to their care, especially when monitoring meropenem levels is not a practical option for many medical centres. After dosing 1 g of meropenem, we found that the plasma meropenem levels for all of our patients remained above the MIC for the entire duration of the SLED session (for at least ~12 h). We therefore suggest dosing meropenem at 1 g every 12 h in this population of ICU patients on SLED.

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Dinucleoside polyphosphates: newly detected uraemic compounds with an impact on leucocyte oxidative burst

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Abstract

Background. Dinucleoside polyphosphates (NpₙN) have pathophysiologic roles in cardiovascular disease and are newly detected uraemic retention solutes. They were retrieved in human plasma, tissues and cells. Although their impact on several cell systems involved in vascular damage (endothelium, smooth muscle cells and thrombocytes) has been evaluated, their effect on different types of leucocytes has never been studied.

Methods. This study evaluates, for the first time, the impact of NpₙN on monocyte, granulocyte and lymphocyte oxidative burst activity at baseline and after stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP) and phorbol 12-myristate 13-acetate (PMA) in whole blood. Diadenosine triphosphate (Ap₃A) to diadenosine hexaphosphate (Ap₆A) were tested to investigate the effect of the number of phosphate groups on reactive oxygen species (ROS) production. The effect of the type of nucleoside...
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was evaluated by comparing adenosine guanosine tetraphosphate, diguanosine tetraphosphate, uridine adenosine tetraphosphate (Up4A) and diadenosine tetraphosphate (Ap4A).

**Results.** This study demonstrated that lymphocytes are especially susceptible to intracellular diadenosine polyphosphates. Depending on the phosphate chain length, different effects were observed. At baseline and with fMLP, Ap3A, Ap4A and Ap5A enhanced lymphocyte-free radical production. In addition, Ap3A, Ap4A and Ap5A increased PMA-stimulated ROS production in lymphocytes. Monocytes and granulocytes parallel the lymphocyte response albeit with an inhibition of Ap6A on granulocytes. Considering Np_nN with four phosphate groups, Up4A showed the most important stimulatory effects on monocytes and Ap4A on lymphocytes.

**Conclusions.** Np_nN mainly have a leucocyte-activating impact, most significant for Ap4A, considering phosphate chain length, and for Up4A, considering the type of nucleosides. These results suggest that the pro-inflammatory effects of Np_nN can contribute to the development of atherosclerosis, probably in the early stages of chronic kidney disease, but their chemical composition affects their activity.

**Keywords:** cardiovascular disease; cell activation; chronic kidney disease; oxidative stress; uraemic toxins

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**Introduction**

In chronic kidney disease (CKD), the risk for premature death, primarily as a result of cardiovascular disease, is high and this risk increases early on in kidney failure [1,2]. Traditional risk factors insufficiently predict cardiovascular outcome in CKD; less traditional risk factors, like inflammation, endothelial dysfunction, oxidative stress, vascular calcification and malnutrition, seem to play an at least as important role [3–6].

When renal function deteriorates, a number of substances, normally excreted in the urine, are retained within the body. In 2003, a list of 90 known uraemic compounds was published and several of these have the potential to induce vascular damage [7]. However, further identification of unknown compounds and the elucidation of pathophysiological mechanisms remain necessary to better understand the process of vessel damage in CKD and to develop specific therapeutic interventions [8–10].

The dinucleoside polyphosphates (Np_nN) are a newly identified group of compounds only recently added to the list of uraemic retention solutes [11]. Np_nN contain two nucleosides interconnected by a variable number of phosphates; with a molecular weight between 800 and 1200 Da, they belong to the so-called middle molecules (molecular structures: see Figure 1) [12].

Specific members of this group have been detected in human plasma, platelets, neuronal cells, endothelial cells, adrenal glands and myocardial tissue [13–16]. In haemodialysis patients, the intracellular diadenosine polyphosphates (Ap_nA) are increased in platelets and released upon activation [17]. Also, renal tubular cells release Ap5A, Ap6A and uridine adenosine tetraphosphate (Up4A), and Up4A is released by the endothelium as well [18].

