Pharmaceutical stabilization of abdominal aortic aneurysms : changing its natural history

Kokje, V.B.C.

Chapter 4

ACTIVATION OF THE VITAMIN D RECEPTOR SELECTIVELY INTERFERES WITH CALCINEURIN-MEDIATED INFLAMMATION: A CLINICAL EVALUATION IN THE ABDOMINAL AORTIC ANEURYSM

Arend Jan Nieuwland[1], Vivianne B.C. Kokje[1], Olivier Koning[2], Jaap F. Hamming[1]; Szuhai K[3], Frans H.J. Claas[4], Jan H.N. Lindeman[1]

Depts. of [1]vascular surgery, [3]molecular cell biology and [4]immunology, Leiden University Medical Center, Leiden; and [2]Dept. Surgery Jeroen Bosch Ziekenhuis, Den Bosch, The Netherlands

Laboratory Investigation (2016) 96. 764-790

ABSTRACT

Introduction

In-vitro and in-vivo studies attribute potent immune regulatory properties to the vitamin D receptor (VDR). Yet, it is unclear to what extend these observations translate to the clinical context of (vascular) inflammation. This clinical study evaluates the potential of a VDR agonist to quench vascular inflammation.

Methods and Results

Patients scheduled for open abdominal aneurysm repair received paricalcitol 1 µg daily during 2-4 weeks prior to repair. Results were compared to matched controls. Evaluation in a parallel group showed that AAA patients are vitamin D insufficient (median plasma vitamin D: 43 [30-62 (IQR)] nmol/L). Aneurysm wall samples were collected during surgery, and the inflammatory footprint studied. The brief paricalcitol intervention resulted in a selective 73% reduction in CD4+ T-helper cell content ($P< 0.024$) and a parallel 35% reduction in T-cell (CD3+) content ($P< 0.032$). On the mRNA level, paricalcitol reduced expression of T-cell associated cytokines IL-2, -4 and 10 ($P<0.019$). No effect was found on other inflammatory mediators. On the protease level, selective effects were found for cathepsin K ($P< 0.036$) and L ($P<0.005$). Collectively these effects converge at the level of calcineurin activity. An effect of the VDR agonist on calcineurin activity was confirmed in a mixed-lymphocyte reaction.

Conclusions

A brief course of the VDR agonist Paricalcitol has profound effects on local inflammation via reduced T-cell activation. The anti-inflammatory potential of VDR activation in vitamin D insufficient patients is highly selective and appears to be mediated by an effect on calcineurin-mediated responses.

INTRODUCTION

Inflammation plays a key role in the progression of the abdominal aortic aneurysm (AAA). The vitamin-D receptor (VDR) is a widely expressed nuclear receptor with an expression pattern that comprises most leucocytes, endothelial cells as well as vascular smooth muscle cells[1,2]. In-vitro studies show that activation of the VDR has strong immune-modulatory (anti-inflammatory) effects, and in the context of atherosclerotic disease it has been proposed that activation of the VDR may quench vascular inflammation. Although the validity of this concept has been well established in animal models, clinical studies consistently fail to show a benefit of VDR activation[3,4]. The basis for this discrepancy is unclear. Possible explanations include differences in inflammatory responses between preclinical disease models and the human situation[5,6]. Moreover, it has been pointed out that observations from situations of true vitamin D deficiency cannot simply be extrapolated to conditions characterized by a (sub) normal vitamin D status[7]. Considering the apparent gap between pre-clinical expectations and outcomes of clinical interventions we decided to explore the anti-inflammatory potential of VDR activation on the human vasculature.

The pathology of AAA is characterized by a broad and intense inflammation that comprises almost all aspects of the native and adaptive immune response[8,9]. In some patients, open surgical repair is indicated, thereby providing access to tissue. As such this procedure provides a unique opportunity to test the anti-inflammatory potential of pharmaceutical interventions.

In the present study, we investigated the anti-inflammatory potential of a 2-4 week treatment with the potent VDR agonist, paricalcitol, in patients scheduled for elective, open AAA repair. Results from this study show that the anti-inflammatory potential of the VDR-agonist paricalcitol in patients with subnormal vitamin D status is restricted to a selective effect on the calcineurin/NFAT axis; an observation that is supported by the ability of paricalcitol to effectively suppress lymphocyte proliferation in the mixed lymphocyte reaction.

