Changes in the human peritoneal mesothelial cells during aging

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The number of older patients admitted to peritoneal dialysis (PD) programmes is growing. At the same time, there is increasing data about the role of mesothelial cells in determining the functional alteration of the peritoneum during PD. However, little is known about the functional changes accompanying the ageing process in mesothelial cells. We aimed to evaluate whether the aging process is accompanied by changes in some functional characteristic of the human peritoneal mesothelial cells (HPMC), which could account for the poor prognosis observed in old patients with PD. HPMCs were isolated from patients undergoing a nonurgent, nonseptic abdominal surgical procedure, without renal, vascular or inflammatory disease. Cytokine levels (by enzyme-linked immunosorbent assay (ELISA)), nitrates + nitrites, and cyclooxygenase (COX) activity (by a chemiluminescence assay), cytokines, COX, nitric oxide synthase (NOS), and nuclear factor (NF)-κB1, two messenger ribonucleic acid (mRNA) gene expressions (by reverse transcriptase (RT)-Multiplex PCR), COX, and NOS promoter gene activities, and NF-κB-dependent transcription (by transient transfection assays) were determined. Our data show a significant increase in cytokines, COX, and NOS activities, and mRNA expression of cytokines, COX-2, inducible nitric oxide synthase (iNOS) and precursors of NF-κB in HPMCs from old people. This was also the case for COX-2 and iNOS promoter gene activities and NF-κB-dependent transcription. There was a positive correlation between the age of the donor's cell and the proinflammatory profile of the HPMCs. Such age-dependent increase (around two–three times) is partially abolished by different antioxidant or free-radical scavengers. Thus, aging is accompanied by the presence of an inflammatory state in HPMCs, which involves the participation of different reactive oxygen species.

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The proportion of old patients with end-stage renal disease is growing, with the increased survival of populations in Western societies. As a consequence, the incidence and prevalence of old patients admitted to the renal replacement therapy programmes are also increasing rapidly.[1–3] Among the therapeutic approaches to such a therapy, PD meets some characteristics (haemodynamic stability, steady-state metabolic control, independence from hospital visits and avoidance of repeated vascular access) that seem to point it as the best suited option for old patients.[4] However, the scarce data comparing the outcomes in old patients undergoing PD vs haemodialysis show, with some few exceptions,[5] a trend toward a worst outcome in PD.[6–9]

Some studies suggest that mesothelial cells can be involved in any of those poorest outcomes. In this regard, a high permeability of the peritoneum has been documented in elderly patients receiving continuous ambulatory PD, which was the cause of a low serum albumin,[10] a marker of the risk of developing peritonitis.[11] Recent data highlighting the role of mesothelial cells in the structural and functional alterations of the peritoneum during PD[12] have shown that mesothelial cells undergo some changes after dialysis is initiated, and that inflammatory factors seem to play some role in this cellular transdifferentiation.

The aging process is universal, it affects every postmitotic cell and is accompanied by relevant morphologic and physiologic changes. *In vitro* models of aging have been developed for several human cell types, including fibroblasts, endothelial cells, keratinocytes, and lymphocytes. Very little is known about the senescence of HPMCs that form the largest cell population in the peritoneal cavity, and play a crucial role in intraperitoneal homeostasis and host defence. The presence of the aging process has also been reported in these cells, indeed with an increased rate compared to cells like the fibroblasts, the other main cellular component of the peritoneum.[13] However, there are very few data about functional changes accompanying the aging process in mesothelial cells. The aim of this study is to evaluate how the aging process is accompanied by changes in some functional characteristics of mesothelial cells. These changes could contribute or partially explain some of the clinical outcomes observed in old people with PD. The aging process

shares many properties with low-grade inflammatory processes (i.e. atherosclerosis), and this may also be the case in this cell type. Even more, this possibility is enhanced by the previously commented observations made in patients receiving PD, and more precisely in the oldest ones. Thus, we have focused our research on two systems closely related to inflammation (nitric oxide (NO) synthase and cyclooxygenase (COX)), and on one of the most ubiquitous transcription factor, nuclear factor κB (NF-κB), which takes part in the inflammatory process, modulating the activity of these two systems. We have also studied some of the potential relationships among these components of the inflammatory response. Finally, we studied the role of one of the most common phenomena associated with aging (oxidative stress) and whether this is involved in the activation of NF-κB and in inflammation. The study was made in human omental mesothelial cells from patients of different ages undergoing a nonurgent, nonseptic abdominal surgical procedure.

RESULTS
Age and cytokines in human mesothelial cells
The levels of interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α were measured in the supernatant of nonstimulated mesothelial cells. In every case, the concentrations were higher in cells from elderly (>65 years old) people

(old cells (OC)) than in younger ones (adult cells (AC)) (18 ± 1.50 vs 6.8 ± 2.8 pg/ml, $P \leqslant 0.001$; 32 ± 5.5 vs 9 ± 5 pg/ml, $P \leqslant 0.001$; 108 ± 24.5 vs 52 ± 10 pg/ml, $P \leqslant 0.001$, for IL-1β, IL-6, and TNF-α, respectively) (Figure 1a). Moreover, there was a significant correlation between the age of the donor and the levels of the basal cytokine production, mainly for IL-1β ($R^2 = 0.926$; $P < 0.0001$), but also for IL-6 ($R^2 = 0.709$; $P < 0.0001$) and TNF-α ($R^2 = 0.723$; $P < 0.0001$) (Figure 1b).

In this same way, the expression of messenger ribonucleic acids (mRNAs) of these cytokines and their receptors were increased in cells from old people (mean age 76 ± 3 years) compared with those obtained from the younger ones (mean age 38 ± 6 years) (Figure 2).

