

*Original Article***Cellular proliferation and second messenger formation altered by dialysis membranes**S. Pacini², S. Aterini¹, M. Salvadori¹, E. Ippolito¹, M. Ruggiero² and M. Amato¹¹Division of Nephrology, Prato Hospital, Prato; ²Institute of General Pathology, University of Firenze, Firenze, Italy**Abstract**

Background. In this study we investigated the effect of different dialysis membranes on the clonal murine haematopoietic cell line 32D cells transmembrane signalling machinery, monitored by 1,2-diacylglycerol (DAG) formation, and on their ability to respond to the physiological growth factor interleukin-3 (IL-3).

Methods. Cells were exposed to dialysers (cuprophane, CU; polysulphone, PS; cellulose diacetate, CA; polyacrylonitrile, AN69; polymethylmethacrylate, PMMA; cellulose triacetate, CT; polyamide, PA; and polycarbonate, PC); they were collected, counted, and treated with physiological amount of IL-3. Cell proliferation was monitored as incorporation of radioactivity in duplicating DNA. DAG was measured by thin-layer chromatography in cell labelled with tritiated glycerol overnight.

Results. CU and PA stimulated cell proliferation in comparison with resting cells. PS and TC did not significantly affect thymidine incorporation either in IL-3-stimulated, or in resting cells. Cells exposed to AN69, PC, and CA showed depressed basal incorporation of thymidine (70, 54 and 56% of controls respectively) but retained the ability to respond to IL-3 in a manner not statistically different from controls. PMMA reduced thymidine incorporation both in basal condition and after IL-3 stimulation CU and PC activated early cell signalling ($1.95 \times$ and $1.31 \times$ respectively, DAG increase over control), while PA and TC depressed DAG generation ($0.38 \times$ and $0.47 \times$ respectively, DAG increase over control). PS, CA, AN69, and PMMA did not stimulate DAG generation.

Conclusions. Alterations of intracellular mitogenic signalling appear to correlate with the ability to make a cell competent for function. These results might help to elucidate the effect of different dialysers, at the molecular level, on the blood cell behaviour *in vivo*.

Key words: biocompatibility; haemodialysis; dialysis membrane; intracellular second messenger; cellular growth; diacylglycerol

Introduction

Cellular mitogens, growth factors, hormones, and cytokines stimulate the transmembrane signalling machinery by triggering a phosphoinositide-specific phospholipase C with subsequent intracellular second messenger inositol trisphosphate (IP3) and diacylglycerol (DAG) generation: the former induces Ca^{2+} mobilization, the latter protein kinase C (PKC) activation, which in turn, by phosphorylating other mitogen-activated protein kinases, may initiate a signal flow that eventually induces cellular response and/or DNA replication.

It has recently been demonstrated that the clonal murine haematopoietic cell line 32D is a suitable experimental model to study *in vitro* the intracellular second messenger (IP3 and DAG) formation following the interaction between dialysers and the cell surface, in a manner that reproduces the behaviour of human leukocytes *in vivo* [1]. The interaction between dialysis membranes and blood cell surface induces a series of biochemical events that deeply influence the resting state of the cell as well as its ability to respond to physiological stimuli. It was postulated that alterations of blood cells following repeated exposure to dialysers may account for a variety of complications frequently observed in the course of chronic dialytic treatment [2,3].

The aim of this study was to provide molecular clues for the understanding of the influence of haemodialysis on cell competency, investigating the effect of different dialysis membranes on the ability of 32D cells to proliferate *in vitro* and to respond to their physiological growth factor Interleukin-3 (IL-3); cell growth was correlated with the activation of the transmembrane signalling machinery, as monitored by DAG formation.

This study suggests that alterations of intracellular mitogenic signalling induced by different dialysers appear to correlate with the ability to proliferate and to respond to mitogenic stimuli.

Methods

32D myeloid immature murine haematopoietic cells (clone 3) were cultured as previously described [1]. They were

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preferred instead of human cells in order to avoid variability due to individual variations between different donors. Furthermore, this clonal haematopoietic cell line mimics normal human leukocytes as far as their response to stimuli is concerned. Finally, they were chosen because of their poor ability to adhere to the plastic material of cell culture plates, resembling circulating blood cells; in fact, these cells can be maintained and proliferate in suspension without adhering, unlike differentiated cells. Interleukin-3 (IL-3) is their physiological growth factor.