MATERIAL AND METHODS

Patient populations

This open proof-of-concept study was approved by the Medical Ethical Committee of the Leiden University Medical Center. Written informed consent was obtained from all patients. Patients scheduled for open AAA repair were eligible for the study. Decision for open-repair was based on anatomical (e.g. neck, elongation), and patients characteristics (e.g. age) and preferences. Patients with impaired liver dysfunction (ALAT >3 times upper limit of the reference values, hypercalcemia and/or hyperphosphatemia, patients on digoxin as well as patients with inflammatory disease or (suspected) so-called inflammatory aortic aneurysms, were excluded from participation in the study. The study was started in November 2008 and the final patient was included in November 2010.

Patients received paricalcitol 1 µg once a day in the 2-4 weeks preceding their planned elective open repair. The final dose was taken in the evening before the surgery. Control AAA wall samples were obtained from the LUMC biobank, these samples were matched for sex, age, maximum AAA diameter and statin use. AAA wall tissue was taken from the anterior-lateral aneurysm wall at the level of the maximal diameter of the aneurysm. All wall samples (viz. samples both study

samples and biobank samples) were collected immediately after opening of the aneurysm sac. Adhering thrombus was carefully removed and wall samples were immediately halved. One half was snap-frozen in CO2-cooled iso-pentane or liquid nitrogen and stored at -80° C until use for mRNA (RT-PCR) and protein (ELISA) analysis. The other half was fixed in formaldehyde (24 hours), decalcified (Kristensens solution, 120 h), and paraffin embedded for histological analysis. All analyses were performed in an investigator-blind fashion.

Assessment of baseline vitamin D status was not foreseen in the study protocol. In the light of the study findings we considered information on the vitamin D status of AAA patients in retrospect relevant. To that end we measured vitamin D levels in available plasma samples from AAA patients who participated in the PHAST study.[10] Selected samples were all from patients from the same study center as the patients from whom aneurysm wall samples were available.

Immunohistochemistry

Slides were incubated overnight with antibodies against myeloperoxidase (MPO; rabbit polyclonal, 1:4000 dilution, DakoCytomation, Heverlee, Belgium), CD3 (polyclonal rabbit, 1:400 dilution, Abcam, Cambridge, UK), CD4 (clone 4B12, 1:200 dilution, DakoCytomation), CD8 (clone C8/144B, 1:200 dilution, DakoCytomation), CD20 (clone L26, 1:1000 dilution, DakoCytomation), CD68 (clone KP6, 1:1200, DakoCytomation), and CD138 (clone B-B4, 1:1000 dilution, Serotec, Oxford, UK)[9].

For each section 6 representative medium power fields (3 photographs for 'medial' and 3 for the 'adventitial layer) were photographed at a 20x magnifier, and the number of positive cells counted.

Semi quantitative mRNA analysis

Total RNA extraction was performed using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads[9]. Copy-DNA was prepared using kit #A3500 (Promega, Leiden, The Netherlands) and quantitative real-time polymerase chain reaction (Taqman system) analysis was performed for human interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL10, Tumor necrosis factor (TNF) -α, Interferon-γ, MCP-1, Perforin , B-lymphocyte-induced maturation protein-1 (BLIMP-1), MMP (matrix metalloproteinase)2, -3, -9 and 12, the Cathepsins K, L and S, on the ABI-7500 Fast system (Life Biosciences, Nieuwerkerk aan den IJssel, The Netherlands) using established primer/probe sets (Assays on Demand, Life Biosciences) and Taqman Gene Expression Master Mix (Life Biosciences). Analyses were performed according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a reference and for normalization.

Protein analysis

Aortic wall tissues were pulverized in liquid nitrogen and homogenized in two volumes lysis buffer (10 mM Tris pH 7.0, 0.1 mM CaCl2, 0.1 M NaCl, 0.25% (v/v) Triton X-100). Samples were subsequently centrifuged at 10,000g for 15 minutes at 4°C, and the supernatant protein extract was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Protein content in thawed protein extracts homogenates was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA). Cytokine/chemokine protein levels in these homogenates were measured by separate ELISAs for IL-6, IL-8

(PeliKane compact kit, Sanquin, Amsterdam, The Netherlands), and MCP-1 (Quantikine kit, R&D Systems, Abingdon, UK).