Age and COX
We evaluated the basal levels of activity of a critical enzyme in the inflammatory processes, COX, measured as relative luciferase units (RLUs), in the whole cell protein extracts of nonstimulated human peritoneal mesothelial cells (HPMCs). The activity of COX was increased in OC compared with AC ($10\,925 \pm 550$ RLUs in OC vs 3261 ± 480 RLUs in AC; $P \leqslant 0.001$) (Figure 3a), showing a strong correlation ($R^2 = 0.835$; $P < 0.0001$) between the age of the donors and the activity of total COX (Figure 3a). We also measured the
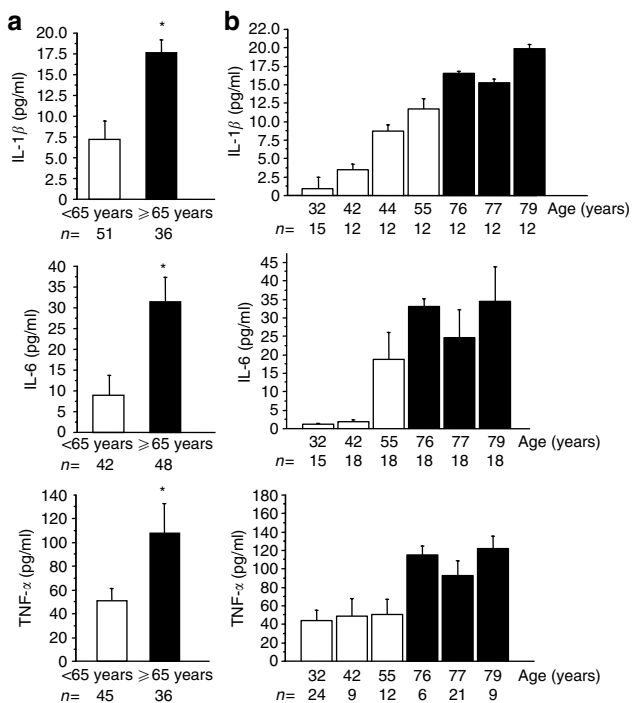
Figure 1 | Production of several proinflammatory cytokines in unstimulated HPMCs. 5×10^5 HPMCs were grown in six-well plates to confluence. The culture medium M-199 was then replaced by vehicle medium, that is, serum-free medium supplemented with 0.1% BSA for 12 h. (**a**) Levels of IL-1β, IL-6, and TNF-α were determined by ELISA assay in cell culture supernatants under the manufacturer's specifications. (**b**) Correlation between age and levels of basal IL-1β, IL-6, and TNF-α, respectively. *$P \leqslant 0.05$ vs the <65 years group.
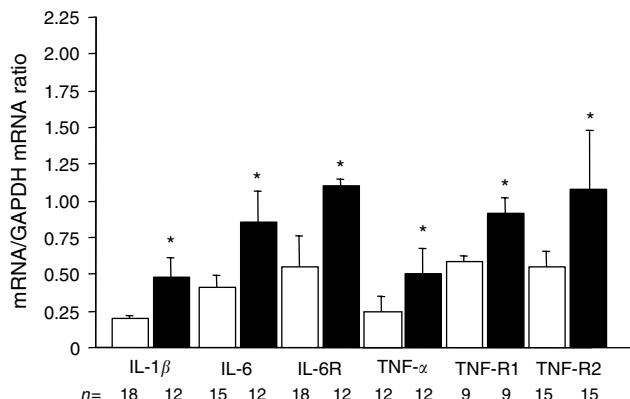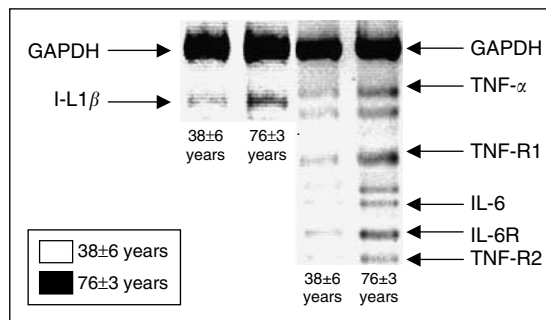
Figure 2 | Age-associated increase of proinflammatory cytokine mRNA gene expressions in unstimulated HPMCs. mRNA gene expressions of IL-1β, IL-6, IL-6R, TNF-α, TNF-R1, and TNF-R2 were carried out by RT-MPCR. A representative blot and densitometric values of the ratios for corresponding mRNAs and GAPDH mRNA are shown. *$P \leqslant 0.05$ vs the <65 years group.
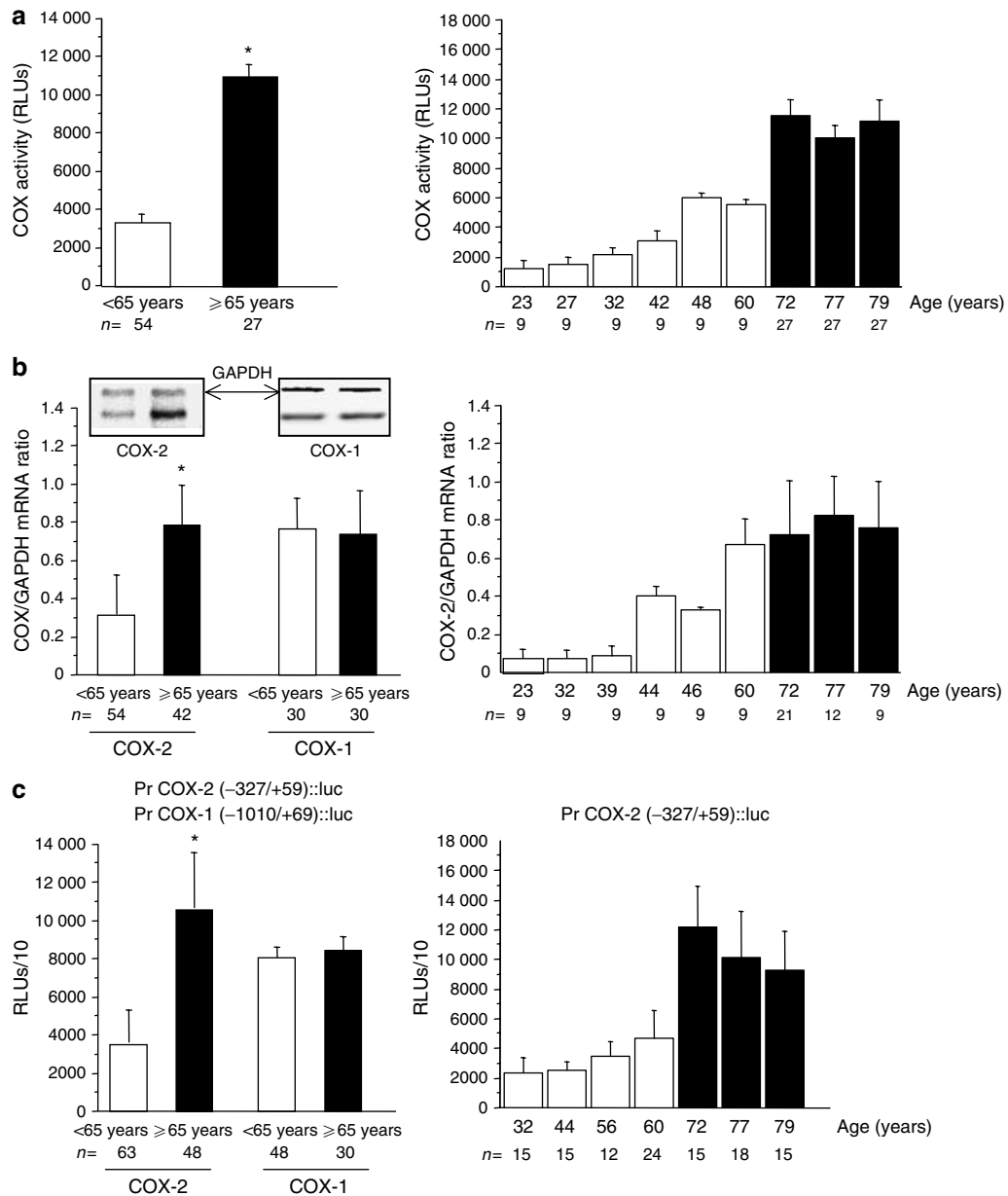
Figure 3 | Effect of age on COX-isoenzyme activity and gene expressions in unstimulated HPMCs. (**a**) Chemiluminescence determination of total COX activity in whole cell protein extracts. (**b**) Representative blots and densitometric values of the ratios for corresponding COX mRNAs and GAPDH are shown. (**c**) Transient transfection assays with 2 μg of each plasmid, phCOX-2($-327/ + 59$)-luc or phCOX-1($-1010/ + 59$)-luc were performed. (**a**, right) Correlation between age and activity of total COX, (**b**, right) values of ratios for COX-2/GAPDH mRNAs and (**c**, right) RLUs of basal COX-2 promoter activity are shown. *$P \leqslant 0.05$ vs the <65 years group.

