cancer–NCIC IND 160. ASCO Annu Meet Proc 2006; 24 Suppl. 18 (Pt I): 3003
45. Witzig TE, Geyer SM, Ghobrial I, et al. Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. J Clin Oncol 2005; 23: 5347-56
46. Mita MM, Mita AC, Chu QS, et al. Phase I trial of the novel mammalian target of rapamycin inhibitor deforolimus (AP23573; MK-8669) administered intravenously daily for 5 days every 2 weeks to patients with advanced malignancies. J Clin Oncol 2008; 26: 361-7
47. Desai AA, Janisch L, Berk LR, et al. A phase I trial of a novel mTOR inhibitor AP23573 administered weekly (wkly) in patients (pts) with refractory or advanced malignancies: a pharmacokinetic (PK) and pharmacodynamic (PD) analysis. ASCO Annu Meet Proc 2004; 22 Suppl. 14: 3150
48. Chawla SP, Tolcher AW, Staddon AP, et al. Updated results of a phase II trial of AP23573, a novel mTOR inhibitor, in patients (pts) with advanced soft tissue or bone sarcomas. ASCO Annu Meet Proc 2006; 24 Suppl. 18 (Pt I): 9505
49. Lee L, Sudentas P, Donohue B, et al. Efficacy of a rapamycin analog (CCI-779) and IFN-gamma in tuberous sclerosis mouse models. Genes Chromosomes Cancer 2005; 42: 213-27
50. Foroncewicz B, Mucha K, Paczek L, et al. Efficacy of rapamycin in patient with juvenile rheumatoid arthritis. Transpl Int 2005; 18: 366-8
51. Bruyn GA, Tate G, Caeiro F, et al. Everolimus in patients with rheumatoid arthritis receiving concomitant methotrexate: a 3 month double-blind, randomized, placebo-controlled parallel-group, proof-of-concept study. Ann Rheum Dis 2008; 67: 1090-5
52. Fernandez D, Bonilla E, Mirza N, et al. Rapamycin reduces activity and normalizes T cell activation-induced calcium fluxing in patients with systemic lupus erythematosus. Arthritis Rheum 2006; 54: 2983-8
53. Teachey DT, Greiner R, Seif A, et al. Treatment with sirolimus results in complete response in patients with autoimmune lymphoproliferative syndrome. Br J Haematol 2009; 145: 101-6
54. Maeda K, Shioi T, Kosugi R, et al. Rapamycin ameliorates experimental autoimmune myocarditis. Int Heart J 2005; 46: 513-30
55. Kerkar N, Dugan C, Rumbo C, et al. Rapamycin successfully treats post-transplant autoimmune hepatitis. Am J Transplant 2005; 5: 1085-9
56. Bonegio RG, Fuhro R, Wang Z, et al. Rapamycin ameliorates proteinuria-associated tubulointerstitial inflammation and fibrosis in experimental membranous nephropathy. J Am Soc Nephrol 2005; 16: 2063-72
57. Heredia A, Amoroso A, Davis C, et al. Rapamycin causes down-regulation of CCR5 and accumulation of anti-HIV beta-chemokines: an approach to suppress R5 strains of HIV-1. Proc Natl Acad Sci U S A 2003; 100: 10411-6
58. Sarkar S, Krishna G, Imarisio S, et al. A rational mechanism for combination treatment of Huntington's disease using lithium and repamycin. Hum Mol Genet 2008; 17: 170-8
59. Mori H, Inoki K, Masutani K, et al. The mTOR pathway is highly activated in diabetic nephropathy and rapamycin has a strong therapeutic potential. Biochem Biophys Res Commun 2009; 384: 471-5
60. Um SH, Frigerio F, Watanabe M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature 2004; 431: 200-5
61. Wang C-Y, Kim H-H, Hiroi Y, et al. Obesity increases vascular senescence and susceptibility to ischemic injury through chronic activation of Akt and mTOR. Sci Signal 2009; 2 (62): ra11
62. Serra AL, Kistler AD, Poster D, et al. Safety and tolerability of sirolimus treatment in patients with autosomal dominant polycystic kidney disease. Nephrol Dial Transplant 2009; 24: 3334-42
63. Simler NR, Howell DC, Marshall RP, et al. The rapamycin analogue SDZ RAD attenuates bleomycin-induced pulmonary fibrosis in rats. Eur Respir J 2002; 19: 1124-7
64. Biecker E, De Gottardi A, Neef M, et al. Long-term treatment of bile duct-ligated rats with rapamycin (sirolimus) significantly attenuates liver fibrosis: analysis of the underlying mechanisms. J Pharmacol Exp Ther 2005; 313: 952-61
65. Shioi T, McMullen JR, Tarnavski O, et al. Rapamycin attenuates load-induced cardiac hypertrophy in mice. Circulation 2003; 107: 1664-70
66. Moses JW, Leon MB, Popma JJ, et al. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. N Engl J Med 2003; 349: 1315-23