Relative tissue MMP2 (pro and activated form) content was assessed by Western blot using anti-MMP2 (sc-10736, Santa Cruz Biotechnology, Dallas USA) and GAPDH (sc-25778, Santa Cruz Biotechnology) for normalization. Blots were visualized using supersignal West femto substrate kit (Life Technologies, Bleiswijk, The Netherlands) and chemiluminiscense visualized and quantified on a Chemidoc Touch Imaging system (Biorad Laboratories, Veenendaal, The Netherlands) .

T-cell proliferation

Mixed Lymphocyte Reactions (MLRs) were set up with 50 µl of 1 × 106 donor PBMCs with 105 irradiated (30 Gy/3000 Rad) HLA-mismatched stimulator cells (antigen-specific stimulus) or donor PBMC were stimulated with the mitogen phytohemagglutinin (PHA, non-antigen specific stimulus) in triplicate in 96-well round-bottomed plates (Greiner Bio-one) in the presence of different concentrations paricalcitol or one the comparators (Tacrolimus or Mycophenolic acid). Proliferation was measured on day 5 by incorporation of 3H-thymidine, which was added during the last 16 h of culture. The results were expressed as the median counts per minute (cpm) for each triplicate culture.

Statistical analysis

Statistical analyses were performed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). For the comparisons, a P-value <0.05 was considered statistically significant. Differences between the groups were evaluated by ANOVA (normally distributed data) or the Kruskal Wallis test in case of non-normally distributed continuous data. As all the observations in the paper fit within a theoretical framework no correction for multiple testing was performed.

RESULTS

Patient population

Baseline characteristics of the pre-operative paricalcitol-intervention group (n=11) and the matched control group (n=11) used in the molecular analysis are shown in table 1. Paricalcitol was well tolerated and there were no drop-outs.

Plasma levels showed that all patients in the parallel AAA cohort are vitamin D insufficient by current standards[11] (median level 43 [30-62 (IQR) nmol/L]).

Paricalcitol intervention

AAAs are characterized by a broad cellular inflammatory component consisting of macrophages, neutrophils, and T- and B-cells[8,9]. Immuno-histochemical analysis showed that paricalcitol reduced the aortic wall T-cell ($CD3^+$) and T-helper cell ($CD4^+$) content ($P< 0.024$ and $P<0.032$ respectively), but did not influence the relative abundance of the other inflammatory cell types (i.e. monocytes/macrophages ($CD68^+$), neutrophils (MPO^+), cytotoxic T-cells ($CD8^+$), B-cells ($CD20^+$) and plasma cells ($CD138^+$)) (Figure 1) or distribution over the media and adventitia (Figure 2).

Evaluation of an effect of paricalcitol treatment on the mRNA levels of inflammatory mediators showed a selective reduction in the Th1/Th2 cytokines IL2, -4 and -10. No effect was seen on

Table 1. Baseline patient characteristics of the paricalcitol intervention study. Median [inter quartile range]

	Paricalcitol n=11	**Controls n=11**
Age (year)	72 [61-76]	72 [67-77]
AAA diameter (mm)	57 [52-62]	57 [51-67]
Male sex	11/12	11/12
(ex) smokers	11/12	10/12
Statin use	10/12	10/12
ACE inhibitor use	3/12	2/12

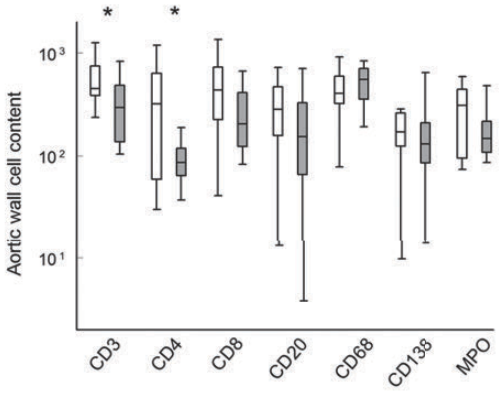

Figure 1. Effect of Paricalcitol on aneurysm wall leucocyte content. Semi-quantitative analysis of aortic wall T-cell (CD3), T-helper cell (CD4), and cytotoxic T-cells (CD8), B-cell) (CD20) monocyte/macrophage (CD68), plasma cell (CD138), and neutrophil (myeloperoxidase (MPO) content. Cell counts are based on reflect the number of positive cells per 6 medium power fields. Cell content is expressed as the number of cells per mm2. Non-treated controls (white bars); Paricalcitol-treated patients (grey bars). Boxplots indicate the inter quartile ranges with the median. The vertical lines represent the range. *P<0.03.