The Np_nN play a role in vasoregulation, neurotransmission and cell signalling [19–21]. Related to the cardiovascular system, Np_nN have been shown to interfere with the function of thrombocytes, endothelium and smooth muscle cells [16,19,22–24]. Despite the fact that leucocytes are also key mediators in vessel damage, studies of Np_nN with these cells are scarce and fragmentary, both regarding the type of cell and the Np_nN variant. Leucocytes play an important role in the immune response. CKD is considered as a state of chronic inflammation characterized by a dual immune response; on one hand, many CKD patients have a baseline status of inflammation, while on the other hand, their immune function upon stimulation is often suppressed, resulting in an increased susceptibility to infection [5,25].

The present study investigates the biological impact of a series of Np_nN on leucocyte function to evaluate their contribution to the uraemic syndrome. Chronic inflammation in CKD is associated with oxidative stress and, since leucocytes are an important source of reactive oxygen species (ROS) [26,27], the effect of the Np_nN was evaluated by measuring changes in leucocyte oxidative burst activity. The effect of the number of phosphate groups on leucocyte oxidative burst activity was studied by testing Ap3A to Ap6A, the subgroup of Np_nN on which the most extensive information is available. Since the most pronounced effects in this analysis were found for Ap4A and in view of the recently demonstrated important vasoconstrictive effects of Up4A [18], a second series of experiments was performed concentrating on known Np_nN with four phosphate groups but a varying type of nucleoside, i.e. Ap4A, adenosine guanosine tetraphosphate (Ap4G), diguanosine tetraphosphate (Gp4G) and Up4A. Finally, the effect of a combination of Ap4A and Up4A was tested.

**Materials and methods**

**Reagents**

High-pressure liquid chromatography (HPLC) water (gradient grade) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany); all other substances were obtained from Sigma-Aldrich (Munich, Germany), unless otherwise specified.

**Synthesis of Np_nN**


Briefly, depending on the type of Np_nN, adenosine 5′-polyphosphates, guanosine 5′-polyphosphates and/or uridine 5′-monophosphate were dissolved in water together with N-[2-hydroxyethyl]-piperazine-N′-2-ethanesulfonic acid, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide and MgCl₂ and incubated at 37°C for 48 h at pH 6.5.

Np_nN were concentrated on a C18 reversed-phase column (Supersphere 100 C18 endcapped, 100 × 2; 1 mm, 4 μm, 10 mm, Merck, Germany) using 40 mM aqueous triethylammonium acetate and were eluted with 30% ACN in water. The lyophilized concentrate of the reversed-phase column was injected on two C18 reversed-phase columns connected in series (Supersphere, 300 × 8 mm, 4 μm, Merck, Germany) and was chromatographed in the displacement mode by use of n-butanol (100 mM). The fractions containing Np_nN of the displacement chromatography were lyophilized and each fraction was chromatographed with...
Fig. 1. Molecular structure of Np₄N: Ap₄A (A), Ap₄G (B), Gp₄G (C) and Up₄A (D).
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an anion exchange column (column: UnoQ (Biorad), 100 × 10 μm, 10 μm; eluent A, 20 mM K2HPO4; eluent B, 20 mM K2HPO4 and 1 M NaCl; gradient: 0–10 min, 0–5% B; 10–115 min, 5–40% B; and 115–120 min, 40–100% B; flow rate, 1.0 mL/min; and UV absorption wavelength, 254 nm). The fractions of the anion exchange chromatography were de-salted by HPLC reversed-phase C18 chromatography. The chromatographically isolated Np nN were lyophilized and stored at −20°C [29].

Sample collection
The present protocol was approved by the local ethics committee after informed consent. Heparinized whole blood from healthy donors, not smoking and not taking any medication, was collected. Additionally, he-parinized whole blood was taken from haemodialysis patients prior to the dialysis session.

