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NEUTROPHILS-DEPENDENT PENTRAXIN-3 AND REACTIVE OXYGEN SPECIES PRODUCTION MODULATES ENDOTHELIAL DYSFUNCTION IN HEMODIALYSIS PATIENTS

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ABSTRACT

Background: The aim of this study was to investigate neutrophil activation and its role in long Pentraxin 3 (PTX3) release and oxidative stress generation during hemodialysis (HD) and to correlate neutrophil PTX3 and oxidant expression with endothelial dysfunction.

Methods: Forty-seven uremic patients on stable HD, 12 healthy subjects and 15 patients with congestive heart failure (III and IV NYHA class), were enrolled. Neutrophils PTX3 protein expression was evaluated by confocal microscopy. L-selectin expression, intracellular PTX3 localization and Reactive Oxygen Species (ROS) generation in human neutrophils were measured by flow cytometry. NADPH-dependent superoxide generation was investigated by chemiluminescence. PTX3 plasma concentrations were measured by ELISA. Endothelial dysfunction was studied by flow-mediated dilation (FMD).

Results: The low baseline levels of FMD significantly improved after HD, but worsened by 24 hours. A significant up-regulation of PTX3 protein expression, localized within secondary granules, was detected in neutrophils isolated at 30' and 240' of HD, along with an increase in L-selectin expression. The up-regulation in intracellular PTX3 in neutrophils was associated with a significant increase in PTX3 plasma concentrations at 240'. HD increased ROS production and NADPH oxidase activity in neutrophils. In a univariate analysis, pre-treatment FMD was inversely correlated with PTX3 expression and ROS generation in neutrophils. In a multivariate analysis, both circulating pre-HD PTX3 and intracellular ROS generation by neutrophils were independent predictors of abnormal FMD.

Conclusions. Neutrophil overexpression of PTX-3 is associated with ROS overproduction and endothelial dysfunction and may represent an emerging marker of vascular damage progression in HD patients.

INTRODUCTION

Endothelial dysfunction predicts cardiovascular events in patients with chronic kidney disease (CKD) (1). This feature is linked to inflammation (2) and may enhance the risk for cardiovascular mortality in hemodialysis (HD) patients.

Despite relevant advances in HD biotechnology, the mortality rate of HD patients with serological signs of chronic inflammation still remains higher than 20% per year (3). Plasma C-Reactive Protein (CRP) has been propounded as a valuable predictive marker of cardiovascular outcome in this setting, and a relationship between CRP and mortality in HD patients has been shown in some studies (4, 5). However, Meuwese et al. showed in two independent European cohorts (MIMICK and NECOSAD), that CRP concentrations do not significantly change during a single HD session and are not associated with mortality (6).

Recent investigations emphasized Pentraxin-3 (PTX3) as a novel marker of systemic inflammation (7). Pentraxins are a superfamily of soluble pattern recognition receptors described by a cyclic multimeric structure (8). PTX3, the prototype long pentraxin, which is highly conserved between mice and humans, differs from classical short pentraxins, including serum amyloid P (SAP) and CRP, in gene organization, cellular source, regulation of the production by inducing stimuli and function (9). SAP and CRP are acute phase proteins generated by the liver for activation of the inflammatory response (10). PTX3 is rapidly generated and secreted by a variety of cell types including fibroblast, dendritic cells, vascular endothelial cells, vascular smooth muscle cells, macrophages and neutrophils (11) in response to proinflammatory molecules, including Toll-like Receptor recruitment, IL-1β, TNF-α, but not IL-6 (12, 13). PTX3 is accumulated in a ready-made form in neutrophils, localized in specific granules, and released in response to recognition of microbial moieties and inflammatory molecules (14). Beyond its expanding importance as an inflammatory marker, PTX3 has numerous additional regulatory functions in tissue repair, angiogenesis, atherosclerotic lesion development, regulation of renal immunopathology and apoptopic cell clearance (15). PTX3 is a biomarker of endothelial dysfunction indicating vascular inflammatory condition in several diseases (16) such as small vessel vasculitis (17). Furthermore PTX3 is characterized as an early marker of acute myocardial infarction in humans (18) and statin treatment significantly decreases its serum

levels (19). Chronic hearth failure (CHF) is associated to endothelial dysfunction. Moreover, functional status and severity of CHF symptoms, defined with NYHA class, are also correlated to more impaired endothelial function (20).
The aim of this study was to investigate neutrophil activation and its role in PTX3 release and oxidative stress generation during HD and to correlate neutrophil PTX3 and oxidant expression with endothelial dysfunction.

MATERIALS AND METHODS

Patient population

After giving a written informed consent according to the declaration of Helsinki, we selected 47 hemodialysis (HD) patients (three times a week for no less than 12 hours/week) for at least 1 year, based on the following inclusion/exclusion criteria: >18 years old; no clinical or laboratory symptoms of diabetes, dysfunction of coagulation system, liver disease, systemic inflammatory disease, vasculitides or neoplasia. In our population, diseases leading to end-stage renal disease were hypertensive nephrosclerosis in 11 patients (23.5%), chronic glomerulonephritis in 9 (18.14%), tubulointerstitial nephritis in 9 (20.14%), polycystic kidney disease in 2 (4.25%), congenital renal disease in 2 (4.25%) and unknown disease in 14 (29.78%). Uremic patients (22 women and 25 men; mean age 62.6±12.9 years) were treated for at least 12 months with synthetic membranes (polyamide, Gambro, Lund, Sweden; polysulphon, Fresenius, Bad Homburg, Germany) with a blood flow ranging from 250 to 320 ml/min. The control group (12 healthy subjects, six women and six men; mean age 56.2±8 years) was matched with HD patients for gender and age. Anticoagulation was performed using 1250 U/h of sodium heparin infusion during HD. No significant difference in the proportions of lymphocytes/monocytes in each sample was observed. Urea reduction rate and Kt/V remained unchanged during the study periods. Dialyzers were used only for one session. The colorimetric LAL assay (Coatest Kabi Vitrum, Stockholm, Sweden) was used for assessing the endotoxin content (< 0.03 EU/ml) in the dialysate. Furthermore, 15 patients (mean age 59.3 ± 7,7 years) with congestive heart failure [CHF], III and IV NYHA class, on medical therapy according to ESC guidelines for acute and chronic heart failure were also enrolled (21).