the mRNA and protein levels of general proinflammatory cytokines (Table 2, Figure 3). Paricalcitol did not influence the expression of the B-cell associated marker BLIMP-1 or the cytotoxic T-cell/NK cell marker perforin. On the protease level paricalcitol reduced the expression of the cathepsins K and L, and increased the expression of MMP2 (Table 2). No effect was found on the expression of other MMPs. The change in MMP2 mRNA expression was not followed by an increase aneurysm wall protein content. On the contrary, VDR activation reduced both pro and activated MMP2 content (relative expression: 1.27 (0.50) vs 0.74 (0.24) (proMMP2) and 0.31 (0.13) vs 0.18 (0.04) (activated MMP2) respectively, P<0.04).

An apparent selective effect on CD4+ T-helper cells, T-helper cell-associated cytokines and a selective effect on cathepsin K and L expression imply an effect of paricalcitol on the Calcineurin/NFAT signalling pathway. We explored such an effect[12] in a mixed lymphocyte reaction. Figure 4 shows that the effect size of paricalcitol mediated T-cell proliferation was almost equal to that of tacrolimus. Cytotoxicity assays showed that this effect is not explained by excess cell death (results not shown).

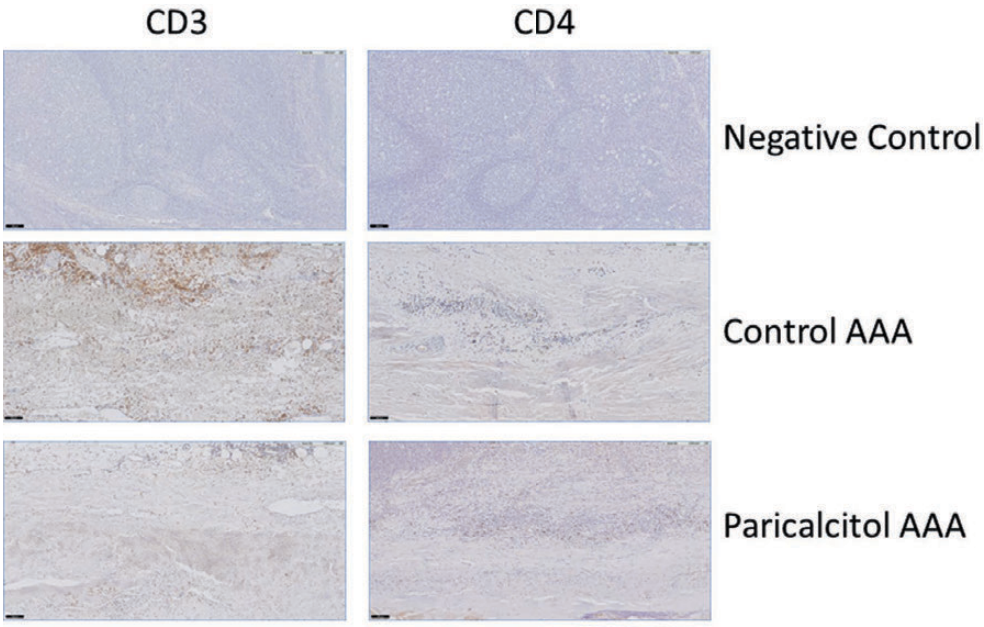

Figure 2. Immunohistological staining for CD3 and CD4 in AAA wall samples from control and paricalcitol-treated individuals. AAA, abdominal aortic aneurysm.

Table 2. Relative mRNA expression of selected inflammatory mediators, proteases, cytokines, and cell activation markers (log transcript level relative to GAPDH (GAPDH=0)). Frozen material was not available from 1 Paricalcitol patient.

