UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

Vitamina D en la hipertensión pulmonar

Vitamin D in pulmonary hypertension

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

María Callejo Arranz

Directores

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Daniel Morales Cano

Madrid

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Universidad Complutense de Madrid
Facultad de Medicina
Dpto. Farmacología y Toxicología

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MENCIÓN DE DOCTORADO INTERNACIONAL

La presente tesis doctoral de Dña. María Callejo Arranz titulada "Vitamina D en la hipertensión pulmonar", realizada en el Departamento de Farmacología y Toxicología, de la Facultad de Medicina de la Universidad Complutense de Madrid, cumple los requisitos exigidos por la Universidad Complutense de Madrid para obtener la mención de Doctor Internacional (R.D. 99/2011):

1. La doctoranda matriculada en el programa de doctorado "Investigación Biomédica" ha realizado una estancia predoctoral en una institución internacional, durante al menos 3 meses:
    - Nombre de la institución: Centre Chirurgical Marie Lannelongue. INSERM U999. Hypertension Artérielle Pulmonaire:Physiopathologie et Innovation Thérapeutique. Paris, Francia.
    - Supervisor: Dr. Frédéric Perros.
    - Duración de la estancia: 95 días (Septiembre-Diciembre 2018).

2. La tesis doctoral ha sido evaluada por dos doctores pertenecientes instituciones internacionales.

3. Un miembro del tribunal evaluador de la tesis doctoral pertenece a un centro de investigación extranjero.

4. La tesis doctoral ha sido redactada en inglés, y al menos, una parte de la defensa será presentada en una de las lenguas habituales para la comunicación científica, distinta a cualquiera de las lenguas oficiales en España.

La presente Tesis Doctoral ha sido realizada en el Laboratorio de Farmacología y Fisiopatología Vascular, del Departamento de Farmacología y Toxicología de la Facultad de Medicina de la Universidad Complutense de Madrid, con vinculación al Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES) y al Instituto de Investigación Sanitaria del Hospital Gregorio Marañón (IiSGM). Y ha sido financiada por los siguientes proyectos de investigación:

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Index

Abbreviations

1,25(OH)$_2$vitD:1α,25-dihydroxyvitaminD_3/ calcitriol

5-HT: serotonin

6MWT: 6-minute walk distance test

18S: 18S ribosomal ribonucleid acid

25(OH)vitD: 25-hydroxyvitamin D_3/calcidiol

ACVR2A: activin A receptor type 2A

ACVR2B: activin A receptor type 2B

ACVRL1/ALK1: activin receptor-like kinase 1

ACh: acetylcholine

ALI: acute lung injury

Ang II: angiotensin II

ANOVA: analysis of variance

AUC: area under curve

B2M: beta-2 microglobulin

BAX: B-cell lymphoma 2 associated X

BCG: bromocresol green

BCL2: B-cell lymphoma 2

BIRC5: baculoviral inhibitor of apoptosis repeat containing 5

BMP: bone morphogenetic proteins

BMP2: bone morphogenetic protein 2

BMP4: bone morphogenetic protein 4

BMP6: bone morphogenetic protein 6

BMPR2/BMPRII: bone morphogenetic protein receptor type 2

BNP: B-type natriuretic peptide

BrdU: 5-bromo-2'-deoxyuridine

BSA: bovine serum albumin

BW: body weight

CAV1: caveolin 1

CCB: calcium channel blocker

CCL5: C-C motif chemokine ligand 5

cDNA: complementary deoxyribonucleic acid

cGMP: cyclic guanosine monophosphate

CI: cardiac index

COPD: chronic obstructive pulmonary disease

CoReg: co-regulator protein

CTEPH: chronic thromboembolic pulmonary hypertension

CYP24A1/24-hydroxylase: cytochrome P450 family 24 subfamily A member 1

CYP27B1/1α-hydroxylase: cytochrome P450 family 27 subfamily B member 1

CYP2R1/vitamin D 25-hydroxylase: cytochrome P450 family 2 subfamily R member 1

DAPI: 4',6-diamidino-2-phenylindole

DBP: vitamin D-binding protein

DDIT4: DNA damage inducible transcript 4

DLCO: diffusing capacity for carbon monoxide

DMEM: dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNase: deoxyribonuclease

dPAP: diastolic pulmonary arterial pressure

DTT: DL-dithiothreitol

EC: endothelial cells

ECE-1: endothelin converting enzyme 1

EFSA: European Food Safety Authority

EGF: epidermal growth factor

ELISA: enzyme-linked immunosorbent assay

Em: membrane potential

eNOS: endothelial nitric oxide synthase

ESC: European Society of Cardiology

ERA: endothelin-receptor antagonist
ERK: extracellular signal-regulated kinase
ERS: European Respiratory Society
ET-1: endothelin-1
FBS: fetal bovine serum
FC: functional class
FDA: Food and Drug Administration
FGF: fibroblast growth factor
FGF-23: fibroblast growth factor-23
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GNB2L1: guanine nucleotide binding protein beta polypeptide 2 like 1
GTP: guanosine triphosphate
HR: heart rate
HPAH: hereditable pulmonary arterial hypertension
HIV: human immunodeficiency virus
HSP70: heat shock protein 70
Hx: hypoxia
IAP: inhibitor of apoptosis family
IC_{50}: half-maximal inhibitory concentration
ICC: immunocytochemistry
IKN: non-inactivating current
IL-1β: interleukin-1β
IL-4: interleukin-4
IL-6: interleukin-6
IL-10: interleukin-10
IL-17: interleukin-17
i.p.: intraperitoneal
IPr: prostacyclin receptor
IPAH: idiopathic pulmonary arterial hypertension
iPTH: intact parathyroid hormone
IQR: interquartilic range
IU: international unit
KCNA5/Kv1.5: potassium voltage-gated channel subfamily A member 5
KCNK3/TASK-1: potassium two pore domain channel subfamily K member 3
Kv: potassium channel voltage dependent
Log: logarithm
LPS: lipopolysaccharide
LV+S: left ventricle plus septum
MAPK: mitogen-activated protein kinase
miRNA/microRNA: micro-ribonucleic acid
mPAP: mean pulmonary arterial pressure
mRNA: messenger ribonucleic acid
mTOR: mammalian target of rapamycin
MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NCBI: National Center for Biotechnology Information
NFAT: nuclear factor of activated T-cells
Nmx: normoxia
NO: nitric oxide
NT-pro BNP: N-terminal fragment of pro-B-type natriuretic peptide
NYHA: New York Heart Association
p21: cyclin-dependent kinase inhibitor 1A
p27: cyclin-dependent kinase inhibitor 1B
p38 MAPK: mitogen activated protein kinase p38
PA: pulmonary artery
PAEC: pulmonary artery endothelial cells
PAP: pulmonary arterial pressure
PASMC: pulmonary artery smooth muscle cells
PAH: pulmonary arterial hypertension
PBS: phosphate buffered saline
PCNA: proliferating cell nuclear antigen
PCR: polymerase chain reaction

PCWP: pulmonary capillary wedge pressure

PDE5: phosphodiesterase type 5

PDE5i: phosphodiesterase type 5 inhibitor

PDGF: platelet-derived growth factor

p-eNOS: phospho-endothelial nitric oxide synthase

PFA: paraformaldehyde

PGI_2: prostacyclin

PH: pulmonary hypertension

PKA: protein kinase A

PTH: parathyroid hormone

PUFA: polyunsaturated fatty acid

PVDF: polyvinylidene fluoride

PVR: pulmonary vascular resitance

qRT-PCR: quantitative reverse transcription polymerase chain reaction

RAP: right atrial pressure

RCAN1: regulator of calcineurin 1

RCT: randomized controlled trial

REHAP: Spanish Registry of pulmonary arterial hypertension

RHC: right heart catheterization

RNA: ribonucleic acid

RNA-Seq: ribonucleic acid sequencing

RPKM: reads per kilobase per million

RV: right ventricle

RVDP: right ventricular diastolic pressure

RVSP: right ventricular systolic pressure

RXR: retinoid-X-receptor

SD: standard deviation

SEM: standard error of mean

sGC: soluble guanylate cyclase

siRNA: small interfering ribonucleic acid

SMAD: decapentaplegic homolog

SMC: smooth muscle cells

SNP: single nucleotide polymorphism

sPAP: systolic pulmonary arterial pressure

Std: standard

TAPSE: tricuspid annular plane systolic excursion

TEI index: myocardial performance

TGF-β: transforming growth factor-β

TGFβR2: transforming growth factor-β receptor 2

Th1: T helper cells type 1

Th2: T helper cells type 2

TL: tibial length

TLR: toll-like receptor

TNF-α: tumor necrosis factor-α

TXA_2: thromboxane A_2

UVB: ultraviolet B radiation

VEGF: vascular endothelial growth factor

VEGFR2: vascular endothelial growth factor receptor type 2

ViDA: vitamin D assessment

VitD: vitamin D

VDR: vitamin D receptor

VDRE: vitamin D response element

VS: versus

VSMC: vascular smooth muscle cell

WHO: World Health Organization

α-SMA: alpha-smooth muscle actin

β-ACTIN: actin beta

Abstract

Introduction

Pulmonary arterial hypertension (PAH) is a vascular chronic disorder, characterized by sustained vasoconstriction, vascular remodelling, thrombosis *in situ* and inflammation. Although there have been important advances in the knowledge of its pathophysiology and consequently of its pharmacological treatments, PAH remains a debilitating, limiting and rapidly progressive disease. In recent years, epidemiological, nutritional studies and animal models have reported an association between nutritional factors and PAH. Moreover, some authors suggest that nutritional intervention may be a new preventive strategy in PAH.

Vitamin D (vitD) deficiency is a worldwide health problem of pandemic proportions. Preliminary studies have suggested that vitD deficiency is more prevalent in PAH patients than in general population. There are some basic and clinic evidences which suggest that hypovitaminosis D may negatively impact on disease progression. The active form of vitD, i.e., calcitriol, exerts its functions through the vitamin D receptor (VDR), which acts as a transcription factor, regulating changes in gene expression. The discovery of VDR in many tissues and cell types that do not participate in calcium and phosphorous homeostasis, (the main functions of calcitriol), led to identify a great variety of functions mediated by VDR, and of potential relevance in the cardiovascular system, such as cell proliferation, differentiation, control of vascular tone or immunomodulation.

Hypothesis and aims

The general **hypothesis** of this Doctoral Thesis is that vitD deficiency is a predisposing or aggravating factor for PAH. The **general aim** of this Doctoral Thesis is to study the relationship between PAH and vitD status and the characterization of VDR in the pulmonary vasculature and its role in PAH. The **specific aims are**: 1) To examine the total, bioavailable and free 25(OH)vitD levels and their prognosis value in patients with PAH. 2) To investigate if vitD deficiency may induce pulmonary vascular dysfunction and/or exacerbate it in an experimental model of PAH and to explore the potential mechanisms involved. 3) To analyse whether recovering optimal vitD levels in an experimental model of PAH previously depleted of vitD improves pulmonary hemodynamics and reverses the pulmonary endothelial dysfunction and the pulmonary ionic remodelling. 4) To examine if vitD deficiency may account for the limited efficacy of sildenafil in some patients with PAH. 5) To study the expression of VDR in the pulmonary vasculature and its role in pulmonary artery smooth muscle cell (PASMC) proliferation.

Methods

First, to determine total, bioavailable and free vitD status and its prognostic value in PAH patients, 67 plasma samples from Spanish patients with idiopathic, hereditable and drug-induced PAH were obtained from the Spanish PAH Biobank and compared to a cohort of 100 healthy subjects. Bioavailable and free vitD and vitD-binding protein (DBP) plasma levels were analysed. Clinical parameters were obtained from the Spanish Registry of PAH (REHAP). Second, to investigate if vitD deficiency may induce or aggravate PAH, male rats were fed with a standard or a vitD-free diet for five weeks. Then, rats were further divided into controls or PAH, which was induced by a single dose of Su5416 (20 mg/Kg) and exposure to chronic hypoxia (10% O_2) for 2 weeks. Hemodynamic, vascular function, histological and molecular approaches were carried out. Third, to study whether restoring vitD levels to an optimal range improves the main features of the pathology, PAH-animals with severe deficit of vit D received a dose of 100.000 IU vitD/Kg plus standard diet for 3 weeks. Hemodynamics, pulmonary arterial tone and pulmonary vascular remodelling were analysed. Forth, the responses to sildenafil and riociguat were studied in isolated pulmonary arteries (PA) from PAH-rats with severe deficit of vitD and after restoring optimal vitD levels. The relationship between vitD status and the response to sildenafil was examined in PAH-patients. And fifth, in order to examine the role of VDR in the pulmonary vasculature and its implication in PAH, the localization, expression and VDR-antiproliferative effects were analysed.

Results

Seventy percent of PAH patients had severe vitD deficiency (total 25(OH)vitD<10 ng/ml) and secondary hyperparathyroidism. PAH patients with total 25(OH)vitD plasma below the median of this cohort (7.17 ng/ml) had worse functional class (FC), lower 6-minute walking distance (6MWD) and reduced survival. DBP and bioavailable 25(OH)vitD plasma levels from PAH patients were downregulated compared to the control cohort. Lower bioavailable 25(OH)vitD levels were associated with more advanced FC, lower exercise capacity and higher risk of mortality. Free 25(OH)vitD plasma levels did not change in PAH patients, but lower values were also associated with high risk of mortality.

In the rat animal model of PAH associated with vitD deficiency, the results were: vitD deficiency had no effect on pulmonary pressure in normoxic rats, indicating that, by itself, it does not trigger PAH. However, it induced several moderate but significant changes in pulmonary arteries PA, typical features of PAH, such as increased muscularization, endothelial dysfunction, attenuated whole potassium current and the acid-sensitive (TASK-1) potassium current, and reduced *Bmp4*, *Bmp6* and *Kcnk3* expression. In rats with PAH induced by Su5416 plus hypoxia,

vitD free diet further increased pulmonary pressure, worsened endothelial function, increased the hyperreactivity to serotonin, and the arterial muscularization, decreased total and TASK-1 potassium currents and caused a further depolarization of PASMC. We have also identified a vitD response element (VDRE) in *KCNK3* promoter conserved in human and mouse and shown that VDR stimulation with calcitriol increased *KCNK3* mRNA expression in human PASMC.

Recovering optimal levels of vitD improved several pathophysiological characteristics of PAH, such as endothelial function measured by the endothelium dependent vasodilator response to acetylcholine, and the activity of TASK-1 potassium channel. However, vitD supplementation did not reduce pulmonary pressure or pulmonary vascular remodelling.

VitD deficiency led to a poor vasodilator response to sildenafil in PA from PAH-rats which can be reverted by restoring vitD levels. By contrast, the response to riociguat was unaffected by the vitD status. Moreover, PAH-patients responders to sildenafil had significantly higher 25(OH)vitD levels than non-responders.