Biochemical analyses

Blood samples were taken from patients and controls in the morning after 12 hours of fasting for the assessment of human serum albumin, C-Reactive Protein (PCR), urea, calcemia, phosphatemia, potassemia, PTH, ferritin, hemoglobin, leukocytes, platelets, total cholesterol, triglycerides, HDL cholesterol, using routine laboratory techniques.

Isolation of peripheral blood human neutrophils (PBN)

Peripheral blood samples were collected before the HD session (Time 0) and from the arterial line at 15, 30 and 240 min (Time 240) after starting HD. The blood samples were collected in sterilezed tubes with K-EDTA as anticoagulant. PBN were isolated from 12 healthy subjects (control) and 12 HD patients at T0-T240. After Ficoll-Paque centrifugation, PBN were divided from erythrocytes by 3% Dextran density gradient centrifugation and centrifuged in gradients of Percoll 60% (GE Healthcare, Milan, Italy) under physiological conditions (280-320 mOs/kg H2O) to enrich for cell populations. Finally, PBN were washed, counted, and resuspended in phosphate-buffered saline to desired concentration. This method was shown to yield samples of >95% PBN with >95% viability. The purity of cells was evaluated by Flow Cytometry acquisition and was >98%. In vivo-activated PBNs were identified by evaluating CD16b, CD18, CD3, CD14 (Beckman Coulter Inc., Brea, CA) and L-selectin (CD62L, Immunotec Inc., Vaudreuil, QC) protein expression on cell surface; only CD62L+ (L-selectin+) - /CD16+ neutrophils were used.

Antibodies

The following primary antibodies were used in this study: rat polyclonal anti-human PTX3 (clone MNB4, Exira Life Sciences Inc., Larsen, Switzerland) for immunofluorescence and FACS analysis; mouse monoclonal anti-human MMP-2 (CA-4001, Novus Biologicals Inc., Littleton, CO) for immunofluorescence; CD62L and CD 16b-FITC (Beckman Coulter Inc.) for FACS analysis. As secondary antibodies we used Alexa Fluor 488-conjugated goat anti-rat IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) for immunofluorescence and PE-labeled anti–rat IgG (clone RG7, Becton Dickinson, East Rutherford, NJ) for FACS analysis.

Cell Immunofluorescence and Confocal Laser Scanning Microscopy

PTX3/MMP2 co-localization was evaluated on PBN cytospins (HD n=5; control n=5) by confocal microscopy. PBN were collected at the start and at the end of the second HD session of the week. Then, cytospins were fixed with 3.7% paraformaldehyde (PFA) for 15 min at room temperature (RT) and quickly permeabilized for 5 min with TRITON X-100 (Sigma-Aldrich) at a 0.25% concentration in PBS (pH 7.4), before specific incubation for 1 h at RT with a blocking solution of 10% Goat Serum (Sigma-Aldrich) and then for 2h with following anti-PTX3 primary antibody at dilution 1:50 and anti-MMP2 at dilution (1:50) respectively in a humidified chamber. After washing, PBNs were incubated for 1 h at RT with Alexa Fluor 488-conjugated goat anti-rat IgG (1:200) and Alexa Fluor 555-conjugated goat anti-mouse IgG (1:200), following secondary antibodies respectively. Labeled cells were washed 4 times with PBS, counterstained with TO-PRO-3 (Molecular Probes, Eugene, Oregon, USA) for 10 min, air-dried, mounted using Gel/Mount aqueous mounting medium (Biomeda, Milan, Italy) and finally closed. Negative controls were obtained missing the antigen specific antibodies. The stained cells were acquired using the Leica TCS SP2 (Leica Microsystems GmbH, Wetzlar, Germany) laser-scanning confocal microscope equipped with helium-neon (633 nm), green-neon (543 nm) and argon krypton (488 nm) lasers. Calculated original magnifications were x 400.

Flow Cytometry

Fifty µl of EDTA peripheral blood from 12 HD patients and 5 controls were labeled with FITC-conjugated CD16b antibody (Beckman Coulter, Milan, Italy). Cells were then washed, fixed and permeabilized with IntraPrepTM Permeabilization Reagent kit, according to the manufacturer's instructions (Beckman Coulter). Unconiugated PTX3 mAb (clone MNB4) was incubated for 25 min at RT. Cells were washed and stained with secondary PE-labeled anti–rat IgG monoclonal antibodies (clone RG7, BD) for 25 min at RT in the dark. Finally, cells were washed twice and resuspended in FACS buffer for acquisition. A FC500 flow cytometer (Beckman Coulter) was used to acquire the stained cells and CXP Software to analyze the data. An isotype-matched monoclonal antibody was

used to determine the area of positivity and a total of 10^4 events for each sample were acquired.

Analysis of oxidative burst in human neutrophils

The oxidative burst was quantified by evaluating of the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma, Milan, Italy) in PBN (HD, n=10 and control, n=5) as previously described (22). Reactive oxygen species (ROS) production is reported in histograms and expressed as the number (%) of positively stained cells.

NADPH oxidase assay in human neutrophils

NADPH oxidase activity was evaluated by chemiluminescence method (lucigenin) in PBNs (CKD, n=5 patients; HD, n=10 patients and control, n=5) as previously described (23).

ELISA Assay for plasma PTX3 levels

Plasma PTX3 levels were quantified using a commercially available sandwich ELISA (Quantikine Human Pentraxin3/TSG-14), according to the manufacturer's instructions (R&D Systems Inc., Minneapolis, MN). The sensitivity of detection was 0.025 ng/ml and the coefficient of variation of both inter- and intra-assay was 5.0%. All plasma samples were run in duplicate and analyzed in the same time.

Evaluation of Flow-Mediated Dilation (FMD)

FMD of the brachial artery was assessed non-invasively, using high-resolution ultrasound in a quiet, air-conditioned environment (22-24°C) (24). The subjects of the study were fasted for at least 8-12 hours. The study was performed using a software of image analysis system, certified by the CNR of Pisa (MVE II).

Statistical Analyses

Data are presented as the mean ± Standard Deviation (SD) and are compared by unpaired t-test analysis or unifactorial ANOVA test, as appropriate. Pearson's correlation test was used to study continuous variables. Differences were considered statistically significant when p value was less than 0.05. Statistical analysis was performed using the StatView Software package (5.0 version; SAS, Inc., Cary, NC).

RESULTS

Clinical data

Table 1 depicts clinical features of patients, healthy subjects and CHF patients included in the study. We did not find any significance difference in demographic and inflammatory status between healthy subjects (Controls), HD patients and CHF patients. HD treatment did not change leukocyte and neutrophil counts at any time.