The following types of dialysers were used: (1) cuprophane (CU) E1S with a surface of 0.60 m² (Fresenius AG, Oberursel, Germany); (2) polysulphone (PS) F-40 with a surface of 0.65 m² (Fresenius AG, Oberursel, Germany); (3) cellulose diacetate (CA) CA-210 with a surface of 2.10 m² (Baxter, Deerfield, Ill. USA); (4) polyacrylonitrile (AN69) Filtral 6 with a surface of 0.60 m² (Hospal Italia, Mirandola, Italy); (5) polymethylmethacrylate (PMMA) Filtryzer B2 0.5 with a surface of 0.50 m² (Toray, Tokyo, Japan); (6) cellulose triacetate (CT) CT-190G with a surface of 1.90 m² (Baxter, Deerfield, Ill. USA); (7) polyamide (PA) Polyflux 160 with a surface of 1.60 m² (Gambro s.p.a., Felino, Italy); and (8) polycarbonate (PC) Pro-600 with a surface of 1.30 m² (Gambro s.p.a., Felino, Italy).

Preliminary experiments performed using the same membranes showed that results were not influenced by varying the filter surface area (data not shown).

Each filter was rinsed with 2 litres of heparinized, isotonic, pyrogen-free saline. The dialysate compartment was tightly closed. After injection of 32D cells (1×10^6 cells/ml of priming volume) into the dialysers, filters remained in a horizontal position upon a table, closed at both ends throughout the experiment. According to previous studies [1], samples were obtained 30 min after cell injection in order to obtain the greatest comparable stimulation for the tested membranes. Immediately before sampling, filters were gently rolled. The first-drawn aliquot of the cell suspension was discarded and a second aliquot was used to study cellular response, in order to obtain random and homogenous samples. 32D cells not exposed to dialysers were used as control. In these experiments 32D cells were suspended in serum-free culture medium, RPMI 1640 (PBI) without L-glutamine, in order to avoid any complement-dependent interference. Every filter was tested in four different experiments, each in triplicate samples. We decided to investigate cell response in no-flow conditions in order to rule out the influence on cellular activation of rolling through the filters.

After being exposed to dialysers, cells were collected, counted and, when described, treated with physiological amount of IL-3. After 20 h, they were labelled for 4 h with [³H] thymidine and cell proliferation was monitored as incorporation of radioactivity in duplicating DNA.

The level of second messengers (either at steady-state or following stimulation) was determined in cells prelabelled to equilibrium with [³H] glycerol, [³H] inositol, [³²P] orthophosphate for 24 h. At the end of the incubation period, a modified Folch extraction was performed by adding ice-cold methanol and chloroform (1/1 v/v) [4,5]. Diacylglycerol and phospholipids were then extracted and separated by thin-layer chromatography [4,5]. Thus, results of diacylglycerol measurements are expressed as the ratio of radioactivity associated with diacylglycerol/radioactivity associated with phospholipids recovered from the thin-layer chromatography plate. An autoradiography of a representative TLC plate is shown in Figure 1. Samples of [³H] inositol-labelled cells were extracted with chloroform/ methanol/ concentrated HCl (100:200:2, v/v) and phases were separated by adding chloroform and

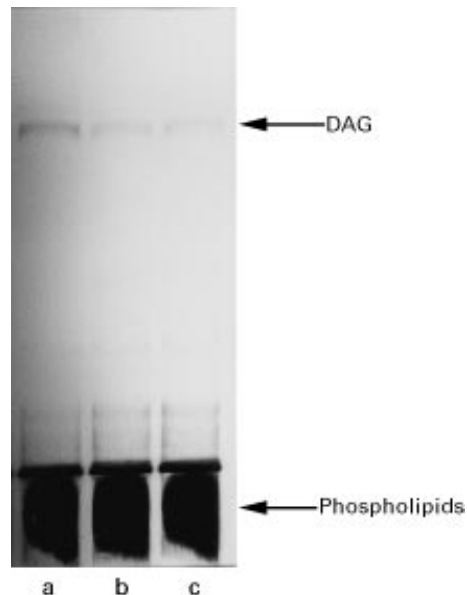


Fig. 1. Representative autoradiography of a thin-layer chromatography plate after separation of DAG from phospholipids. DAG formation in 32D cells challenged with: a, cuprophane; b, polycarbonate; c, control.