	Control n=11	Paricalcitrol n=10	p
IL1β	-1.49 [-1.65 – -1.30]	-1.23 [-1.47 – -0.99]	0.122
IL-2	-1.42 [-1.62 – -1.26]	-1.98 [-2.30 – -1.90]	0.000056
IL-4	-2.76 [-3.17 – -2.51]	-3.49 [-3.61 – -3.01]	0.019
IL-6	-0.32 [-1.10 – 0.03]	-0.77 [-1.08 – -0.46]	0.595
IL8	-0.44 [-1.28 – -0.21]	-0.42 [-0.71 – -0.18]	0.295
IL-10	-1.77 [-2.06 – -1.53]	-2.13 [-2.28 – -2.00]	0.006
MCP-1	-0.05 [-0.75 – 0.44]	-0.36 [-0.55 – -0.15]	0.421
TNFα	-1.32 [-2.17 – -0.87]	-1.01 [-1.21 – -0.90]	0.188
Interferon γ	-2.18 [-2.65 – -1.62]	-1.57 [-1.98 – -1.21]	0.119
MMP2	-0.41 [-0.99 – -0.11]	0.03 [-0.17 – 0.18]	0.011
MMP3	-1.61 [-1.89 – -1.09]	-1.51 [-2.06 – -1.09]	0.929
MMP9	-0.94 [-1.34 – -0.20]	-0.54 [-0.59 – -0.22]	0.105
MMP12	-1.02 [-1.76 – -0.44]	-0.66 [-0.88 – -0.50]	0.106
Cathepsin K	-1.16 [-1.41 – -0.94]	-0.90 [-1.07 – -0.83]	0.036
Cathepsin L	-0.14 [-0.26 – 0.10]	-0.43 [-0.56 – -0.19]	0.005
Cathepsin S	-0.74 [-1.03 – -0.31]	-0.41 [-0.56 – -0.32]	0.079
Perforin	-1.68 [-2.71 – -0.74]	-0.95 [-1.07 – -0.92]	0.057
BLIMP	-1.19 [-2.56 – 0.14]	-0.57 [-0.81 – -0.41]	0.125

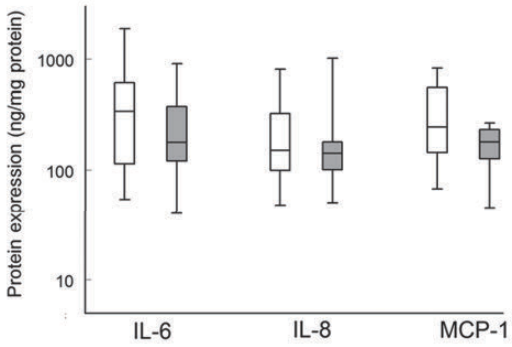

Figure 3. Similar aneurysm wall protein interleukin-6, interleukin-8, and monocyte chemo-attractant protein-1 content in non-treated controls (white bars) and Paricalcitol-treated patients (grey bars). Boxplots indicate the inter quartile ranges with the median. The vertical lines represent the range. No differences were found between the two groups (ANOVA). IL, interleukin.

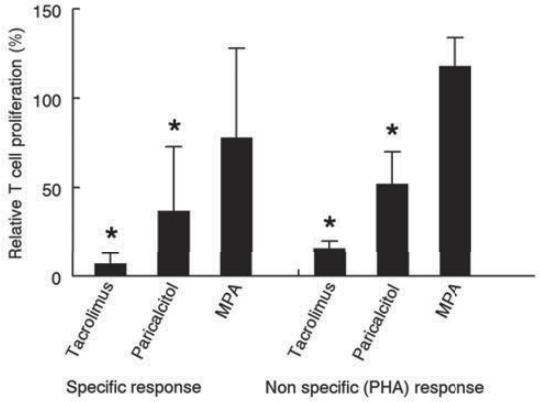

Figure 4. Relative effects of tacrolimus, paricalcitol and mycophenolic acid (MPA) on the specific and non-specific T-cell response in a mixed lymphocyte reaction (control condition = 100%). *P<0.04. PHA=phytohemagglutinin.

DISCUSSION

This clinical study shows that a brief 2-4 week intervention with the VDR agonist paricalcitol exerts clear effects on aspects of vascular inflammation in the AAA. The selective effect on T-helper cells, the proteases cathepsin K and L, and the results of the mixed lymphocyte response are consistent with a selective effect on the calcineurin/NFAT axis.