VDR is expressed in the nucleus and in the cytoplasm of PASMC. VDR is downregulated in lungs and PASMC from PAH patients, without changes in endothelial cells. After 48 hours of calcitriol treatment, VDR expression was strongly upregulated time-dependently in PASMC from controls and PAH patients *in vitro*. Immunofluorescence assay revealed that in the absence of calcitriol, VDR is in the cytosol and nucleus from PASMC, while after calcitriol treatment, VDR is mainly translocated to the cell nucleus, where it is physiologically functional. Calcitriol-VDR mediated an antiproliferative effect in PASMC from controls and PAH patients via SURVIVIN inhibition, partially by BMPR2 signalling pathway but not via *KCNK3*.

Conclusions

1. Vitamin D deficiency is very prevalent in PAH-patients and low levels of total 25(OH)vitD are associated with worse prognosis.
2. Severe deficit of vitamin D does not induce PAH in normoxic rats but leads to several moderate changes in PA characteristics of PAH such as endothelial dysfunction, as well as vascular and ionic remodelling.
3. Vitamin D deficiency in PAH-rats further increased mean pulmonary arterial pressure and worsened pulmonary vascular disease.
4. Restoring vitamin D levels in previously depleted rats reverts endothelial dysfunction and the activity of TASK-1 channels and the vasodilator response to sildenafil in rats. Low levels of 25(OH)vitD were also associated to an insufficient response to PDE5 inhibitors in PAH patients.

5. Vitamin D receptor is expressed in human PASMC and it is downregulated in the lungs and PASMC, but not in PAEC from PAH patients. Calcitriol can restore the receptor expression and enhance its nuclear localization and function *in vitro*. Calcitriol modulates the expression and consequently the activity of several genes dysregulated in PAH and exerts an antiproliferative effect in PASMC by opposing the actions of Survivin and by modulating the BMP signalling pathway.
6. Taken together, the data suggest that vitamin D deficiency aggravates most pathological features of PAH and may play a pathophysiological role in clinical PAH. Restoring optimal vitamin D levels in patients with severe deficiency would represent a very feasible, safe, easy, economic and promising coadjuvant therapy to improve the symptoms, quality of life and prognosis of PAH patients.

Resumen

Introducción

La hipertensión arterial pulmonar (HAP) es una enfermedad vascular crónica, caracterizada por una vasoconstricción sostenida, remodelado vascular, trombosis *in situ* e inflamación en las arterias pulmonares (AP). A pesar de los importantes avances en el conocimiento de su fisiopatología y consecuentemente la identificación de terapias farmacológicas, la HAP continúa siendo una enfermedad debilitante, limitante y progresiva. En los últimos años, estudios epidemiológicos, nutricionales, así como modelos animales han identificado una asociación entre factores nutricionales e HAP; incluso algunos autores sugieren que la intervención nutricional podría ser una nueva estrategia preventiva en pacientes con HAP.

La deficiencia de vitamina D (vitD) es un problema de salud a nivel global de proporciones pandémicas, incluso se estima que es más prevalente en pacientes con HAP que en la población en general. Existen algunas evidencias que sugieren que la deficiencia de vitD puede tener un impacto negativo en progresión de dicha patología. La forma activa de la vitD, denominada calcitriol ejerce sus funciones a través del receptor de vitD (VDR), que actúa como un factor de transcripción, regulando la expresión de genes diana. El descubrimiento de VDR en diversos tejidos y células, que no participan en la homeostasis del calcio y el fósforo (las principales funciones del calcitriol), permitió la identificación de una gran variedad de funciones mediadas por VDR con gran relevancia a nivel cardiovascular, como, por ejemplo, la regulación de la proliferación y diferenciación celular, control del tono vascular e inmunomodulación.

Hipótesis y objetivos

La **hipótesis general** de la Tesis Doctoral es que la deficiencia de vitD es un factor predisponente o agravante de la hipertensión pulmonar. El **objetivo general** de esta Tesis Doctoral es estudiar la relación que existe entre la HAP y el estatus de vitD y la caracterización del receptor de vitD en la vasculatura pulmonar y su papel en la HAP. El objetivo general a su vez se divide en **objetivos específicos**: 1) determinar los niveles de vitD total, biodisponible y libre y su valor pronóstico en pacientes con HAP; 2) investigar si la deficiencia de vitD puede inducir o agravar el desarrollo de HAP en un modelo experimental; 3) investigar si la recuperación de niveles óptimos de vitD en animales con PAH que previamente tenían déficit de vitD mejora la fisiopatología característica de la HAP; 4) examinar si la deficiencia de vitD puede explicar la eficacia parcial del sildenafilo en algunos pacientes con HAP; y 5) estudiar el papel de VDR en arterias pulmonares y su efecto en la proliferación de células musculares lisas de AP.

Métodos

Primero, se determinaron los niveles de vitD total, biodisponible y libre y la proteína de unión a la vitD (DBP) y su valor pronóstico en 67 muestras de plasma de pacientes con HAP idiopática, hereditaria o inducida por fármacos, obtenidas del Biobanco Español de HAP y comparados con una cohorte de 100 sujetos sanos, sin enfermedad cardiovascular. Las variables clínicas se obtuvieron del Registro Español de HAP (REHAP). Segundo, para investigar si la deficiencia de vitD puede inducir o agravar la HAP, ratas macho fueron alimentadas con dieta estándar de animalario o una dieta libre de vitD durante 5 semanas. Posteriormente, los animales fueron divididos en controles o con HAP, inducida a través de una dosis de Su5416 (20 mg/Kg) y la exposición crónica a hipoxia (10% O_2) durante 2 semanas. Se llevaron a cabo estudios hemodinámicos, de función vascular, histológicos y moleculares. Tercero, para estudiar si restaurar niveles óptimos de vitD mejora la fisiopatología de la HAP, animales con HAP y déficit severo de vitD recibieron una dosis de 100.000 UI vitD/Kg y dieta estándar durante 3 semanas. Se llevaron a cabo estudios de hemodinámica, electrofisiología, reactividad vascular e histología pulmonar. Cuarto, las respuestas vasodilatadoras a sildenafilo y riociguat se estudiaron en AP de ratas con HAP y déficit severo de vitD y tras recuperar niveles óptimos de vitD. La relación entre los niveles de vitD y una respuesta eficaz al tratamiento con sildenafilo se examinó en pacientes con HAP. Y quinto, para estudiar el papel del receptor de vitD en la vasculatura pulmonar y su implicación en la HAP, su localización, expresión y efectos antiproliferativos fueron analizados.

Resultados

El setenta por ciento de los pacientes con HAP presentó un déficit severo de vitD (total 25(OH)vitD<10 ng/ml) e hiperparatiroidismo secundario. Los pacientes con HAP con niveles plasmáticos de 25(OH)vitD total por debajo de la mediana de esta cohorte (7.17 ng/ml) presentaron peor clase funcional, menor distancia recorrida en el test de la marcha de los 6 minutos y menor supervivencia. Los niveles plasmáticos de DBP y 25(OH)vitD biodisponible también estaban reducidos en pacientes con HAP. Niveles bajos de 25(OH)vitD biodisponible se asociaron con clase funcional más avanzada, menor capacidad al ejercicio y un mayor riesgo de mortalidad. Los niveles de 25(OH)vitD libre eran similares en los dos grupos, pero valores bajos también se asociaron con un mayor riesgo de mortalidad.

En el modelo animal de HAP asociado a un déficit severo de vitD, los resultados obtenidos fueron: la deficiencia de vitD no causó un incremento de la presión pulmonar en aquellos animales expuestos a condiciones de normoxia, lo que indica que la deficiencia de vitD *per se* no causa HAP. Sin embargo, sí indujo algunos cambios moderados pero significativos en

las AP, característicos de la HAP. Por ejemplo, incremento de la muscularización de las AP, disfunción endotelial, atenuó la corriente de potasio TASK-1 en miocitos de AP, así como una disminución en la expresión génica de *Bmp4*, *Bmp6* and *Kcnk3*. En ratas con HAP, el déficit severo de vitD incrementó aún más la presión pulmonar, así como empeoró la función endotelial, incrementó la vasoconstricción a serotonina, la muscularización de las AP y disminuyó la corriente TASK-1 y una mayor despolarización en miocitos de AP, en comparación con los animales con HAP y dieta estándar. Además, se identificó un elemento de respuesta a vitD en el promotor de *KCNK3*. La estimulación de VDR con calcitriol incrementó significativamente la expresión génica de *KCNK3* en células musculares lisas de AP humanas.

Restaurar los niveles de vitD, en animales con HAP y déficit de vitD, mejoró algunas de las características fisiopatológicas de la HAP, como la función endotelial, analizada por la respuesta vasodilatadora inducida por acetilcolina, y la actividad del canal de potasio TASK-1. Sin embargo, la suplementación con vitD no redujo la presión pulmonar ni el remodelado pulmonar vascular en dichos animales.

La deficiencia de vitD produjo una pobre respuesta vasodilatadora al sildenafilo en AP de ratas con HAP, revertiéndose con la suplementación de vitD. Por el contrario, la respuesta a riociguat no se modificó tras el tratamiento con vitD. Además, los pacientes con HAP con una respuesta eficaz a sildenafilo presentaban unos niveles de 25(OH)vitD significativamente superiores a aquellos pacientes que no responden al tratamiento con sildenafilo.

El receptor de vitD se expresa en el núcleo y citoplasma de células musculares lisas de AP (CMLAP). VDR está disminuido en pulmones y CMLAP de pacientes con HAP. A las 48 h del tratamiento con calcitriol, la expresión de VDR estaba marcadamente aumentada tiempo-dependiente en CMLAP de donantes y pacientes con HAP. En ausencia de calcitriol, VDR se encuentra en el citosol y en el núcleo. Tras el tratamiento con calcitriol, VDR se transloca al núcleo, donde es esencialmente funcional. VDR ejerce un efecto antiproliferativo en CMLAP de controles y pacientes con HAP a través de la inhibición de survivina, parcialmente por la vía de señalización de BMP, pero no está mediado vía KCNK3.

Conclusiones

1. La deficiencia de vitamina D es muy prevalente en pacientes con HAP y niveles bajos de 25(OH)vitD se asocian con un peor pronóstico.
2. El déficit severo de vitD no induce HAP en ratas control, pero sí induce algunos moderados pero significativos cambios en las arterias pulmonares, característicos de la HAP, como disfunción endotelial y remodelado vascular e iónico.

3. La deficiencia de vitD en ratas con HAP agravó la presión arterial pulmonar y empeoró la fisiopatología de la HAP.
4. La suplementación de vitD en animales con PAH y déficit de vitD revertió la disfunción endotelial, la actividad TASK-1 y la respuesta vasodilatadora a sildenafilo. Bajos niveles de 25(OH)vitD se asociaron con una respuesta insuficiente a sildenafilo en pacientes con HAP.
5. El receptor de vitD se expresa en CMLAP humanas y está disminuido en pulmón y en CMLAP de pacientes con HAP. *In vitro*, el calcitriol puede restaurar la expresión de su receptor y potenciar su localización nuclear así como su función. El calcitriol modula la expresión y consecuentemente la actividad de varios genes desregulados en HAP y ejerce un efecto antiproliferativo en CMLAP a través de la inhibición de las acciones de la survivina y modulando la vía de señalización de BMP.
6. Estos datos sugieren que el déficit de vitD agrava las principales características patológicas de la HAP y por ello puede tener un papel fisiopatológico en la clínica de la HAP. La restauración de niveles óptimos de vitD en pacientes con déficit severo de vitD podría ser una terapia coadyuvante factible, fácil, segura, económica y prometedora, que podría mejorar los síntomas, la progresión de la HAP y la calidad de vida de estos pacientes.

Introduction

1. PULMONARY ARTERIAL HYPERTENSION

1.1. Definition

Pulmonary circulation is a closed circuit between the heart and the lungs, and it is almost completely separated from the systemic circulation. Its major role is gas exchange. Physiologically, pulmonary circulation is a high flow and low resistance system that accommodates the entire cardiac output at approximately one sixth of the systemic pressure [1]. Normal mean pulmonary arterial pressure (mPAP) is 14.0 ± 3.3 mmHg at rest, being 20 mmHg the upper limit of normal mPAP. A potential complication of pulmonary circulation is pulmonary hypertension (PH), due to a rise in mPAP and pulmonary vascular resistance (PVR). PH is a chronic vascular disorder in which the right ventricle (RV) adapts to the increased afterload. However, this compensatory mechanism is insufficient and results in RV failure and eventually death [2-4]. Since the 1st World Symposium on Pulmonary Hypertension in 1973 until 2019, pulmonary hypertension was defined as mPAP > 25 mmHg at rest, measured by right heart catheterization [4]. This definition has been revised in the 6th World Symposium on Pulmonary Hypertension for the first group of PH (see next section), i.e. Pulmonary arterial hypertension (PAH). PAH is now defined as a mean pulmonary arterial pressure > 20 mmHg by right heart catheterization, normal left atrial pressure and pulmonary vascular resistance ≥ 3 Wood units [2].

1.2. Classification. Diagnosis

A clinical classification categorizes PH into five groups according to their pathophysiological mechanisms, clinical presentation, hemodynamic characteristics and treatment strategy [2, 4]. Group 1, Pulmonary Arterial Hypertension (PAH) is also subclassified into idiopathic, familial, associated with other disorders, infections, or resulting from drug or toxin exposure (Table 1) [4, 5].

In Europe, the prevalence PAH is in the range of 15-60 subjects per million population and an incidence of 5-10 cases per million per year [6, 7]. PAH normally manifests in the 30s to 40s, but it can also be diagnosed in children, in whom prognosis is more severe [8]. PAH is more frequent in females, with a ratio in women to men ranging between 2:1 and 4:1 [7], although male sex is associated with worse prognosis [6, 7].

Table 1. Clinical classification of PAH

1. Pulmonary arterial hypertension (PAH)
   - 1.1. Idiopathic (IPAH)
   - 1.2. Hereditable (HPAH)
     - 1.2.1. BMPR2 mutation
     - 1.2.2. Other mutation
   - 1.3. Drug or toxin-induced PAH
   - 1.4. Associated with
     - 1.4.1. Connective tissues diseases
     - 1.4.2. HIV infection
     - 1.4.3. Portal hypertension
     - 1.4.4. Congenital heart diseases
     - 1.4.5. Schistosomiasis

PAH: pulmonary arterial hypertension; IPAH: idiopathic PAH; HPAH: hereditable PAH: BMPR2: bone morphogenetic protein receptor type 2; HIV: human immunodeficiency virus

Because of the non-specific symptoms of PAH, such as fatigue, weakness and dyspnea, the time between patient reporting the onset of symptoms and the definitive diagnosis by right heart catheterization is considerably delayed (approximately one-two years), which may impact on the prognosis and therapy [7, 9]. In the clinical context, several non-invasive predictors are used to evaluate the prognosis of PAH, i.e. New York Heart Association (NYHA) and World Health Organization (WHO) functional class (FC; Table 2), 6-minute walk distance test (6MWT), diffusing capacity for carbon monoxide (DLCO), B-type natriuretic peptide (BNP) and the N-terminal fragment of pro-BNP (NT-pro BNP) levels [4, 10]. Currently, the parameter non-invasive risk score has been proposed. It is a simplified version of the 2015 ESC/ERS (European Society of Cardiology and European Respiratory Society) risk assessment score and it is calculated using three non-invasive low-risk criteria: NYHA FC I-II, 6MWD > 440 m, BNP < 50 ng/l or NT-proBNP < 300 ng/l [11].