water. The upper phase contained the water-soluble inositol phosphates which were separated by ion-exchange chromatography or by high-pressure liquid chromatography [6]. Incorporation of radioactive labelling in a typical experiment was as follows. Diacylglycerol: 17991 ± 401 ; glycerol-containing phospholipids: 2514130 ± 100021 (c.p.m. \pm SE, $n=8$, of radioactivity associated with diacylglycerol or phospholipids spots on TLC plate). Inositol phosphates: 3722 ± 161 ; inositol-containing phospholipids 33926 ± 1601 (c.p.m. \pm SE, $n=8$, of radioactivity recovered in the total inositol lipid fraction, and of an aliquot (100 μ l) of the organic phase of modified Folch extraction). In order to minimize error due to variability in cell number, labelling procedure and extraction efficiency, the ratios between diacylglycerol and phospholipids, and between inositol phosphates and phosphoinositides, were taken into account in making calculations.

Statistical analysis

Statistical analysis was performed with SPSS for Windows. All data are presented as means \pm SEM and compared by means of ANOVA. *P* values less than 0.05 were considered to be significant.

Results

Second messenger generation

Qualitatively identical results were obtained measuring either inositol phosphate and phosphatidic acid formation, or polyphosphoinositide breakdown (data not shown); the concurrent generation of DAG, IP3 and phosphatidic acid implies early activation of phospholipase C.

DAG generation in 32D cells after being in contact with different dialysis membranes is presented in

Figure 2. Results are given as times increase over control, untreated, cells.

CU strongly activated early cell signalling (1.95; DAG,-fold increase over untreated cells), while PC induced a non-significant rise in DAG formation (1.31; DAG,-fold increase over untreated cells). PA and CT lowered DAG generation, even though not significantly (0.38 and 0.47 respectively; DAG,-fold increase over control). Intracellular second messenger generation in 32D cells challenged with PS, CA, AN69 and PMMA was not substantially affected.

Cellular growth

In Figure 3, 32D cell growth after exposure to different dialysis membranes is presented. CU and, to a lesser

extent, PA directly stimulated cell proliferation in comparison with control cells not exposed to membranes. PS and CT did not significantly affect thymidine incorporation in resting cells. Cells exposed to CA, AN69, PMMA, and PC showed depressed basal incorporation of thymidine (56, 70, 56 and 54% of controls respectively).

32D cell growth induced by stimulation with IL-3 is shown in Figure 4. In control cells, i.e. in cells not exposed to membranes, IL-3 boosted proliferation by approximately 15-fold, labelled as 100%. In cells previously exposed to CU and PMMA, response to IL-3 stimulation was significantly curtailed, expressed as percentage variation in the incorporation of radioactivity in duplicating DNA in comparison with control cells. Cells which had been in contact with the other

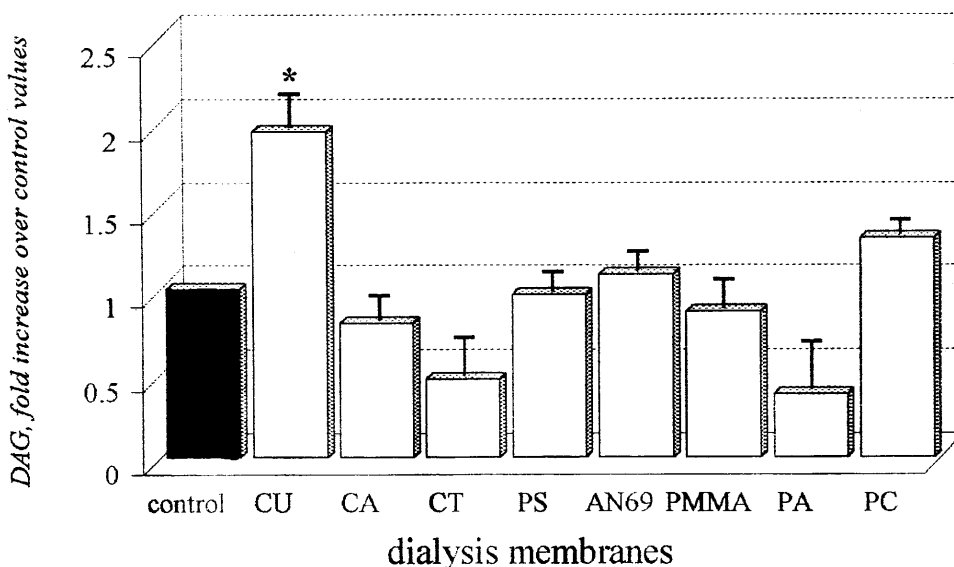


Fig. 2. Diacylglycerol formation in 32D cells after their exposure to different dialysis membranes (* $P < 0.01$ versus control).