Reports on the anti-inflammatory potential of VDR agonists are multiple and diverse. Reported mechanisms include, among others quenching of NFκB[13] and MAPK[14,15] activation, as well as interference with macrophage activation[16] or the Ang-2-Tie-2-MLC kinase cascade[17]. Using the abdominal aortic aneurysm as a clinical example of comprehensive vascular inflammation[8,9] we show that the effects of VDR activation through the vitamin D analogue paricalcitol are restricted, and include an effect on the aneurysm wall T-helper (CD4+) cell content and its associated cytokines,

as well as an effect on the expression of the cysteine proteases cathepsins K and L. The approximate 75% reductions in both T-helper cell content and in the T-helper cell related cytokines IL-2, 4 and 10 suggests that the reduction in cytokine is expression is primarily reflects a reduction of aortic wall cell content rather than an effect on cell activation. For logistic reasons assessment of vitamin D status was not included in the study protocol. As of the remarkable findings we considered information on the vitamin D status of AAA patients relevant. To address this point we assessed vitamin D plasma levels in plasma samples from a group of AAA patients in this trials' study center who participated in the PHAST study. All these patients were vitamin D insufficient, viz. none of the AAA patients tested was vitamin D deficient or sufficient[11].

The observed selective effects on T-cell activation and the (contrasting) effect on the cysteine proteases cathepsin K and L, converge at the level of the calcineurin/NFAT axis; such an effect that has been clearly demonstrated for vitamin D in in-vitro studies[18,19]. A NFAT-1c responsive element has been described for the Cathepsin L promoter region[20], as such the reduced cathepsin L expression may reflect reduced promoter activity. An alternative (but non-exclusive) explanation is that the reduced cathepsin L expression mirrors a reduction in CD4 content. Increased cathepsin K expression seemingly contrasts with the findings for cathepsin L. Yet, cathepsin K expression is in part regulated by NFAT-2[21], a factor that in contrast to NFAT-1c requires phosphorylation rather than de-phosphorylation (NFAT1c) for nuclear transfer. Hence, it has been pointed out that calcineurin inhibitors promote NFAT-2 activity[22]. No effect was found of VDR agonist on RANKL expression[23], (results not shown). Opposed associations have been reported for a link between MMP2 expression and the calcineurin axis, as such it is unclear whether the increased MMP2 expression links to an effect on this axis or, alternatively reflects a separate effect of VDR activation or a statistical type I error[24,25]. This latter possibility is supported by reduced MMP2 protein content in paricalcitol treated patients.

In two previous studies, we have shown that brief 2-4 week pre-operative interventions with doxycycline and the ACE-inhibitor Ramipril profoundly reduced aortic wall inflammation through, respectively, effects on AP-1 (doxycycline)[26] and NFκB[27] pro-inflammatory pathways. Similarly, it was found that statins dose-dependently reduce NFκB-driven inflammation in the aneurysm wall[28]. Lacking clear effects on other inflammatory pathways, VDR activation appears highly selectively influencing NFAT mediated inflammation. Our observations not necessary exclude an effect of VDR activation on other pathways as reported in experimental studies. Yet, such effects may only be apparent when studying vitamin D deficient individuals, and are missed in the real for life situation with most patients having suboptimal (i.e. with plasma levels beyond 20 nmol/L as found in AAA patients) or normal vitamin D levels.

The observed selective effect of VDR activation on NFAT mediated inflammation implies a role for VDR agonists in pathologies with established benefit from NFAT inhibition inhibitors, either as an low-toxic, moderate potent mono therapy, or alternatively as an add-on therapy, allowing tempering of calcineurin inhibitor dose; thereby potentially limiting the negative side effects of this class of compounds.

A critical point is whether the observed effects of VDR activation are beneficial in the context of AAA disease or vascular disease in general (atherosclerosis). Although cathepsin K[29] and L[30] have both been implicated in the process of aneurysm formation[31], an apparent dominant role in AAA

progression is challenged by the observation that reductions in cathepsin K and L expression during respectively statin[28] and ACE-inhibitor therapy[27] are not followed by an effect on aneurysms growth. It is unclear whether and if the effects of VDR activation on T-helper cell content will influence AAA disease. A role for T-helper cells in the context of AAA disease remains elusive[32] with reported observations from animal studies being inconclusive[33,34], and clinically accelerated aneurysm progression during intense immune suppression[35].

Along these lines the potential benefit of VDR activation in the context of atherosclerotic disease remains unclear with clear benefits in preclinical studies, and consistent epidemiological association between vitamin D (sub) deficiency and manifest atherosclerotic disease[2], but very limited evidence for a benefit of vitamin D supplementation on manifestations of atherosclerotic disease[3,4].