In vitro effect of dinucleoside polyphosphates on the leucocyte oxidative burst
Experimental set-up. The present study was performed according to a standardized protocol for in vitro testing of uraemic retention solutes de-scribed by Cohen et al. [30], generated after ample discussion among the experts of the European Uraemic Toxin (EUTox) Work Group. This monograph recommends testing the highest reported uraemic concentra-
tion. It is of note, however, that current data on Np nN concentrations are still limited. Up till now, only Jankowski et al. described an increased intracellular amount of Ap nA in platelets and an increased release by plate-
ettes of haemodialysis patients upon activation. A maximum release of 154 ± 59 fg Ap nA/platelet (range from 95 to 213 fg Ap nA/platelet) was described [17] and, since whole blood contains 4 × 10⁵ platelets/μL, we calculated a mean local concentration of 61.6 ng Ap4A/μL blood (range from 38.0 to 85.2 ng Ap4A/μL blood) or 72.2 μM (range from 45.4 to 101.9 μM). In the in vitro set-up, therefore, a concentration of 100 μM was tested for each Np nN, which, based upon the current knowledge, ra-ther reflects local concentrations of Np nN after release from their storage cells than their plasma concentration. These values also correspond to the local concentrations reported by Ogilvie [31].

The lyophilized Np nN were reconstituted separately in saline (0.9% NaCl, Baxter) in a 10-fold concentrated stock solution of 1 mM and stored at −20°C and, upon testing, they were diluted 1:10 in heparinized whole blood.

Oxidative burst. To evaluate the effect of the Np nN on the production of ROS in leucocytes, the Bursttest (Phagoburst®) (Orpegen Pharma, Hei-delberg, Germany) was applied after a 10-min incubation period at 37°C in the presence of the separate Np nN, as described previously [32].

The oxidative burst activity of the leucocytes was measured at baseline, after moderate stimulation with N-formyl-methionine-leucine-
phenylalanine (fMLP, 0.83 μM) and after strong stimulation with phorbol 12-myristate 13-acetate (PMA, 1.35 μM). Samples were analysed with the FACScan® flow cytometer (Becton Dickinson). Experiments were re-peated eight (Ap nA) or six (Np nN) times.

FACScan analysis. Using the CellQuest Pro™ software, 10 000 events were counted in every sample. Based on their light scatter properties, monocytes, granulocytes and lymphocytes were gated separately. Within these gates, the percentage of rhodamine-positive cells (%) was evaluated for the baseline and fMLP-stimulated samples. The mean fluorescence intensity (MFI) per cell was considered as a measure for the oxidative burst activity after PMA stimulation.

Endotoxin concentration
To exclude that an effect due to lipopolysaccharide (LPS) contamination was observed, all experimental solutions causing a stimulation of the oxida-tive burst were checked by means of the Limulus Amebocyte Lysate (LAL) QCL-1000 test, a quantitative kinetic and chromogenic assay (Cambrex Bio Science, Walkersville, MD, USA). The detection limit of this assay is 0.005 endotoxin units (EU)/mL.

Statistical analysis
Data are expressed as the mean ± SEM. Normality was checked with the Kolmogorov–Smirnov test in combination with descriptive statistics. Sta-
tistics were performed using a paired t-test for the in vitro data. A P-value of <0.05 was considered significant.

Results
Effect of a varying number of phosphate groups on the leucocyte oxidative burst activity
Effects of Np nN with a varying number of phosphate groups linking the two nucleoside moieties are described. To evaluate whether the number of phosphate groups plays a role in the effect of the Np nN on leucocyte oxidative burst activity, four Ap nA with three to six phosphate groups were tested.

At baseline, 4.65 ± 0.10% of the monocytes, 4.76 ± 0.07% of the granulocytes and 2.63 ± 0.53% of the lymphocytes (n = 8) produced ROS. As presented in Figure 2A, incubation with Ap3A and Ap5A induced a signifi-
cant rise (P < 0.05, n = 8) in free radical production in all three leucocyte types. For both monocytes and lympho-
cytes also, Ap6A resulted in a significantly increased per-
centage of rhodamine-positive cells. Finally, for Ap7A, no significant effects were observed.

A moderate stimulation of the Bursttest with fMLP re-
sulted in a significant increase of the ROS production for monocytes and granulocytes in the saline condition as well as in the presence of Ap6A. However, only Ap5A affected the fMLP-induced ROS production in comparison to the saline condition in monocytes. The oxidative burst in lympho-
cytes remained unaffected by fMLP as previously de-
monstrated and the Ap6A showed an enhanced activity versus saline which was comparable with the effects ob-
served in baseline cells (Figure 2B) [33].

