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Arterial 'inflammaging' drives vascular calcification in children on dialysis

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Abstract

Children on dialysis have a cardiovascular mortality risk equivalent to the elderly and develop medial vascular calcification, an age-associated pathology. We hypothesized that premature vascular ageing contributes to calcification in children with renal failure.

Vessels from children on dialysis showed elevated oxidative DNA damage and senescence markers p16 and p21 and treatment of vessel rings *ex vivo* with calcifying media increased oxidative DNA damage. Vascular smooth muscle cells (VSMCs) cultured from dialysis vessels exhibited persistent DNA damage, impaired DNA damage repair and accelerated senescence. In response to calcifying conditions dialysis VSMCs showed increased osteogenic differentiation and calcification correlating with activation of the pro-inflammatory senescence-associated secretory phenotype (SASP). Blockade of ATM-mediated DNA damage signalling reduced both inflammation and calcification, consistent with a role for the SASP in calcification. Clinically, children on dialysis showed elevated circulating levels of key osteogenic SASP factors, BMP2, OPG and IL6 that correlated with increased vascular stiffness and coronary artery calcification.

These data imply that dysregulated mineral metabolism drives vascular 'Inflammaging' by promoting oxidative DNA damage, premature senescence and activation of a pro-inflammatory SASP. Drugs that target DNA damage signalling or senolytics may be therapeutic agents for the prevention of vascular calcification.

Key Words: dialysis, senescence, vascular smooth muscle cells, aging, calcification.

Introduction

Age is the dominant risk factor for cardiovascular disease and medial vascular calcification is a prevalent, age-associated pathology. Medial calcification is also a prominent pathology in patients with chronic kidney disease (CKD) and progresses rapidly in patients on dialysis[1-3]. Medial calcification is associated with increased vascular stiffening and cardiac work load, poor coronary perfusion and sudden cardiac death and is thought to be responsible for the high cardiovascular mortality observed in CKD patients[4]. Significantly, even children and adolescents on dialysis develop vascular calcification and have a vastly elevated risk for cardiovascular mortality when compared to the normal age matched population. Strikingly, the risk in adolescence is equivalent to that of the very elderly in the general population[2, 5]. The clear association between ageing and vascular calcification in the general population has led to the suggestion that CKD patients may exhibit accelerated vascular ageing however, so far, there is little direct molecular evidence to support this notion[6, 7].

A key event leading to cellular ageing is the accumulation of un-repairable or persistent DNA damage[8, 9]. DNA damage increases with age and factors such as oxidative stress can accelerate ageing in part, by promoting oxidative DNA damage[10-12]. Persistent DNA damage signalling, via key transducers such as the kinase ataxia-telangiectasia mutated (ATM), promotes upregulation of the checkpoint cell cycle inhibitors p16 (*CDKN2A/INK4a*) and/or p21 (*CDKN1A*) leading to cell cycle arrest and ultimately to cellular senescence[13]. Senescent cells are viable but can no longer contribute to repair processes. Importantly, they display an inflammatory phenotype termed the senescence associated secretory phenotype (SASP) characterized by the secretion of an array of pro-inflammatory cytokines, and growth factors as well as proteases that can act in a paracrine fashion to influence remote cells and tissues[14]. DNA damage and cellular senescence have recently been reported to promote osteogenic differentiation of VSMCs suggesting there may be a direct link between ageing and

calcification, however, the mechanisms involved as well as evidence for this process *in vivo* is still limited[15, 16].

In CKD non-traditional risk factors such as dysregulated calcium (Ca) and phosphate (P) metabolism accelerate vascular calcification by promoting VSMC death and osteogenic differentiation[17, 18]. There is also evidence to suggest that dysregulated mineral metabolism, and in particular elevated P, can drive premature ageing[19]. Fibroblast Growth Factor 23 (FGF23) and its obligate co-receptor Klotho are major physiological regulators of Ca and P metabolism[20]. Mice deficient in either of these proteins develop an array of age-associated pathologies including osteoporosis, vascular calcification and premature death in the context of hypercalcemia, hyperphosphatemia and vitamin D dysregulation [21, 22]. Importantly normalization of mineral metabolism can alleviate premature ageing in these models. Elevated P is also associated with increased cardiovascular calcification and mortality in ageing populations[23] however, the molecular events linking ageing and calcification with dysregulated mineral metabolism are not understood.

Children make an ideal model for studying accelerated ageing. They are not confounded by long-term exposure to environmental stresses that complicate the interpretation of 'ageing' measures in adults. In the context of CKD, vascular damage and calcification occurs almost exclusively due to the complications of renal failure, rather than smoking, dyslipidaemia or pre-existing cardiovascular disease that are prevalent in adults with CKD. Dysregulated mineral metabolism is a key cause of vascular calcification in children on dialysis[24] and we recently reported accumulation of the ageing biomarker prelamin A in the calcified arteries of these children[15, 25]. Prelamin A interferes with DNA damage repair leading to accelerated VSMC senescence and activation of the SASP[15, 26]. This toxic nuclear protein also accumulates in the calcified vasculature of aged adults and is causal in the induction of accelerated vascular

calcification and stiffening in children with the premature ageing disorder Hutchinson-Gilford Progeria Syndrome[27, 28].

We hypothesized that vessels from children with CKD are prematurely aged and that persistent DNA damage leading to premature senescence may be a key event in driving accelerated calcification. We examined evidence for vascular ageing and senescence in children both *in vitro* and *in vivo* and correlated these with clinical vascular measures. We found direct evidence for premature VSMC ageing and define a potential role for DNA damage signalling and 'inflammaging' in driving vascular calcification in children with CKD.

Results

Vessels from CKD children show premature vascular ageing

Medium-sized muscular arteries were harvested from children in pre-dialysis CKD stage 5 (CKD5) and on dialysis (CKD5D), as well as healthy controls (Table S1). An antibody to 8-oxo-dG that recognises oxidatively modified DNA showed that vessels from control patients had low levels of oxidative DNA damage as expected in young children. In contrast, vessels from children with CKD5-5D showed significantly elevated levels of oxidative DNA damage (Fig 1A). Compared to controls, vessels from CKD5-5D patients also showed elevated levels of the senescence marker p21. However, p16 was significantly increased only in dialysis vessels with levels in CKD5 patients highly variable (Figure 1 B,C).

VSMCs from children on dialysis show elevated levels of DNA damage and premature senescence in vitro

VSMCs were explanted from control and CKD5-5D vessels and compared during serial passaging *in vitro*. There was marked variability in growth potential between different CKD5-

5D isolates (Table S2 and Figure S1) but commonly, VSMCs explanted from CKD patients grew more slowly and senesced at earlier passages than control cells (control; passage >30 vs CKD; passage 16.4+/-10.1) (Figure 2A and B). Senescence was demonstrated using senescence associated β-galactosidase (SAβG) staining and observation of an enlarged, flattened, cell morphology (Figure 2B). On Western blot VSMCs approaching senescence showed elevated levels of p16 with variable levels of p21 suggesting senescence was mediated by p16 *in vitro* (Figure 2C).

To assess whether the limited growth potential of CKD VSMCs was associated with DNA damage, immunofluorescence staining for the DNA damage response (DDR) signalling markers γH_2AX and pATM/ATR substrate was performed. VSMCs cultured from dialysis patients showed increased DNA damage compared with CKD5 or controls at equivalent passage number (Figure 2D,E). Elevated levels of DNA damage signalling in dialysis VSMCs was confirmed by Western Blot performed on a limited number of cell isolates that had not been subjected to freeze/thaw cycles during culture, in order to limit any potential bias towards selection of healthy cells. Levels in control and CKD5 VSMCs were low and equivalent (Figure 2F). Comet assays were used to determine whether the elevated DNA damage signalling observed in dialysis VSMCs was due to unrepaired DNA double strand breaks. This was confirmed as comet tails were observed in dialysis VSMCs and were mainly absent in control cell nuclei (3.5 +/- 1.3% control versus 37.1+/-1.2% dialysis) (Figure 2G).

Ca and P induce oxidative stress and DNA damage in VSMCs.

To test whether dysregulated mineral metabolism might contribute to DNA damage, control VSMCs were treated with calcifying media containing elevated levels of Ca/P. Increased levels of DNA damage, shown by γH_2AX nuclear foci and protein on Western Blot

were observed at levels equivalent to those induced by hydrogen peroxide and doxorubicin, both of which induce oxidative DNA damage (Figure 3A-C). Lucigenin assays demonstrated that calcifying media induced hydrogen peroxide production in VSMCs suggesting oxidative DNA damage may be responsible for the increased levels of DNA damage signalling observed (Figure S2).

To examine further the role of Ca/P in the induction of DNA damage, vessel rings from control and CKD patients were exposed to calcifying medium for 7 days *ex vivo* and levels of 8-oxo-dG staining quantified. Vessel rings from control patients were relatively resistant to the induction of oxidative DNA damage and did not show a significant elevation in staining. In contrast, VSMCs in CKD5-5D showed significantly elevated 8–oxo-dG staining after Ca/P treatment, with dialysis vessels showing the greatest accumulation over the time period tested (Figure 3D,E).

To explore the mechanism of increased susceptibility to DNA damage observed in dialysis VSMCs we treated control and dialysis VSMCs with an acute dose of doxorubicin and examined levels of DNA damage before and after washout, to quantify the efficiency of DNA damage repair. The DNA damage response was similar in control and dialysis VSMCs after 2 hours, however, 24 hours after washout control cells showed a greater efficiency of repair evidenced by fewer γH_2AX foci (Figure 4A). At this stage dialysis VSMCs also showed evidence for persistent DNA damage with a higher percentage of cells retaining >5 53BP1 nuclear foci consistent with a reduced repair capacity in these cells (Figure 4B).