basal mRNA expression of the two isoenzymes separately, COX-1 and COX-2. They showed a different behaviour. While we did not find any change in the ratio mRNA COX-1/mRNA GADPH (0.76 ± 0.23 arbitrary units in OC vs 0.78 ± 0.13 arbitrary units in AC, $P = 0.82$) (Figure 3b), the ratio mRNA COX-2/mRNA GADPH increased nearly two-and-a-half fold in OC compared with the cells obtained from the younger donors (0.79 ± 0.21 in OC vs 0.32 ± 0.22 in AC; $P \leqslant 0.001$) (Figure 3b). As in cytokines, there was a strong correlation ($R^2 = 0.69$; $P < 0.0001$) between the age of the donors and the expression of COX-2 mRNA (Figure 3b, right).

To further characterize this finding, and by using transient transfection techniques, we also studied the activity of two reporter plasmids bearing the human COX-1 and a short ($-327/ + 59$ bp) human COX-2 promoter. As in mRNA, the activity of the luciferase-based promoter construct was essentially the same at any age for COX-1 (8080 ± 258 RLUs in OC vs 7800 ± 426 RLUs in AC, $P = 0.69$) (Figure 3c), but showed a remarkable dependency upon age in the case of this short COX-2 promoter construct, increasing its activity in OC around three-fold as compared to the mesothelial cells from people younger than 65 years old ($10\,501 \pm 3101$ RLUs

in OC vs 3446 ± 1788 RLUs in AC; $P \leqslant 0.001$) (Figure 3c) and showing a good correlation ($R^2 = 0.551$; $P < 0.0001$) among those variables (age and COX-2 promoter–reporter activity) (Figure 3c, right).

Age and NO system

The third component of the inflammatory response that we have studied is the NO system. For this purpose, we initially evaluated the levels of NO measured as nitrites + nitrates (NOx) for 24 h at the cell culture supernatants of non-stimulated HPMCs and the expression and activity of NO synthases as the activity of their promoter constructs in transiently transfected HPMCs. As with COX, we separately studied the constitutive (endothelial nitric oxide synthase (eNOS)) and the inducible (inducible nitric oxide synthase (iNOS)) isoenzymes. In every case we studied the basal
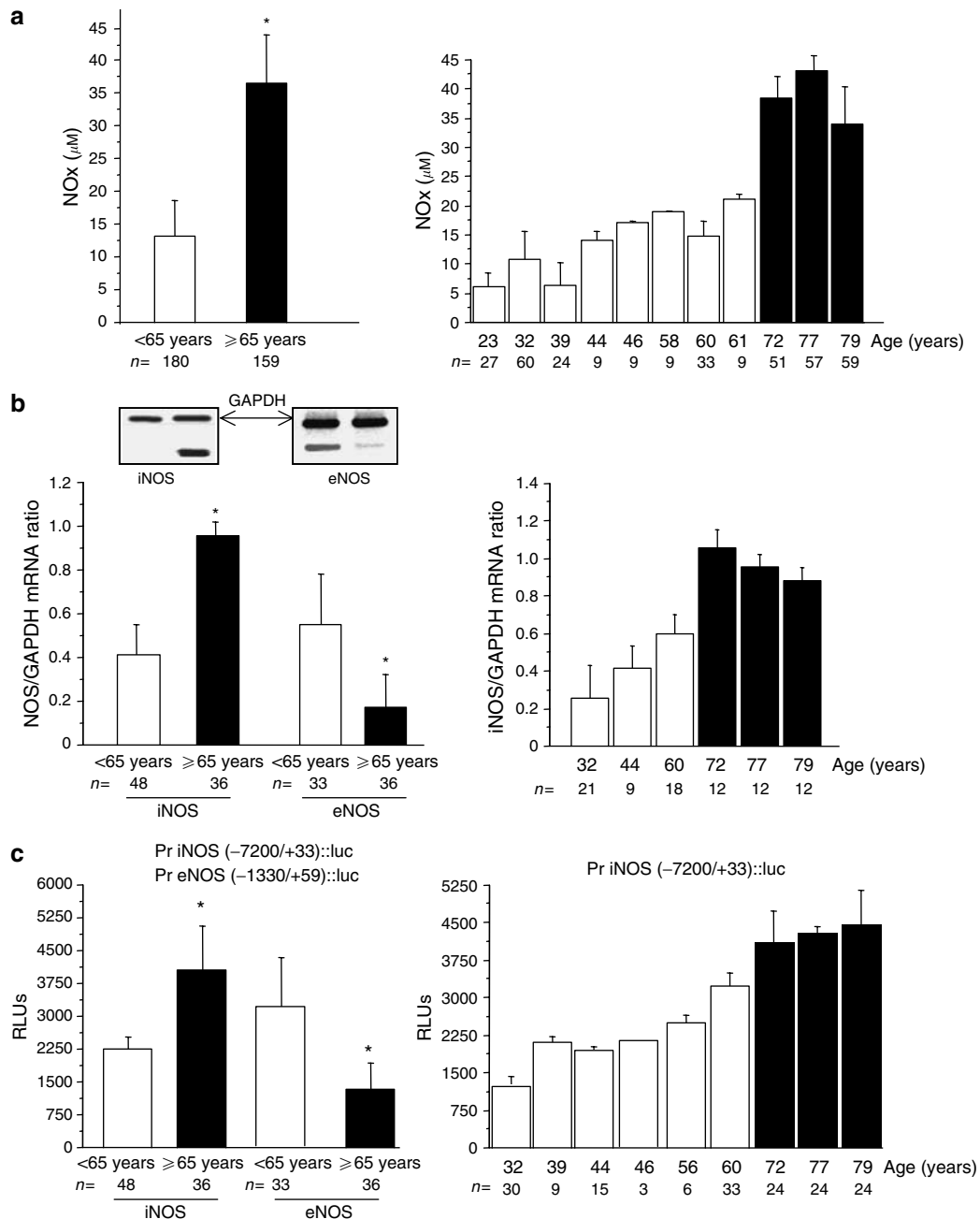
Figure 4 | Effect of age on NO production and on NOS-isoenzyme gene expressions in unstimulated HPMCs. (**a**) NOx measurements were made in unstimulated HPMC supernatants. (**b**) Representative blots and densitometric basal values of the ratios for corresponding NOS mRNAs and GAPDH were performed by RT-MPCR. (**c**) Transient transfection assays with 2 μg of each of p7.2-hiNOS-luc or p1.01-heNOS-luc and reporter plasmids were performed. (**a**, Right) correlation between age and NOx levels, (**b**, right) values of ratios for iNOS/GAPDH mRNAs, and (**c**, right) values for iNOS promoter activity are shown. *$P \leqslant 0.05$ vs the <65 years group.