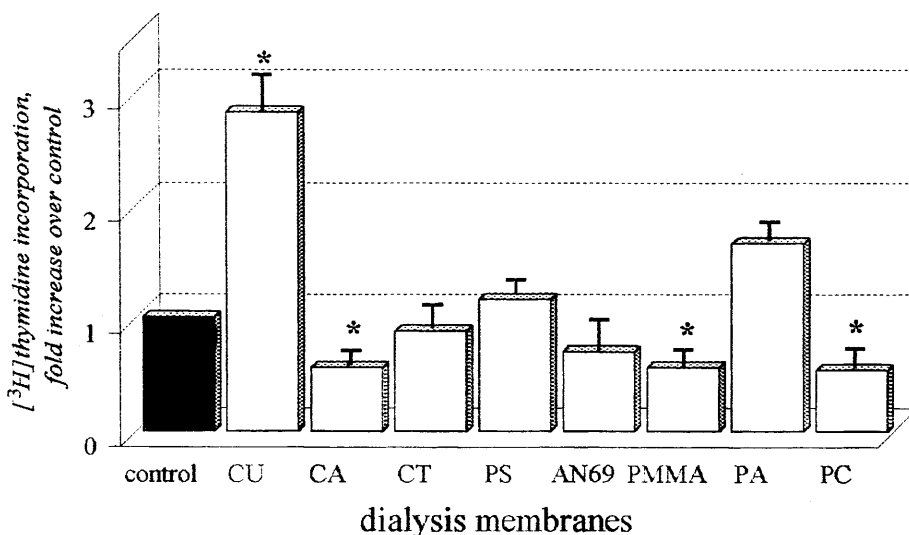


Fig. 3. 32D cell growth, monitored as incorporation of [³H] thymidine in duplicating DNA, after exposure to different dialysis membranes. (* $P < 0.01$ versus control).

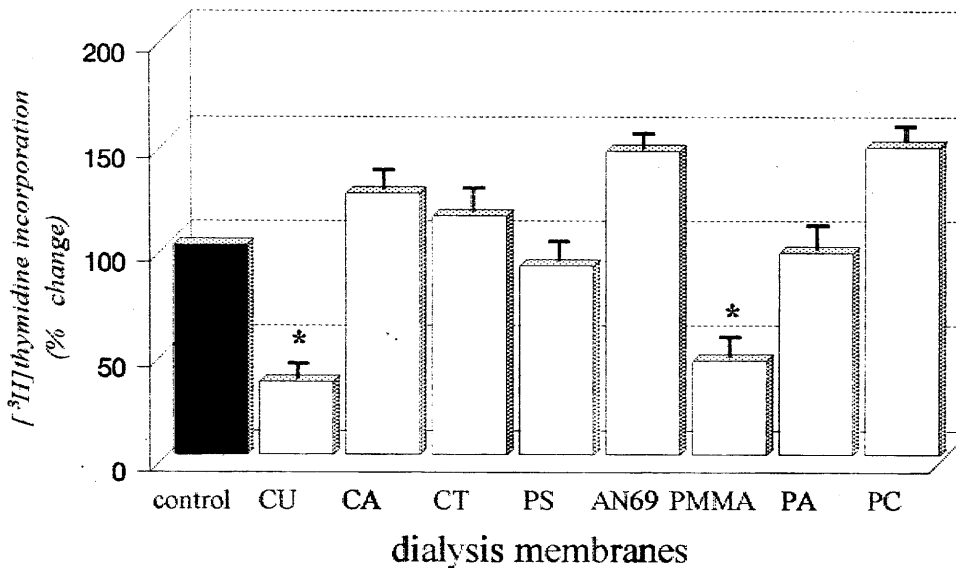


Fig. 4. Effect of IL-3 on proliferation of 32D cells previously exposed to dialysis membranes. Results are expressed as percentage change in the incorporation of [³H] thymidine in duplicating DNA, in comparison with untreated cells, labelled as 100% (**P* < 0.01 versus controls).

tested membranes, CA, CT, PS, AN69, PA, and PC, retained their ability to respond to IL-3 in a manner similar to unexposed cells.