DNA damage and senescence drive increased calcification and the pro-inflammatory SASP in dialysis VSMCs

Given the clear differences in the DNA damage response between control and dialysis VSMCs we next used qRT-PCR to compare gene expression at equivalent early passage numbers between these two cell types. At baseline in normal media, levels of the senescence marker p16 were not different. However, VSMCs from dialysis patients had decreased levels of the differentiation marker α-SM actin and elevated expression of the osteogenic morphogen BMP2, as well as Runx2 and BSP, although these did not reach significance (Figure 5A). In response to calcifying media VSMCs from dialysis patients showed greater mineralization (Figure 5B). This was associated with increased expression of the osteogenic marker Runx2 and dramatically elevated BMP2 expression compared to controls (Figure 5C).

We previously identified BMP2 as a SASP factor in adult aortic VSMCs[15] so we next tested whether elevated Ca/P might accelerate senescence in dialysis VSMCs. SAβG staining showed a small but significant increase in the number of senescent cells after short-term treatment (16 hours) with calcifying media in dialysis but not in control VSMCs. Of note, treatment with P alone was sufficient to increase the number of senescent cells (Figure 6A and B). Western blot showed that Ca/P media consistently increased the expression of p16 in dialysis VSMCs, in contrast to control VSMCs where no pattern was observed (Figure 6C). Western blot also confirmed elevated BMP2 in dialysis compared to control VSMCs in both normal and calcifying conditions (Figure S3).

To examine in more detail the inflammatory SASP profile of control and dialysis VSMCs, antibody arrays were performed comparing conditioned media from equivalent early passage cells. A number of previously identified SASP factors were found to be abundantly secreted by VSMCs including the cytokines IL6, IL8, OPG and MCP-1 and the protease inhibitors TIMP1 and TIMP2 (Figure S4A,B). Dialysis VSMCs secreted elevated levels of a number of these factors compared to control cells including IL6, IL8 and OPG and also secreted elevated levels of the chemokines CSF2, CXCL1 and CXCL3 although of these, only CXCL3

was relatively abundant (Figure S4B,C). qRT-PCR analysis was used to verify that BMP2, IL6 and OPG were all more highly expressed by dialysis VSMCs compared with controls and increased secretion of IL6 and OPG was confirmed using ELISA (Figure 7C and Figure S5).

To determine whether DNA damage signalling acting upstream of the inflammatory SASP was responsible for the increased calcification observed in dialysis VSMCs, both dialysis and control cells were treated with either an siRNA or a chemical inhibitor (Ku55933) targeting ATM, a key signalling kinase in the DNA damage response. ATM inhibition reduced the accelerated calcification observed in dialysis VSMCs (Figure 7A and B). Importantly the reduced calcification observed in dialysis VSMCs was mirrored by the pattern of expression of the SASP markers BMP2, IL6 and OPG; ATM inhibition significantly decreased their expression in dialysis cells consistent with their activation in response to persistent DNA damage (Figure 7C).

CKD children show elevated levels of circulating SASP factors correlating with vascular ageing measures.

Three of the SASP factors elevated in dialysis VSMCs, BMP2, OPG and IL6, have previously been shown to play a role in regulating calcification[15]. Therefore we quantified the serum levels of these factors in children with CKD5-5D, correlating these biochemical measures with vascular scans. Serum BMP2, OPG and IL6 levels did not depend on age or gender in children. However, both IL6 and BMP2 levels were significantly higher in dialysis versus the CKD5 group (Figure 8A,B), and IL6 levels increased with increasing time on dialysis (Figure S6A). The circulating levels of BMP2 and OPG correlated with arterial stiffness measured by aortic pulse wave velocity, a surrogate marker of medial calcification (Figure 8C,D). Moreover, levels of IL6 correlated with both BMP2 and OPG suggesting

increases in these cytokines may occur concomitantly (Figure S6B,C). Importantly, comparing CKD children with coronary artery calcification measured by multi-slice CT scan (n = 8) against those who did not show calcification (n = 16), there was an increase in these secreted SASP factors in patients with overt calcification (Figure 8E) suggesting that the paracrine release of these factors may be clinically relevant.

Discussion

In this study we show that vessels from children with CKD exhibited features of premature ageing *in vivo* including oxidative DNA damage and elevated senescence markers and these ageing indices persisted upon culture of VSMCs *in vitro*. Ca/P treatment was shown to increase DNA damage in CKD vessel rings *ex vivo* and to drive senescence of dialysis VSMCs *in vitro*. The persistent DNA damage observed in dialysis VSMCs was associated with increased osteogenic differentiation and calcification driven in part, by activation of the pro-inflammatory SASP. Importantly, a number of these SASP factors, previously identified as pro-calcific, correlated with increased PWV and were significantly increased in the serum of children with clinically measurable calcification. Taken together, these data suggest that the paracrine secretion of inflammatory/osteogenic factors by aged VSMCs may be an important driver of arterial stiffening and vascular calcification in CKD. We suggest that the onset of VSMC senescence may be a key 'set-point' driving the rapid progression of this age associated pathology in young CKD patients. Importantly it may be that only a few senescent VSMCs are required to initiate this cascade.

VSMCs from children with CKD show hallmarks of premature ageing

We demonstrated that CKD and in particular, the dialysis environment, is associated with increased oxidative DNA damage and premature senescence of VSMCs *in vivo*. While studies have shown that oxidative DNA damage and the senescence markers p16 and p21 are elevated in atherosclerotic plaque and adult vessels from CKD patients[11, 29, 30] this is the first study to show increased levels of these markers in vessels from young children, who are free of confounding factors including atherosclerosis, providing compelling evidence that the vascular pathology observed in these patients is closely associated with premature ageing[31, 32]. Our data suggests that oxidative DNA damage accrual begins in predialysis CKD5, as levels of 8-oxo-dG and p21 were significantly elevated at this stage, but is accelerated in the dialysis milieu leading to unrepaired DNA damage and stress induced premature senescence a hallmark of which is p16 accumulation[29,31].

Dysregulated mineral metabolism promotes oxidative DNA damage and premature senescence in dialysis VSMCs.

A major signal driving oxidative DNA damage was Ca and P stress. Osteogenic media induced oxidative DNA damage in CKD5-5D vessel rings cultured *ex vivo,* with control vessels showing some resistance to the same stimulus. This is consistent with our previous studies showing that dialysis vessels, and to a lesser extent CKD5 vessels, undergo VSMC death, osteogenic differentiation and calcification when cultured *ex vivo* in Ca/P media, providing further support for a link between premature ageing and calcification[24, 33]. There are a number of potential mechanisms, that are not mutually exclusive, that may explain why dialysis patient VSMCs show increased susceptibility to Ca/P stress. We showed that osteogenic media induced DNA damage and accelerated senescence of dialysis VSMCs *in vitro* and that these cells showed delayed DNA damage repair compared with control cells. Delayed repair may be due in part to the reported increased levels of prelamin A in dialysis VSMCs as this nuclear toxin

delays DNA damage repair and accelerates senescence[15, 26] by sequestering and compromising nuclear import of essential DNA repair factors[26, 34]. Dialysis VSMCs also display decreased oxidant defenses[35] and this, as well as mitochondrial damage,[36] could lead to further elevations in reactive oxygen species. Of note, P alone was able to promote senescence of dialysis VSMCs and a recent study showed that P may interfere with the expression of a key longevity gene Sirtuin1, which deacetylates and inactivates p53, allowing cells to survive DNA damage[37]. In addition key longevity genes expressed in VSMCs such as Klotho are intimately linked with P metabolism and oxidative stress[38] and these proteins are downregulated in response to ageing and disease suggesting further studies on the regulation and role of these factors in VSMCs are warranted[39, 40].

Aged VSMCs show increased inflammation, osteogenic differentiation and calcification

The SASP is a storm of inflammatory cytokines released by cells in response to persistent DNA damage signalling[41, 42] and previous studies have shown that senescent adult aortic VSMCs *in vitro* upregulate and secrete potent osteoinductive and inflammatory factors such as BMP2 and IL6 as part of the SASP response[43-45]. In this study, we showed that VSMCs from children on dialysis, without additional stimulation, expressed and secreted elevated levels of these same SASP osteoinductive factors and that Ca/P stimulation increased levels further. Furthermore, elevated levels of SASP inflammatory markers correlated with increased osteogenic differentiation and calcification of dialysis VSMCs while blocking ATM-mediated DNA damage signalling reduced inflammation and calcification. SASP factors including IL6 and OPG have been reported to be elevated in both adult and paediatric dialysis patient cohorts[31, 46, 47] while in adult CKD populations increased circulating levels of BMP2 have been reported correlating with increased serum 8-oxo-dG levels and vessel wall stiffness[48]. It is plausible that the systemic release of cytokines from prematurely aged vascular cells may induce

a vicious cycle of inflammation and calcification in CKD patients[42]. In support of this hypothesis, elevated serum IL-6 levels in hemodialysis patients were found to associate with aortic calcification and to predict cardiovascular death[49, 50]. Moreover, IL6 was a significantly better predictor of mortality risk than other inflammation markers such as C-reactive protein, albumin or TNFα[51]. The presence of SASP factors in the serum of patients on dialysis may also explain studies *in vitro* where dialysis serum promotes calcification of VSMCs and this is independent of serum Ca/P levels[52].