Effects of HD on brachial artery reactivity

The low baseline FMD values observed in HD patients significantly (<0.001) improved after HD (pre 4.48±1.34%, post 6.76±1.43%), but slightly decreased by 24 hours (6.02± 2.02%) (**Figure 1)**. No difference in FMD was observed between HD patients before dialysis and patients with CHF (4.63±1.24%, p=ns).

Neutrophils expression and circulating levels of PTX3 during HD session

Spontaneous activation of purified neutrophils ($CD16b^+$) was evaluated on cells isolated at the beginning and during dialytic treatment by examining the expression of CD62L (L-selectin), a leukocyte adhesion molecule for endothelium; only $CD62L^+/CD16^+$ neutrophils were analyzed. A slight reduction of CD62L expression was observed after 15 min (T15) and 30 min (T30) of HD. This reduction was only transient, and CD62L expression increased significantly after 240 min (T240) of HD (p=0.013 vs T0) (**Figure 2A**).

To evaluate the ability of neutrophils to synthesize PTX3, we investigated intracellular PTX3 expression on whole blood by FACS analysis gating on neutrophils ($CD16b^+$, $CD18^+$). We observed a rapid and significant increase in PTX3 expression at 30 min of HD (p=0.0003 vs T0) that became even more relevant at the end of HD (p=0.00001 vs T0) (**Figure 2B**).

The intracellular localization and the PTX3 expression in neutrophils isolated at T0 and at T240 were investigated by confocal microscopy. We observed a clear co-localization of PTX3 and MMP2 specific fluorescence in secondary granules, particularly at the end of HD (**Figure 2C**).

At the beginning of HD circulating PTX3 levels were significantly higher (2.4±0.6 ng/ml) in HD population than healthy subjects (1.1±0.2 ng/ml, p=0.003) (**Figure 2D**). Interestingly, we observed a further increase in PTX3 levels at the end of the dialytic session (3.8±1.0 ng/ml; p=0.005 compared to T0).

NADPH-dependent ROS generation in neutrophils

We measured ROS production in neutrophils freshly isolated from HD patients and healthy subjects. ROS generation was significantly greater (p=0.002) in HD patients than in controls **(Figure. 3A).** At T240, HD patients showed a significantly increase in ROS production compared with T0 (p=0.02). Pre-incubation with the ROS scavenger, N-acetylcysteine (NAC) and NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) significantly inhibited ROS production (data not shown). To investigate the role of NADPH oxidase in ROS generation, we tested its activity in neutrophils of HD patients. NADPH dependent superoxide generation was significantly increased in neutrophils of HD patients compared with controls (p=0.01) (**Figure. 3B**).

Relationship between intracellular PTX3, oxidative stress and endothelial dysfunction

FMD value pre-HD was inversely and significantly correlated with intracellular levels of pre-HD PTX3 (R^2=0.57; p=0.0001) and ROS production by neutrophils (R^2=0.633 p=0.0001) (Figure **4A-B**). Intracellular pre-HD PTX3 was directly correlated with ROS production by neutrophils (R^2=0.41; p=0.002) (**Figure 4C**). Table 2 shows the association between pre-HD FMD and the main clinical and experimental variables by linear regression analysis. In a multivariate analysis, both circulating pre-HD PTX3 and ROS production by neutrophils were independent predictors of abnormal FMD in HD patients (p=0.009) (Table 3).

DISCUSSION

In the present study we showed for the first time that PTX3 released from neutrophils is involved in endothelial dysfunction in HD patients and we provided evidence that this effect is associated to NADPH dependent ROS production.

PTX3 has emerged as a key acute-phase protein associated with inflammation in cardiovascular disorders, including heart failure, atherosclerosis, acute coronary syndromes, and peripheral vascular diseases (25). Moreover PTX3 serum concentrations are associated with increased carotid intima-media thickness (cIMT) in patients with high cardiovascular risk (26-27). More importantly, the predictive value of PTX3 appears to be independent of other risk factors, including markers of the same superfamily, including CRP. It was recently observed that the rise in PTX3 after a single HD session was larger than for other plasma inflammatory biomarkers (28). We observed a significant increase in circulating PTX3 plasma levels during dialysis supporting the idea that PTX3 could be an early indicator of the activation of innate immune response (29). Simultaneously, we observed a rapid and significant increase in PTX3 expression in neutrophils with a clear co-localization of PTX3 and MMP2 (collagenase IV) in specific secondary granules at the end of HD. In our study HD treatment increased intracellular PTX3 expression. The PTX3 storage in specific secondary granules may support the hypothesis that this inflammatory molecule can be secreted upon appropriate stimulation (14), including blood–membrane contact during HD. L-Selectin (CD62L) is expressed on most leukocytes and appears to mediate lymphocyte binding to endothelial venules of peripheral lymph nodes, as well as lymphocyte, neutrophil and monocyte attachment to endothelium at areas of inflammation (30). Our results demonstrate that the increase in CD62L neutrophil expression is a dialysis-related phenomenon. CD62L is important for leukocyte attachment to endothelium at sites of inflammation, leading to endothelial dysfunction and atherosclerosis (31). Endothelial dysfunction is linked to inflammation and may increase the risk for cardiovascular disease (CVD) mortality in dialysis patients (32). Chronic inflammation associated with uremia may induce an increase in oxidative stress in HD patients (33).

Furthermore, the HD treatment itself is a source of oxidative stress by generating ROS through activation of circulating neutrophils (34). Oxidative stress plays a key role in the initiation and progression of atherosclerosis, and scavengers of ROS decrease LDL oxidation and reduce plaque development (33**)**. NADPH oxidase is the main enzyme involved in superoxide radicals generation. The enzyme has a key role in neutrophils and monocytes host defense. An increased phagocytic NADPH oxidase activity and elevated circulating oxidized LDL has been shown in patients with metabolic syndrome (35).

In addition, the overexpression of NADPH oxidase in vascular cells from atherosclerotic lesions supports the role for this enzyme in the pathogenesis and progression of atherosclerosis (36). Finally, NADPH oxidase–dependent superoxide production is increased in mononuclear cells from patients with CKD and HD treatment (37,38).

In this study, a ROS overproduction and a significant upregulation of NADPH-dependent superoxide generation was observed in neutrophils isolated from HD patients suggesting a pivotal role of “activated neutrophils” in generating an oxidative status in these patients. In addition, we observed a close association between PTX3 expression and ROS generation in circulating neutrophils at the beginning of dialysis.