Discussion

In the present study we describe the effect of cellulosic and synthetic dialysis membranes on intracellular second messenger generation and spontaneous or induced cellular proliferation in clonal haematopoietic cells which reproduce normal human leukocyte behaviour. Polyphosphoinositide turnover, a marker of cellular activation, initiates intracellular signal flow which eventually triggers gene transcription and DNA duplication. IP₃ and DAG generation parallels the cell adhesion to the dialyser and the time-related modulation of the leukocyte adhesion molecule expression. We previously demonstrated that IL-3 stimulated proliferation and the formation of DAG in normal 32D cells, and that DAG was constitutively elevated in transformed 32D cells which grew independently of IL-3 stimulation [7,8]. We also demonstrated that stimuli which caused 32D cell proliferation triggered an increase of DAG that was also related with the ability of the cells to efficiently repair DNA damage and sustain proliferation [8,9]. These results, obtained in the same cell clones that were used for the experiments with the dialysers, clearly demonstrate that IL-3 induces the formation of DAG in 32D cells, being DAG the only mitogenic second messenger involved in IL-3 stimulation [7], and that DAG formation is causatively linked with the induction of DNA synthesis, in 32D cells as well as in a number of other cell systems, including human tissues [5]. The present results trace a temporal relationship between cellular contact with the dialyser and cellular activation. The

previously reported cuprophane ability to directly activate early cell signalling [1] has been further sustained by the stimulation of growth in 32D cells after their exposure to this membrane; the stimulation with the cellular physiological growth factor (IL-3) has brought about a blunted response due to the cell's inability to cope with further stimuli. Apart from CU, among the other tested membranes, IL-3-dependent growth stimulation was reduced only by contact with PMMA. It has been reported that PMMA, unlike PS and AN69, activates granulocytes through a complement-independent mechanism [10]. Since no DAG formation was observed after 32D cell incubation with PMMA, the polyphosphoinositide turnover does not appear to be involved. However, cellular functions are tuned by cross-communication between different signalling systems. It could be speculated that PMMA triggers other biochemical pathways which exert a negative control on cell growth, preventing the cell from committing itself to proliferate even though stimulated with the physiological growth factor, namely IL-3. As far as the observation that polycarbonate (PC) filter stimulates DAG production and inhibits thymidine incorporation is concerned, it might be related to the interesting property of such a membrane to uncouple the DAG-dependent growth signal. These data confirm that dialysis membranes influence cellular functions at the molecular level to different degrees. In particular, it is worth pointing out that the present results can be attributed uniquely to the cell-dialysis membrane interaction, because all the experiments were carried out in a serum-free medium and in no-flow conditions, ruling out the influence of any serum component or haemodynamic effect.

In vitro experiments are controlled, short-term, acute studies and wariness should be applied in matching laboratory findings with clinical evidences. Among

patients with acute renal failure needing haemodialysis, more encouraging results have been reported when synthetic membranes, in comparison with cuprophane membrane, were used [11–13]. These results have been attributed to the well known properties of cellulosic membranes, unlike synthetic ones, in triggering complement cascade and neutrophil activation, causing a delayed recovery from acute renal failure. On the other hand, multicentre, controlled, randomized clinical trials comparing polysulphone and polyacrylonitrile with cuprophane have not found any significant difference between the tested membranes as far as the patient's intradialytic well-being and the acute complications of haemodialysis were concerned [14,15]. Moreover, the preservation of residual renal function in patients entering the haemodialysis programme does not appear to be influenced by the choice of the dialysis membranes, either polyacrylonitrile, polysulphone, or cuprophane [16]. In chronic renal failure therapy, the use of synthetic as compared to cellulosic membranes has been associated with better clinical results [13,17], although not confirmed by other studies [18,19].

The present results are in keeping with our previous observation that different dialysers show distinct effects on cell adhesion and transmembrane signalling: alterations of intracellular mitogenic signalling appear to correlate with the ability to proliferate, to respond to mitogenic stimuli and ultimately, to make a cell competent for function. Synthetic membranes seem to be less detrimental for cellular functions as compared with cuprophane: however the quality and the dose of the dialytic treatment delivered might be as important as the material used. Actually the role of comorbid states, haemodynamic parameters, blood pressure control or nutritional status should be taken into account when evaluating dialysis patients' responses to treatment. Whatever the case, these results might help to elucidate the effect of different dialysers, at the molecular level, on the blood cell; however, since *in vitro* experiments do not always mirror patient's behaviour, this study precludes conclusions on dialyser biocompatibility under *in vivo* conditions and controlled prospective clinical trials are recommended so as to provide a definite answer to the question of the role of bio(in)compatibility in treating acute and chronic renal failure patients.