Limitations and Clinical implications

This study has a number of limitations that need to be addressed before taking any findings forward to clinical interventions. Although children provide the best model for investigating ageing processes tissue retrieval is difficult, hence, the study was limited by small sample size. A key question that was not fully addressed was whether the factors driving premature ageing were present in predialysis CKD5 or at even earlier stages of CKD. Although the findings suggest that DNA damage does begin predialysis this needs to be confirmed using a larger patient cohort. Moreover, the origin of the cells from different vascular beds, children of different ages and dialysis vintage, the inherent heterogeneity of the CKD population and the phenotypic modulation that takes place *in vitro,* were all reflected in the behaviour of the VSMCs studied, making it impossible to draw further conclusions. The clinical studies were also limited by small sample size and the lack of a control group. The correlations with PWV and CAC suggest that inflammation closely follows the onset of vessel stiffening and worsens when overt calcification is present. However extrapolating from peripheral arteries to coronary arteries should be viewed with caution although it is likely that in children, who are free of atherosclerosis, coronary calcification is also likely to be medial.

Despite these limitations these findings may have important clinical implications because they do point to a mechanism whereby elevated oxidative DNA damage in vessels, probably starting in predialysis CKD, promotes a cascade of senescence, inflammation and calcification. Studies have shown that oxidant stress occurs in CKD before dialysis therapy is initiated[53] and increases according to duration of dialysis treatment[54, 55] and it is likely that in addition to Ca and P, other factors present in the dialysis environment contribute to oxidative stress[56, 57]. These agents are likely to have a cumulative effect over time as evidenced by the increased senescence markers found in dialysis vessels, thus highlighting the need to monitor and maintain Ca and P levels within the age-appropriate range starting from CKD5[58]. It also highlights that conventional clinical interventions, aimed at correcting circulating risk factors, must be complimented by alternate techniques and that drugs targeting alternate pathways such as the DNA damage response or senescent cells (senolytics) may be potential therapies to slow the progression of vascular calcification and associated 'Inflammaging" in this patient group[59, 60].

Materials and Methods- (extended methods in Supplement)

Patient Samples

The study was conducted at Great Ormond Street Hospital, London, with full ethical approval from the Institutional Review Board (12/LO/1186). Medium-sized muscular arteries, omental (OM), mesenteric (MA) or inferior epigastric (IE), routinely removed and discarded in the course of planned surgery were collected from CKD5-5D patients at the time of catheter insertion or renal transplantation and compared with disease-free, age-matched children (controls) undergoing intra-abdominal surgery. The demographic details and baseline calcium load in the vessel wall are shown in Table S1. Freshly isolated vessel rings were paraffin

embedded or cultured *ex vivo* in control or calcifying media for 7 days as previously described[33]. VSMCs were grown by explant culture as previously described[61]. Cells were counted in triplicate at each passage to ensure equivalent plating densities and to determine growth rates and used between passages 4-10 for comparisons. All experiments, except where indicated, were performed on at least 3 independent VSMC isolates from each patient group (Table S2).

Antibody Array

Antibody arrays (RayBio, human cytokine antibody array VI and VII, catalog No. AAH-CYT-6/-7) were performed using conditioned media from control (n=2) and dialysis (n=2) patient VSMCs. Signals were normalized and quantified using densitometry and the signal ratio of control versus dialysis calculated. ELISA or qRT-PCR for verification was performed as previously[15].

Clinical Studies

Simultaneous vascular imaging and blood tests were performed in children with CKD5 (n = 11) and those on dialysis (n = 24). Vascular measures were performed in all children above 5 years of age (n=8 CKD5 and n=23 dialysis patients). Applanation tonometry for aortic PWV and 64-slice spiral CT scan were performed using methods previously described.[28] Vascular scans were performed by a single blinded operator before a mid-week session of hemodialysis or after overnight cycling peritoneal dialysis. Serum levels of BMP2, IL-6 and OPG were determined using ELISA (R & D Systems) according to manufacturer's instructions.

Statistical Analysis

Shapiro-Wilk test was used to test for normalcy of data before one-way ANOVA or Kruskal-Wallis tests were used to determine the significance of differences among groups as appropriate. Student's t or Mann-Whitney U tests were used to assess differences between means as appropriate. Spearman correlation tests were used for correlation analyses. Statistical Package for Social Sciences Software (SPSS) and GraphPad software were used for statistical computations. A p value < 0.05 was considered to indicate a significant difference.

Figure Legends

Figure 1. Vessels from children with CKD show elevated levels of oxidative DNA damage and senescence markers. (A) Immunohistochemistry and quantification for 8-oxo-dG showed a significantly increased percentage of VSMCs with oxidative DNA damage in vessels from predialysis (CKD5) and dialysis (CKD5D) patients compared with controls. Positive nuclei are arrowed, boxed inset shows enlargement. Bar=100μm (B) Immunohistochemistry showing increased p21 and p16 nuclear staining in predialysis (CKD5) and dialysis (CKD5D) vessels compared with controls. Note the nuclear staining (arrowed and insets). Bar=50μm (C) The percentage of positively stained cells/nuclei for p21 was significantly higher in both CKD5 and CKD5D when compared to control vessels while p16 was only significantly elevated in dialysis vessels. (Graphs show mean +/- SE, ANOVA) M: media, Ad: adventitia.

Figure 2. VSMCs from dialysis vessels senesce early and show elevated levels of DNA damage *in vitro*. (A-C). VSMCs grown from dialysis patients showed premature senescence compared to cells from controls as shown by (A) slower growth rates (B) SAβG staining, and (C) elevated levels of p16 at equivalent passage number. Representative data from n=3 control and dialysis isolates is shown. Bar=50μm (D) Immunofluorescence staining for γH_2AX and pATM/ATR shows dialysis VSMCs have increased DNA damage signalling compared to control cells at equivalent early (P6) passage (positive nuclei arrowed). Bar=10μm (E) The percentage of γH_2AX and pATM/ATR positive nuclei was significantly higher in dialysis VSMCs compared to both control and predialysis (CKD5). Each bar represents mean ± SE, (*** $p < 0.001$, dialysis vs control and dialysis vs pre-dialysis, ANOVA). (F) Western blot showing increased γH_2AX and pATM/ATR protein levels in dialysis (n=3, patients 04,34,51)

VSMCs compared to CKD5 (n=2, patients 07,40) and control (n=2, patients 20,39) VSMCs at equivalent passage number. P, passage number. (G) Comet assays confirm increased DNA damage in dialysis VSMCs with an increased percentage of cells with comet tails (arrowed in inset showing dialysis VSMCs) compared to control cells at equivalent passage (* $p < 0.05$, n = 3, Students t-test). Bar=10μm

Figure 3. Elevated Ca/P caused oxidative stress and increased oxidative DNA damage in CKD vessels. (A) Immunofluorescence staining for γH_2AX and pATM/ATR indicates that Ca/P promoted DNA damage (arrows indicate positive nuclei). Bar=10μm. (B) The percent of γH_2AX and pATM/ATR positive nuclei in response to Ca/P media is similar to doxorubicin and hydrogen peroxide treatments and significantly higher compared to baseline VSMCs. Each bar represents mean ± SE (*** $p < 0.001$ compared to baseline). Bar=10μm (C) Western blotting shows an increase in γH_2AX and pATM/ATR protein levels in calcifying media equivalent to doxorubicin and hydrogen peroxide treatments. (D) Immunohistochemistry for 8-oxo-dG shows that calcifying media promotes an increase in 8-oxo-dG staining in CKD5-5D vessels. (E) Quantification shows that calcifying media significantly increases 8-oxo-dG positive nuclei in CKD5 ($p = 0.04$; n = 4) and dialysis ($p = 0.001$; n = 4) vessels with a greater increase in dialysis vessels despite similar baseline levels of oxidative DNA damage. The effect of Ca/P on control VSMCs was not significant ($p = 0.08$; n = 4).

Figure 4. Dialysis VSMCs showed impaired DNA damage repair. (A,B) Immunofluorescence showing elevated levels of DNA damage in dialysis VSMCs (arrowed) 24 hours after washout of the DNA damage agent doxorubicin. In contrast control VSMCs are mostly repaired at this stage. Quantification shows both γH2AX and 53BP1 foci are elevated. A

high percentage of cells with >5 53BP1 foci is indicative of persistent DNA damage and failure of repair. n=3 iolates/group. ANOVA (*p<0.05, **p<0.01). Bar=10μm.

Figure 5. VSMCs from dialysis patients show increased osteogenic differentiation and calcification. (A) qRT-PCR shows that VSMCs from dialysis patients have reduced expression of SM markers and increased expression of osteogenic markers when compared to control VSMCs. Each bar represents mean ± SE, Students t-Test. (B) Alizarin Red staining shows VSMCs from dialysis vessels are more prone to calcify than VSMCs from control vessels in response to calcifying medium for 7 days. Each bar represents mean ± SE. (* $p < 0.05$, ANOVA). (C) qRT-PCR shows that VSMCs from dialysis patients have significantly elevated mRNA expression of the osteogenic markers Runx2 and BMP2 in response to calcifying media when compared to control media. Each bar represents mean ± SE , (* $p < 0.05$, ANOVA).