67. Morice MC, Serruys PW, Sousa JE, et al. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. N Engl J Med 2002; 346: 1773-80
68. Sehgal SN. Immunosuppressive profile of rapamycin. Ann N Y Acad Sci 1993; 696: 1-8
69. Martel RR, Klicius J, Galet S. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can J Physiol Pharmacol 1977; 55: 48-51
70. Calne RY, Collier DS, Lim S, et al. Rapamycin for immunosuppression in organ allografting [letter]. Lancet 1989; II (8656): 227
71. Morris RE, Meiser BM. Identification of a new pharmacologic action for an old compound. Med Sci Res 1989; 17: 877-81
72. Neff GW, Montalbano M, Tzakis AG. Ten years of sirolimus therapy in orthotopic liver transplant recipients. Transplant Proc 2003; 35 (3 Suppl.): 209-16S
73. Douros J, Suffness M. New antitumor substances of natural origin. Cancer Treat Rev 1981; 8: 63-87
74. Houchens DP, Ovejera A, Riblet SM, et al. Human brain tumor xenografts in nude mice as a chemotherapy model. Eur J Cancer Clin Oncol 1983; 19: 799-805
75. Applegate RJ, Hermiller JJ, Sanz M, et al. Comparison of everolimus-eluting and paclitaxel-eluting coronary stents in patients with two treated vessels: 2-year results from the SPIRIT III randomised trial. EuroIntervention 2010; 6: 437-46
76. Park DW, Kim YH, Yun SC, et al. Comparison of zotarolimus-eluting stents with sirolimus- and paclitaxel-eluting stents for coronary revascularization: the ZEST (comparison of the efficacy and safety of zotarolimus-eluting stent with sirolimus-eluting and paclitaxel-eluting stent for coronary lesions) randomized trial. J Am Coll Cardiol 2010; 56: 1187-95
77. Murgia MG, Jordan S, Kahan BD. The side effect profile of sirolimus: a phase I study in quiescent cyclosporine-prednisone-treated renal transplant patients. Kidney Int 1996; 49: 209-16
78. Hong J, Kahan BD. Sirolimus-induced thrombocytopenia and leukopenia in renal transplant recipients: risk factors, incidence, progression and management. Transplantation 2000; 69: 2085-90
79. Babinska A, Markell MS, Salifu MO, et al. Enhancement of human platelet aggregation and secretion induced by rapamycin. Nephrol Dial Transplant 1998; 13: 3153-9
80. MacDonald AS, for the Rapamune Global Study Group Transplant Service. A randomized trial of sirolimus, cyclosporine and prednisone vs cyclosporine-prednisone alone in recipients of mismatched first kidney grafts: results at one year [abstract]. 25th Annual Meeting of the American Society of Transplant Surgeons; 1999 May 19-21; Chicago (IL)
81. Kahan BD, for the USA Rapamune Study Group. Pivotal phase III multicenter, randomized, blinded trial of sirolimus versus azathioprine in combination with cyclosporine and prednisone in primary renal transplants [abstract]. 25th Annual Meeting of the American Society of Transplant Surgeons; 1999 May 19-21; Chicago (IL)
82. Paul SR, Bennett F, Calvetti JA, et al. Molecular cloning of a cDNA encoding interleukin 11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. Proc Natl Acad Sci U S A 1990; 87 (19): 7512-6
83. Quesniaux VF, Wehrli S, Steiner C, et al. The immunosuppressant rapamycin blocks in vitro responses to hematopoietic cytokines and inhibits recovering but not steady-state hematopoiesis in vivo. Blood 1994; 84: 1543-52
84. Tabernero J, Rojo F, Burris H, et al. A phase I study with tumor molecular pharmacodynamic (MPD) evaluation of dose and schedule of the oral mTOR-inhibitor everolimus (RAD001) in patients (pts) with advanced solid tumors [abstract]. J Clin Oncol 2005; 23 Suppl. 16: 3007
85. Vanrenterghem Y, Ponticelli C, Morales JM, et al. Prevalence and management of anaemia in renal transplant recipients: a European survey. Am J Transplant 2003; 3: 835-45
86. Shah N, Al-Khoury S, Afzali B, et al. Posttransplantation anaemia in adult renal allograft recipients: prevalence and predictors. Transplantation 2006; 81: 1112-8
87. Afzali B, Al-Khoury S, Shah N, et al. Anaemia after renal transplantation. Am J Kidney Dis 2006; 48: 519-36
88. MacDonald AS. A worldwide, phase III randomized controlled, safety and efficacy study of a SRL/cyclosporine regimen for prevention of acute rejection in recipients of primary mismatched renal allografts. Transplantation 2001; 71: 271-80
89. Kahan BD. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomized multicentre study. The Rapamune US Study Group. Lancet 2000; 356: 194-202
90. Augustine JJ, Knauss TC, Schulak JA, et al. Comparative effects of sirolimus and mycophenolate mofetil on erythropoiesis in kidney transplant patients. Am J Transplant 2004; 4: 2001-6
91. Larson TS, Dean PG, Stegall MD, et al. Complete avoidance of calcineurin inhibitors in renal transplantation: a randomized trial comparing sirolimus and tacrolimus. Am J Transplant 2006; 6: 514-22
92. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. N Engl J Med 2007; 357: 2562-75
93. Toso Ch, Meeberg GA, Bigam DL, et al. De novo sirolimus-based immunosuppression after liver transplantation for hepatocellular carcinoma: long-term outcomes and side effects. Transplantation 2007; 83: 1162-8
94. Demirjian S, Stephany B, Abu Romeh IS, et al. Conversion to sirolimus with calcineurin inhibitor elimination vs. dose minimization and renal outcome in heart and lung transplant recipients. Clin Transplant 2009; 23: 351-60
95. Vitko S, Margreiterb R, Weimarc W, et al. Three-year efficacy and safety results from a study of everolimus versus mycophenolate mofetil in *de novo* renal transplant patients. Am J Transplant 2005; 5: 2521-30
96. Webster AC, Lee VWS, Chapman JP, et al. Target of rapamycin inhibitors (sirolimus and everolimus) for primary immunosuppression of kidney transplant recipients: a systematic review and meta-analysis of randomized trials. Transplantation 2006; 81: 1234-48
97. Bellmunt J, Szczylik C, Feingold J, et al. Temsirolimus safety profile and management of toxic effects in patients