Recently Witasp et al showed that adipose tissue PTX3 mRNA levels are associated with endothelial dysfunction in patients with CKD (39). In addition, they demonstrated an inverse correlation between the vasal response and PTX3 overexpression in the endothelium of subcutaneous arteries. Interestingly, the absence of correlations between basal vascular tone and other inflammatory markers, including IL-6, TNF-α, CD68 and MCP1, suggests that PTX3 per se plays a pivotal role in the regulation of endothelial function. In addition, the observation that PTX3 weakly correlates to these inflammatory markers supports the hypothesis that increased PTX3 may linked to endothelial dysregulation in uremic patients (39).

PTX3 could exacerbate endothelial dysfunction, at least partially, through IKK/IκB/NF-κB activation and overexpression of iNOS and NO (40). In the present study the endothelial function was assessed by the most common non-invasively

techniques, FMD of the brachial artery, which represents the “gold standard” for clinical research on the role of the endothelium in the cardiovascular physiopathology. For the causal relationship with clinical end point and the ability to anticipate the clinical benefits in intervention studies, the FMD can be considered as a surrogate marker of atherosclerosis (41). A recent study showed that plasma PTX3 is significantly correlated with endothelial function assessed by FMD in patients with clinically stable Coronary Artery Disease (42). Furthermore, the changes in PTX3 and FMD also correlated, indicating that PTX3 levels are a potential biomarker for predicting endothelial dysfunction. Finally, multivariate stepwise regression analysis showed that PTX3, but not hsCRP, is an independent factor associated with FMD. Furthermore Yilmaz MI et al (43) demonstrated that PTX3 and intima-media thickness increased, whereas FMD and soluble TNF-like weak inducer of apoptosis (sTWEAK) decreased across CKD stages. Both PTX3 and sTWEAK appeared as strong determinants of FMD in multivariate analysis; in a model excluding sTWEAK, circulating levels of PTX3 were directly associated with cardiovascular outcomes independently of basic confounders, but this association was lost after adjustment for FMD (43). In our study we found that FMD value before HD was inversely and significantly correlated with intracellular pre-HD PTX3 and ROS production by neutrophils, suggesting for the first time the crucial role of uremic neutrophil as an intermediate node in PTX3-pathways triggering endothelial aberrations and cardiovascular disease. In a multivariate analysis, both circulating pre-HD PTX3 and intracellular ROS production by neutrophils were independent predictors of abnormal FMD, supporting the hypothesis that neutrophil derived oxidative stress may represent a link between chronic inflammation and endothelial dysfunction in HD patients. We did not observe a significant association between post-HD PTX3 and FMD since the latter at this time point is considerably influenced by the change in extracellular volume featuring the post-HD period. This influence is suggested also by the rapid decline in FMD levels in the following hours. In addition, the increase in PTX3 at the end of HD session is unlikely to affect immediately FMD since all the mechanisms hypothesized need time to finally influence endothelial cell function.

Ultimately, our finding is of particular interest because PTX3 may be a potential therapeutic target in inflammation- or atherosclerosis-related diseases. In this

regard, short-term angiotensin-converting enzyme inhibitor treatment significantly improved endothelial function and normalized both PTX3 and urinary protein excretion in type 2 diabetic proteinuric patients (44). In addition, the improvement in FMD after combined therapy with the renin–angiotensin system and calcium channel blockers was independently associated with both PTX3 and sTWEAK normalization in type 2 diabetic hypertensive patients (43). The pharmacological relevance of targeting PTX3 in relation to clinical benefits would represent therefore a main subject of investigation for further studies. It is well known that chronic heart failure is associated with endothelial dysfunction and this latter represents a predictive factor of disease progression, cardiac death and hospitalization (45). In particular, in heart failure patients endothelial dysfunction has been related to NYHA functional class, and congestive CHF subjects showed a worst vascular function (46). In this study we found an impairment of endothelial function in CHF patients. Interestingly, the FMD values of this group were similar to those of pre-HD patients.

CONCLUSIONS

Increased neutrophil overexpression of PTX3 and ROS are associated with an impaired endothelial function and may represent an emerging inflammatory marker involved in the progression of vascular damage in HD patients.

TABLE 1. Clinical and laboratory data in healthy control subjects (Control), patients with congestive heart failure (CHF) and hemodialysis patients (HD).

	Control	CHF	HD	P-value
Number	12	15	47	**/**
Gender (M/F)	6/6	10/5	25/22	NS
Age (years)	57.2 ± 8	59.3±7.7	62.6 ± 12.9	NS
Time on dialysis (months)	0	0	33.9 ± 32.4	/
CVD (Yes/No)	0/12	15/15	19/29	0.0001
BMI (kg/m2)	22.9± 0.9	21.9± 0.8	21.7 ± 1.01	0.01
Phosphorus (mg/dl)	4,12 ± 0,5	4,22 ± 0.4	4.9 ± 1.2	0.02
Calcium (mg/dl)	9.24 ± 0.4	9.44± 0,5	9.03 ± 0.7	NS
PTH (ng/ml)	43.25 ± 16.9	42.25 ± 15.9	392.9 ± 198.3	0.0001
Cholesterol total (mg/dl)	155.8 ± 22.4	160.9±14.3	159.8 ± 35.3	NS
Cholesterol HDL (mg/dl)	46.1 ± 7.4	47.9±8,51	42.9 ± 14.8	NS
Triglycerides (mg/dl)	80.1 ± 21.2	159.6±16.70	148.2 ± 73.5	0.003
hs-CRP (mg/dl)	0.4 ± 0.29	0.5 ± 0.39	0.9 ± 1.1	NS
Ferritin (ng/ml)	107.2 ± 27.8	118.9±10.0	390.4 ± 264.5	0.0005
Hemoglobin (g/dl)	13.9 ± 0.4	12.4±1.2	11.1 ± 1.7	0.0001
Leukocytes (x10^3)	6.8 ± 1.6	6.6±1.4	6.4 ± 1.9	NS
Platelets (x10^3)	220.2 ± 60.2	216.5±24.8	219.8 ± 56.7	NS
Systolic blood pressure (mmHg)	128.2 ± 13.4	117.7±15.1	131.1 ± 24.3	NS
Diastolyc blood pressure (mmHg)	76.5 ± 11.6	73.0±10.5	78.6 ± 13,5	NS
SIV	9.9 ± 0.6	11.9±1.5	11.7 ± 1.8	0.002

Data are presented as mean±SD. M/F indicates males/females; CVD, cardiovascular disease; BMI, body mass index; PTH, Parathyroid hormone; hsCRP, high-sensitivity C-Reactive Protein; SIV, interventricular septum.