Despite great advances in the knowledge of its clinical requirements and managements, PAH continues to be a very debilitating, rapidly progressive and fatal disease with a low survival rate; one- and three-year survival rates around 87 and 67%, respectively [4, 12, 13]. In addition to poor prognosis, limitations in functional status affect the patient's quality of life, daily activities and employment [13, 14]. Patients also feel isolated and a high prevalence of depression and anxiety in PAH patients has been described [15, 16].

Table 2. Functional classification of pulmonary hypertension in adults based on The New York Heart Association classification and WHO

Functional class	Characteristics
FC I	Patients with pulmonary hypertension buy without resulting limitation of physical activity. Ordinary physical activity does not cause dyspnea or fatigue, chest pain or syncope.
FC II	Patients with pulmonary hypertension resulting in slight limitation of physical activity. They are comfortable al rest. Ordinary physical activity causes dyspnea or fatigue, chest pain or syncope.
FC III	Patients with pulmonary hypertension resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes dyspnea or fatigue, chest pain or syncope.
FC IV	Patients with pulmonary hypertension with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

1.3. Aetiology

Several genetic and environmental factors have been identified to be involved in the development and progression of PAH [4, 5, 17]. In the Western world, idiopathic PAH (IPAH), i.e., without any familial history or known triggering factor, is the most common subtype (30–50% of all cases of PAH), followed by connective tissue disease-associated PAH, congenital heart disease-associated PAH, and heritable PAH [6].

Related to familial PAH, genetic analysis identified the first mutation in 2000: a heterozygous germline mutation in *BMPR2*, gene encoding bone morphogenetic protein receptor type 2, a member of the transforming growth factor-β (TGF-β) superfamily [17]. It is well-stablished that mutations in *BMPR2* can be detected in approximately 70% of cases of heritable PAH and they are also identified in 10–20% of IPAH [18]. In addition, mutations in other genes related to BMPR2 signalling axis have been discovered [17]: *ACVRL1/ALK1* (Activin receptor-like kinase 1), *ENG* (endoglin), and *SMAD9* (decapentaplegic homolog 9), as well as the intracellular calcium regulator CAV1 (Caveolin-1) [17, 19]. *BMPR2* mutation is the major genetic predisposing factor for PAH. The variants in other genes have a modest contribution to the disease [17]. Despite hemodynamics seem to be similar between familial PAH and IPAH cases, patients with hereditable PAH associated with *BMPR2* or *ACVRL1* mutations are diagnosed at a younger age, with more severe haemodynamic abnormalities, as well as reduced survival with current pharmacological therapy [17].

Other important mutations identified in PAH patients are those in *KCNK3* gene, which encodes the potassium channel TASK-1 [20], and in *KCNA5* gene, which encodes the voltage-dependent potassium channel Kv1.5 [21]. Mutations in *KCNK3* were the first channelopathy identified in PAH [20]. Dysfunctional TASK-1 potassium channel is a hallmark in hereditable and sporadic cases of PAH [22, 23]. The prevalence of *KCNK3* mutations is 1.3% in IPAH and 3.2% in familial PAH [20]. In addition to the heterozygous *KCNK3* mutations, there is also a homozygous mutation in *KCNK3*, which is associated with an aggressive form of hereditary PAH [24].

In addition, numerous drugs and substances have been involved in the development of PAH, including anorexigens, selective serotonin reuptake inhibitors, interferons, antiviral therapies, chemotherapeutic agents, and tyrosine kinase inhibitors such as dasatinib, which has the strongest evidence in drug-induced PAH [4, 25, 26]. Furthermore, PAH is also associated with other systemic disorders, such as connective tissue diseases and portal hypertension; congenital heart diseases, and infections, like human immunodeficiency virus (HIV) and schistosomiasis [4].

In summary, with the exception of idiopathic PAH, in all forms of the disease, there is a known factor involved in its etiopathogeny, including mutations, systemic diseases, congenital heart defects, infections, drugs, and toxins. However, none of them by itself can trigger the disease and the need for a second hit has been proposed. In fact, *BMPR2* mutations present low penetrance: only 42% of the women and 14% of the men carrying the mutation develop the disease. Thus, female sex is the single most important factor influencing the penetrance of *BMPR2* mutations in PAH. Additional factors influencing this incomplete penetrance can be genetic, epigenetic and/or environmental [19, 27]. Similarly, about 30% of patients with scleroderma and 0.5% of HIV patients develop PAH [28, 29].

1.4. Pathophysiology

The initial trigger leading to PAH is still poorly understood. The pathogenesis of PAH is complex and involves a wide variety of interconnected dysfunctional molecular and cellular processes. The main pathophysiological mechanisms of PAH are sustained vasoconstriction, endothelial dysfunction, pulmonary vascular remodelling, *in situ* thrombosis, and inflammation. These deranged processes occur mainly in distal pulmonary arteries (PA) and alter PA structure and function [3, 30, 31] (Figure 1).

Sustained vasoconstriction and endothelial dysfunction, one of the early events of all forms of PAH, are due to an altered production of endothelial vasoactive mediators. These include decreased vasodilator and antiplatelet factors such as nitric oxide (NO) and prostacyclin (PGI_2), and increased vasoconstrictors and/or prothrombotic factors such as endothelin-1 (ET-1), serotonin (5-HT), thromboxane (TXA_2), angiotensin II (Ang II), and diverse growth factors such

as PDGF (platelet-derived growth factor), FGF-2 (fibroblast growth factor-2) and EGF (epidermal growth factor) which also contribute to a hyperproliferative and procoagulant state [3, 30-32].

Contributing to this dysregulated pulmonary vasoreactivity, ionic remodelling is also a key feature of PAH, involved in the onset and exacerbation of the disease. The downregulation of potassium channels, notably Kv1.5 [33, 34] and TASK-1 [22, 23] in the earliest stages of the disease results in a more depolarized membrane potential in pulmonary arterial smooth muscle cells (PASMC) in PAH patients and experimental models of PAH, leading to increased intracellular calcium and consequently PASMC vasoconstriction and also PASMC proliferation [35, 36]. Notably, several key pulmonary vasoconstrictors like ET-1 and TXA_2, acute and chronic hypoxia and reactive oxygen species which decreased NO bioavailability, inhibit the activity of these potassium channels [34, 37, 38].

As previously mentioned, one of the hallmarks in PAH is the impairment of BMPR2 signalling pathway, not only due to mutations. Nongenetic downregulation of BMPR2 is observed in idiopathic and many secondary forms of PAH, including hypoxia [39-41]. Because BMPR2 is involved in the control of vascular cell proliferation, dysregulation of this pathway, with an imbalance in bone morphogenetic proteins (BMP) and TGF-β signalling, results in a proliferative state in pulmonary arteries [42, 43]. In pulmonary arterial endothelial cells (PAEC), the altered BMPR2 signalling pathway seems to be more susceptible to apoptosis and promotes endothelial-to-mesenchymal transition [44-46]. Moreover, BMPR2 disturbance causes a phenotypic switch to a hyperproliferative state in PASMC and resistance to the growth-suppressive effects of the receptor ligands, i.e., BMPs [47, 48].

In situ thrombotic events are common in PAH and contribute to the hypercoagulable phenotype and narrowing of the pulmonary arteries as well [31]. Likewise, altered immune mechanisms also play a significant role in the pathogenesis of PAH. Pulmonary vascular lesions in PAH patients and animal models reveal a recruitment of inflammatory cells such as T- and B-lymphocytes, macrophages, dendritic cells, and mast cells [3, 30]. In addition, there are abnormal circulating levels of certain cytokines, such as IL-1β, IL-6, IL-17, TNF-α, and CCL5. Notably, some of these cytokines correlate with a worse prognosis in PAH patients [49-51].

Epigenetic mechanisms, such as those mediated by microRNAs, have also been proposed to play an important role in the development and progression of PAH [52]. Multiple studies have demonstrated a direct link between microRNAs dysregulation and PAH development [33, 53, 54].

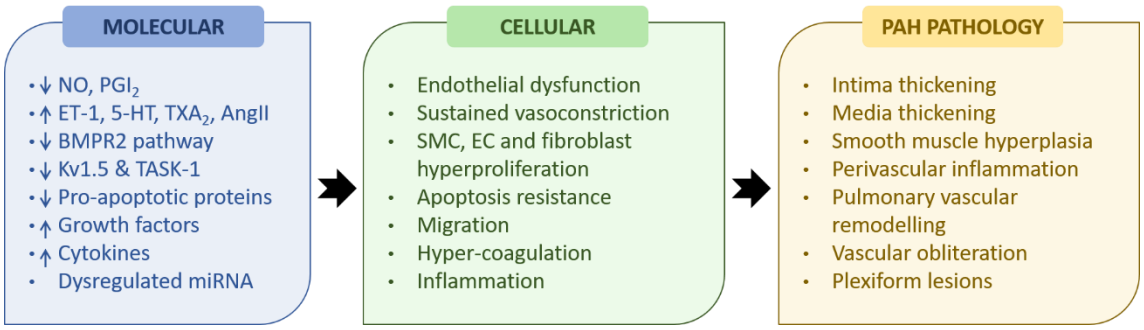

Figure 1. Molecular and cellular processes involved in the pathophysiology of PAH. *The most common molecular disturbances in PAH (left panel) are: decreased vasodilators (NO, PGI_2), increased vasoconstrictors (ET-1, 5-HT, TXA_2, AngII), downregulation of BMPR2 signalling pathway, potassium channels (Kv1.5, TASK-1), pro-apoptotic proteins (BCL-2, p53), increased growth factors and cytokines (IL-6, IL-1β) and dysregulated miRNA. Consequently, many cellular alterations occur: endothelial dysfunction, sustained vasoconstriction, cellular proliferation, apoptosis resistance and migration, hyper-coagulation and inflammation. These progressive processes drive the pulmonary vascular remodelling in pulmonary arteries, generating the plexiform lesions.*

Excessive PASMC and PAEC proliferation and resistance to apoptosis due to paracrine growth factors, dysregulation of BMPR2 signalling pathway, dysfunctional potassium channels, rise of anti-apoptotic proteins, increased cytokine production and dysregulated microRNAs, among other factors, lead to pulmonary vascular remodelling and smooth muscle hyperplasia [3, 30, 31, 55]. This complex interplay among the different processes culminate in the narrowing and the obliteration of PA by enlarged intima and media layers [30, 56] and the formation of proliferating vascular structures called plexiform lesions [56, 57] (Figure 2). In the early pathological stage, abnormal contraction and vascular remodelling become predominant, following by gradual stenosis and obstructions of pulmonary vessels with medial and then intima thickening. And finally, complex lesions consisting of plexiform lesions, space-occupying lesions and vasculitis [56-58].

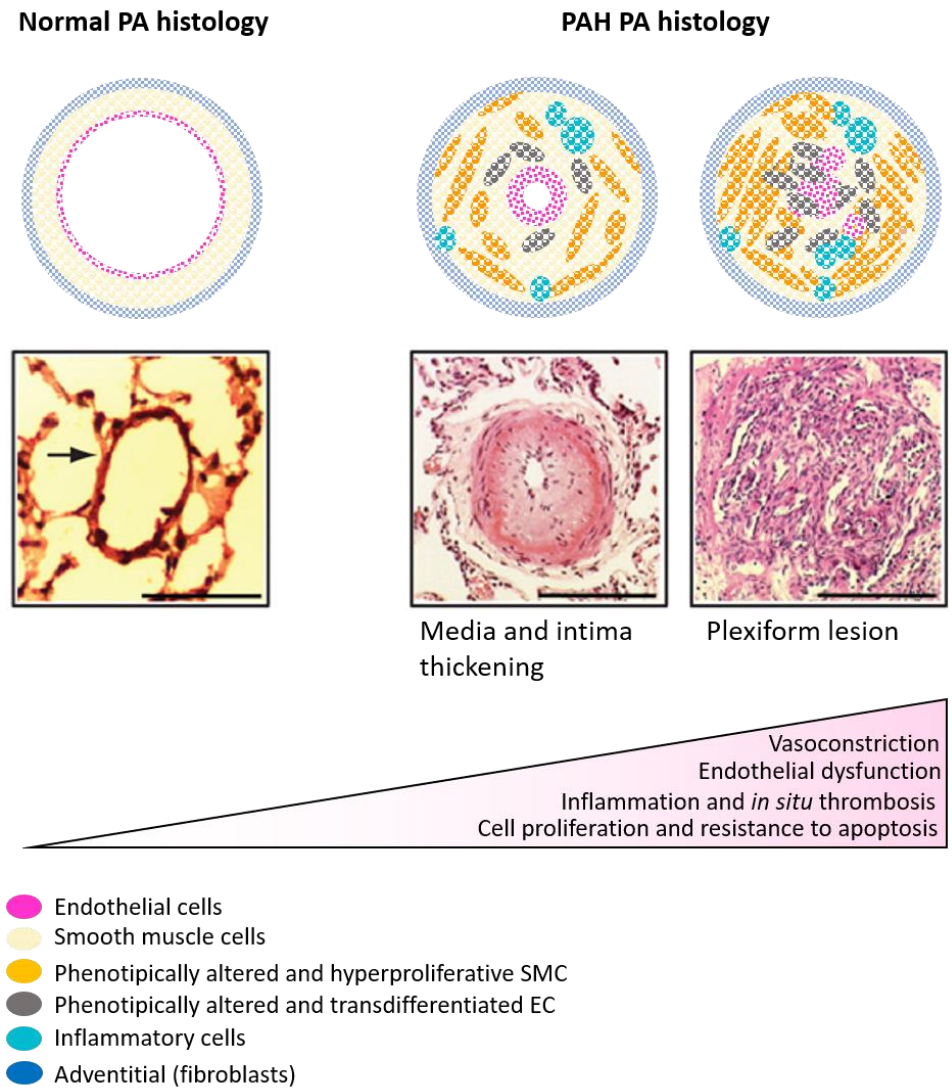

Figure 2. Schematic progression of pulmonary arterial remodelling in PAH. *Endothelial dysfunction and vasoconstriction mainly in distal pulmonary arteries (PA) followed by exaggerated proliferation and resistance to apoptosis, in situ thrombosis and inflammation contributes to the pulmonary vascular remodelling and finally the formation of plexiform lesions. These structural changes progressively result in the occlusion of the pulmonary vascular lumen. Images of histology come from the article carried out by Sutendra et al.* [59]*. Reprinted with permission from AAAS (American Association for the Advancement of Science). As they described, haematoxylin and eosin staining of distal PA shows a cross section from a healthy control (left panel), with a normal thin-walled vessel (arrow) around a wide lumen, surrounded by alveoli. Right panels correspond to sections from idiopathic PAH patient, which show, from left to right, intima and media thickening and second, a plexiform lesion showing anarchic growth of cells that completely obliterates the vascular lumen. Scale bar, 100 µm.*

1.5. Current pharmacological therapies

Over the last decades, intensive research on the cellular and molecular mechanisms and signalling pathways has provided a better understanding of the pathophysiology of PAH and consequently the identification of different effective pharmacological treatments. Unfortunately, a definitive cure does not exist for PAH and available therapies remain essentially palliative. Currently, the five classes of therapies approved for PAH target the Ca^{2+} entry and the three main dysfunctional endothelial pathways: nitric oxide (NO), prostacyclin and endothelin-1 pathways (Figure 3) [60, 61]. The imbalance of these vasoactive mediators, involved in the control of pulmonary vascular tone, plays a critical role in the pathogenesis and progression of this disease [60, 61].