with advanced renal cell carcinoma and poor prognostic features . Ann Oncol 2008; 19 (8): 1387-92

98. Cahill BC, Troy K, Crompton JA, et al. Early experience with sirolimus in lung transplant recipients with chronic allograft rejection. J Heart Lung Transplant 2003; 22: 169-76
99. Friend P, Russ G, Oberbauer R, et al. Incidence of anaemia in sirolimus-treated renal transplant recipients: the importance of preserving renal function. Transplant Int 2007; 20: 754-60
100. Russ G, Schena FP, Oberbauer R, et al. Incidence and treatment of anaemia after conversion to sirolimus vs continuation of calcineurin inhibitors in renal allograft recipients: 2-year results from the CONVERT trial. American Society of Nephrology Annual Meeting: Renal Week 2006; 2006 Nov 14-19; San Diego (CA). J Am Soc Nephrol 2006; 17: 18A
101. Sanchez Fructuoso A, Calvo N, Moreno MA, et al. Study of anaemia after late introduction of everolimus in the immunosuppressive treatment of renal transplant patients. Transpl Proc 2007; 39: 2242-4
102. Masetti M, Rompianesi G, Montalt R, et al. Effects of everolimus monotherapy on haematological parameters and iron homeostasis in de novo liver transplant recipients: preliminary results. Transplant Proc 2008; 40: 1947-9
103. Bouscary D, Pene F, Claessens YE. Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood 2003; 101: 3436-43
104. Cruz R, Hedden L, Boyer D, et al. S6 kinase 2 potentiates interleukin-3-driven cell proliferation. J Leukoc Biol 2005; 78: 1378-85
105. Jaster R, Bittorf T, Klinken SP, et al. Inhibition of proliferation but not erythroid differentiation of J2E cells by rapamycin. Biochem Pharmacol 1996; 51: 1181-5
106. Kim MJ, Mayr M, Pechula M, et al. Marked erythrocyte microcytosis under primary immunosuppression with sirolimus. Transplant Int 2006; 19: 12-8
107. Maiorano A, Stallone G, Schema A, et al. Sirolimus interferes with iron homoestasis in renal transplant recipients. Transplantation 2006; 82: 908-12
108. Thaunat O, Beaumont C, Chatenoud L, et al. Anaemia after late introduction of sirolimus may correlate with biochemical evidence of a chronic inflammatory state. Transplantation 2005; 80: 1212-9
109. Augustine JJ, Rodriguez V, Padiyar A, et al. Reduction in erythropoietin resistance after conversion from sirolimus to enteric coated mycophenolate sodium. Transplantation 2008; 86: 548-53
110. Fishbane S, Cohen DJ, Coyne DW, et al. Posttransplant anaemia: the role of sirolimus. Kidney Int 2009; 76: 376-82
111. Fibach E, Bianchi N, Borgatti M, et al. Effects of rapamycin on accumulation of a-, b- and c-globin mRNAs in erythroid precursor cells from b-thalassaemia patients. Eur J Haematol 2006; 77: 437-41
112. Mischiati C, Sereni A, Lampronti I, et al. Rapamycin-mediated induction of *c*-globin mRNA accumulation in human erythroid cells. Br J Haematol 2004; 126: 612-21
113. Zuccato C, Bianchi N, Borgatti M, et al. Everolimus is a potent inducer of erythroid differentiation and gamma-globin gene expression in human erythroid cells. Acta Haematol 2007; 117: 168-76
114. Sofroniadou S, Kassimatis T, Goldsmith D. Anaemia, microcytosis and sirolimus: is iron the missing link? Nephrol Dial Transplant 2010; 25: 1667-75
115. Nicolas G, Bennoun M, Devaux I, et al. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. Proc Natl Acad Sci U S A 2001; 98: 8780-5