After stimulation with PMA, 97.1 ± 1.51% of the leu-
cocytes produced ROS and, therefore, the MFI was evalu-
ated for these samples. As demonstrated in Figure 2C, Ap4A further enhanced the oxidative burst activity in monocytes, while Ap6A inhibited it in granulocytes. The most significant effects were seen in the lymphocytes where Ap3A, Ap5A and Ap6A significantly enhanced free radical production.

The present study demonstrate that, depending on the number of phosphate groups and the type of leucocyte, Ap nA has a different impact on the oxidative burst ac-
20°C [29].
served in monocytes, while Ap$_4$G and Gp$_4$G both inhibited the ROS production in granulocytes.

The monocytic fMLP-stimulated oxidative burst activity was enhanced in the presence of Up$_4$A, while no effects on the fMLP-stimulated Bursttest in granulocytes were seen. None of the compounds with four phosphate groups, except for Ap$_4$A, showed an effect on lymphocytes, neither at baseline nor after fMLP stimulation (Figure 3B).

As shown in Figure 3C, Up$_4$A caused, in parallel with Ap$_4$A, a significant rise in free radical production in PMA-stimulated monocytes and lymphocytes. No effect was seen on the PMA-stimulated granulocytes or after incubation with Ap$_4$A and Gp$_4$G.

By studying the effect of different types of nucleosides (Np$_n$N), it can be concluded that, again, different effects are observed in the different types of leucocytes under study. The most important effects are observed in the presence of Up$_3$A, especially in monocytes and with Ap$_3$A in lymphocytes. However, after PMA stimulation, Up$_3$A induces a significantly higher response compared to Ap$_3$A in both cell types.

**Effect of the combination Ap$_4$A and Up$_4$A**

Because of their most prominent effects, the effect on leucocyte ROS production of a combination of Ap$_4$A and Up$_4$A was evaluated (each at 100 μM). Although the above-described results were confirmed in blood from healthy donors, no cumulative effect of the compounds could be demonstrated (data not shown).

In contrast, when the Ap$_4$A and Up$_4$A mixture was added to whole blood obtained from haemodialysis patients, collected just before the start of haemodialysis, no significant effects on the ROS production could be observed (data not shown).

**Endotoxin concentration**

All solutions tested were checked for their endotoxin concentration by performing the LAL test. None of them had a LPS concentration above 0.05 EU/mL, which corresponds to 4 pg LPS/mL, except for Ap$_6$A containing 0.26 EU/mL.

When testing LPS in the Bursttest at this concentration, no effect was observed however. Therefore, it can be ex-

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**Fig. 2.** Effect of the Ap$_n$A ($n = 3–6$) on the oxidative burst activity of monocytes, granulocytes and lymphocytes at baseline (A) and after fMLP stimulation (B), expressed as %, and after PMA stimulation, expressed as MFI (*P < 0.05, **P < 0.01 versus saline; n = 8).
cluded that the effects observed are attributable to endotoxin contamination.

**Discussion**

The present study evaluated the role of the Np$_n$N as newly identified uraemic retention solutes in CKD-related inflammation and atherogenesis. To the best of our knowledge, it is the first time that the impact of different Np$_n$N on oxidative burst activity of several types of leucocytes was tested, at baseline as well as after stimulation. The effect of the number of phosphate groups was studied in the most studied subgroup of Np$_n$N, the Ap$_n$A ($n=3$–6). Based upon the results, the effect of the type of nucleoside was studied on Np$_n$N with four phosphate groups but a varying type of nucleoside, i.e. Ap$_n$A, Ap$_n$G, Gp$_n$G and Up$_n$A. Also, the effect of a combination of Ap$_4$A and Up$_4$A was tested.

Mainly, a pro-inflammatory effect of the Np$_n$N on leucocytes was found. Depending on the number of phosphate groups, lymphocytes were especially susceptible to the Ap$_n$A, with Ap$_4$A inducing a marked stimulation of the oxidative burst activity in cells at baseline, but also after fMLP and after PMA stimulation (Figure 2). Considering the type of nucleoside, Up$_4$A was shown to exert the most significant stimulatory effects on the basal and fMLP-activated monocytes (Figure 3A and B) and after PMA stimulation in both monocytes and lymphocytes (Figure 3C). A combination of both Ap$_4$A and Up$_4$A revealed a comparable, but not a cumulative, effect on ROS production by normal leucocytes, whereas on uraemic leucocytes, no effect on ROS production could be observed.