The pharmacological treatment related to the arginine-derived NO synthesis by the endothelial nitric oxide (eNOS) pathway includes the cyclic nucleotide phosphodiesterase type 5 (PDE-5) inhibitors such as sildenafil and tadalafil, which potentiate the action of endogenous NO and promote vasodilation [6, 60, 61]. Soluble guanylate cyclase (sGC) also acts in the NO signalling pathway catalysing the transformation of GTP (guanosine triphosphate) to cGMP (cyclic guanosine monophosphate) in PASMC. The sGC (soluble guanylate cyclase) stimulator riociguat promotes the synthesis of cGMP favouring vasodilation and inhibiting cell proliferation. The action of riociguat is independent of NO availability. Available prostacyclin-related therapies include synthetic prostacyclin as epoprostenol (the first therapy approved for PAH in 1995), prostacyclin analogues as treprostinil and iloprost and the most recent prostacyclin receptor (IPr) agonist, named selexipag [60, 61], resulting in PA vasodilation. Third, endothelin-1 receptor antagonists (ERAs) include bosentan, macitentan and ambrisentan [6, 60, 61]. Blocking ET-1 signalling pathway inhibits pulmonary vasoconstriction.

Despite the current approved drugs as monotherapy have shown a favourable impact on clinical, functional and hemodynamic outcomes, disease progression is frequently observed. At the 5th World Symposium of PH in 2013 and based on the high level of evidence gathered from numerous randomized, controlled trials (RCT), the use of sequential combination therapy (two or more agents which simultaneously target different dysregulated pathways) was proposed, at least in PAH patients with inadequate response to monotherapy, and possible first-line therapy in patients with advanced disease (NYHA FC III/IV) [4, 62-64]. In addition, to achieve greater therapeutic response, currently, initial combination therapy at the time of diagnosis is recommended [4, 62-64]. Moreover, triple combination regimens are also considered in severe PAH, when double therapy fails [60, 65]. However, the disease is often resistant to treatment and pharmacological therapies remain unsatisfactory. Thus, lung transplantation continues being the best option for patients in whom pharmacological treatment fails [4, 61, 66].

Moreover, it should be noted that current pharmacological therapies mainly act at restoring the imbalance of vasoactive mediators and do not directly target the pulmonary vascular remodelling, cell proliferation as well as inflammation, observed in PAH patients and experimental models of PAH. Several clinical trials are currently exploring the efficacy and safety of different anti-inflammatory agents in PAH [3, 67]; these include tocilizumab, a humanised anti-IL-6 receptor antibody (ClinicalTrials.gov NCT02676947) in PAH patients; and rituximab, a chimeric anti-human CD20 (ClinicalTrials.gov NCT01086540) in patients with systemic sclerosis-associated PAH [68]. Another developing drug target is BMPR2 signalling. To date, the only treatment that has been used to target BMPR2 signalling in clinical trials is FK506, also named tacrolimus, which is currently in phase 2 (ClinicalTrials.gov NCT01647945). FK506 is an inhibitor of calcineurin, that has also been shown to upregulate BMPR2 expression [69, 70]. Sotatercept, a TGFβ inhibitor which corrects dysregulated activin–growth differentiation factor signalling, has also shown promising preliminary effects in PAH (ClinicalTrials.gov, NCT03496207).

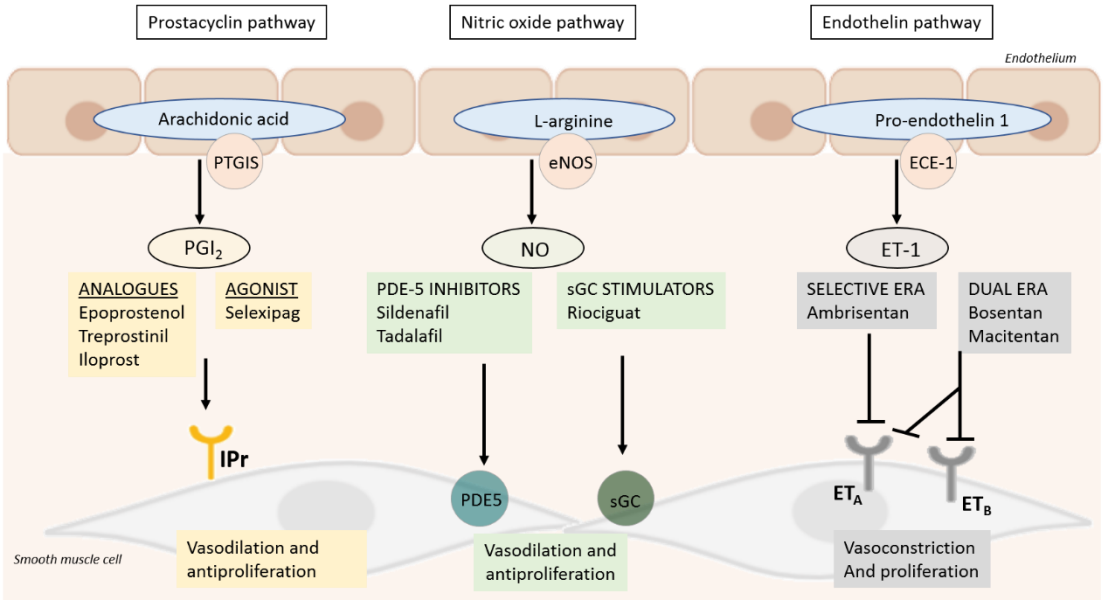

Figure 3. Molecular targets of approved PAH drug therapies: the prostacyclin, nitric oxide and endothelin pathways*. PAH is characterized by a decreased production of vasodilatory nitric oxide (NO) and prostacyclin (PGI_2) and upregulation of vasoconstrictor endothelin-1 (ET-1). PGI_2 is a product of arachidonic acid synthetized by the PGI_2 synthase (PTGIS). The PGI_2 pathway can be enhanced by prostanoid analogues or PGI_2 receptor (IPr) agonists. Arginine-derived NO synthesis by the endothelial nitric oxide synthase (eNOS) can be enhanced by the inhibition of phosphodiesterase 5 (PDE5) or by the stimulation of soluble guanylate cyclase (sGC). Pro-endothelin-1 by the endothelin converting enzyme 1 produced ET-1. The ET-1 pathway can be blocked by either selective or non-selective ET-1 receptor antagonists (ERAs), ET_A or ET_B.*

1.6. Non-pharmacological therapies. Impact of nutrition in PAH

In addition to specific treatments, non-pharmacologic approaches can be also strategies considered in PAH patients. For instance, supervised exercise rehabilitation programs can be recommended. Exercise therapy improves exercise tolerance, functional capacity and quality of life [4, 71] Moreover, some authors have reported that micronutrient deficiencies may be linked to fatigue and exercise intolerance in PAH patients [72]. Interestingly, associations between nutritional factors and PAH have recently been reported in both human epidemiological studies and animal models [72-74]. There are several pieces of evidence that nutritional deficiencies such as vitamin D (vitD) and iron are largely more prevalent in PAH patients than in general population, and they may trigger or aggravate the disease progression [75-77]. Other dietary components such as dietary polyphenols like quercetin, genistein and resveratrol may also affect the progression of the disease in animal models of PAH [78-80]. In addition, n-3 polyunsaturated fatty acids (PUFAs), vitamin C, vitamin E, melatonin and coenzyme Q10 may theoretically have an effect in PAH but there is no experimental or clinical evidence to support it [74]. Moreover, the diet is a critical regulator of the composition and function of the gut microbiota [81]. Notably, in recent years the role of gut microbiota and its interplay with the diet and the host immune system is emerging in multiple cardiovascular and respiratory diseases including PAH [82, 83]. However, to date, most of these evidences are based on animal models, observational studies and small series of uncontrolled trials. Therefore, robust RCT would be required to establish cause-effect relationships.

In light of these evidences, targeted nutritional and lifestyle interventions could have a great clinical importance in PAH patients. In fact, lately, it has been reported that multiple-target nutritional intervention with extra protein, leucine, fish oil and oligosaccharides can be a new strategy in PAH patients [84]. Moreover, dietary interventions are one of the first steps in the treatment of cardiovascular diseases [85]. Nevertheless, the ESC and the ERS Guidelines [4] have not yet stablished specific recommendations for dietary habits, nutrient supplementation and lifestyle recommendations for patients with PAH.

2. VITAMIN D

2.1. Metabolism

Vitamin D (vitD) is a fat-soluble vitamin that acts as a steroid hormone. It was discovered by Elmer McCollum as an essential nutrient for the prevention of rickets [86, 87]. Thanks to the discovery of vitD, rickets disappeared as a major medical problem [88].

The main source of vitD is derived from endogenous synthesis in the skin under the influence of solar ultraviolet B radiation (UVB; 290-315 nm); the liver-derived precursor 7-dehydrocholesterol is converted to pre-vitamin D. Food intake is a minor source of vitD, around 20%. The two main isoforms of vitD are vitamin D_3 (cholecalciferol), which it is synthetized in the skin and is contained in animal food, and vitamin D_2 (ergocalciferol), which the human body cannot synthetize, and it is obtained from fungi [89]. The inactive precursor from the skin or diet undergoes a two-step activation process to become biologically active [87, 90] (Figure 4). In the circulation, vitD metabolites are mainly transported by the vitamin D-binding protein (DBP). The first step is the 25-hydroxylation in the liver by the enzyme CYP2R1, resulting in the 25-hydroxyvitamin D_3 (25(OH)vitD), also called calcidiol, which has partial activity. Several studies have pointed to the essential role of CYP2R1 in the vitD metabolism. CYP2R1 mutations result in vitD-dependent rickets [91] and CYP2R1 knock-out mice have lower 25(OH)vitD levels [92]. Subsequently, the second hydroxylation occurs mainly in the kidney. 25(OH)vitD metabolites does not simply diffuse into cells. At least in the kidney, cell-surface receptor megalin uptake the 25(OH)vitD-DBP complex into renal proximal cells [87, 90, 93]. Once internalized, 25(OH)vitD is thought to dissociate from DBP for delivery to the mitochondria where it can be metabolized to the active metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2vitD_3$), also called calcitriol by the 1α-hydroxylase enzyme also named CYP27B1 [87, 90] (Figure 4). Consistent with this fact, mice lacking CYP27B1 gene and megalin$^{-/-}$ mice are unable to adequately metabolize 25(OH)vitD to $1,25(OH)_2$vitD and consequently develop vitD deficiency and rickets accompanied with poor survival [90, 93, 94].

Although calcitriol is the active metabolite of vitamin D, calcidiol is the best circulating biomarker of vitamin D status because the calcitriol half-life is shorter than that of calcidiol, about one day versus 3 weeks [87, 90].

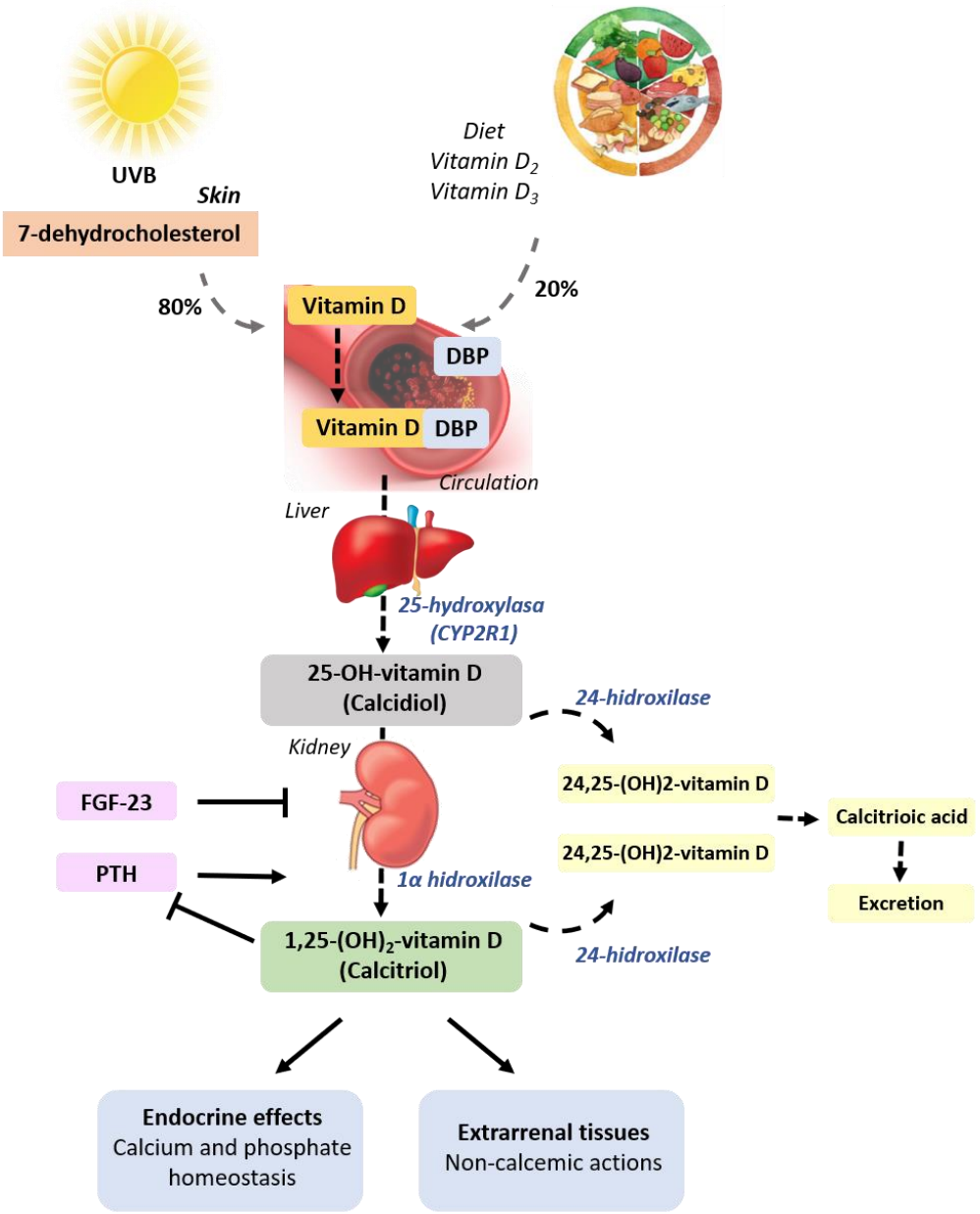

Figure 4. Metabolism of vitamin D.