Figure 6. Dialysis VSMCs show increased senescence in response to calcifying media. (A and B) VSMCs were incubated in control, Ca (2.7mM), P (2.5mM) or Ca/P supplemented serum-free medium for 16 hours and stained for SA-βG. Increased staining was observed in response to elevated Ca/P or P alone in dialysis VSMCs (*** $p < 0.001$, ANOVA). Bar= 100μm (C) Western blot shows that in response to elevated Ca/P p16 is increased in dialysis VSMCs but not in control VSMCs. p21 levels did not consistently change in either group. Blots representative of n=3 isolates/group.

Figure 7. Inhibition of DNA damage signalling reduces calcification and expression of SASP factors in dialysis VSMCs. (A and B) Control and dialysis VSMCs were treated with osteogenic media in the presence or absence of the ATM inhibitor Ku55933. Alizarin red

staining shows that after 5 days dialysis but not control VSMCs had calcified and calcification of dialysis cells was decreased by ATM inhibition. (*p<0.05 ANOVA) (C) qRT-PCR showing levels of expression of the SASP factors BMP2, IL6 and OPG in control and dialysis VSMCs. Note the higher levels of these factors in dialysis VSMCs at baseline. ATM inhibition using siRNA decreased elevated levels of the SASP factors BMP2, OPG and IL6 in dialysis VSMCs (pink) with little or no effect on control VSMCs (green). Effectiveness of ATM siRNA knockdown compared with control siRNA was equivalent for both control and dialysis VSMCs. Mean +/- SE, ANOVA, *p<0.05.

Figure 8. Children with CKD show elevated SASP factors correlating with vascular stiffness. (A and B) Serum levels of BMP2 and IL6 in children with CKD5 (n = 11) and those on dialysis (n = 24) showing significantly higher levels of both markers in children on dialysis. In children with CKD5 and those on dialysis who were above 5 years of age (n = 31), BMP2 and OPG correlated with aortic pulse wave velocity (PWV) measured by applanation tonometry (C and D), showing significant correlation between these markers and PWV. (E) Coronary artery calcification (CAC) was measured by multi-slice CT scan and patients were divided into 2 groups: those with calcification (n = 8) and those who did not have calcification (n = 16). The median (range) CAC score was 149 (43 to 2019) in the calcification group and 7/8 calcified patients were on dialysis. Children with CAC had significantly higher levels of BMP2, OPG and IL6 compared to those without calcification (p<0.0001; Multiple ANOVA). Comparing individual factors both BMP2 and IL6 were significantly elevated in the calcified group while OPG was not (p values for unpaired t-test are indicated on legend).

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Disclosures: None to disclose.

Supplementary Material

Supplementary Figure Legends

Supplementary Figure S1: VSMCs from dialysis patients senesce early in culture. Graph showing the spread for passage number at senescence for VSMC isolates from control and CKD patients as listed on Supplementary Table S2. Note isolates listed as growing for >30 passages had not yet undergone senescence at this stage. Only a limited number of CKD5 predialysis isolates were tested so further analyses were mostly restricted to CKD5D dialysis isolates.

Supplementary Figure S2: Induction of oxidative stress in VSMCs treated with osteogenic Ca/P media. Lucigenin chemiluminescence assays shows that Ca/P media stimulates reactive oxygen species (ROS) production in VSMCs cultured in serum free conditions to a similar level as hydrogen peroxide treatment. Control shows VSMCs in BSA only. Data shown for two different VSMC isolates (A and B) and performed in triplicate.

Supplementary Figure S3. VSMCs from children on dialysis upregulate p16 in response to extracellular P and Ca/P. A. VSMCs from control and dialysis patients were exposed to control and calcifying media containing elevated levels of Ca and P. Two experimental pairs of samples are shown. Set 1 (left side): 1 year old male control patient (renal artery) passage 11 and 1 year old dialysis patient (renal artery) passage 10. Set 2 (right side): 8 month old control patient (omental artery) passage 8 and 9 year old dialysis patient (inferior epigastric artery) passage 7. Note that dialysis VSMCs have higher base line levels of p16 and a trend to increase p16 expression in response to P and Ca/P media. In contrast p21 levels show no clear pattern of response. BMP2 levels are also elevated in dialysis VSMCs regardless of treatment. B. Quantification of Western blots by densitometry shows a clear trend for Ca/P to increase p16 protein levels in dialysis patient VSMCs exposed to short term Ca/P treatment. Mean +/- SD. n=4 isolates/group.

Supplementary Figure S4: Antibody Array analysis shows dialysis VSMCs secrete increased levels of SASP factors compared with controls. (A). Representative image of an antibody array showing pattern of cytokine secretion by control and dialysis VSMCs. Arrow indicates example of elevated cytokine in dialysis sample. (B). Table showing ranking of the abundance of selected SASP factors based on density normalized to control samples on each array. (C). Table showing the fold increase in SASP factors secreted from dialysis VSMCs compared to control.

Supplementary Figure S5: ELISA shows dialysis VSMCs secrete elevated levels of osteogenic factors compared with control cells. ELISA showing increased levels of IL6 and OPG in the conditioned media (CM) of two age-matched representative cell isolates from a control 8.5 year old male (8.5M) and dialysis 9 year old male (9M) patient measured at the same passage number.

Supplementary Figure S6: Children with CKD show increased serum levels of osteogenic factors correlating with time on dialysis. In children on dialysis (n = 24) IL6 levels increased with increasing time on dialysis (A). IL6 levels also showed a modest correlation with both BMP2 and OPG (B and C).

Supplementary Tables

Supplementary Table S1. Patient vessel samples analysed by immunohistochemistry.

Supplementary Table S2. Patient samples used in VSMC growth studies *in vitro.*

Supplementary Materials and Methods

Supplementary References

Supplementary information is available at Kidney International's website.

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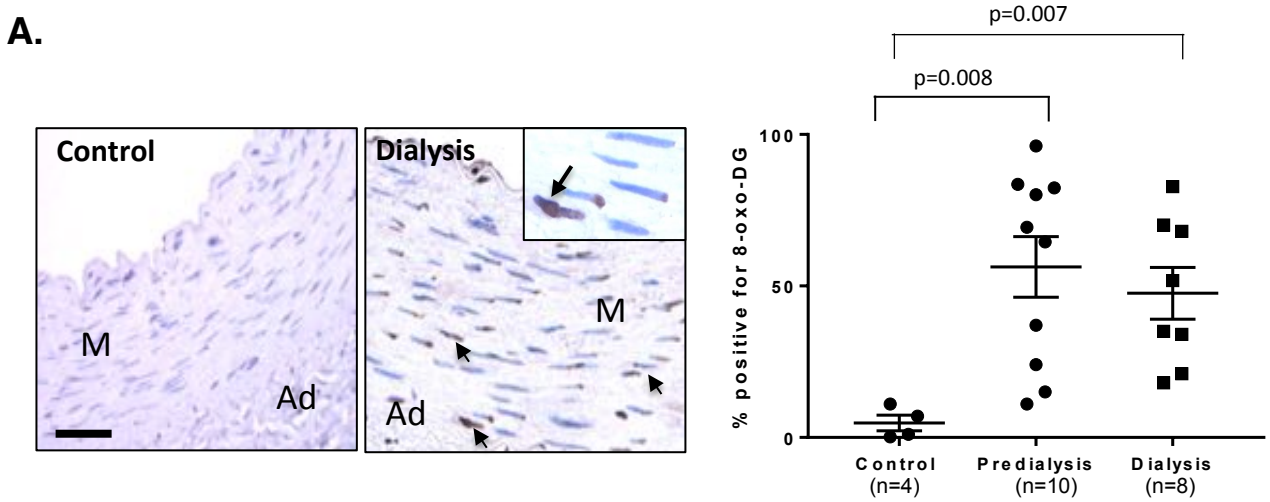
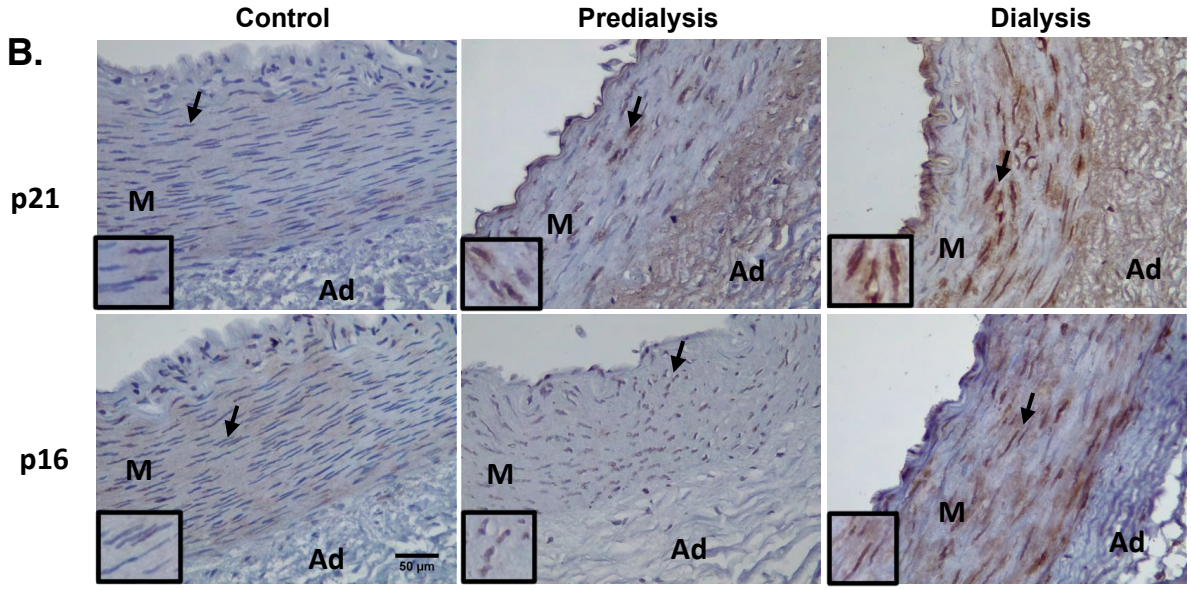