Oxidative stress plays an important negative role in the cardiovascular outcome of the CKD patient and oxidative stress is present already in the early stages of CKD [27,34]. The present study illustrates that Np$_n$N, especially the ones containing four phosphate groups, may contribute to this pro-inflammatory status.

Also, the type of nucleoside can be at the origin of diverse effects, as is described for vasoconstriction of arteries where
one adenine seemed crucial and sufficient because of the equipotent effect of ApnA and ApnG, whereas in contrast, GpnG were inactive [24]. In the present study, it was shown that the presences of adenosines and uridines have a stimulatory effect on lymphocytes and monocytes, whereas guanosines have an inhibitory effect on granulocytes.

The observation that an increase of NpnN in an extreme uraemic milieu does not induce additional ROS production by uraemic leucocytes, as was observed for normal leucocytes, leads to the assumption that the effects observed with healthy donor blood mirrors the importance of their effect in the early stages of CKD playing a role in the initial phase of the atherosclerotic process.

The observed inhibitory effect in the presence of ApnA and the G-containing NpnN can be related to the enhanced susceptibility of the CKD patient for infection [23]. Opposite effects related to the phosphate chain length in the NpnN have already been previously demonstrated in vascular injury [24].

The specificity of the nucleosides for the different leucocyte subpopulations is most probably due to variability in their purinergic P2 receptor expression patterns for NpnN. There are two main families of P2 receptors, P2X and P2Y. P2X receptors form plasma membrane channels selective for monovalent and divalent ions and do not require intracellular messengers. P2Y, on the other hand, are G protein-coupled receptors and, upon activation, inositol 1,4,5-triphosphate is generated and intracellular Ca\(^{2+}\) is released, followed by a Ca\(^{2+}\) influx from outside the cell [36]. Each of these receptors has a series of subtypes and, as recently reviewed in haematopoietic cell lines, many of them are present in leucocytes [37]. Next, a series of interactions of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) with P2Y receptors on leucocytes were described during vascular injury [38]. The type of P2 receptors playing a role in the currently observed effects on ROS production can differ among the type of NpnN tested, the cell type and the stimulus. The use of inhibitors like suramin, a P2 purinergic antagonist, α,β-methylene ATP, a P2X1 inhibitor or other selective antagonists should elucidate this in the future.

Data on the effects of NpnN on leucocytes had been, up to now, limited to the evaluation of the effect of some ApnA in neutrophils. It was found that ApnA and ApnA are able to prime the respiratory burst if followed by further fMLP stimulation in isolated neutrophils and this effect was Ca\(^{2+}\)-dependent with NpnN concentrations above 50 μM. The priming effects appeared to be maximal when the ApnA were added 1 min before fMLP and at 600–800 μM [39]. The same group also found that ApnN (n = 3–6) delayed neutrophil apoptosis [40,41]. They also suggested that neutrophils express P2 receptors with different binding affinities to mononucleotides and dinucleotides [42]. Vartanian et al. found that interferons induce an accumulation of ApnA in both a monocyte and a granulocytic human cell line due to an accumulation of the enzyme tryptophanyl-tRNA synthetase [43].

According to the standardized protocol for in vitro testing of uraemic retention solutes developed by the EUTox Work Group, the NpnN were tested at their highest estimated uraemic concentration [30]. Considering the still limited available data on concentrations of NpnN in CKD, the NpnN concentration was based upon the release of ApnA by thrombocytes from haemodialysis patients [17]. NpnN are released by endothelial cells and platelets, which are both cell types playing a role in atherogenesis. Hence, it is likely that, intravascularly, leucocytes are locally exposed to similar NpnN concentrations especially if the producing cells are activated, as is the case for thrombocytes and endothelium in uraemia [44].

The present data were obtained by performing acute experiments with an exposure time of only 10 min. In vivo, the chronic exposure in CKD is, however, continuous, especially in atherosclerotic lesions, which contain macrophages, lymphocytes as well as thrombocytes, and thus the damaging impact is conceivably more persistent [26]. Of note, another possibility that should be considered is that the damaging effect of NpnN in vivo is attenuated by compensatory, regulatory and/or repairing mechanisms. Although such mechanisms are certainly at play, it should be taken into account that the net in vivo result of these interactions is still essentially pro-inflammatory.