Table 2. Association between inflammation, endothelial dysfunction and oxidative stress markers using linear regression analysis.

	Anagraphic age (years) P-value	Dialytic age (months) P-value	hs-CRP (mg/dL) P-value
FMD pre-HD (%)	0.0033	< 0.0001	< 0.0001
PTX3 concentrations pre-HD (ng/mL)	0.018	< 0.0001	< 0.0001
Intracellular pre-HD PTX3 (% positive cells)	< 0.0001	< 0.0001	< 0.0001
ROS production by neutrophils (% positive cells)	0.0012	< 0.0001	< 0.0001

Table 3. Multiple regression analysis for FMD.

	Coefficient	Std. Error	Std. Coeff.	t-value	P-value
Intercept	13.808	1.835	13.808	7.525	<0.0001
Age (years)	-0.017	0.016	-0.131	-1.053	0.3114
Time on dialysis (months)	0.0006	0.009	0.104	0.625	0.5429
hs-CRP (mg/dL)	-3.91E-4	0.219	-2.206E-4	-0.002	0.9986

Plasma PTX3 concentrations pre- HD (ng/mL)	**-0.556**	**0.308**	**-0.237**	**-1.808**	**0.0937**
Intracellular pre-HD PTX3 (% positive cells)	**-0.044**	**0.031**	**-0.261**	**-1.435**	**0.1750**
Intracellular ROS production	**-0.111**	**0.036**	**-0.615**	**-3.059**	**0.0091**

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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FIGURE LEGENDS

Figure 1. **Measure of the endothelial dysfunction assessed by flow-mediated dilation (FMD) in uremic patients on stable haemodialysis (HD) treatment and congestive heart failure patients.** FMD of brachial artery was assessed non-invasively, using high-resolution ultrasound. Congestive CHF patients showed similar FMD values to pre-HD patients. HD treatment increased FMD from 4.23±1.6% to 7.03±3.3% ($p<0.0001$). These changes returned to baseline by 24h (5.59±1.9%, $p=0.05$); p values were calculated by t-test.
*$p=0.0001$ vs Pre-HD; $^{\$}p=0.016$ vs pre-HD

Figure 2 A. Expression of CD62L on neutrophils surface during hemodialysis session. The graph represents the mean±SD of CD62L expression level determined by FACS, in neutrophils isolated from 12 HD enrolled at the beginning (T0, Pre-HD Session), after 15-min (T15), 30-min (T30) and at the end of HD treatment (T240, Post-HD Session). HD was associated with a rapid, but not significant reduction, in CD62L at 15- (T15) and 30-min (T30) samples, respectively. CD62L expression levels increased significantly after 240 min dialysis (T240) ($p=0.013$ vs T0). P values were calculated by t-test. The shaded line represents the normal expression level.

Figure 2 B. Production of intracellular PTX3 by neutrophils during a dialysis session. The graph represents the mean±SD of intracellular PTX3 protein level determined by FACS, in neutrophils isolated from 12 HD enrolled at T0, T15, T30 and T240. HD was associated with a clear PTX3 specific fluorescence at the beginning of dialytic session (T0). Thirty minutes of dialysis 30' (T30) caused a significantly increase of PTX3 expression ($p=0.0003$ vs T0), which further increased at T240 ($p=0.00001$ vs T0); p value were calculated by t-test. The shaded line represents the normal expression level.

Figure 2 C. Coexpression of PTX3 and MMP2 in HD patients and healthy subjects (Control). Representative image of neutrophil-specific granules isolated from HD patients at T0 (panel A), T240 (panel B) and Control (panel C), showing a colocalization of PTX3 (green) and MMP-2 (red) in specific granules at T240, by double immunofluorescence. Nuclei were stained with topro (Blue). The arrows indicate the enlarged cells in the zoom. Magnification x 400. Control group (n=5); HD T0 (n=5); HD T240 (n=5).

Figure 2 D. Plasma PTX3 levels in blood samples collected by healthy subjects (Control) and HD patients.
The histograms represent the mean±SD of PTX3 concentrations, determined by ELISA, in plasma of 12 controls and 47 HD patients enrolled at the beginning (T0) and at the end of HD treatment (T240). Mean plasma PTX3 levels were significantly higher at T0 as compared to controls ($p=0.003$). A further significant ($p=0.02$)

increase in plasma PTX3 levels was observed at T240 as compared to T0; p values were calculated by t-test.

Figure 3 A. ROS production in neutrophils isolated from healthy subjects (Control) and hemodialysis (HD) patients.
Intracellular ROS levels were investigated by DCF-DA in neutrophils isolated from 10 healthy subjects (controls), and 10 HD patients enrolled at the beginning (T0) and at the end of HD treatment (T240), as described in Material and Methods section. A significant higher generation of intracellular ROS was observed at T0 compared to controls (p=0.002). At T240 HD patients showed significantly (p=0.02) higher levels of intracellular ROS generation than T0; p values were calculated by t-test.

Figure 3 B. NADPH-dependent superoxide generation in HD patients and Controls. NADPH oxidase activity was measured by lucigenin-enhanced chemiluminescence in neutrophils isolated from 10 HD patients and 12 healthy subjects (Controls). NADPH oxidase activity was significantly increased in neutrophils from HD patients versus control both at T0 (p=0.006) and T240 (p=0.002); p values were calculated by t-test.

Figure 4. Correlation between FMD value pre-HD, intracellular pre-HD PTX3 and ROS production by neutrophils haemodialysis (HD) patients. FMD value pre-HD was inversely and significantly correlated with intracellular pre-HD PTX3 (R^2=0.57; p=0.0001) **(A)** and ROS production by neutrophils (R^2=0.633; p=0.0001**) (B)**. Intracellular pre-HD PTX3 was directly correlated with ROS production by neutrophils (R^2=0.41; p=0.002) **(C)**.