116. Ganz T, Nemeth E. Iron imports: IV. Hepcidin and regulation of body iron metabolism. Am J Physiol Gastrointest Liver Physiol 2006; 290: G199-203
117. Donovan A, Lima CA, Pinkus GS, et al. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. Cell Metab 2005; 1: 191-200
118. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 2004; 306: 2090-3
119. Ganz T. Molecular control of iron transport. J Am Soc Nephrol 2007; 18: 394-400
120. Pigeon C, Ilyin G, Courselaud B, et al. A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. J Biol Chem 2001; 276: 7811-9
121. Pak M, Lopez MA, Gabayan V, et al. Suppression of hepcidin during anemia requires erythropoietic activity. Blood 2006; 108: 3730-5
122. Nemeth E, Valore EV, Territo M, et al. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003; 101: 2461-3
123. Nicolas G, Chauvet C, Viatte L, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002; 110: 1037-44
124. Means Jr RT, Krantz SB. Inhibition of human erythroid colony-forming units by interferons alpha and beta: differing mechanisms despite shared receptor. Exp Hematol 1996; 24: 204-8
125. Theurl I, Mattle V, Seifert M, et al. Dysregulated monocyte iron homeostasis and erythropoietin formation in patients with anemia of chronic disease. Blood 2006; 107: 4142-8
126. Park CH, Valore EV, Waring AJ, et al. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 2001; 276: 7806-10
127. Kulaksiz H, Gehrke SG, Janetzko A, et al. Pro-hepcidin: expression and cell specific localisation in the liver and its regulation in hereditary haemochromatosis, chronic renal insufficiency, and renal anaemia. Gut 2004; 53: 735-43
128. Taes YE, Wuyts B, Boelaert JR, et al. Prohepcidin accumulates in renal insufficiency. Clin Chem Lab Med 2004; 42: 387-9
129. Thaunat O, Beaumont C, Lechaton S, et al. Late introduction of sirolimus induces anaemia in renal transplant recipients [letter]. Transplantation 2007; 15: 1283
130. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 2003; 102: 783-8
131. Kemna E, Tjalsma H, Laarakkers C, et al. Novel urine hepcidin assay by mass spectrometry. Blood 2005; 106: 3268-70
132. Tomosugi N, Kawabata H, Wakatabe R, et al. Detection of serum hepcidin in renal failure and inflammation by using protein chip system. Blood 2006; 108: 1381-7

133. Murphy AT, Witcher DR, Luan P, et al. Quantitation of hepcidin from human and mouse serum using liquid chromatography tandem mass spectrometry. Blood 2007; 110: 1048-54

134. Kobold U, Dulffer T, Dangl M, et al. Quantification of hepcidin-25 in human serum by isotope dilution micro-HPLC-tandem mass spectrometry. Clin Chem 2008; 54: 1584-6

135. Li H, Rose MJ, Tran L, et al. Development of a method for the sensitive and quantitative determination of hepcidin in human serum using LC-MS/MS. J Pharmacol Toxicol Methods 2009; 59: 171-80

136. Ganz T, Olbina G, Girelli D, et al. Immunoassay for human serum hepcidin. Blood 2008; 112: 4292-7

137. Zaritsky J, Young B, Wang HJ, et al. Hepcidin: a potential novel biomarker of iron status in chronic kidney disease. Clin J Am Soc Nephrol 2009; 4: 1051-6

138. Koliaraki V, Marinou M, Vassilakopoulos TP, et al. A novel immunological assay for hepcidin quantification in human serum. PLoS ONE 2009; 4: e4581

Correspondence: Dr *David Goldsmith*, MA, FRCP, Department of Renal Medicine and Transplantation, Guy's and St Thomas' NHS Foundation Trust, King's Health Partners AHSC, London SE1 9RT, UK.
E-mail: david.goldsmith@gstt.nhs.uk