A.
Control
Dialysis
M
Ad
M
Ad
p=0.007
p=0.008
% positive for 8-oxo-DG
100
50
0
Control
(n=4)
Predialysis
(n=10)
Dialysis
(n=8)


B.
Control
Predialysis
Dialysis
p21
p16
M
Ad
50 µm

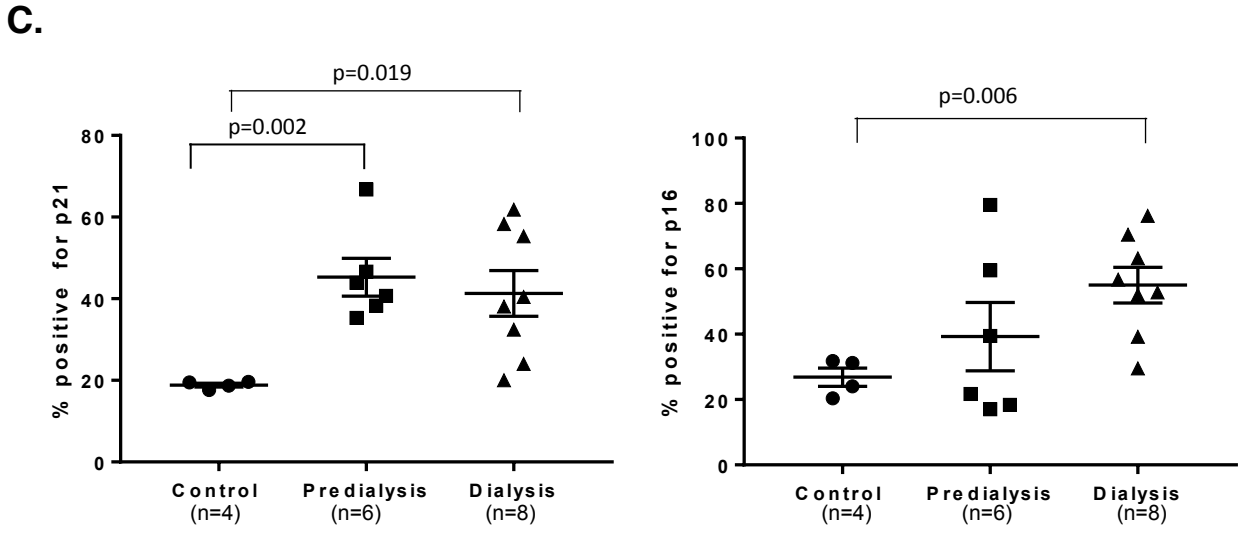

C.
p=0.019
p=0.002
% positive for p21
80
60
40
20
0
Control
(n=4)
Predialysis
(n=6)
Dialysis
(n=8)
p=0.006
% positive for p16
100
80
60
40
20
0
Control
(n=4)
Predialysis
(n=6)
Dialysis
(n=8)

Figure 1.

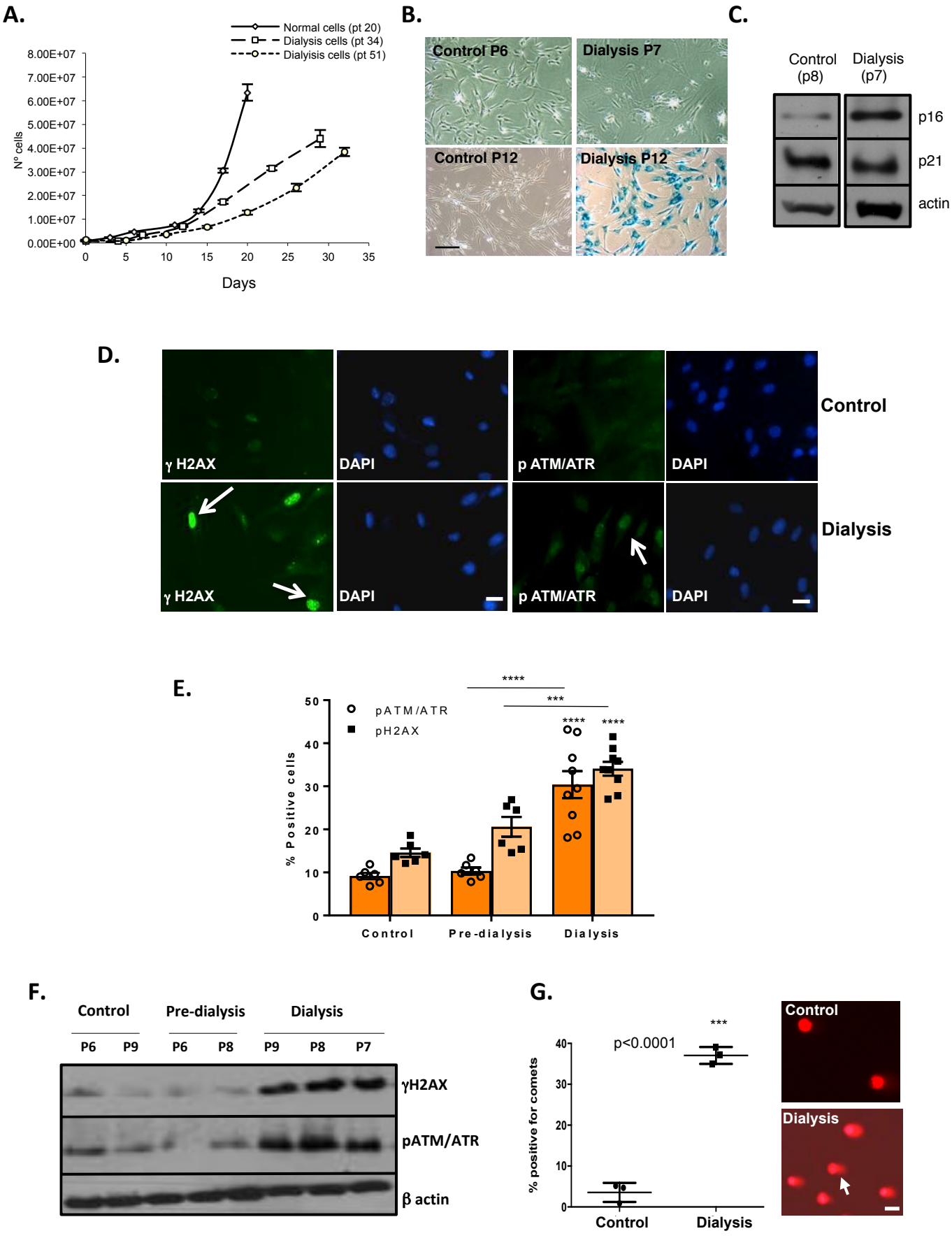

A.
Normal cells (pt 20)
Dialysis cells (pt 34)
Dialyisis cells (pt 51)
8.00E+07
7.00E+07
6.00E+07
5.00E+07
4.00E+07
3.00E+07
2.00E+07
1.00E+07
0.00E+00
Nº cells
0
5
10
15
20
25
30
35
Days
B.
Control P6
Dialysis P7
Control P12
Dialysis P12
C.
Control (p8)
Dialysis (p7)
p16
p21
actin
D.
γ H2AX
DAPI
p ATM/ATR
DAPI
Control
γ H2AX
DAPI
p ATM/ATR
DAPI
Dialysis
E.
pATM/ATR
pH2AX
% Positive cells
50
40
30
20
10
0
****
***
****
****
Control
Pre-dialysis
Dialysis
F.
Control
Pre-dialysis
Dialysis
P6
P9
P6
P8
P9
P8
P7
γH2AX
pATM/ATR
β actin
G.
% positive for comets
40
30
20
10
0
p<0.0001
***
Control
Dialysis
Control
Dialysis

Figure 2.