This interventional study has a number of limitations. The study is not placebo controlled and small. Yet given the progressive decline in open AAA repair procedures, interventional studies like this become more and more difficult to perform. We have chosen for an individual matching procedure of cases and control material from our tissue bank; this approach reduces clinical variation in the study and removes potential strong confounders, and is very suited to observe subtle differences in pathways. However, the matched design does not circumvent biases as in the double blinded randomized trials. The consistent data from the different platforms used in this study make a type I-error extremely unlikely. A larger sample size would obviously have resulted in smaller confidence intervals, but this is getting more and more complicated in an era of endovascular aneurysm repair. We cannot exclude that minor effects on other inflammatory pathways are missed due to a type II statistical error. Yet, if such effects exist, they presumably compare weakly to the effect exerted on the NFAT pathways, and to pleiotropic effects on NFκB and AP-1 signalling exerted by respectively statins[28] and ACE-inhibitors[27], and doxycycline[26]. A further limitation of the study is that evaluation of baseline vitamin D status was not included in the study protocol. We therefore assessed vitamin D levels in plasma from AAA patients participating PHAST study who were included in same centre as the patients in this study[10]. Without any exception all patients in this AAA group had insuffient vitamin D plasma levels; as such it is unclear whether and how the observations from this study translate to vitamin D deficient and sufficient individuals.

In conclusion the anti-inflammatory potential of VDR activation in vitamin D insufficient individuals is highly selective and appears mediated by an effect on the NFAT/Calcineurin axis.

REFERENCE

1. Hayes CE, Nashold FE, Spach KM, et al. The immunological functions of the vitamin D endocrine system. Cell Mol Biol (Noisy-le-grand). 2003; 49: 277-300.
2. Norman PE, Powell JT. Vitamin D and cardiovascular disease. Circ Res. 2014; 114: 379-393.
3. Thadhani RI, Manson JE. Vitamin D: too soon to turn out the lights? Arterioscler Thromb Vasc Biol. 2013; 33: 2467-2469.
4. Lavie CJ, Lee JH, Milani RV. Vitamin D and cardiovascular disease will it live up to its hype? J Am Coll Cardiol. 2011; 58: 1547-1556.
5. Seok J, Warren HS, Cuenca AG, et al. Inflammation and Host Response to Injury, Large Scale Collaborative Research Program. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A. 2013; 110: 3507-12.
6. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol. 2004; 172: 2731-2738.
7. Shapses SA, Manson JE. Vitamin D and prevention of cardiovascular disease and diabetes: why the evidence falls short. JAMA. 2011; 305: 2565-2566.
8. Rizas KD, Ippagunta N, Tilson MD 3rd. Immune cells and molecular mediators in the pathogenesis of the abdominal aortic aneurysm. Cardiol Rev. 2009; 17: 201-210.
9. Lindeman JH, Abdul-Hussien H, Schaapherder AF, et al. Enhanced expression and activation of pro-inflammatory transcription factors distinguish aneurysmal from atherosclerotic aorta: IL-6 and IL-8-dominated inflammatory responses prevail in the human aneurysm. Clin Sci (Lond). 2008; 114: 687-697.
10. Meijer CA, Stijnen T, Wasser MN, et al. Pharmaceutical Aneurysm Stabilisation Trial Study Group. Doxycycline for stabilization of abdominal aortic aneurysms: a randomized trial. Ann Intern Med. 2013; 159: 815-23.
11. Dawson-Hughes B, Heaney RP, et al. Estimates of optimal vitamin D status. Osteoporos Int. 2005; 16: 713-716.
12. Batiuk TD, Kung L, Halloran PF. Evidence that calcineurin is rate-limiting for primary human lymphocyte activation. J Clin Invest. 1997; 100: 1894-901.
13. Chen Y, Zhang J, Ge X, et al. Vitamin D receptor inhibits nuclear factor κB activation by interacting with IκB kinase β protein. J Biol Chem. 2013; 288: 19450-19458.
14. Zhang Y, Leung DY, Richers BN, et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J Immunol. 2012; 188: 2127-2135.
15. Nonn L, Peng L, Feldman D, et al. Inhibition of p38 by vitamin D reduces interleukin-6 production in normal prostate cells via mitogen-activated protein kinase phosphatase 5: implications for prostate cancer prevention by vitamin D. Cancer Res. 2006; 66: 4516-4524.
16. Korf H, Wenes M, Stijlemans B, et al. 1,25-Dihydroxyvitamin D3 curtails the inflammatory and T cell stimulatory capacity of macrophages through an IL-10-dependent mechanism. Immunobiology. 2012; 217: 1292-1300.
17. Kong J, Zhu X, Shi Y, et al. VDR attenuates acute lung injury by blocking Ang-2-Tie-2 pathway and renin-angiotensin system. Mol Endocrinol. 2013; 27: 2116-2125.
18. Takeuchi A, Reddy GS, Kobayashi T, et al. Nuclear factor of activated T cells (NFAT) as a molecular target for 1alpha,25-dihydroxyvitamin D3-mediated effects. J Immunol. 1998; 160: 209-218.
19. Alroy I, Towers TL, Freedman LP. Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. Mol Cell Biol. 1995; 15: 5789-5799.
20. Guha M, Pan H, Fang JK, et al. Heterogeneous nuclear ribonucleoprotein A2 is a common transcriptional coactivator in the nuclear transcription response to mitochondrial respiratory stress. Mol Biol Cell. 2009; 20: 4107-4119.
21. Troen BR. The regulation of cathepsin K gene expression. Ann N Y Acad Sci. 2006; 1068: 165-72.