The definition/specification of NpnN as uraemic retention solutes is mainly based on the following observations: NpnN are endogenous compounds with a strong impact on physiologic and pathophysiologic processes in the cardiovascular system [21,22,45]; NpnN are released by different cell types involved in atherosclerosis, the major cause of death in CKD (e.g. thrombocytes, endothelial cells) [15,18]; platelets from haemodialysis patients have an increased intracellular ApnA concentration and an increased release [17]; UpnA plasma concentration is increased in juvenile hypertensives versus normotensives and UpnA is described to affect glomerular filtration rate [22,45]. Uraemic plasma concentrations per se have, however, been rarely reported. The quantification of NpnN in CKD (Stage 2–5) and in uraemic patients before and after haemodialysis is currently under investigation in different ongoing studies. Based upon the increased release of ApnA from platelets from haemodialysis patients [17], local concentrations of NpnN were calculated to rise up to 100 μM.

Based on their molecular weight ranging from 800 to 1200 Da, the NpnN are classified as middle molecules. In addition, dinucleosides were shown to be protein bound [13]. Because of their physicochemical characteristics, NpnN are expected to be difficult to remove by dialysis, bringing along the need for more advanced removal strategies such as online haemodiafiltration, adsorption and/or pharmaceutical interventions in the function of the involved pathophysiological mechanism [10,12].

**Conclusion**

In conclusion, this study describes, for the first time, the effect of a series of NpnN on leucocyte oxidative burst activity in relation to uraemia. Depending on the length of the phosphate chain, the type of nucleoside and the type of leucocyte, a different impact on the oxidative burst activity at baseline and after stimulation was observed. In general, these data, especially showing a pro-inflammatory...
effect, suggest that Np,nN are likely to be involved in the development of atherosclerosis, probably in the early stages of CKD.

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Conflict of interest statement. None declared.

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Who should be referred for a fistula? A survey of nephrologists

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Abstract

Background. There is marked variation in the use of the arteriovenous fistula (AVF) across programmes, regions and countries not explained by differences in patient demographics or comorbidities. The lack of clear criteria of who should or should not get a fistula may contribute to this, as well as barriers to creating AVFs.

Methods. We conducted a survey of Canadian and American nephrologists to assess the patient variables considered to determine the timing and type of access requested. Perceived barriers and absolute contraindications to access were also collected.

Results. An immediate referral for a fistula was more highly preferred when patients are <65 years old, have minimal comorbidities or have no history of failed access. In older patients, and in those with increased comorbidities or a previously failed fistula, US nephrologists selected arteriovenous grafts as an alternative to the fistula, while Canadian nephrologists selected primarily catheters. Referral for vascular mapping was more common in the USA than in Canada. Gender did not influence the timing or the type of access. Perceived barriers to establishing a mature fistula included patient refusal for creation (77%) or cannulation (58%), delay in decision regarding dialysis modality (71%), wait time for surgical creation (55%) and high failure-to-mature rate (52%). We found that 27% of Canadian and 43% of American nephrologists indicated no absolute contraindications for permanent vascular access.

Conclusions. This study demonstrated marked variability in timing and criteria used to select patients for referral for a vascular access between nephrologists practicing within Canada and the USA. Establishing minimal eligibility criteria for fistulae is an important area of future research.

Keywords: arteriovenous fistula; central venous catheter; haemodialysis; vascular access

Introduction

The establishment and maintenance of vascular access in patients present major challenges in haemodialysis (HD) for the management of end-stage renal failure. Current practice guidelines recommend an arteriovenous fistula (AVF) as the preferred access for lower mortality and complication rates compared to central venous catheters (CVCs) and arteriovenous grafts, and that patients be referred for an AVF if their estimated glomerular filtration rate (eGFR) is <15 ml/min and progressive [1,2]. The Fistula First initiative promulgated these vascular access guidelines, resulting in a marked decline in grafts and an increase in both catheters and AVF in the USA [3]. However, studies within Australia, Canada and Europe have shown a trend towards increased CVC use, which is not fully explained by patient demographics or comorbidities [4–6].