The renal endocrine production of $1,25(OH)_2vitD_3$ is tightly regulated by parameters of bone and mineral metabolism, with the purpose of maintaining an adequate serum calcium concentration. This transcriptional feedback involves the parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF-23). The production of $1,25(OH)_2vitD$ by the CYP27B1 enzyme is stimulated by PTH and is inhibited by the action of FGF-23. Moreover, $1,25(OH)_2vitD$ itself inhibit PTH released. Another key element of vitD metabolism is the enzyme 24-hydroxylase or CYP24A1, which is responsible for the catabolism of $1,25(OH)_2vitD$ in most tissues. $1,25(OH)_2vitD$ indirectly regulates its own levels via CYP24A1 [87, 90] and mutations in this gene result in infantile hypercalcemia [95].

It is now recognized that various extrarenal tissues as epidermis, prostate, breast, lung, colon and many cell types also expressed 1α-hydroxylase enzyme (CYP27B1), and therefore are able to contribute to circulating levels of $1,25(OH)_2$vitD [87, 90] (Figure 5). Although megalin-dependent uptake of DBP is well-stablish in renal cells, it is not yet clearly defined whether a similar mechanism is utilized by other vitD target tissues [96]. Outside the kidney, megalin complex is expressed in several tissues, including mammary glands, placenta, parathyroid glands, and osteoblasts and even breast cancer cells, in which 1α-hydroxylase is also presented [96]. This local production serves as an autocrine and paracrine factor with cell-specific functions and it is not subject to the same feedback controls as renal production. Extrarenal 1α-hydroxylase is probably regulated by cytokines and growth factors [87, 90].

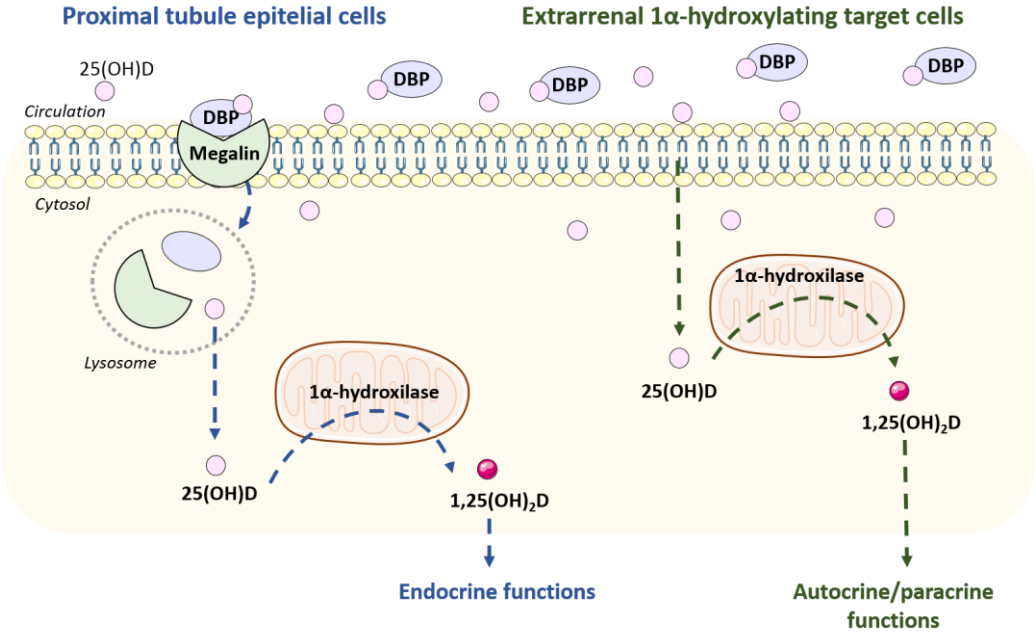

Figure 5. Renal and extrarenal 1α -hydroxylase (CYP27B1) and the production of $1,25(OH)_2$vitD. *In the kidney, cell-surface receptor megalin in the proximal tubule epithelial cells take up 25(OH)vitD bound to DBP (left), while in extrarenal cells only non-bound 25(OH)vitD can diffuse (right). Once internalized, 25(OH)vitD is converted into $1,25(OH)_2$vitD in the mitochondria.*

2.2. Transport of vitD metabolites: Vitamin D-binding protein

In the circulation, vitD metabolites are mainly transported by the glycoprotein DBP. DBP is mainly produced in the liver and it is expressed in other tissues such as kidney and fatty acid; body fluids, like serum, peritoneal fluid and cerebrospinal fluid; and also on the surface of several cell types such as neutrophils, contributing to macrophage activation [97].

Human serum concentrations of DBP are usually 20 times higher than the total amount of vitD metabolites, with a half-life of 2.5-3.0 days [98]. VitD ligands are poorly soluble in aqueous media, and their binding to DBP promotes their solubility and transport, with the highest affinity for 25(OH)vitD and lower affinity for $1,25(OH)_2vitD_3$ [99]. About 85-90% of 25(OH)vitD is bound to DBP, 10-15% are bound to albumin and only a very small fraction circulates free, less than 1% and potentially biologically active [100]. According to the Free Hormone Hypothesis, only the unbound fraction can enter the cells and perform biological actions. Albumin-bound 25(OH)vitD dissociates rapidly and is also biologically available in tissues. Therefore, bioavailable 25(OH)vitD is defined as free 25(OH)vitD plus albumin-bound 25(OH)vitD [101]. DBP serves as a critical reservoir for vitD metabolites reducing the risk of vitD deficiency when epidermal production or intake is low [101].

DBP-bound vitD metabolites have limited access to target cells [90]. First, the proportion of total vitD metabolites in plasma that is unbound or free is dependent on the total DBP, vitD concentration, as well as their binding affinity. And second, as mentioned above, most extra-renal tissues do not appear to express megalin, that means that these tissues are more likely to acquire 25(OH)vitD that is not bound to DBP, i.e. bioavailable or free 25(OH)vitD [90, 96]. Given this evidence of what is the biologically active fraction of vitD, numerous studies have suggested that measuring levels of bioavailable vitD instead of total vitD may be a more accurate biomarker of vitD status [100]. Moreover, DBP gene is highly polymorphic, with three common variants, which present different ability to bind to 25(OH)D and can affect its functions [100, 102].

DBP levels are not regulated by vitD levels [103]; in fact, DBP-null mice lack any symptoms of rickets, despite its very low total levels of 25(OH)vitD [90, 101, 104]. However, DBP concentration is reduced in several conditions such as liver disease, nephrotic syndrome and malnutrition and it is increased during pregnancy and oestrogen therapy [90, 105].

There is also an intracellular transport network for vitD, but it is not stablished for all target cells. This intracellular trafficking of vitD metabolites is mediated by HSP70 (heat shock protein 70), that promotes the cellular uptake and distribution within the cell. Once in the cell, HSP70 functions to transport 25(OH)vitD to the mitochondria for its conversion to $1,25(OH)_2vitD_3$ and subsequently it facilitates the movement of $1,25(OH)_2vitD_3$ into the nucleus for binding to the vitamin D receptor (VDR) [106, 107].

2.3. Vitamin D receptor

$1,25(OH)_2vitD_3$ or calcitriol exerts its function through the VDR. As other steroid receptor family members, VDR acts as a transcription factor, and it is found in nearly all cells, although at variable levels [108]. Cytosolic VDR binds calcitriol with high affinity and specificity, even at sub-nanomolar concentrations, and then heterodimerizes with the retinoid-X-receptor (RXR). The integrity and structure of VDR-RXR complex is essential for it to be functional. After that, the VDR-RXR complex translocates rapidly to the nucleus along microtubules and interacts with the vitamin D response elements (VDRE) on the promoter region of targets genes, resulting in changes in gene expression [89, 90, 109] (Figure 6). Genes generally have multiple VDRE binding sites and its activity may vary in different cells and different species. Over 200 genes are regulated directly or indirectly by $1,25(OH)_2vitD$. VDR not only regulates the expression of mRNAs but also of several miRNAs, indirectly regulating the expression of other genes [110]. Additionally, non-genomic actions of $1,25(OH)_2vitD$ have been also described, involved in transmembrane calcium transport [109]. In fact, VDR can accommodate two types of ligand configurations, a conformation with genomic activity and an alternative one for rapid an non-genomic responses [106], which results in different biological activity.

Interaction between VDR and VDRE in targeted genes is controlled by co-activator and co-repressor proteins, which lead to positive or negative transcriptional regulation of gene expression, respectively. Transactivation is the process in which co-activators stimulate the transcription of the target gene, and in contrast, transrepression is a down-regulation of VDR-target gene transcription (Figure 6). The co-regulators (Co-reg), such as histone acetylase, methyltransferases and other transcription factors, influence VDR function and $1,25(OH)_2vitD$ responses in a cell-and tissue-specific manner [109].

VDR expression can be modulated by the presence of its own ligand $1,25(OH)_2vitD$. This homologous regulation or autoregulation occurs in a wide variety of type cells [111, 112]. VDR expression is upregulated in response to calcitriol. This autoregulation can be caused by increased VDR gene transcription, concordant with the presence of VDRE in the promoter region of VDR gene, and /or by stabilization of VDR, due to decreased rate of receptor degradation [113-115]. In addition to $1,25(OH)_2vitD$, many factors regulate VDR expression, including growth factors, PTH, glucocorticoids, oestrogens, calcium and a variety of transcription factors [95].

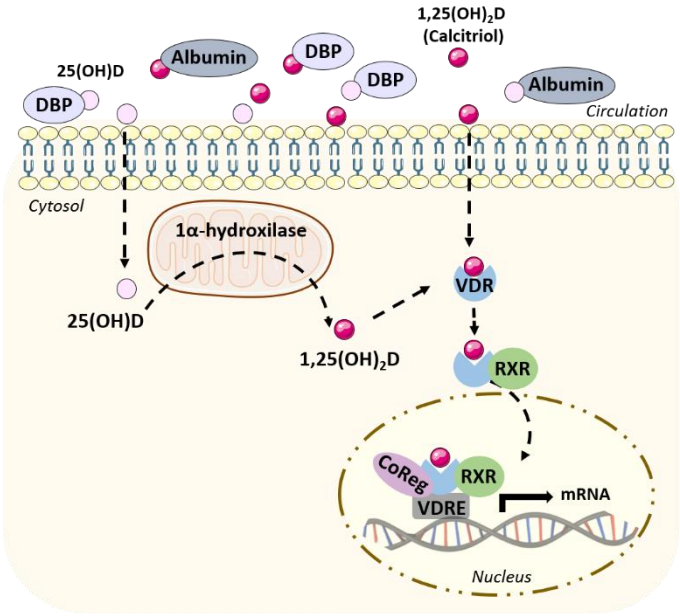

Figure 6. Steps of vitamin D receptor (VDR) activation. *25(OH)vitD and 1,25(OH)$_2$vitD circulates bound to DBP, albumin or free. Metabolites taken up by target cells are either targeted to the mitochondria to synthesize 1,25(OH)$_2$vitD or bound to VDR in the cytoplasm. The 1,25(OH)$_2$vitD-VDR complex heterodimerizes with RXR and translocate to the nucleus. The VDR-RXR heterodimer binds specific sequences called vitamin D response elements (VDREs) in the promoter regions of the target genes, resulting in changes in mRNA expression. The interaction between VDR and VDRE is under control of co-activator and co-repressor proteins (Co-Reg).*

Traditionally, VDR has been characterized as a nuclear steroid receptor. In recent years, confocal and electronic microscopy revealed that there is an equilibrium between nuclear and cytoplasmic VDR [116, 117]. In the absence of ligand, a substantial proportion of VDR reside in the cytoplasm. 1,25(OH)$_2$D induces gradually VDR translocation to the nucleus in a time-dependent manner [116, 118]. This phenomenon is observed in several cell types such as cancerous and vascular cells [119-121]. Moreover, some studies have identified that VDR may be in the membrane and can co-localize with caveolin-1 in caveolae to mediate the non-genomic actions [95].

2.4. Vitamin D receptor actions

It is well-known that vitD is a central regulator of blood calcium and phosphate concentrations and bone mineralization in order to preserve skeletal functions and integrity, by actions at intestine, bone, parathyroid and kidney [87, 122]; the so-called calcaemic actions.

In the intestine, vitD is essential to absorb dietary calcium and phosphate. Calcitriol induces a rapid calcium and phosphate uptake and transport through the epithelial calcium channel

TRPV6 (Transient receptor potential cation channel subfamily V member 6) and Na-Pi cotransporter, respectively, and finally delivery to the bloodstream. Moreover, vitD is essential for the development and maintenance of a mineralized skeleton. 1,25$(OH)_2$vitD exerts an anabolic effect necessary to sustain bone-forming activity. 1,25(OH)2vitD also enhances renal calcium reabsorption. In addition, in the kidney, 1,25$(OH)_2$vitD plays a tight control on its own homeostasis through simultaneous suppression of 1α-hydroxylase and stimulation of 24-hydroxylase and induces megalin expression in the proximal tubule [90, 123]. Conversely, vitD deficiency is associated with hypocalcaemia accompanied with high levels of PTH that results in bone turnover, higher bone loss and ultimately higher risk of osteoporosis or rickets and fractures [122].

VDR protein are found in several tissues and different cell types from those involved in calcium-phosphate regulation. The non-calcemic roles of 1,25$(OH)_2$vitD were discovered through molecular biology and genomic techniques as gene chip arrays and gene knockout mice and accumulating nutritional and epidemiological evidence of vitD deficiency and abnormalities unrelated to calcium homeostasis. Over the past few years an extensive research to the non-classic actions of vitD has been carried out. The functions of VDR in these other tissues and cells is a topic of ongoing investigation, as the expression levels of VDR in different cells are quite variable and can change with the stage of cell differentiation [106]. Non-calcemic actions mediated by VDR include the modulation of immune responses, antiproliferative effects, cellular differentiation and apoptosis in a various type cells, as cancerous cells, vascular smooth muscle and endothelial cells (Figure 7) [124-126].

Nearly all cells of the immune system express VDR, notably macrophages, dendritic cells and activated T cells [125]. The cells of the immune system are highly responsive to calcitriol [127-130]. VitD can modulate the innate and adaptative immunes responses, with a shift of Th1 (T helper cells type 1) toward Th2 (T helper cells type 2) and T regulatory cells. In fact, VDR-null mice show impaired Th1 and macrophage responses [131, 132]. Calcitriol is capable of inhibiting proinflammatory cytokines such as interleukin-6 (IL-6), IL-1, tumour necrosis factor-α (TNF- α) and there is some evidence of the role of calcitriol in the attenuation of Toll-like receptor (TLR)-mediated inflammation. On the contrary, 1,25$(OH)_2$vitD upregulated anti-inflammatory interleukins as IL-4 and IL-10 [124, 125, 133].