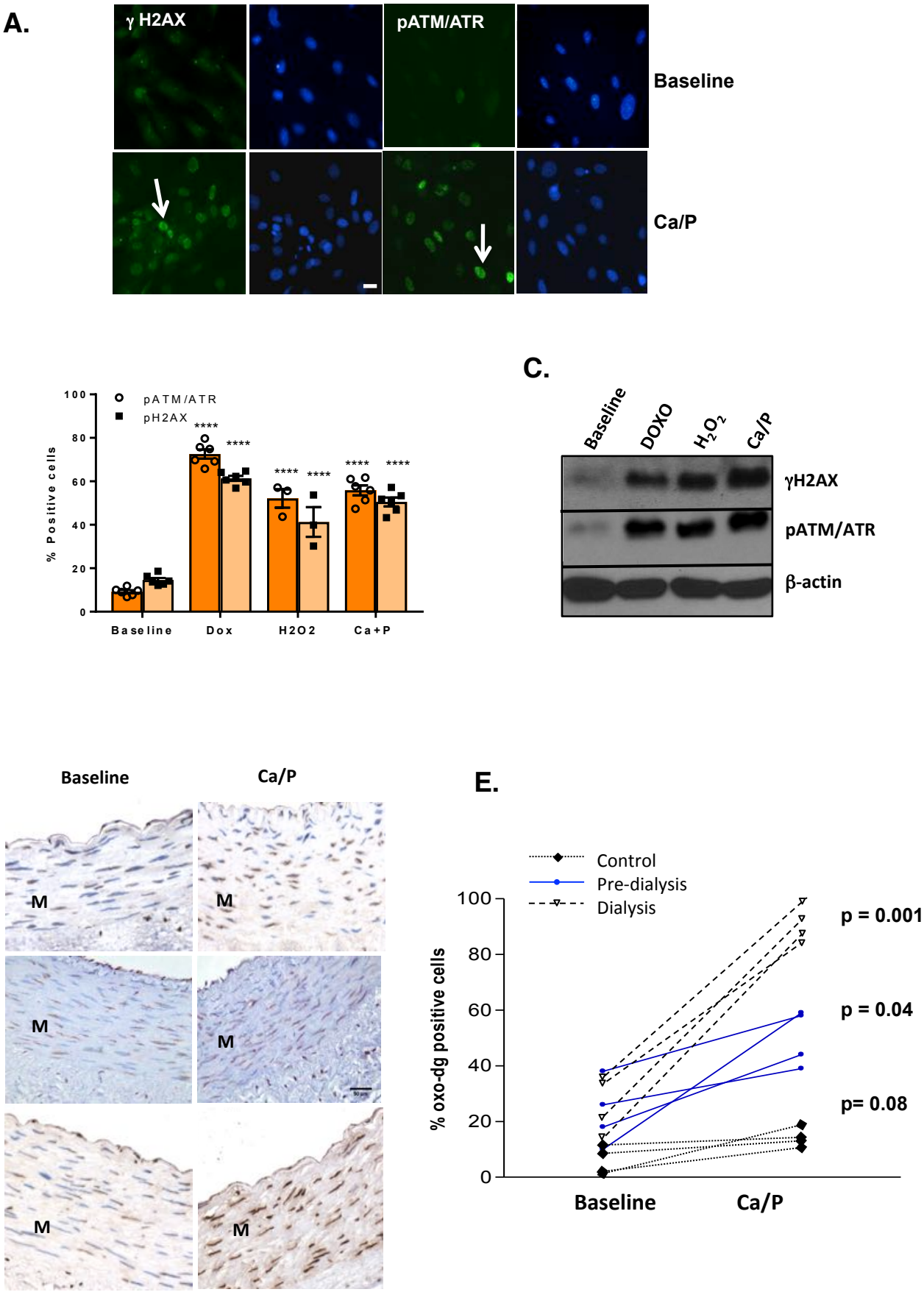

A.
γ H2AX
pATM/ATR
Baseline
Ca/P
B.
pATM/ATR
pH2AX
% Positive cells
Baseline
Dox
H2O2
Ca+P
C.
Baseline
DOXO
H2O2
Ca/P
γH2AX
pATM/ATR
β-actin
D.
Baseline
Ca/P
Control
Predialysis
Dialysis
M
E.
Control
Pre-dialysis
Dialysis
% oxo-dg positive cells
p = 0.001
p = 0.04
p= 0.08
Baseline
Ca/P

Figure 3.

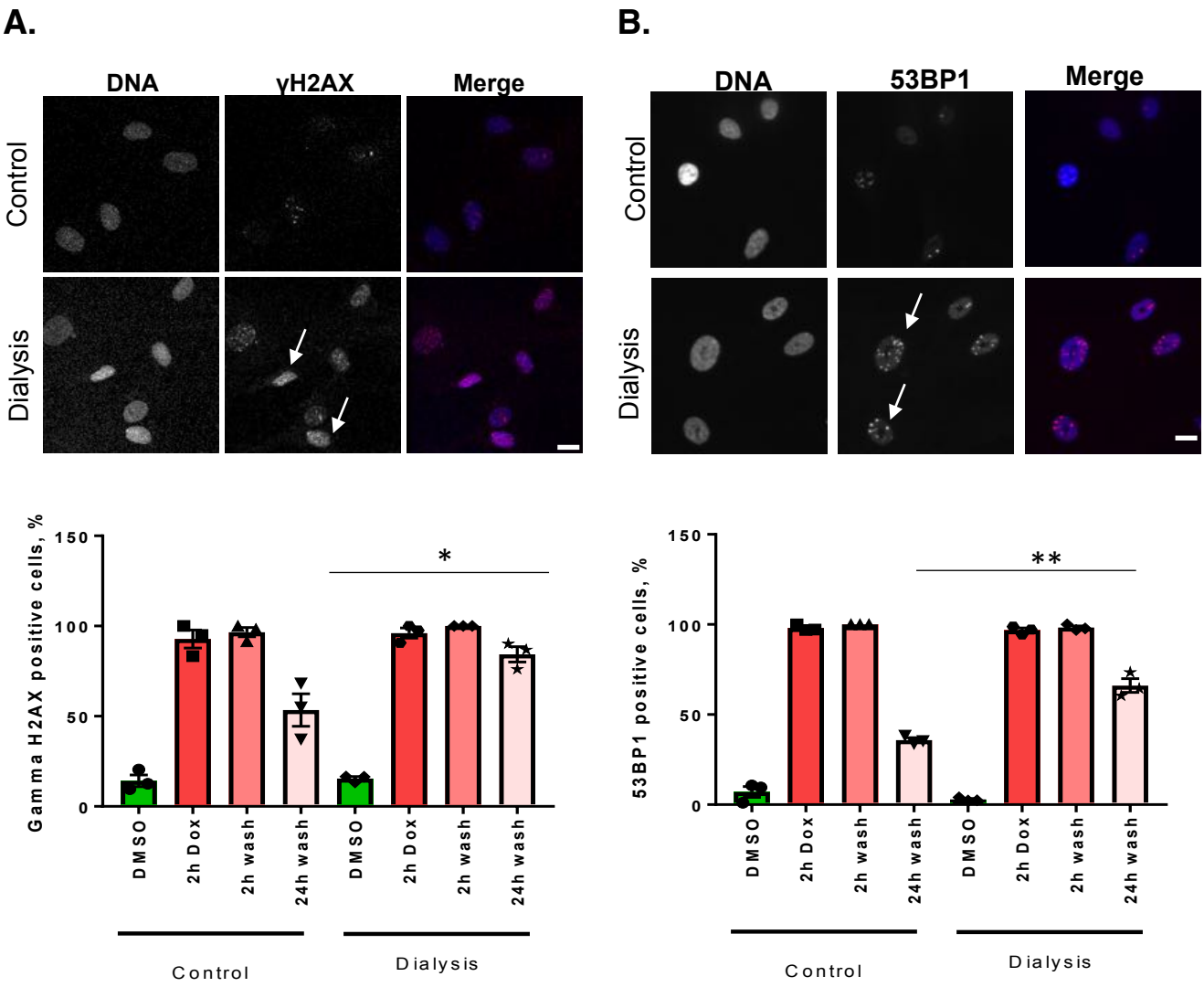
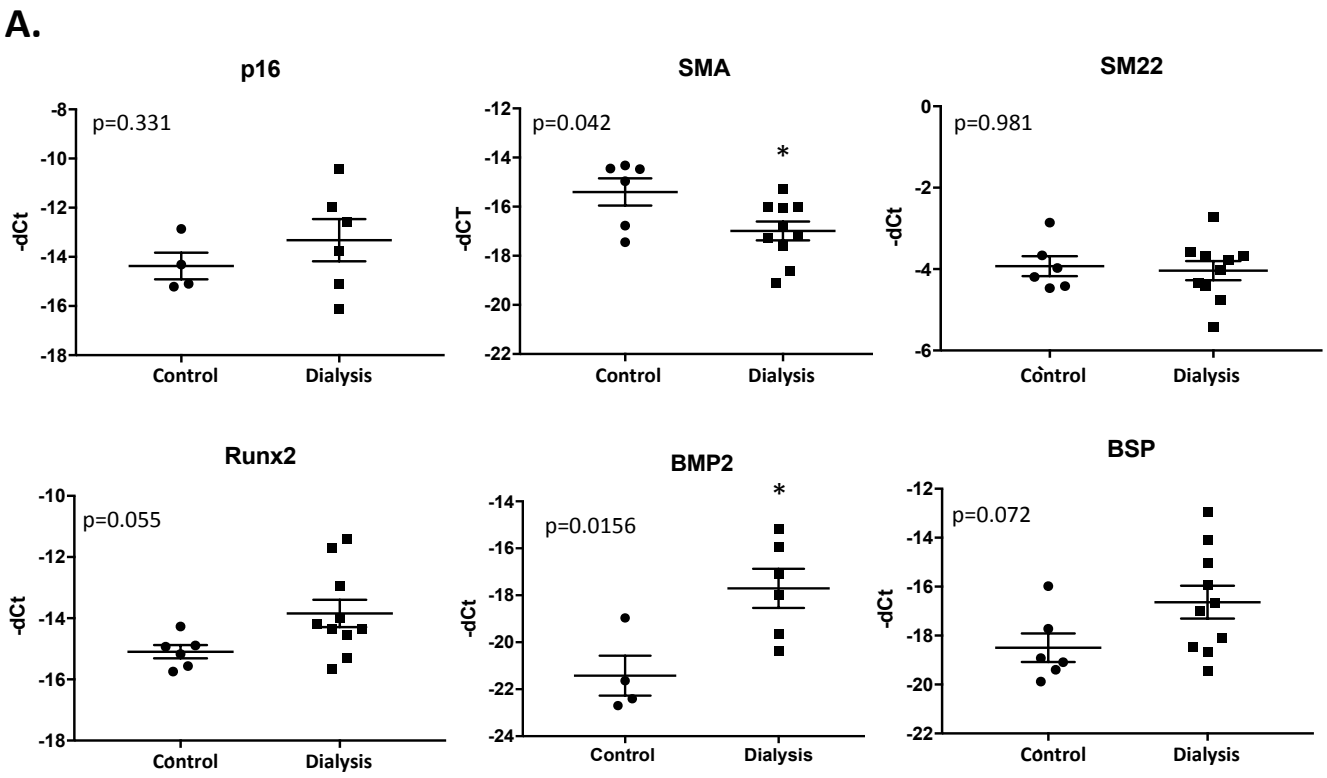
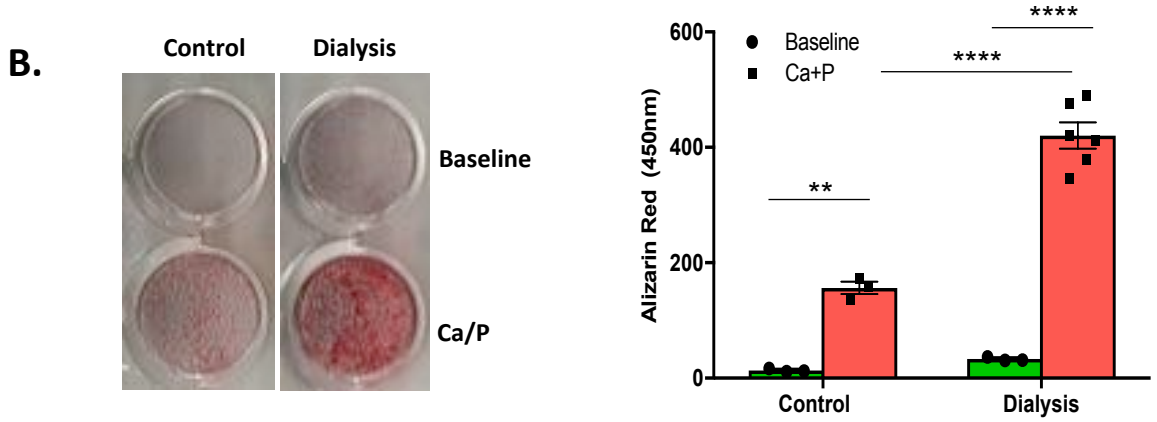