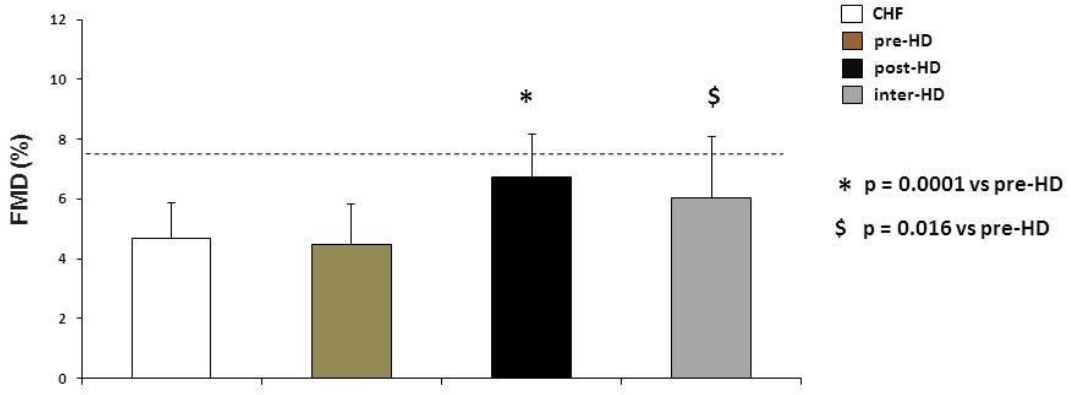

Figure 1

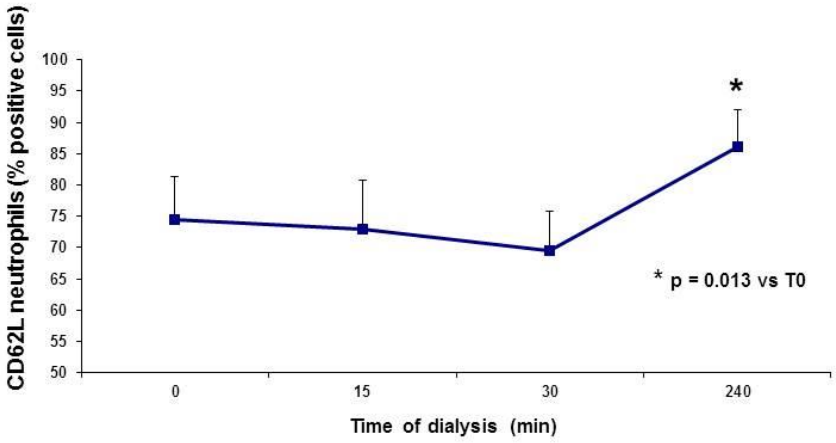

Figure 2A

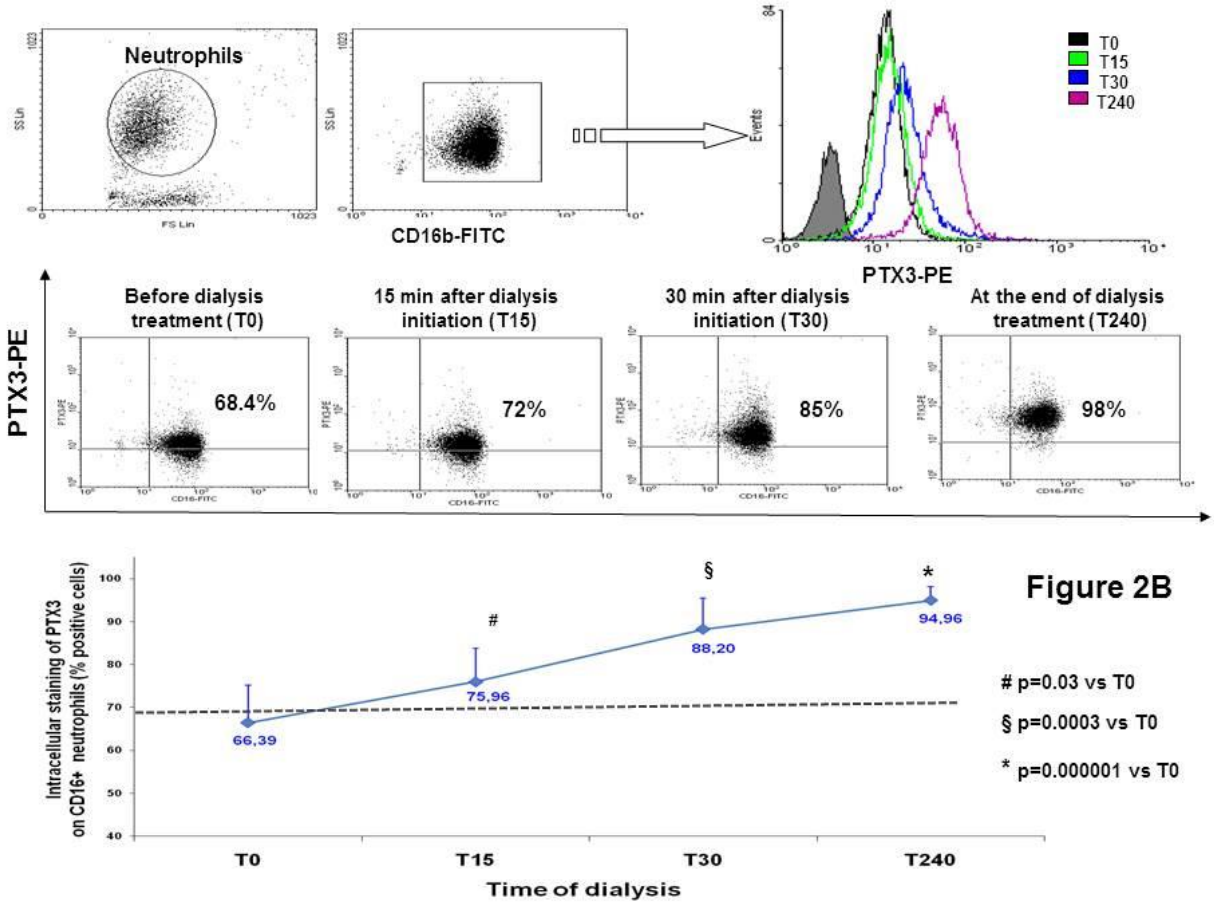

Figure 2B

\# p=0.03 vs T0

§ p=0.0003 vs T0

* p=0.000001 vs T0

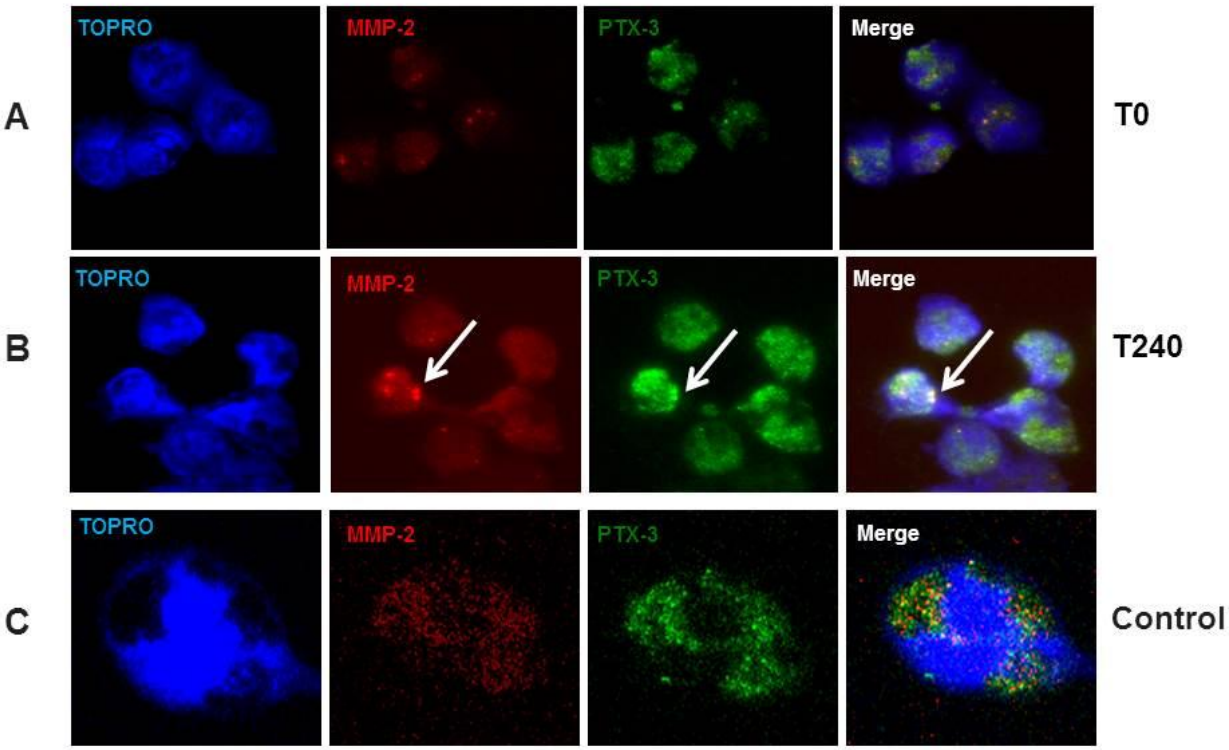
A
TOPRO
MMP-2
PTX-3
Merge
T0
B
TOPRO
MMP-2
PTX-3
Merge
T240
C
TOPRO
MMP-2
PTX-3
Merge
Control