Moreover, VDR has been shown to control the expression of several genes associated with cellular proliferation and differentiation [133, 134]. For instance, numerous studies have identified the inhibitory cell growth effect mediated by 1,25$(OH)_2$vitD in several types of cancerous cells [135]. While many epidemiological studies suggest an inverse correlation between vitD status and malignancies in human [135], no spontaneous tumours are observed in the VDR null

mice [132]. But, these mice exhibit hyperproliferation of colonic cells and increased breast and skin cancer in response to chemical carcinogens [132].

The antiproliferative effects of $1,25(OH)_2$vitD are mediated by multiple mechanisms including the regulation of growth factors, cell cycle and signalling pathways. For example, vitD induces gene transcription of the cyclin-dependent kinase inhibitor p21 and p27 or stimulates TGFβ signalling pathway [90, 124, 134]. In addition, calcitriol also induces apoptosis in hyperproliferative disorders through the modulation of BCL2 and BAX expression [90].

Likewise, it has been identified that $1,25(OH)_2$vitD also inhibit cardiomyocyte, vascular smooth muscle and endothelial cell proliferation and maturation [125, 136]. Furthermore, calcitriol has also other beneficial effects on the cells of the arterial wall and cardiomyocytes. For instance, active vitD metabolites modulate vascular tone, enhance NO release, decrease reactive oxygen species, improve flow-mediated vasodilatation and promote endothelial repair [89, 125]. Interestingly, in mice with endothelial specific knockout of the VDR gene, vascular function is significantly altered [137] and VDR null mice also exhibit a prothrombotic state because of the downregulation of both antithrombin and thrombomodulin [138].

Continuing from a cardiovascular perspective, $1,25(OH)_2$vitD acts as a negative endocrine regulator of the renin-angiotensin system. In fact, VDR-null and CYP27B1 knockout mice show a marked elevated levels of renin and increased angiotensin II production, associated with systemic hypertension and cardiac hypertrophy, which can be reversed by calcitriol treatment [132, 139, 140]. Moreover, on the heart, VDR activation modulates cardiac contractility and promotes antihypertrophic effects [89, 132, 141]. The antihypertrophic effects of VDR may be mediated by suppression of the calcineurin/NFAT/RCAN1 pathway [125, 141].

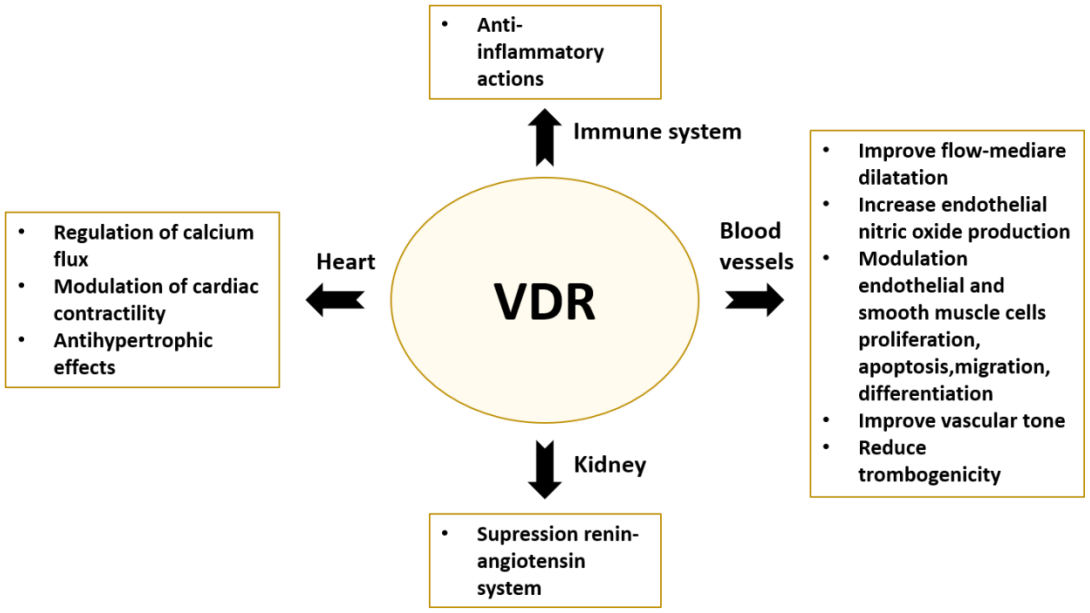

Figure 7. Vitamin D receptor (VDR)-mediated endocrine functions on cardiovascular system.

Furthermore, the finding of CYP27B1 protein in pancreatic cells suggest the possibility of an autocrine control of insulin secretion by calcitriol. The potential antidiabetic properties of $1,25(OH)_2$vitD become apparent in experimental animal models with vitD deficiency, which show abnormal insulin secretion and insulin resistance [142, 143]. It has also been identified that VDR activation participates in the regulation of lipid metabolism and in the control of muscle function and nervous system [144, 145].

2.5. Definition and prevalence of vitamin D deficiency

Although $1,25(OH)_2$vitD is the biologically active form of vitD, 25(OH)vitD is the ideal circulating biomarker of vitD status. There are several reasons. The half-life of circulating $1,25(OH)_2$vitD is only 4-6 hours, while 25(OH)vitD has a relatively long half-life, around 2-3 weeks [87, 90, 146]. Thus, circulating levels of $1,25(OH)_2$vitD are a thousand-fold less than those of 25(OH)vitD. Moreover, 25(OH)vitD is the vitD metabolite in highest concentration in the blood [95, 146].

There is controversy about the definition of optimum serum level of 25(OH)vitD and vitD deficiency [147-149]. The Endocrine Society, FDA (Food and Drug Administration), EFSA (European Food Safety Authority), the National Academy of Medicine and Vitamin D council have their own cut-off values for severe deficiency, deficiency or insufficiency and sufficiency. Contributing also to the variability of the current guidelines, it is not clear if 25(OH)vitD levels that are optimal for Caucasians, are the same as those for Black Africans, Hispanics or Asians [95].

This lack of consensus and the variability between the different assays used to measure 25(OH)vitD make the assessment of vitD status complex. This is particularly relevant in those extrarenal tissues that synthetize $1,25(OH)_2$vitD, because of the local concentrations of vitD metabolites outside the serum compartment cannot be easily measured *in vivo* [147-149].

Despite the non-standardization of vitD cut-off levels, there is increasing agreement that the optimal circulating 25(OH)vitD level should be 20 ng/ml (50 nmol/l) or above, which are associated with optimal physiological function [95, 150]. In an international effort to provide a consistent set of guidelines, experts from endocrinology, paediatrics, nutrition, epidemiology and public health have defined cut-off values for vitD. Thus, less than 20 ng/ml (50 nmol/l) indicates vitD deficiency, often referred to as inadequate or insufficient, and it has been associated with a variety of adverse health consequences. In addition, severe vitD deficiency, at 10 ng/ml (25 nmol/l) appears to cause calcium malabsorption, resulting in elevated bone turnover and ultimately bone loss [146, 150-152]. Moreover, the upper limit of normal has also been questioned. Vitamin D intoxication is defined as 25(OH)vitD higher than 100 ng/ml, that is associated with hypercalcemia (Figure 8) [146, 152].

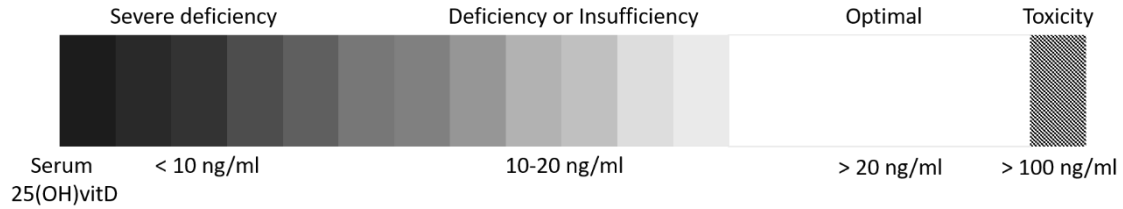

Figure 8. Cut-offs of vitamin D status*. Optimal 25(OH)vitD concentrations should be 20 ng/ml, associated with optimal physiological function. Deficiency or insufficient of vitD, in a range of 10-20 ng/ml has been associated with a variety of adverse health consequences. Less than 10 ng/ml of 25(OH)vitD indicates severe deficit. VitD intoxication is defined up to 100 ng/ml.*

Despite variation in the methodology used to determined 25(OH)vitD levels or the threshold selected, it is clear that vitD deficiency is common worldwide. Epidemiological studies estimate that insufficient or deficient vitD is found in 30-50% of the general population, that means ~ 3 billion ($3 \cdot 10^9$) people worldwide [153, 154]. Many of the causes of vitD deficiency have been identified, such as inadequate cutaneous synthesis due to insufficient sun exposure, inadequate dietary intake, dark skin, and even modern human cultures in part of increasingly indoor lifestyle. Other noted risk factors include aging, obesity, renal or hepatic diseases, disorders that affect fat absorption and even medications [153-155].

As mentioned previously, low vitD values are correlated with several pathological conditions. Vitamin D deficiency has long been associated with adverse bone consequences as osteomalacia in adults and rickets in children, populations at higher risk of bone fractures. Patients with osteomalacia due to vitD deficiency develop muscle pain and weakness [122, 146, 151, 152]. Rickets or osteomalacia are also dependent on calcium intake. It is defined calcium sufficiency as > 500 mg/day, insufficient as 300-500 mg/day, and deficiency as < 300 mg/day.

Moreover, since the discovery of non-calcemic actions mediated by VDR, vitD deficiency has been also related with other pathologies as higher cancer risk, infections, autoimmune, metabolic, neuropsychological, respiratory and cardiovascular diseases [125, 151, 156, 157]. Therefore, some researchers suggest that vitD deficiency might be a general marker of poor health [89].

2.6. Vitamin D requirements and treatment strategies

Sun exposure can provide an adequate amount of vitD, which is stored in body fat and released during the winter, when vitD production is reduced. Exposure of arms and legs for 5 to 30 minutes, depending on season, latitude, the time of the day, and skin pigmentation, between the hours of 10 a.m. and 3 p.m. twice a week is often adequate [151, 153]. However, exposure to sunlight does not seem to be a viable approach, due to the relationship between UV exposure with skin cancer. Sun avoidance and sunscreen use reduce skin exposure to UV radiation and thereby reduce skin vitD synthesis [151]. It is estimated that approximately 3000 IU are obtained with a sun exposure of 5 to 10 minutes daily. Among foods, oily fish have the highest content of vitD, which ranges from 100 to 1000 IU per 1 kg, while milk contain up to 100 IU per 1 cup. Every 100 IU of vitD ingested daily, 25(OH)D levels increased by about 1 ng/ml [153, 154].

Both, American and European Guidelines recommend and adequate daily intake of vitD depending on age [158] (Table 3).

Table 3. Daily recommendation of vitD according to age

Age group	**Daily recommendation (IU)**
Infants 0- 12 months	400 IU
Children 1-13 years	600 IU
Adolescent 14-18 years	600 IU
Adults 19-70 years	600 IU
Adults > 70 years	800 IU
Pregnancy & lactation	600 IU

Subjects diagnosed with vitD deficiency require higher doses of vitD than those recommended for the general population [158]. Vitamin D supplementation is indicated to treat vitD deficiency, rickets in children, osteoporosis in adults and to prevent fractures in postmenopausal women and elderly population. Most patients with osteoporosis are currently treated with bisphosphonates, and vitD metabolites are normally used in combination to promote intestinal absorption of calcium, and even in combination with calcium. The therapeutic dose and the duration of the supplementation usually vary depending on the patient's age, body weight and the severity of vitD deficiency [158-160]. For instance, the therapeutic dose in severe depletion should be 1.000-10.000 IU/day (~ 50.000 IU/week) with a duration from 1-3 months. After reaching an optimal 25(OH)vitD concentration, levels are periodically monitored [159].

2.7. Vitamin D deficiency in cardiovascular and respiratory diseases

Several meta-analyses of prospective studies and many preclinical studies in *Vdr*$^{-/-}$ *and* *Cyp27b*$^{-/-}$ mice have consistently shown that vitD deficiency is associated with increased all-cause and cardiovascular risk and mortality [161, 162], such as systemic hypertension, coronary artery disease, stroke, heart failure, myocardial infarction or type 2 diabetes [89, 122, 125]. Remarkably, *Vdr and Cyp27b1* null mice exhibit higher blood pressure and cardiac hypertrophy due to an upregulation of renin-angiotensin system, and these abnormalities can be reversed by calcitriol administration or inhibiting the renin-angiotensin system[132, 139, 163]. Moreover, low 25(OH)vitD levels have also been associated with endothelial dysfunction, but this association has not been consistently found in all studies [164].

Nevertheless, despite promising pre-clinical and epidemiological studies associated with low 25(OH)vitD levels, the impact of vitD supplementation in cardiovascular diseases remains controversial and so far, supplements have not succeeded in proving a benefit. It is well-stablished that vitD supplementation is effective in decreasing PTH levels, by contrast, large meta-analyses of RCT have reported no significant effect of vitD treatment on systolic and diastolic blood pressure. Similarly, vitD supplementation had no significant clinical effects on left ventricular ejection fraction or 6-minute walking distance, neither significant functional effects on endothelial dysfunction and arterial stiffness [89, 122, 165, 166]. If vitD has clinically relevant effects on cardiovascular system, the effect sizes are likely to be small compared with those of stablished treatments, such as angiotensin inhibitors or vasodilators.

Some authors claim that vitD supplementation does not provide a beneficial and causative role in cardiovascular diseases, and therefore, vitD deficiency could simply be a general marker of poor health in patients with chronic cardiovascular diseases [165]. Alternatively, others consider that RCT have not been optimally designed and point some methodological limitations. For instance, previous RCT were heterogenous in terms of the accuracy of serum 25(OH)vitD assays, the cut-of values used to define vitD deficiency, the adequacy of the dosages and dosing regimens, and they did not take into account the genetic variations and polymorphisms in VDR and DBP genes and even the selection of the study population and their sociodemographic characteristics [165].

Remarkably, novel results published by a European consortium indicate that the highest cardiovascular mortality occurs at 25(OH)D levels less than 12 ng/ml, values considered as severe deficit of vitD. Therefore, considering only those patients with severe deficit of vitD, some recent RCT have shown that vitD supplementation significantly reduced risk of death [167] while did not show a reduction in cardiovascular events in individuals with initial 25(OH)vitD levels in the range of insufficiency [166, 168]. In light of these results, The Vitamin D Assessment (ViDA) study

suggest that future RCT of vitD supplementation should mainly recruit participants with very low vitD levels, to shed light on the current uncertainties and controversies [168].