situation, not using substances to stimulate or modulate the activity or expression of any of them.

The activity of NO synthase, measured by the levels of NOx, was increased in OC compared with AC (36.42 ± 7.99 vs $13.62 \pm 5.55\,\mu\text{M}$ in AC; $P \leqslant 0.001$) (Figure 4a). This increased activity was due to iNOS. The ratio iNOS mRNA/GAPDH mRNA was higher in cells from old donors (0.958 ± 0.10 arbitrary units) than in cells from younger adult people (0.412 ± 0.26 arbitrary units; $P \leqslant 0.001$) (Figure 4b). On the contrary, eNOS activity not only did not increase but also showed a statistically significant decrease. The ratio eNOS mRNA/GAPDH mRNA was lower in OC than in AC (0.21 ± 0.07 vs 0.49 ± 0.15 arbitrary units; $P = 0.011$) (Figure 4b). In agreement with these data, we found a significant correlation among age and NOx levels ($R^2 = 0.796$; $P < 0.0001$), iNOs ($R^2 = 0.664$; $P < 0.0001$) (Figure 4a and b, right), and eNOS mRNA levels ($R^2 = 0.43$; $P < 0.01$) (data not shown).

Supporting these findings, the activity of the iNOs and eNOS luciferase-based promoter constructs was parallel to that shown by the respective mRNA gene expressions. Thus, iNOs promoter activity was higher in advancing age (4151 ± 1080 vs 2275 ± 350 RLUs; $P < 0.001$) (Figure 4c, left),

with a positive correlation among age and promoter activity ($R^2 = 0.653$; $P < 0.0001$) (Figure 4c, right), while eNOS promoter activity was lower in cells from old people compared with cells from younger adult people (1225 ± 400 vs 3105 ± 766 RLUs; $P < 0.001$; $R^2 = 0.703$; $P < 0.0001$) (Figure 4c, left, and data not shown, respectively).

Age and NF-κB

We also studied one of the main transcription factors involved in the inflammatory response, the NF-κB. For this purpose, we determined NF-κB-dependent trancription basal activity in OC as well in AC transiently transfected with p5 × NF-κB-luc. Once again, we detected an increased age-dependent NF-κB activity. Mean values were higher in OC than in AC ($18\,750 \pm 3500$ vs 7000 ± 1700 RLUs; $P < 0.001$), and a strong correlation was observed between age and NF-κB-dependent trancriptional activity ($R^2 = 0.853$; $P < 0.0001$) (Figure 5a, right).

We additionally analysed the effect of age on mRNA levels of p50/105 (NF-κB1) and p49/100 (NF-κB2) in HPMCs. The best-characterized form of NF-κB is comprised of a heterodimer of 50 kDa (p50/NF-κB1) and 65 kDa (p65/RealA) proteins. RT-Multiplex PCR (MPCR) assays revealed
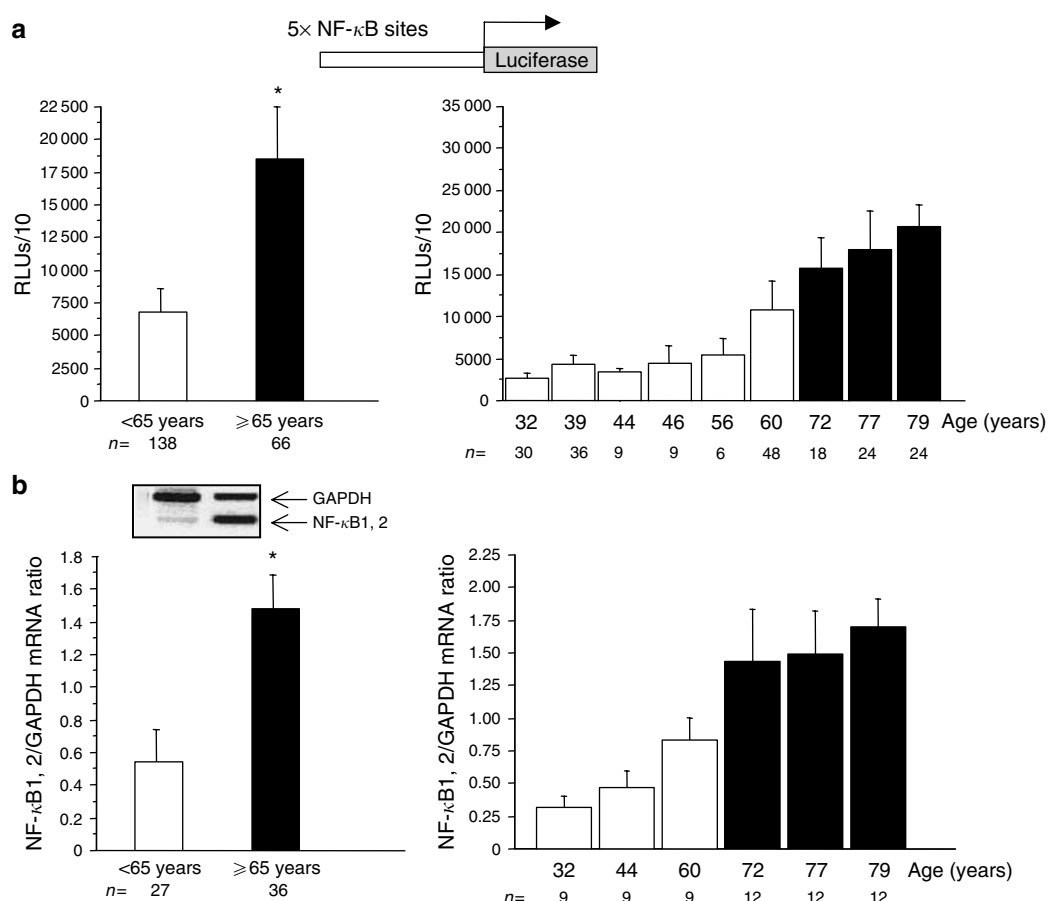
Figure 5 | Effect of age on NF-κB-dependent transcription and NF-κB1, 2 mRNA gene expression in unstimulated HPMCs. (a) Cells were transiently transfected with 2 μg of p5 × NF-κB-luc reporter plasmid. **(b)** A representative blot and densitometric values of the ratios for corresponding NF-κB1, 2, and GAPDH mRNAs are shown. **(a**, right) Correlation between cell donor's age and RLU values for NF-κB-dependent transcription activity and **(b**, right) values of ratios for NF-κB1, 2/GAPDH mRNAs are shown. *$P \leqslant 0.05$ vs the <65 years group.