Figure 2C

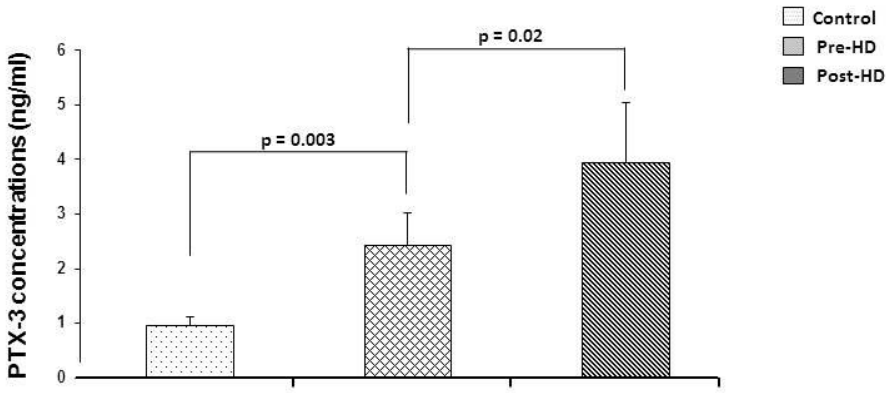

Figure 2D

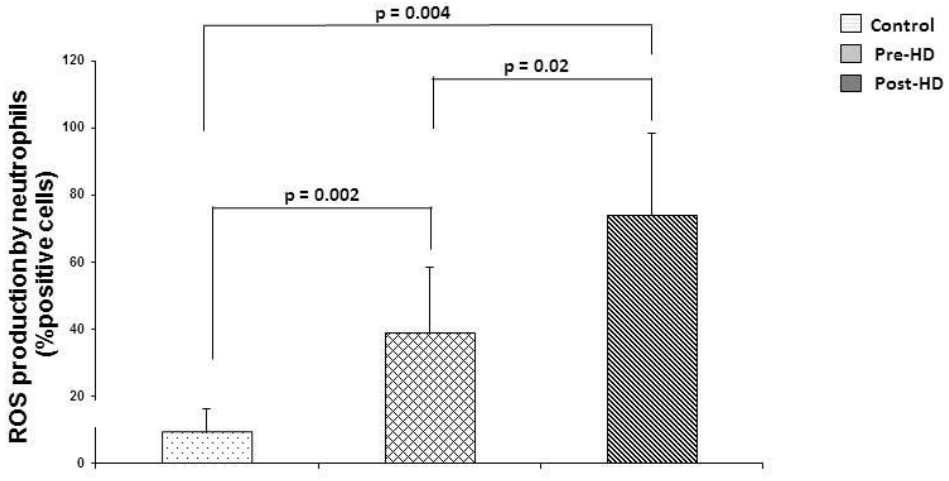

Figure 3A

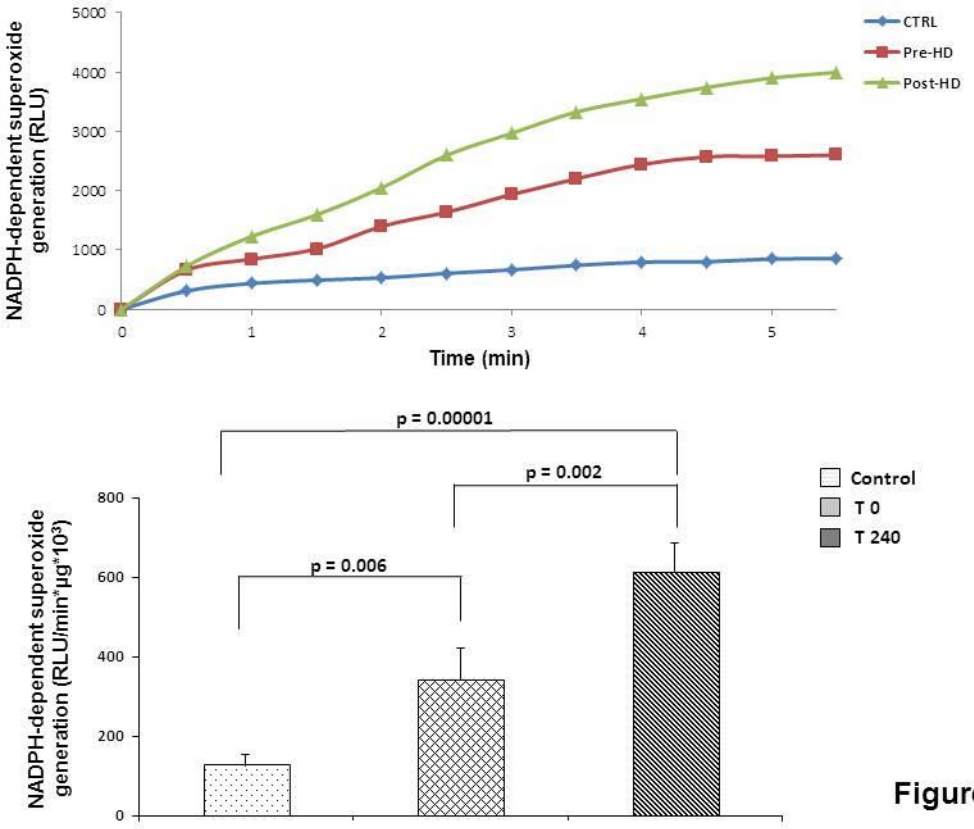
NADPH-dependent superoxide generation (RLU)
5000
4000
3000
2000
1000
0
0
1
2
3
4
5
Time (min)
CTRL
Pre-HD
Post-HD
p = 0.00001
p = 0.002
p = 0.006
NADPH-dependent superoxide generation (RLU/min*µg*10³)
800
600
400