A.
DNA
γH2AX
Merge
Control
Dialysis
Gamma H2AX positive cells, %
150
100
50
0
*
DMSO
2h Dox
2h wash
24h wash
DMSO
2h Dox
2h wash
24h wash
Control
Dialysis
B.
DNA
53BP1
Merge
Control
Dialysis
53BP1 positive cells, %
150
100
50
0
**
DMSO
2h Dox
2h wash
24h wash
DMSO
2h Dox
2h wash
24h wash
Control
Dialysis

Figure 4.


A.
p16
p=0.331
-dCt
Control
Dialysis
SMA
p=0.042
-dCT
*
SM22
p=0.981
Runx2
p=0.055
BMP2
p=0.0156
BSP
p=0.072


B.
Control
Dialysis
Baseline
Ca/P
Alizarin Red (450nm)
Baseline
Ca+P
**
****

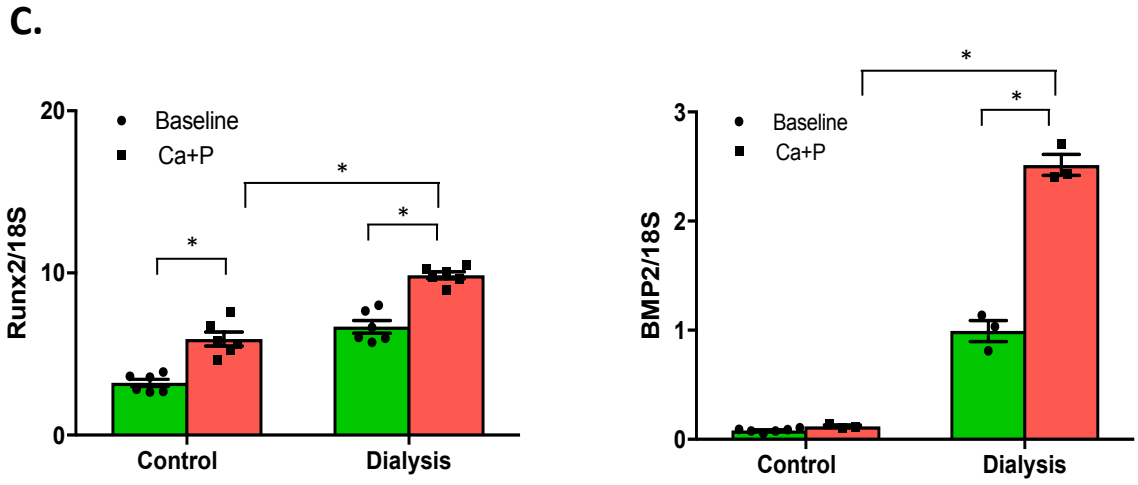
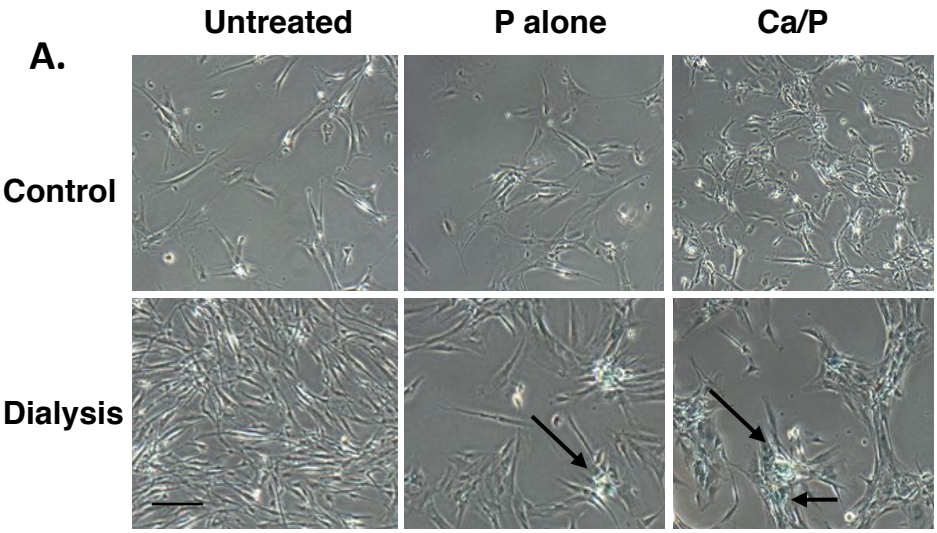
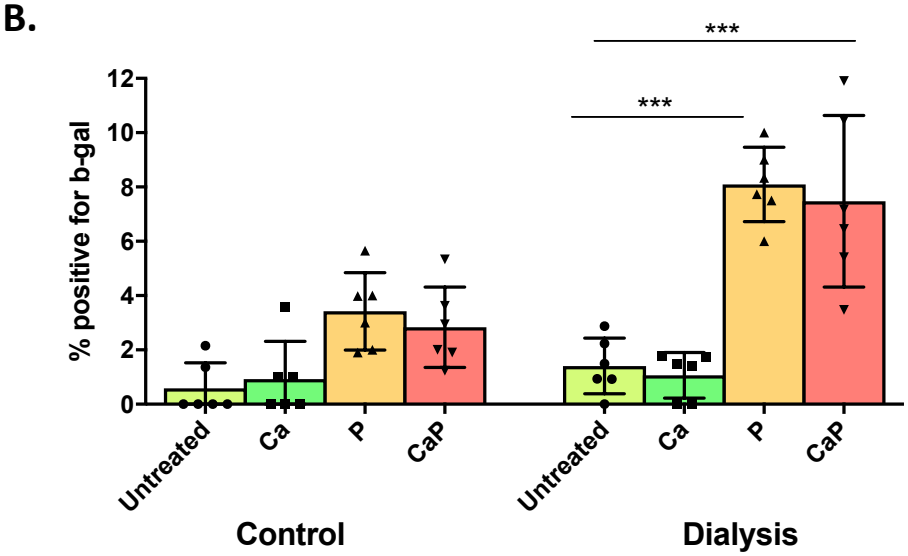

C.
Runx2/18S
BMP2/18S
Baseline
Ca+P
*
Control
Dialysis

Figure 5.