an increased band ratio NF-κB1, 2 mRNAs/GAPDH mRNA in OC vs AC (1.48 $\pm$ 0.31 vs 0.54 $\pm$ 0.25 arbitrary units, respectively, $P < 0.001$) (Figure 5b), and a significant correlation was observed between age and NF-κB1, 2 mRNA gene expressions ($R^2 = 0.806$; $P < 0.0001$) (Figure 5b, right).

Effect of reactive oxygen species scavengers, indomethacin and pyrrolidine dithiocarbamate

Finally, we tried to establish a cause–effect relationship among the several substances shown to be involved in the changes observed in HPMCs with aging. To elucidate the possible mechanism(s) acting as mediator(s) on the age-dependent increased activity of NOx, NF-κB-dependent transcription and iNOS or COX-2 luciferase-based reporters observed in HPMCs from old people, we evaluated the effect of scavenging reactive oxygen species (ROS), the inhibition of COX and the inhibition of the NF-κB-dependent transcription on NF-κB activity, iNOS promoter activity and NOx levels in cells isolated from the elderly. The hand Right side of Figure 6 shows that all of the ROS scavengers used, indomethacin, as well as pyrrolidine dithiocarbamate (PDTC), reduced in a very similar percentage the activity of NF-κB-dependent transcription, iNOS promoter and NOx levels in OC (mean age 76 $\pm$ 3 years). In the experimental conditions used, neither PDTC nor all ROS scavengers (used at the same concentrations as those used in the experiments
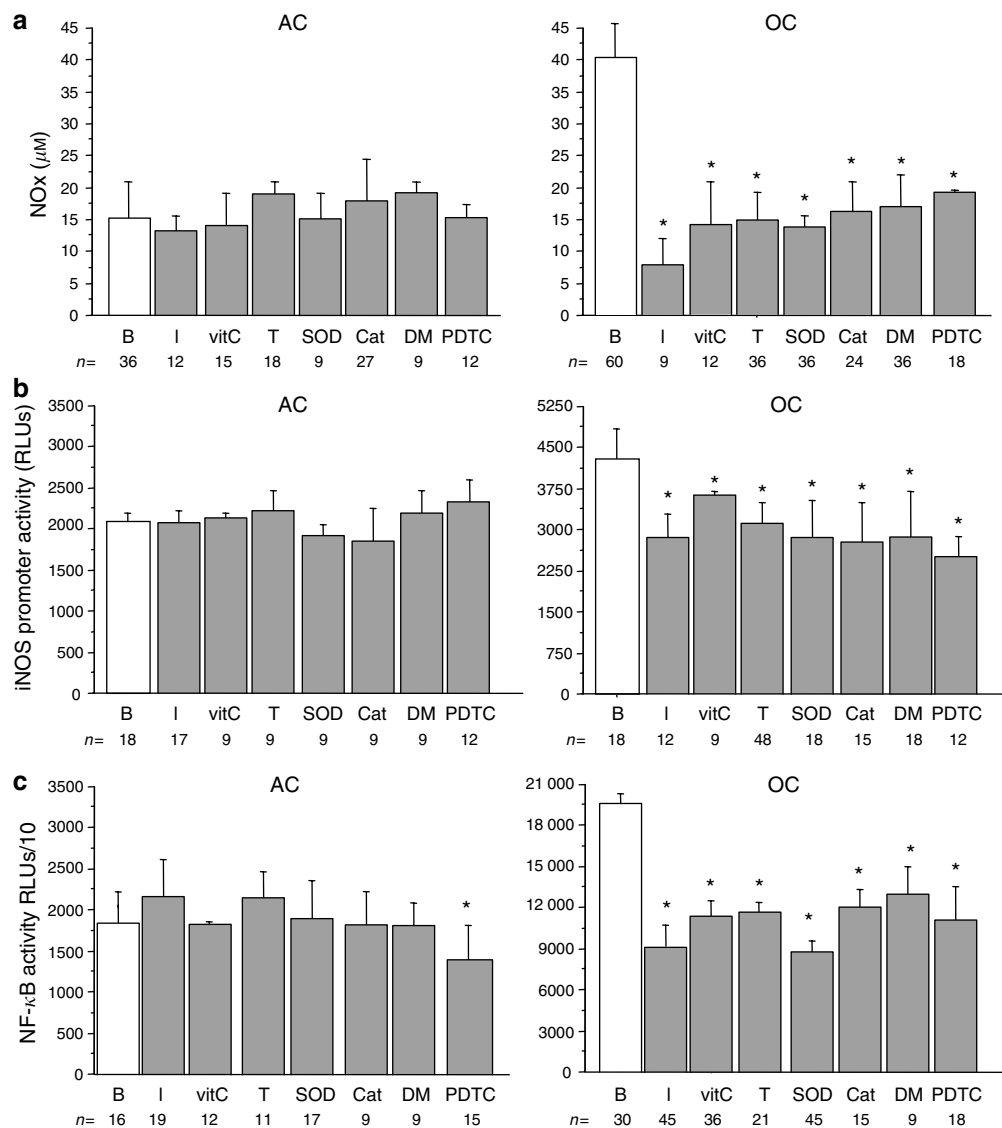
Figure 6 | Effect of different substances on the changes observed in HPMCs in the elderly. (a–c, right) HPMCs from the elderly (OC, mean age 76 $\pm$ 3 years) and **(a–c,** left) younger adults (AC, mean age 38.5 $\pm$ 17.5) were transiently transfected with 2 μg of the luciferase-based reporter plasmids (p7.2-hiNOS-luc, or p5 $\times$ NF-κB-luc) and incubated during 12 h with several antioxidant agents, such as Cu–Zn SOD (200 U/ml), Tempol (T, 100 μM), vitamin C (vitC, 10 μM), dimethylthiourea (DM, 1 mM), Catalase (Cat, 200U/ml), indomethacin (I, 10 μM) or NF-κB inhibitor PDTC (100 μM). **(a)** Following treatment with the specified agents, cell culture supernatants were used to measure NOx, **(b, c)** while the cells were harvested and lysed with passive lysis buffer for luciferase determination as per the manufacturer's instructions. *$P \leqslant 0.05$ vs untreated cells (B, basal).

carried out in OC) significantly modified basal NF-κB transcriptional activity, basal iNOS promoter activity and basal NOx levels in younger adults with AC (mean age 38.5 ± 17.5 years) (Figure 6a–c, left).