200
0
Control
T 0
T 240

Figure 3B

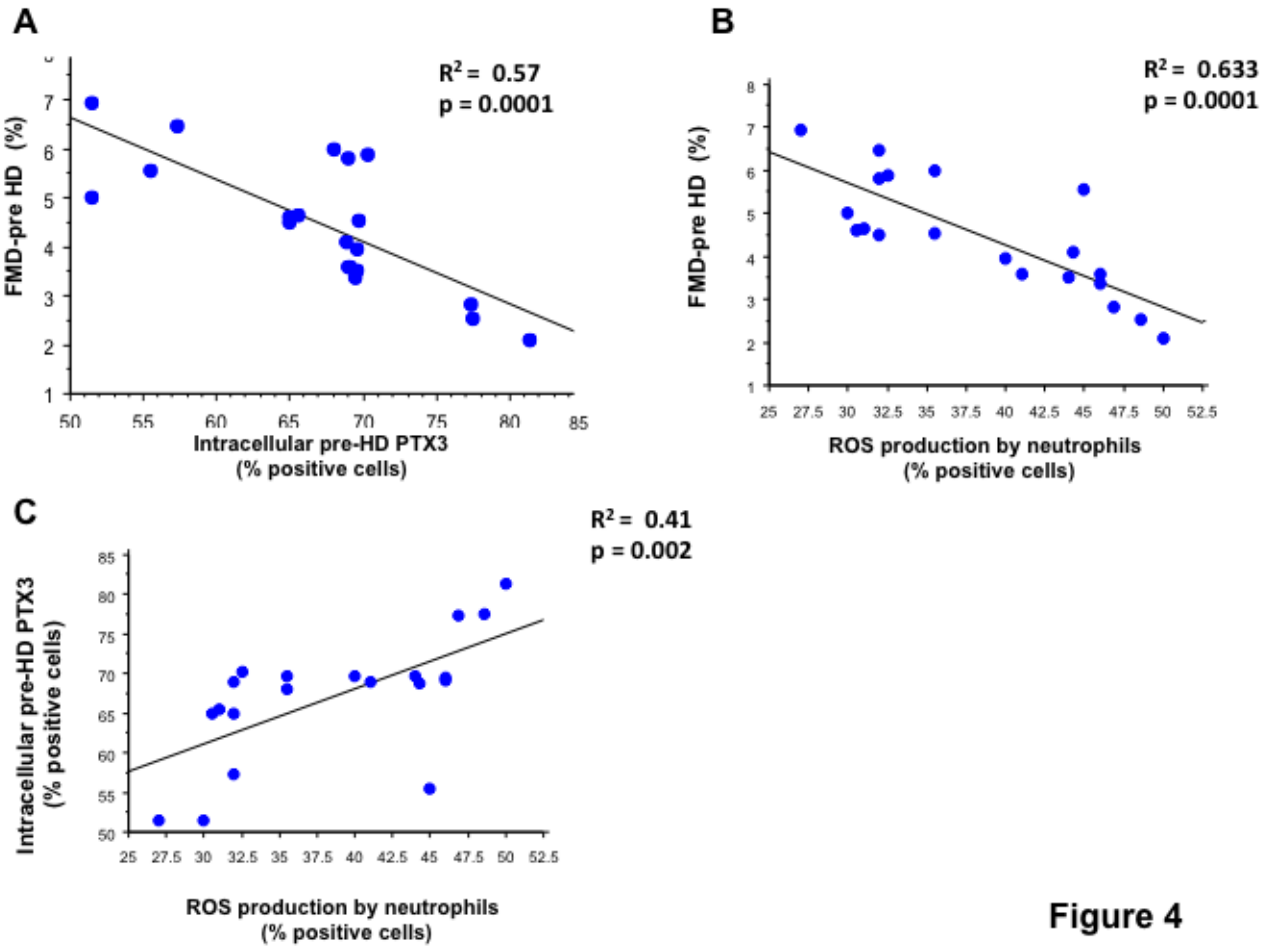
A
R² = 0.57
p = 0.0001
FMD-pre HD (%)
Intracellular pre-HD PTX3
(% positive cells)
B
R² = 0.633
p = 0.0001
FMD-pre HD (%)
ROS production by neutrophils
(% positive cells)
C
R² = 0.41
p = 0.002
Intracellular pre-HD PTX3
(% positive cells)
ROS production by neutrophils
(% positive cells)

Figure 4