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References

- Aterini S, Ippolito E, Salvadori M, Pacini S, Ruggiero M, Amato M. Second messenger formation altered by different dialysis membranes in human leukocytes. *Kidney Int* 1994; 46: 461–466
- Betz M, Haensch GM, Rauterberg EW, Bommer J, Ritz E. Cuprammonium membranes stimulate interleukin 1 release and arachidonic acid metabolism in monocytes in the absence of complement. *Kidney Int* 1988; 34: 67–73
- Hakim RM. Recent advances in the biocompatibility of haemodialysis membranes. *Nephrol Dial Transplant* 1995; 10 [Suppl 10]: 7–11
- Ruggiero M, Srivastava SK, Fleming TP, Ron D, Eva A. NIH/3T3 fibroblasts transformed by the *dbl* oncogene show altered expression of bradykinin receptors. Effect of inositol lipid turnover. *Oncogene* 1989; 4: 767–771
- Casamassima F, Pacini S, Dragotto A, Anichini M, Chiarugi V, Ruggiero M. Intracellular diacylglycerol: a mitogenic second messenger proposable as a marker of transformation in squamous cell carcinoma of the lung. *Lung Cancer* 1996; 15: 161–170
- Lapetina EG, Siliò J, Ruggiero M. Thrombin induces serotonin secretion and aggregation independently of inositol phospholipids hydrolysis and protein phosphorylation in human platelets permeabilized with saponin. *J Biol Chem* 1985; 260: 7078–7083
- Ruggiero M, Wang LM, Pierce JM. Mitogenic signal transduction in normal and transformed 32D hematopoietic cells. *FEBS Lett* 1991; 291: 203–207
- Pacini S, Santucci MA, Milano M *et al.* Increased formation of diacylglycerol in tumor cells that are resistant to ionizing radiations. *Radiat Oncol Invest* 1994; 2: 20–24
- Pacini S, Ruggiero M, Casamassima F *et al.* Study of second messenger levels and of sugar catabolism enzyme activities in transformed cells resistant to ionizing radiations. *Biochem Mol Biol Int* 1995; 37: 81–88
- Böhler J, Donauer J, Birmelin M, Schollmeyer PJ, Hörl WH. Mediators of complement-independent granulocyte activation during haemodialysis: role of calcium, prostaglandins and leukotrienes. *Nephrol Dial Transplant* 1993; 8: 1359–1365
- Schiffl H, Lang SM, König A, Strasser T, Haider MC, Held E. Biocompatible membranes in acute renal failure: prospective case-controlled study. *Lancet* 1994; 344: 570–572
- Hakim RM, Wingard RL, Parker RA. Effect of the dialysis membrane in the treatment of patients with acute renal failure. *N Engl J Med* 1994; 331: 1338–1342
- Hakim RM, Tolkoff-Rubin N, Himmelfarb J, Wingard RL, Parker RA. A multicenter comparison of bioincompatible and biocompatible membranes in the treatment of acute renal failure. (abstract) *J Am Soc Nephrol* 1994; 5: 394
- Bergamo Collaborative Dialysis Study Group. Acute intradialytic well-being: results of a clinical trial comparing polysulphone with cuprophane. *Kidney Int* 1991; 40: 714–719
- Collins DM, Lambert MB, Tannenbaum JS, Oliviero M, Schwab SJ. Tolerance of hemodialysis: a randomized prospective trial of high-flux versus conventional high-efficiency hemodialysis. *J Am Soc Nephrol* 1993; 4: 148–154
- Caramelo C, Alcazar R, Gallar P *et al.* Choice of dialysis membrane does not influence the outcome of residual renal function in haemodialysis patients. *Nephrol Dial Transplant* 1994; 9: 675–677
- van Ypersele de Strihou, Jadoul M, Malghem J, Maldague B, Jamart J. Effect of dialysis membrane and patient's age on signs of dialysis-related amyloidosis. *Kidney Int* 1991; 39: 1012–1019
- Charra B, Calzavara E, Ruffet M *et al.* Survival as an index of adequacy of dialysis. *Kidney Int* 1992; 41: 1286–1291
- Bonomini V, Colli G, Feliciangeli G, Mosconi G, Scolari MP. Long term results: cellulosic versus synthetic membranes. *Contrib Nephrol* 1995; 113: 120–134

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