DISCUSSION

In this study, we describe that aging is accompanied by the presence of several significant changes in the functional profile of the HPMCs regarding their activity as a cell potentially involved in the generation of inflammatory events within the peritoneum. These changes seem to be causally related among them, as suggested by the effect of inhibiting COX on the NO system, and the participation of ROS as well.

The capacity of peritoneal cells from animals[14] and in HPMCs[15,16] to produce several cytokines in basal or stimulated conditions has been described some years ago. More recently, the same has been shown for another system involved in the tissue inflammatory response, the NO system.[17] Although inflammation has been implicated in several pathological scenarios, including atherosclerosis, its potential role in the changes observed in physiological processes such as aging is less known. Many experimental data have confirmed the role of inflammation during the aging process in several tissues, but there are no data about that topic in the human peritoneum.

To our knowledge, this is the first report investigating the differences in some characteristics related to inflammation according to the age of the cell's donor in the absence of inflammatory stimuli *in vivo* or *in vitro*. In our study, we show that in basal, unstimulated conditions, HPMCs show an inflammatory phenotype that is age dependent. These changes have been observed in the absence of any known condition promoting inflammatory changes. Moreover, people from whom peritoneum and mesothelial cells were obtained suffered from the same nonseptic surgical procedures. Excluding their surgical diagnosis, they did not have any other relevant medical condition, including cardiovascular diseases or risk factors, nor were they taking drugs that could potentially interfere with our results, including nonsteroidal anti-inflammatory drugs and antioxidants. Thus, the main difference between the samples was the age of the persons they were obtained from.

These changes embrace several components and molecules of the inflammatory cascade. The mechanisms taking part in these changes are modulated pretranscriptionally, as shown by the experiments of transitory transfection using different luciferase-based promoter constructs. In this regard, an increase in the activity of the promoter genes of the two systems studied (nitric oxide synthase (NOS) and COX) has been clearly shown in cells from old people, but not in those from adult persons. Moreover, the two components of those systems that exhibited some changes were the ones usually involved in the inflammatory responses iNOS and COX-2, while the mainly constitutive enzymes did not show any change or, indeed, decreased their expression. Furthermore, this change in the expression of mRNA has functional

implications, as shown by the increased activity of COX and NOx production as well in HPMCs from old donors, as compared with those from younger adult ones.

NF-κB plays a central role in the mechanisms controlling the inflammatory responses in several ways.[18,19] Indeed, NF-κB increased its activity and also showed a higher expression of NF-κB1, and/or NF-κB2 mRNAs, in cells from old subjects than in cells from younger adults, paralleling the results observed with cytokines, COX-2, and iNOS. NF-κB seems to exert a regulatory role in this process, as shown by the changes induced by the preincubation of HPMCs with its inhibitor PDTC on the NO system. Moreover, the regulatory mechanisms controlling this transcriptional factor are diverse, ranging from ROS to molecules whose synthesis is regulated by the same NF-κB (iNOS, COX). In HPMCs, NF-κB activity was decreased by several ROS scavengers, but also by indomethacin, suggesting that there are many regulatory mechanisms involved in these changes raising complex routes with their activities being regulated by one another. With our data it is not possible to establish a linear source of activation. On the contrary, our data suggest that oxidative stress and inflammation can positively modulate one other, acting as a mutual amplifying mechanism, with NF-κB playing a key role in this mechanism of activation. This nonlinear regulatory mechanism of the inflammatory processes is usual in other tissues,[19] but also in HPMCs,[20] suggesting that once the process is initiated the participating and signaling mechanisms are very unspecific, whatever was the trigger.

These findings may have clinical significance, raising a potential explanation for the increased complications related to PD in old people. It is remarkable that among the predictive factors for the development of a decrease in filtration capacity, peritoneal inflammation is one of the most relevant.[21,22] In this regard, the functional longevity of the peritoneum in patients requiring PD is highly related to the rate of peritoneal inflammation.[21] The presence of an inflammation in basal conditions due to age may account for the higher rate of filtration failure and infectious complications in old patients with PD, compromising the election of dialysis modality in spite of the best ratings obtained by PD when patients are asked about their preferences.[23] However, the finding that one of the two main components of the peritoneal cells (mesothelial cells) shows an inflammatory phenotype as it ages jointly with the fact that senescence is more prominent in these cells than in other cells of the peritoneum (fibroblasts)[13] also offers some opportunities for therapeutic intervention. These potential therapeutic interventions, targeted to limit the inflammatory component, may range from drug regimens (including anti-inflammatory therapies) to the election of dialysis solutions.[12]

CONCLUSION

In summary, we show that aging is accompanied by the presence of an inflammatory state in HPMCs, reaching its maximal activity in old people. This inflammatory state is regulated by common and well-known mechanisms, offering opportunities

to therapeutic interventions to improve the outcomes in old patients with end-stage renal disease and receiving PD.

MATERIALS AND METHODS
Materials
Culture plastic -ware was obtained from Corning-Costar (New York, NY, USA). M199 medium with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid/Hank's salt, L-glutamine and streptomycin/penicillin solution were from Biochrom KG (Berlin, Germany). Phosphate-buffered saline, foetal calf serum (FCS) and trypsin-ethylenediamine tetraacetate (EDTA) solution were from Amresco (Solon, OH, USA), Biological Industries (Beit-Hamek, Israel) and GIBCO BRM (Paisley, UK), respectively. Monoclonal antibodies against E-cadherin and von willebrand's factor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and DAKO laboratories (Hamburg, Germany), respectively. Taq DNA polymerase and DNTP mixtures were from EGOGEN (Barcelona, Spain). Unless otherwise stated, all other reagents and antibodies were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Establishment of HPMC culture
HPMCs were isolated from segments of omental tissue (1 g pieces) from 15 different donors (mean age 54.66 ± 5.08; range 23–79 years) undergoing nonurgent, nonseptic abdominal surgery (Table 1), using methods described previously.[24] Donors were free of any renal, cardiovascular, inflammatory or peritoneal disease, and were not taking anti-inflammatory drugs or antioxidants. The study was approved by the Research Committee and by the Ethics Committee of Clinical Research as well, both at the Hospital Universitario de Getafe in Madrid.