In the context of respiratory diseases, vitD deficiency is also highly prevalent [169]. Specifically, in respiratory system, lower plasma levels of vitD have been associated with a faster decline in lung function and with a higher risk of develop respiratory diseases such as chronic obstructive pulmonary disease (COPD), asthma, cystic fibrosis and respiratory infections [170, 171]. While many epidemiological and experimental studies highlight the association between vitD and lung diseases, the underling mechanisms between vitD and the pathogenesis of these diseases have not been clear yet. Asthmatic, COPD and cystic fibrosis patients with insufficient vitD levels present higher chances of exacerbations. In this context, several studies have pointed to the immunomodulatory actions of vitamin D. In respiratory tract, vitD appears to inhibit pulmonary inflammatory responses while enhances adaptive immune system [169, 172-174]. Therefore, vitD could be a potential candidate to treat or to prevent exacerbations in these patients. In fact, two recent studies carried out by Jolliffe et al. showed that vitD supplementation substantially reduce the rate of moderate and severe asthma and COPD exacerbations, in those patients with baseline 25(OH)vitD levels below 10 ng/ml [175, 176].

2.8. Vitamin D receptor in the lung

Previous studies have identified that VDR is already expressed in the lung during the late stage of fetal development and vitD is required for the lung growth in utero [177]. In the lung, VDR has been mainly identified in airway cells such as bronchial epithelial and SMC cells, as well as lung fibroblast and pulmonary immune cells [178-181]. Human bronchial cells also express CYP27B1 and CYP24A1, so these cells are able to locally produce and inactivate $1,25(OH)_2$vitD, which contribute to the autocrine and paracrine levels [181, 182]. The functionality of VDR in human bronchial SMC was demonstrated by an increase in its expression after stimulation with calcitriol [183].

The downregulation of VDR has been associated with several lung diseases, including asthma, COPD, pulmonary fibrosis, respiratory tract infections and lung cancer [174, 184]. For example, VDR expression decreased with increasing histologic lung cancer grade in bronchial biopsies [179]. Moreover, in recent years a few potential single nucleotide polymorphisms (SNP) have been identified in VDR. These studies support that VDR polymorphisms are associated with higher susceptibility to suffer lung cancer [185], respiratory infections [186], asthma [187] and COPD [97]. Likewise, polymorphisms in other genes involved in the vitD pathway that encode the proteins responsible for vitD metabolism, transport and signalling have been also identified [97, 186]. In fact,

some authors have suggested that VDR polymorphisms, rather than vitD levels, might be a biomarker of respiratory diseases, like asthma [188, 189].

In addition to the airway anatomic compartment, there is some evidence of VDR in the PA vasculature. For instance, VDR mRNA transcripts and protein were detectable in human, rat and mice PAEC [177, 190]. In this context, VDR status has only been studied in experimental models after exposure to the endotoxin lipopolysaccharide (LPS). The expression of VDR and enzymes involved in its metabolism CYP27B1 and CYP24B1 was reduced in fetal PAEC from an experimental model of pulmonary bronchopulmonary dysplasia after antenatal exposure to LPS [177]. The decrease of VDR content in the fetal rat lung seems to cause abnormal lung structure [177].

Furthermore, to our knowledge there is no data about the presence of VDR in PASMC. However, a few effects of vitD on PASMC have been described [191], so it can be assumed that VDR is expressed and it is functional in PASMC.

3. VITAMIN D IN PAH

3.1. Background

In the context of PAH, there is some basic and clinical evidence suggesting a role for vitD in the pathophysiology of the disease. To clarify whether vitD levels could be involved in PAH progression, Tanaka et al. treated PAH rats with a diet containing 10.000 UI/Kg of vitD [192]. Notably, in this study, they found that vitD supplementation in PAH rats improved survival and attenuated some typical features in PAH as right ventricle remodelling, assessed by Fulton index (ratio of right ventricle weight to left ventricle plus septum weight) and medial wall thickness of muscular pulmonary arteries. Despite these benefits of vitD, it did not decrease pulmonary arterial pressure [192]. Moreover, in an *in vitro* setup, calcitriol treatment inhibited the hypoxia-induced proliferation and migration in rat pulmonary artery endothelial cells via miR-204/TGFβ/Smad signalling pathway. Specifically, calcitriol suppressed the expression of *Tgfbr2*, *α-SMA* and *Smad7* and induced miR-204, *p21* and *Smad2* expression [193]. In the same study, similar results were found in an *in vivo* rat model. Remarkably, intraperitoneal calcitriol administration (20 mg/Kg) partly reversed the rise in mPAP and Fulton index induced by 3 weeks of hypoxia [193].

In the clinical arena, Ulrich et al. showed that secondary hyperparathyroidism is highly prevalent in PAH patients [194]. Physiologically, decreased serum 25(OH)vitD results in increased PTH levels in order to maintain adequate serum calcium concentrations. Therefore, low vitD status in PAH patients could be the reason for the elevated PTH. Later, epidemiological studies demonstrated that vitD deficiency is quite prevalent in PAH patients [192, 195-197]. In the prospective study carried out by Demir et al., PAH patients presented much lower vitD levels (median of 6.79 ng/ml), considered as severe deficit of vitamin D (<10 ng/ml of serum 25(OH)vitD) than controls (18.76 ng/ml) [195]. In line with this result, Tanaka and colleagues found that in a cohort of PAH patients, 39 out of 41 (95.1%) presented vitD insufficient and 25 patients (61%) showed deficient levels [192]. Similar results were found in the recent study carried out by Atamañuk et al. [197], in which vitD deficiency prevalence was higher in PH patients with different aetiologies as compared to control subjects and even to patients with left ventricle failure. The relationship between vitD deficiency and PAH prognosis was evaluated. Serum 25(OH)vitD levels were negatively correlated with mPAP assessed by right heart catheterization, and a significant positive correlation with cardiac output was found [192]. Likewise, patients in functional class III-IV had lower levels of vitD than those in functional class I-II (86.7 *vs* 40.5 %) and presented reduced exercise tolerance, measured by 6MWD test [197].

The potential benefits of vitD replacement on clinical outcomes has been also studied [196]. Twenty-two PAH patients were enrolled in a prospective uncontrolled longitudinal study. All

PAH patients received vitD at a dose of 50.000 IU weekly for 3 months. In addition to the rise of serum 25(OH)vitD levels from 14±9 to 69±31 ng/ml, remarkably, vitD supplements improved the 6MWD test by around 80 meters and right ventricle size. Mean PAP estimated by echocardiography was reduced from 79 ± 25 to 69 ± 23 mmHg but this effect did not reach statistical significance. NT-pro BNP and functional class were also unchanged after vitamin D therapy [196].

All these data strongly suggest that vitD may have a beneficial effect in PAH patients and that vitD deficiency may have detrimental effects in these patients. However, the therapeutic use of vitD in this context has not been validated in randomized clinical trials and the impact of vitD deficiency on pulmonary circulation is not completely clear.

3.2. Potential VDR targeted genes altered in PAH

As previously mentioned, vitD exerts its biological functions through VDR, regulating the expression of its target genes. Interestingly, some of the genes regulated by VDR are of special interest because they are altered in PAH. In other words, VDR can upregulate the expression of some genes downregulated in PAH or *vice versa*, VDR can downregulate genes upregulated in this pathology (Figure 9).

It has been extensively reported that calcitriol binds VDR and control the growth of a wide variety of normal and malignant cell types, which is mediated via different pathways and mechanisms. One of VDR targeted genes inhibiting cellular growth is *BIRC5*, which encodes the antiapoptotic protein survivin [198-200]. Survivin is unfrequently expressed in healthy adult tissue, and it is selectively expressed in most common human hyperproliferative disorders. Increased survivin expression correlates with advanced tumour proliferation and subsequently the grade of carcinoma and poor patient survival [199, 201, 202]. Notably, it has been demonstrated that vitD compounds induce cellular growth inhibition and apoptosis at least partially dependent on survivin downregulation in several cancer cell lines [198-200]. Likewise, in the context of PAH, survivin is increased in serum and pulmonary arteries from both patients and animal models [202, 203]. It is considered that increased survivin level is a feature of irreversible PAH and its pharmacological inhibition has been proposed as a novel therapeutic strategy [204].

Other downregulated gene by VDR and relevant in PAH is the gene encoding IL-17. As described above, inflammation is a key pathophysiological factor in the development and progression of PAH. T helper 17 cells and their secreted proinflammatory IL-17 contribute to pulmonary vascular remodelling [49, 205]. Those agents targeting IL-17 may be a potential novel anti-inflammatory therapeutic strategy for the clinical treatment of PAH [205]. One of these possible agents could be vitD. *In vivo* and *in vitro*, vitD treatment reduce serum IL-17 levels in

autoimmune diseases [206, 207] and in respiratory diseases [208]. In asthmatic patients, calcitriol repressed IL-17 secretion through VDR at transcription level [209].

By contrast, one of the upregulated genes by VDR with great significance in PAH is *KCNK3*, encoding the TASK-1 potassium channel. Reduced *KCNK3* mRNA expression and TASK-1 activity are key and early events in the pathogenesis of heritable and non-heritable PAH in both patients as well as experimental models. This dysregulation contributes to PASMC vasoconstriction and proliferation [19, 22]. Contrary to ET-1 [210], which has been shown to inhibit TASK-1 function, some experimental compounds enhance its activity and increase TASK-1 current in PASMC [20, 211]. Notably, *in vitro* experiments revealed that calcitriol increases the expression of this channel in human coronary artery smooth muscle cells and in fresh slices from breast cancer [212, 213]. However, the effect of calcitriol on TASK-1 activity is currently unknown. It would be expected that the increased expression may lead to enhanced TASK-1 currents.

In addition, calcitriol treatment has been reported to increase the expression of the BMPR2 ligands *BMP4* and *BMP6* in coronary artery SMC and in dermal fibroblast [213, 214]. It is well-known that the downregulation of the BMPR2 pathway critically promotes disturbances in vascular cellular growth, such as PASMC and PAEC proliferation and vascular remodelling in all forms of PAH [215, 216]. Rescuing BMP signalling pathway is a potential therapeutic strategy under study. Indeed, several drugs have been defined such as FK506, as well as BMP ligands [217]. Therefore, if vitD enhance BMP ligands in PAH, calcitriol may hold a considerable therapeutic promise.

Furthermore, *RCAN1*, encoding for the NFAT inhibitor calcipressin-1 [218] and *DDIT4* [219], an mTOR inhibitor, are VDR-targeted genes. In PAH, the over stimulation of calcineurin/NFAT and the protein kinase mTOR signalling pathways are also involved in PASMC proliferation and pulmonary vascular remodelling [220, 221]. VDR activation can reduce cardiac hypertrophy via suppression of the calcineurin/NFAT/RCAN1 pathway [141].

Although it has been recognized a variability of VDRE sequences depending on the VDR-targeted gene, no VDRE sequences have yet been identified in the promoter region of the genes above mentioned to be altered in PAH [222].

In addition of these VDR target genes detailed in this section, there is already evidence that vitD modulates the expression of *Tgfbr2*, *Smad2*, *Smad7* and *p21* in a rat model of PAH [193]. The fact that VDR can regulate the expression of some genes dysregulated in PAH, implies that vitD and its receptor can play a role in the physiopathology of PAH.

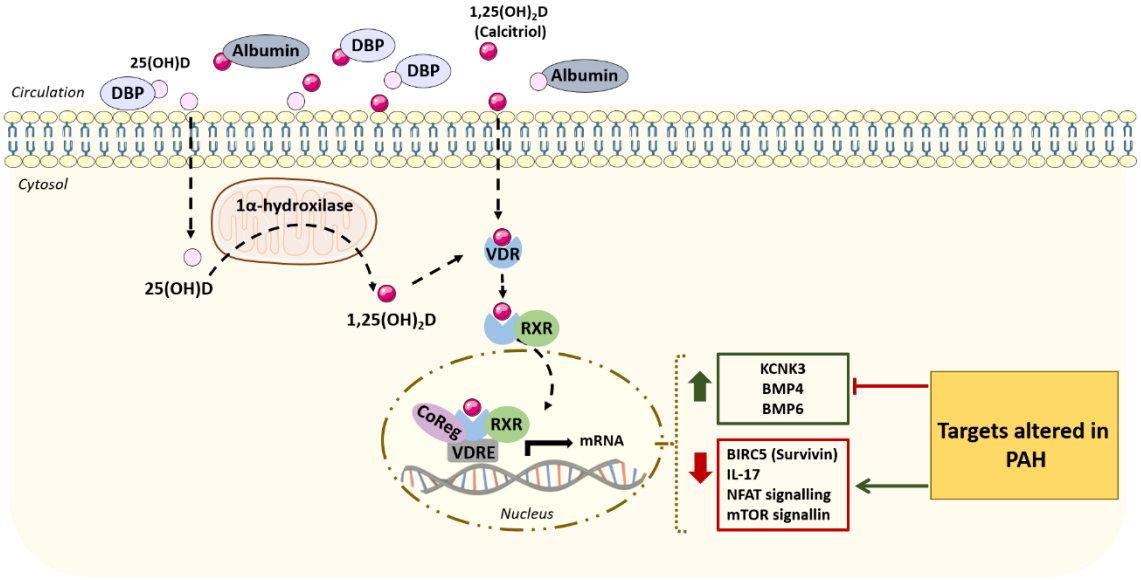

Figure 9. Potential VDR targeted genes altered in PAH. *Once the 1,25(OH)$_2$vitD-VDR-RXR complex is set up, in the nucleus binds to vitamin D response elements and regulate the mRNA expression of target genes. Some of VDR-targeted genes are involved in the pathology of PAH, i.e., KCNK3, BMP4, BMP6, BIRC5, IL-17, NFAT and mTOR signalling.*

Hypothesis & objectives

Currently, vitD deficiency is a global pandemic. To date, there is some evidence that indicate that reduced levels of vitD are more prevalent in PAH patients than in the general population. However, it is unclear whether there is causal relationship between these reduced levels and the development and progression of PAH.

Moreover, current classifications of vitD status are based on total vitD concentrations. In the circulation, about 85% of total vitD is bound to DBP, about 15% is bound to albumin and only less than 1% is free. Bioavailable vitD is defined as free plus albumin-bound vitD and DBP acts as a reservoir for vitD. However, several studies have reported that bioavailable or free vitD, biologically available in tissues, may be a better biomarker for vitD status. However, the relationship between total, bioavailable and free vitD and clinical outcomes in PAH patients have not been studied.

Furthermore, vitD binds with high affinity and specificity to its receptor, VDR which acts as a transcriptional factor, regulating target gene expression. The discovery of VDR in vascular cells, including endothelial and smooth muscle cells strongly suggest that vitD may play a role in a great variety of processes of potential relevance in cardiovascular diseases, such as: cell proliferation, differentiation and apoptosis; cell adhesion; oxidative stress; angiogenesis and immunomodulatory activity. Remarkably, some of the genes regulated by VDR are of special interest because they are impaired in PAH. VDR can upregulate the expression of some genes downregulated in PAH or *vice versa*, VDR can downregulate genes upregulated in this disease. Of note, the presence, expression, modulation and effects of VDR on pulmonary vasculature have not been investigated.

The general **hypothesis** of this Doctoral Thesis is that vitamin D deficiency is a predisposing or aggravating factor for pulmonary hypertension.