22. Ortega-Pérez I, Cano E, Were F, et al. c-Jun N-terminal kinase (JNK) positively regulates NFATc2 transactivation through phosphorylation within the N-terminal regulatory domain. J Biol Chem. 2005; 280: 20867-78.

23. Balkan W, Martinez AF, Fernandez I, et al. Identification of NFAT binding sites that mediate stimulation of cathepsin K promoter activity by RANK ligand. Gene. 2009; 446: 90-98.

24. Bianchi R, Rodella L, Rezzani R. Cyclosporine A up-regulates expression of matrix metalloproteinase 2 and vascular endothelial growth factor in rat heart. Int Immunopharmacol. 2003; 3: 427-433.

25. Yoo SA, Park BH, Park GS, et al. Calcineurin is expressed and plays a critical role in inflammatory arthritis. J Immunol. 2006; 177: 2681-2690.

26. Lindeman JH, Abdul-Hussien H, van Bockel JH, et al. Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells. Circulation. 2009; 119: 2209-2216.

27. Kortekaas KE, Meijer CA, Hinnen JW, et al. ACE inhibitors potently reduce vascular inflammation, results of an open proof-of-concept study in the abdominal aortic aneurysm. PLoS One 2014; 9: e111952.

28. van der Meij E, Koning GG, Vriens PW, et al. A clinical evaluation of statin pleiotropy: statins selectively and dose-dependently reduce vascular inflammation. PLoS One. 2013; 8: e53882

29. Sun J, Sukhova GK, Zhang J, et al. Cathepsin K deficiency reduces elastase perfusion-induced abdominal aortic aneurysms in mice. Arterioscler Thromb Vasc Biol. 2012; 32: 15-23.

30. Sun J, Sukhova GK, Zhang J, et al. Cathepsin L activity is essential to elastase perfusion-induced abdominal aortic aneurysms in mice. Arterioscler Thromb Vasc Biol. 2011; 31: 2500-2508.

31. Abdul-Hussien H, Soekhoe RG, Weber E, et al. Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases. Am J Pathol. 2007; 170: 809-817.

32. Curci JA, Thompson RW. Adaptive cellular immunity in aortic aneurysms: cause, consequence, or context? J Clin Invest. 2004; 114: 168-171.

33. Xiong W, Zhao Y, Prall A, et al. Key roles of CD4+ T cells and IFN-gamma in the development of abdominal aortic aneurysms in a murine model. J Immunol. 2004; 172: 2607-2612.

34. Uchida HA, Kristo F, Rateri DL, et al. Total lymphocyte deficiency attenuates AngII-induced atherosclerosis in males but not abdominal aortic aneurysms in apoE deficient mice. Atherosclerosis. 2010; 211: 399-403.

35. Lindeman JH, Rabelink TJ, van Bockel JH. Immunosuppression and the abdominal aortic aneurysm: Doctor Jekyll or Mister Hyde? Circulation. 2011; 124: e463-465.