HPMCs were routinely cultured in M199 containing 1 g/l of D-glucose and supplemented with 10% FCS, 100 μg/ml streptomycin, 100 U/ml penicillin and 2.5 μg/ml amphotericin. At confluence, HPMCs were passaged using a 0.02% EDTA–0.05% trypsin solution and split in 1:2 ratio. Mesothelial cell characterization was based on both cell morphology and indirect immunofluorescence staining of several human mesothelial markers (Table 2). Human aortic smooth muscle cells, human umbilical vascular endothelial cells and human

Table 2 | HPMC characterization

Markers	HPMC	HAEC	HUVEC	HASMC
Factor VIII (vWF)	+/− (diffuse)	+++ (granular)	+++ (granular)	Negative
α-Actin	Negative	Negative	Negative	+++
E-Cadherin	++	Negative	Negative	
VE-Cadherin	+++	++	++	
Vimentin	+++	++	++	
Cytokeratin 8	++	++	++	
Cytokeratin 18	++	++	++	
PECAM-1(CD31)	Negative	++		

HPMC, human peritoneal mesothelial cells; hAEC, human aortic endothelial cells; HUVEC, human umbilical vascular endothelial cells; HASMC, human aortic smooth muscle cells.

aortic endothelial cells, used as controls of the different markers tested, were obtained using methods described previously.[25,26] All experiments were performed in passage 3–5. The morphologic and staining features of the cells remain stable during these passages.

Ribonucleic acid isolation and RT-MPCR assays
Total RNA from HPMC (10^5–10^6 cells in 90-mm diameter Petri dishes) was obtained using RNAquous®, from Ambion, Inc. (Austin, TX, USA), following the manufacturer's instructions. RT and MPCR were performed following manufacturer's instructions (Maxim Biotech. Inc., San Francisco, CA, USA). In all, 1μg of complementary deoxyribonucleic acid derived from RNA was used from each Multiplex polymerase chain reaction. MPCR kit has been designed to direct the simultaneous amplification of specific open reading frame regions of different human NOS genes and GAPDH (hNOSG-MPCR) or different human COX genes, NF-κB (NF-κB1, 2) and GAPDH (hTNF-M052G-MPCR), or several cytokines genes such as IL-6, IL-1β, TNF-α, IL-6R, TNF-R1, TNF-R2, and GAPDH genes (h-Inflammation-M053G-MPCR). Levels of mRNA were normalized to GAPDH transcript levels and expressed in relative densitometric units, using a Chemi-Imager 5.5 from AlphaInnotec, CA, USA.

Reporter plasmids
The reporter plasmids used were $5 \times$ NF-κB-luc (Stratagene, La Jolla, CA, USA), and different luciferase-based reporter plasmids corresponding to the 5′ flanking regulatory regions of human iNOS (7.2 kb hiNOS-luc)[27] or human eNOS (1.33 kb heNOS-luc)[28] or short human COX-2 (phPES2-327/+59)[29] or human COX-1 genes (phPES1 −1010/+69)[29].

Transient transfection experiments
Transient transfection experiments were performed as described previously.[30] The transfection mixture consisted of 2μg of the above-mentioned plasmids incubated with 75 μl of DMEM and 7.5 μl of Superfect® (Quiagen Gmbh, Hilden, Germany) in vehicle medium, following the manufacturer's instructions. Following treatment with the specified agents, HPMCs were harvested and lysed with passive lysis buffer and the extracts were assayed with a luciferase reporter system (Promega; Madison, WI, USA). Luciferase activity was expressed as RLUs.[30]

Measurement of nitrite plus nitrate as NOS activity
Nitrite plus nitrate production (NOx) was measured by an ozone-chemiluminescence method in confluent HPMC (5×10^5 cells), using a NO detector (NOA® 280 analyzer, Sievers, Boulder, CO, USA). A standard curve was generated by injections of known

Table 1 | Characteristics of the patients

Age	Gender	Diagnosis[a]	Drugs
23	M	Hepatic hydatidosis	No
32	F	Rectal cancer	No
39	F	Cholelitiasis	Omeprazol Lorazepam Fluoxetin
42	F	Rectal cancer	No
44	F	Rectal cancer	No
46	F	Colonic cancer	No
55	M	Colonic cancer	No
56	M	Gastric cancer	No
58	BRM	Pancreatic cancer	No
60	M	Rectal cancer	No
61	M	Rectal cancer	Omeprazol
72	F	Colonic cancer	Lorazepam Metamizol
76	F	Cholelitiasis	Bromazepam
77	M	Pancreatic cancer	No
79	M	Gastric cancer	Omeprazol

[a]Patients with a diagnosis of cancer were free of metastatic disease and peritoneal involvement.
F, female; M, male.

concentrations of sodium nitrate. The levels of nitrite plus nitrate in the cell culture supernatant were normalized to protein concentration ($1\,\mu g$). Protein contents of the cell culture supernatants and whole cell extract were determined using the BCA assay (Pierce, Rockford, IL, USA).

Measurement of COX activity

Confluent cells were used to measure total COX activity by the Cyclooxygenase Activity Kit (Stressgen Biotech, Madison, WA), detecting the peroxidative activity of COX enzymes in protein extract homogenates, as specified by the manufacturers, by a specific chemiluminescence's substrate. Light emission is directly proportional to COX activity in the sample. Results are expressed as RLUs normalized to protein content ($1\,\mu g$).

Preincubation experiments

Some experiments were performed to elucidate the way by which aging modified inflammatory mediators and effectors. For this purpose, HPMCs were transiently transfected with the different luciferase reporter plasmids described and afterwards incubated during 12 h with several antioxidant agents (200 U/ml Cu–Zn superoxide dismutase, $100\,\mu M$ tempol, $10\,\mu M$ vitamin C, 1 mM dimethylthiourea and 200 U/ml catalase), indomethacin ($10\,\mu M$) and PDTC ($100\,\mu M$). Following treatment with the specified agents, cell culture supernatants were used to measure NOx, while cells were harvested and lysed with passive lysis buffer for luciferase determination under the manufacturer's instructions.

Determination of cytokine levels

Cytokine levels in confluent cell (10^5 cells) culture supernatants were determined using human TNF-α, IL-1β, and IL-6 Instant ELISAs (The Bender Medsystems, Vienna, Austria), by generating a standard curve provided by the manufacturer and normalized to protein content ($1\,\mu g$).