A.
Untreated
P alone
Ca/P
Control
Dialysis


B.
% positive for b-gal
12
10
8
6
4
2
0
***
***
Untreated
Ca
P
CaP
Untreated
Ca
P
CaP
Control
Dialysis

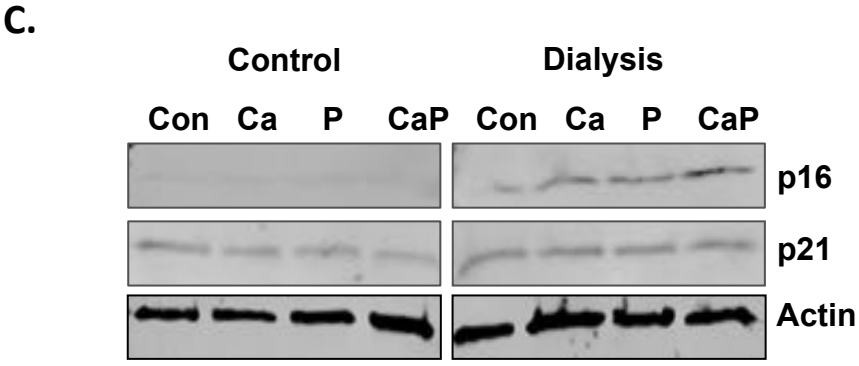

C.
Control
Dialysis
Con
Ca
P
CaP
Con
Ca
P
CaP
p16
p21
Actin

Figure 6

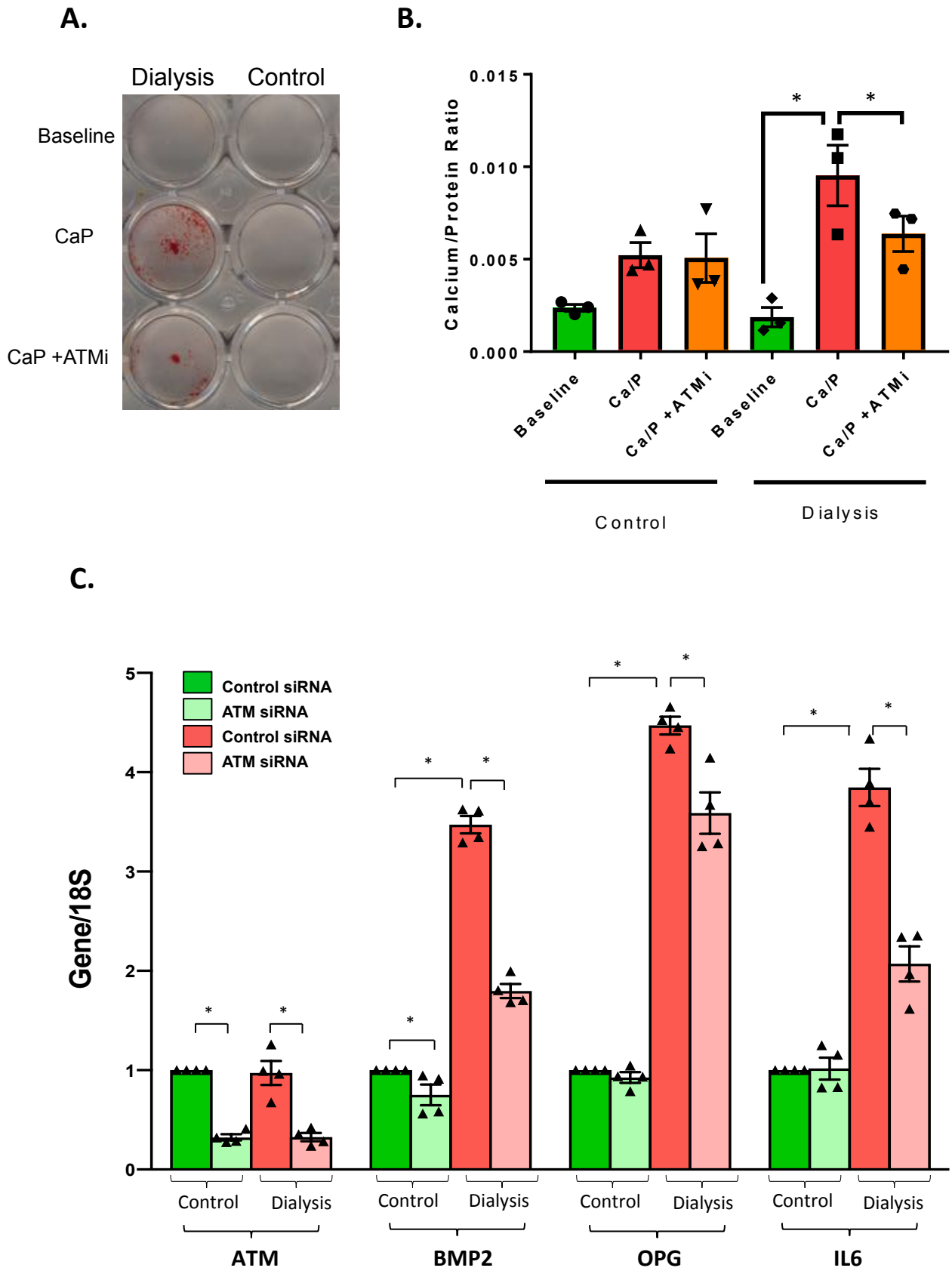

A.
Dialysis
Control
Baseline
CaP
CaP +ATMi
B.
Calcium/Protein Ratio
0.015
0.010
0.005
0.000
Baseline
Ca/P
Ca/P +ATMi
Baseline
Ca/P
Ca/P +ATMi
Control
Dialysis
C.
Gene/18S
Control siRNA
ATM siRNA
Control siRNA
ATM siRNA
Control
Dialysis
ATM
BMP2
OPG
IL6

Figure 7

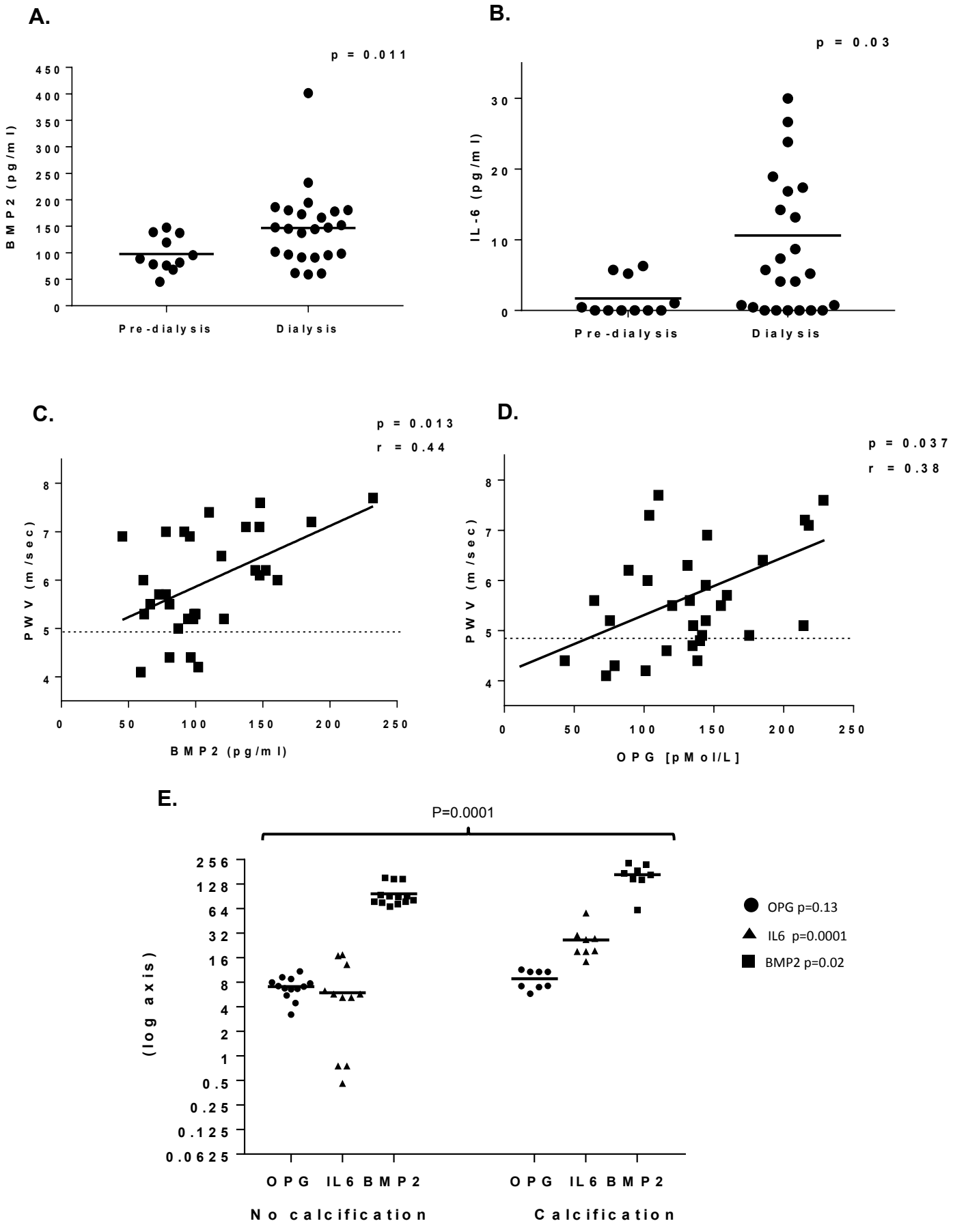

A.
p = 0.011
BMP2 (pg/ml)
Pre-dialysis
Dialysis
B.
p = 0.03
IL-6 (pg/ml)
Pre-dialysis
Dialysis
C.
p = 0.013
r = 0.44
PWV (m/sec)
BMP2 (pg/ml)
D.
p = 0.037
r = 0.38
PWV (m/sec)
OPG [pMol/L]
E.
P=0.0001
(log axis)
OPG IL6 BMP2
No calcification
Calcification
OPG p=0.13
IL6 p=0.0001
BMP2 p=0.02

Figure 8.