Statistical analysis

Results are expressed as mean $\pm$ s.e.m. The statistical analysis was carried out using the StatView statistics programme (Abacus Concepts, Inc., Berkeley, CA, USA). Differences in the mean or in the variance were evaluated using the unpaired Student t-test or factorial analysis of variance, as required. The results obtained from the separate experiments conducted in every cell line were plotted according to the age of the donors, and a simple regression analysis was carried out to establish the potential correlation between age and each variable of interest. A two-sided P-value < 0.05 was considered statistically significant. 'n' denotes the number of experiments performed in triplicate, using cells obtained from at least three different donors.

REFERENCES

1. Letourneau I, Ovimet D, Dumont M *et al.* Renal replacement in end-stage renal disease patients over 75 years old. *Am J Nephrol* 2003; **23**: 71–77.

2. McDonald SP, Russ GR, Kerr PG, Collins JF. Australia and New Zealand dialysis and transplant registry. ESRD in Australia and New Zealand at the end of the millennium: a report from the ANZDATA registry. *Am J Kidney Dis* 2002; **40**: 1337–1339.

3. Feest TG, Rajamahesh J, Byrne C *et al.* Trends in adult renal replacement therapy in the UK: 1982–2002. *QJM* 2005; **98**: 21–28.

4. Dimkovic N, Oreopoulos DG. Chronic peritoneal dialysis in the elderly. *Semin Dial* 2002; **15**: 94–97.

5. Harris SA, Lamping DL, Brown EA, Constantinovici N. North Thames Dialysis Study (NTDS) group. Clinical outcomes and quality of life in elderly patients on peritoneal dialysis versus hemodialysis. *Periton Dial Int* 2002; **22**: 463–470.

6. Winkelmayer WC, Glynn RJ, Mittleman MA *et al.* Comparing mortality of elderly patients on hemodialysis versus peritoneal dialysis: a propensity score approach. *J Am Soc Nephrol* 2002; **13**: 2353–2362.

7. Collins AJ, Weinhandl E, Snyder JJ *et al.* Comparison and survival of hemodialysis and peritoneal dialysis in the elderly. *Semin Dial* 2002; **15**: 98–102.

8. De Vecchi AF, Maccario M, Braga M *et al.* Peritoneal dialysis in nondiabetic patients older than 70 year: comparison with patients aged 40–60 years. *Am J Kidney Dis* 1998; **31**: 479–490.

9. Kadambi P, Troidle L, Gorban-Brennan N *et al.* APD in the elderly. *Semin Dial* 2002; **15**: 430–433.

10. Nakamoto H, Imai H, Kawanishi H *et al.* Low serum albumin in elderly continuous ambulatory peritoneal dialysis patients is attributable to high permeability of peritoneum. *Adv Periton Dial* 2001; **17**: 238–243.

11. Wang Q, Bernardini J, Piraino B, Fried L. Albumin at the start of peritoneal dialysis predicts the development of peritonitis. *Am J Kidney Dis* 2003; **41**: 664–669.

12. Yáñez-Mó M, Lara-Pezzi E, Selgas R *et al.* Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. *N Engl J Med* 2003; **348**: 403–413.

13. Thomas E, al-Baker E, Dropcova S *et al.* Different kinetics of senescence in human fibroblasts and peritoneal mesothelial cells. *Exp Cell Res* 1997; **236**: 355–358.

14. Yao V, Platell C, Hall JC. Peritoneal mesothelial cells produce inflammatory related cytokines. *ANZ J Surg* 2004; **74**: 997–1002.

15. Riese J, Denzel C, Zowe M *et al.* Secretion of IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 alpha, and TNFalpha by cultured intact human peritoneum. *Eur Surg Res* 1999; **31**: 281–288.

16. Donudevani A, Rapoport J, Konforty A *et al.* Human peritoneal mesothelial cells synthesize IL-1 alpha and beta. *Kidney Int* 1994; **46**: 993–1001.

17. Chen JY, Chiu JH, Chen HL *et al.* Human peritoneal cells produce nitric oxide: induction by cytokines. *Periton Dial Int* 2000; **20**: 772–777.

18. Orange JS, Geha RS. Finding NEMO: genetic disorders of NF-κB activation. *J Clin Invest* 2003; **112**: 983–985.

19. Yan ZQ, Sirsjö A, Bochaton-Piallat ML *et al.* Augmented expression of inducible NO synthase in vascular smooth muscle cells during aging is associated with enhanced NF-κB activation. *Arterioscler Thromb Vasc Biol* 1999; **19**: 2854–2862.

20. Lee SK, Kim JH, Yang WS *et al.* Exogenous nitric oxide inhibits VCAM-1 expression in human peritoneal mesothelial cells. Role of cyclic GMP and NF-κB. *Nephron* 2002; **90**: 447–454.

21. Selgas R, Fernandez-Reyes MJ, Bosque E *et al.* Functional longevity of the human peritoneum: how long is continuous peritoneal dialysis possible? Results of a prospective medium long-term study. *Am J Kidney Dis* 1994; **23**: 64–73.

22. Chung SH, Heimbürger O, Stenvinkel P *et al.* Association between inflammation and changes in residual renal function and peritoneal transport rate during the first year of dialysis. *Nephrol Dial Transplant* 2001; **16**: 2240–2245.

23. Rubin HR, Fink NE, Plantinga LC *et al.* Patient ratings of dialysis care with peritoneal dialysis vs hemodialysis. *JAMA* 2004; **291**: 697–703.

24. Chung-Welch N, Patton WF, Shepro D, Cambria RP. *Microvasc Res* 1997; **54**: 121–134.

25. Peiró C, Lafuente N, Matesanz N *et al.* High glucose induces cell death of cultured human aortic smooth muscle cells through the formation of hydrogen peroxide. *Br J Pharmacol* 2001; **133**: 967–974.

26. Gimbrone Jr MA. Culture of vascular endothelium. *Prog Hemost Thromb* 1976; **3**: 1–28.

27. Taylor BS, de Vera ME, Ganster RW *et al.* Multiple NF-κB enhancer elements regulate cytokine induction of human inducible nitric oxide synthase gene. *J Biol Chem* 1998; **273**: 15148–15156.

28. Cieslik K, Lee LH, Tang JL, Wu KK. Transcriptional regulation of endothelial nitric oxide synthase by an interaction between casein kinase 2 and protein phosphatase 2A. *J Biol Chem* 1999; **274**: 34669–34675.

29. Inoue H, Yokoyama C, Hara S *et al*. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* 1995; **270**: 24965–24971.

30. Peiró C, Matesanz N, Nevado J *et al*. Glycated human oxyhaemoglobin activates nuclear factor-κB and activator protein-1 in cultured human aortic smooth muscle. *Br J Pharmacol* 2003; **140**: 681–690.