The **general aim** of this Doctoral Thesis is to study the relationship between pulmonary hypertension and vitamin D status and the characterization of the vitamin D receptor in the pulmonary vasculature and its role in PAH. The general objective is further subdivided into the specific aims:

1) To examine the total, bioavailable and free 25(OH)vitD levels and their prognosis value in patients with PAH.

2) To investigate if vitD deficiency may induce pulmonary vascular dysfunction and/or exacerbate it in an experimental model of PAH and to explore the potential mechanisms involved.

3) To analyse whether recovering optimal vitD levels in an experimental model of PAH previously depleted of vitD improves the hemodynamic, the pulmonary endothelial dysfunction and the pulmonary ionic remodelling.

4) To examine if vitD deficiency may account for the limited efficacy of sildenafil in some patients with PAH.

5) To study the role of VDR in the pulmonary vasculature.

Results

Chapter 1

Total, bioavailable, and free vitamin D levels and their prognostic value in pulmonary arterial hypertension

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Total, Bioavailable, and Free Vitamin D Levels and Their Prognostic Value in Pulmonary Arterial Hypertension

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Abstract: *Introduction*: Epidemiological studies suggest a relationship between vitamin D deficiency and cardiovascular and respiratory diseases. However, whether total, bioavailable, and/or free vitamin D levels have a prognostic role in pulmonary arterial hypertension (PAH) is unknown. We aimed to determine total, bioavailable, and free 25-hydroxy-vitamin D (25(OH)vitD) plasma levels and their prognostic value in PAH patients. *Methods*: In total, 67 samples of plasma from Spanish patients with idiopathic, heritable, or drug-induced PAH were obtained from the Spanish PH Biobank and compared to a cohort of 100 healthy subjects. Clinical parameters were obtained from the Spanish Registry of PAH (REHAP). *Results*: Seventy percent of PAH patients had severe vitamin D deficiency (total 25(OH)vitD < 10 ng/mL) and secondary hyperparathyroidism. PAH patients with total 25(OH)vitD plasma above the median of this cohort (7.17 ng/mL) had better functional class and higher 6-min walking distance and TAPSE (tricuspid annular plane systolic excursion). The main outcome measure of survival was significantly increased in these patients (age-adjusted hazard ratio: 5.40 (95% confidence interval: 2.88 to 10.12)). Vitamin D-binding protein (DBP) and albumin plasma levels were downregulated in PAH. Bioavailable 25(OH)vitD was decreased in PAH patients compared to the control cohort. Lower levels of bioavailable 25(OH)vitD (<0.91 ng/mL) were associated with more advanced functional class, lower exercise capacity, and higher risk of mortality. Free 25(OH)vitD did not change in PAH; however, lower free 25(OH)vitD (<1.53 pg/mL) values were also associated with high risk of mortality. *Conclusions*: Vitamin D deficiency is highly prevalent in PAH, and low levels of total 25(OH)vitD were associated with poor prognosis.

Keywords: pulmonary arterial hypertension; vitamin D; survival; prognosis

1. Introduction

Pulmonary arterial hypertension (PAH) is currently defined by an abnormal increase in pulmonary vascular resistance not due to left-heart, respiratory, or thromboembolic disease [1]. It is a disorder with

a significant burden in terms of severity and prevalence, and it entails a poor prognosis. The etiology of the disease is complex and multifactorial, and it preferentially affects women, exerting substantial impact on their quality of life, i.e., reduced functional ability, greater oxygen requirements, and an increased risk of mortality [2]. Despite the advances in the knowledge of PAH, there is a need to improve its prevention and treatment [2,3]. Several non-invasive predictors are used to evaluate the prognosis of pulmonary hypertension (PH), i.e., New York Heart Association (NYHA) functional class, reduced 6-min walk test (6MWT), diffusing capacity for carbon monoxide (DLCO), and B-type natriuretic peptide (BNP) and the N-terminal fragment of pro-BNP (NT-pro-BNP) levels [4].

Vitamin D (vitD) is a fat-soluble vitamin that is obtained from dermal synthesis following exposure to sunlight or from the diet. VitD in the liver is converted into 25-OH-cholecalciferol (25(OH)vitD), the most abundant circulating form of vitD. Generally, 25(OH)vitD plasma measure serves as indicator of the vitD status. 25(OH)vitD is further metabolized in the kidney into the most active metabolite, 1-α-25,dihydroxycholecalciferol, also known as calcitriol. It plays a crucial role in the regulation of calcium and phosphorous metabolism, and its deficiency leads to bone diseases [5]. VitD is also involved in cellular growth, metabolism, and innate and adaptive immune responses [5,6]. There is no consensus on the threshold levels for 25(OH)vitD deficiency, its assessment, and its treatment, and clinical practice is inconsistent. However, even using conservative thresholds in population surveys, an important proportion of the global population shows vitD deficiency due to insufficient solar light exposure and/or reduced dietary intake [7].

In recent years, observational studies showed an association between low vitD levels and increased cardiovascular risk, respiratory diseases, and all-cause mortality [5,8–12]. However, new randomized controlled trials with moderate or high doses of vitD rendered dissimilar results. For instance, vitD supplements failed to demonstrate systemic antihypertensive effects or to reduce cardiovascular events [13–15], but succeeded in reducing asthma [16] and chronic obstructive pulmonary disease (COPD) [17] exacerbations in patients with baseline 25(OH)vitD levels lower than 25 nmol/L (10 ng/mL)

About 85% of total 25(OH)vitD is bound to vitD-binding protein (DBP), about 15% is bound to albumin, and only less than 1% is free [18]. Albumin-bound 25(OH)vitD dissociates rapidly and is also biologically available in tissues, and DBP acts as a reservoir for vitD. Thus, bioavailable 25(OH)vitD is defined as free 25(OH)vitD plus albumin-bound 25(OH)vitD [19]. Current classifications of vitD status are based on total 25(OH)vitD concentrations. However, several studies reported that bioavailable 25(OH)vitD may be a better biomarker for vitD status. For example, free and bioavailable 25(OH)vitD levels may be useful predictors for the prognosis of patients with coronary artery disease [20]. By contrast, other studies refuted this view [21,22].

Some preliminary evidence suggests that vitD deficiency is common in PAH [23–26]. Herein, we aimed to examine the total, bioavailable, and free 25(OH)vitD levels and their relationship with relevant clinical variables in PAH. The main hypothesis of the study was that lower levels of total 25(OH)vitD predicted reduced survival.

2. Experimental Section

2.1. Study Design and Participants

Human samples were provided from Biobank repositories, and the study was approved by the research ethics committees of Hospital Gregorio Marañón (REF. 123/16), the Spanish PH Biobank, and the Biobanco Vasco. Informed consent from donors was obtained in all cases. We carried out a multicenter, observational case–control study. The PAH cohort included all PAH patients with idiopathic, hereditary, and drug-induced PAH, whose plasma samples were deposited at the Spanish Biobank of PH at the IDIBAPS (Barcelona, Spain). It included 68 patients, but one was excluded because 25(OH)vitD could not be determined. According to international guidelines [4], at the time of sampling, PAH is defined in the Biobank as a mean pulmonary arterial pressure (mPAP) of more than 25 mm Hg, with pulmonary capillary wedge pressure (PCWP) less than 15 mm Hg. Samples from

another control cohort were obtained from the Biobanco Vasco (Bilbao, Spain) which included plasma from 100 subjects, paired by sex with the PAH cohort; with no known cardiovascular disease (two were excluded because 25(OH)vitD could not be determined). No clinical data were available from the control group.

2.2. Measurements of Total 25(OH)vitD and Intact Parathyroid Hormone (iPTH)

Total 25(OH)vitD was measured using a chemiluminescence immunoassay (ADVIA Centaur® VitD Total assay, Siemens Healthcare Diagnostics) which conforms with the National Institutes of Health CDC VitD Standardization Certification Program at the Clinical Biochemistry Service, Gregorio Marañon Hospital. Values below the detection limit of the technique (4.2 ng/mL) were found in 11 samples (all from the PAH cohort) and were replaced by the limit value divided by $\sqrt{2}$ (i.e., 2.97 ng/mL) as reported (https://analytics.ncsu.edu/sesug/2003/SD08-Croghan.pdf). VitD deficiency was defined as values of 25(OH)vitD below 20 ng/mL (equivalent to 50 nM), and severe deficiency was considered as values below 10 ng/mL. Plasma intact parathyroid hormone (iPTH) was measured by immunoassay using the same platform as above. This iPTH assay has standardization traceable to the World Health Organization's standard preparation code 79/500. iPTH could not be measured in three control and 10 PAH samples. Values within 10–55 pg/mL were considered in the normal range.

2.3. Measurements of DBP and Albumin

Plasma DBP levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (R&D Systems; Minneapolis, MN, USA). Values of DBP are expressed in μg/mL (250 μg/mL equal to 4 μM). Two PAH samples were discarded because DBP could not be determined. Albumin plasma concentrations were tested by the colorimetric Bromocresol Green (BCG) method (Sigma-Aldrich, MAK124). The intensity of the color, measured at 620 nm, is directly proportional to the plasma albumin concentration. Albumin levels are expressed in g/dL (0.1 g/dL is equivalent to 15 μM).

2.4. Calculation of Bioavailable 25(OH)vitD and Free 25(OH)vitD

Plasma bioavailable and free 25(OH)vitD were calculated using total 25(OH)vitD, DBP concentration, albumin levels, and affinity constants for albumin and DBP, as previously reported [27]. Total 25(OH)vitD can be defined as follows: total 25(OH)vitD = free 25(OH)vitD + albumin-bound 25(OH)vitD + DBP-bound 25(OH)vitD. Free 25(OH)vitD can be assessed via an indirect method, using a formula, which was first described by Bikle et al. [28] and later used by many authors [20,29].

$$\text{Free } 25(\text{OH})\text{vitD} = \frac{\text{Total } 25(\text{OH})\text{vitD}}{1 + \text{Kalb}\times\text{albumin} + \text{KDBP} \times \text{DBP}}$$

The vitD bioavailable concentration can also be calculated using the following mathematical formula:

$$\text{Bioavailable } 25(\text{OH})\text{vitD} = [(\text{Kalb} \times \text{albumin}) + 1] \times \text{Free } 25(\text{OH})\text{vitD}$$

Kalb is the affinity constant for 25(OH)vitD and albumin binding (6×10^5 M^{-1}), whereas KDBP is the affinity constant for 25(OH)vitD and DBP binding (7×10^8 M^{-1}). Total and free 25(OH)vitD, albumin concentration, and DBP levels are expressed in mol/L.

2.5. Clinical Variables

We obtained the following clinical data of the PAH patients described above from the Spanish Registry of PAH (REHAP) [30] which is linked to the Biobank: PAH subgroup, date of birth, sex, weight, height, date of death, date of lung transplantation, and whether the patient was alive on 31 January 2017. The following parameters were obtained from the REHAP at the nearest date within six months

of the date of plasma sampling for the Biobank: New York Heart Association functional class, diffusing capacity of the lung for carbon monoxide (DLCO), 6-min walking distance (6MWD), BNP, pro-BNP, cardiac catheterization (mPAP, PCWP, right-atrial pressure, cardiac output, cardiac index), parameters from echo (TAPSE, systolic PAP, myocardial performance or TEI index), oxygen, and drug therapy. In order to analyze if vitD levels affect the prognosis of PAH patients, we categorized all patients into two groups above or below the median from total (7.17 ng/mL), bioavailable (0.91 ng/mL), free (1.53 pg/mL) 25(OH)vitD, and DBP (337.3 µg/mL). The predefined main endpoint was an increased survival or a reduction in mPAP for total 25(OH)vitD levels. However, there were very few data from right-heart catheterizations (<35%) in the registry within the predefined time frame chosen of six months. Thus, survival was finally the single main endpoint of the study. The non-invasive risk score [4], a simplified version of the 2015 European Society of Cardiology (ESC) and the European Respiratory Society (ERS) risk assessment score, was calculated using three noninvasive low-risk criteria: NYHA functional class I–II, 6MWD < 440 m, BNP < 50 ng/L, or NT-proBNP < 300 ng/L [31].

2.6. Statistical Analyses

Analyses were performed using GraphPad Software v7 (GraphPad Software Inc., San Diego, CA, USA). All data were analyzed with non-parametric statistics. Two-sample comparisons were analyzed using Mann–Whitney test, and data are presented as scatter plots and medians. Multiple-sample comparisons, NYHA functional class, risk assessment, and percentage of patients were analyzed by chi-square for trends, and data are represented as tables of contingency. Survival curves were analyzed using the Kaplan–Meier method and compared by the log-rank test. Treatments in PAH patients were compared using Fisher's exact test. The Cox proportional hazard model was used to assess hazard ratios of the survival, i.e., the main outcome, and compared with the Wald test using Stata (version 15). Unadjusted and age-adjusted hazard ratios were calculated. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Total 25(OH)vitD Plasma Levels

The characteristics of PAH patients at diagnosis are shown in Table 1. Plasma sampling for the Biobank was carried out 3.8 (0.9) years (median and interquartile range (IQR)) after diagnosis. The control group, comprising 98 sex-matched patients without known cardiovascular disease, showed lower than recommended total 25(OH)vitD plasma levels (median (IQR): 12.27 (9.11–18.08) ng/mL; Figure 1A). Nevertheless, the group with PAH had significantly lower total 25(OH)vitD values (7.17 (5.15–10.84) ng/mL, Figure 1A) than controls. The levels were similar for the idiopathic and heritable PAH subgroups (6.97 (5.09–10.45), n = 58, and 7.30 (7.04–11.44), n = 7, respectively) and higher in the only two patients that had drug-induced PAH (11 and 15 ng/mL). The deficiency was present in both women (7.49 (5.46–10.84) ng/mL, n = 53) and men (5.93 (3.51–9.59) ng/mL, n = 14) with PAH ($p > 0.05$ vs. women, Mann–Whitney test). Therefore, 35% and 70% of the control subjects and PAH patients, respectively, had severe deficiency (Figure 1C). As expected, severe vitD deficiency in PAH was accompanied by significantly higher levels of iPTH than in the control group (Figure 1B). Thus, 70% of PAH patients showed hyperparathyroidism (i.e., iPTH > 55 pg/mL, Figure 1D).

Table 1. Patient characteristics at diagnosis.

	PAH	Controls
N	67	98
Idiopathic	58	-
Heritable	7	-
Drug-induced	2	-
Age (years)	47 (31.5–62)	39 (29–47)
Female (%)	53 (79%)	80 (81%)
mPAP (mm Hg)	52 (43–61)	-
Cardiac index (L/min/m^2)	2.2 (1.8–2.5)	-
DLCO (mL/min/mmHg)	64 (47.5–73.5)	-
6MWD (m)	412 (336–474)	-
Functional class (I, II, III, IV)	1, 23, 40, 3	-

Data are median (interquartile range, IQR). PAH: pulmonary arterial hypertension; mPAP: mean pulmonary arterial pressure; DLCO: CO diffusing lung capacity; 6MWD: six-minute walk distance.

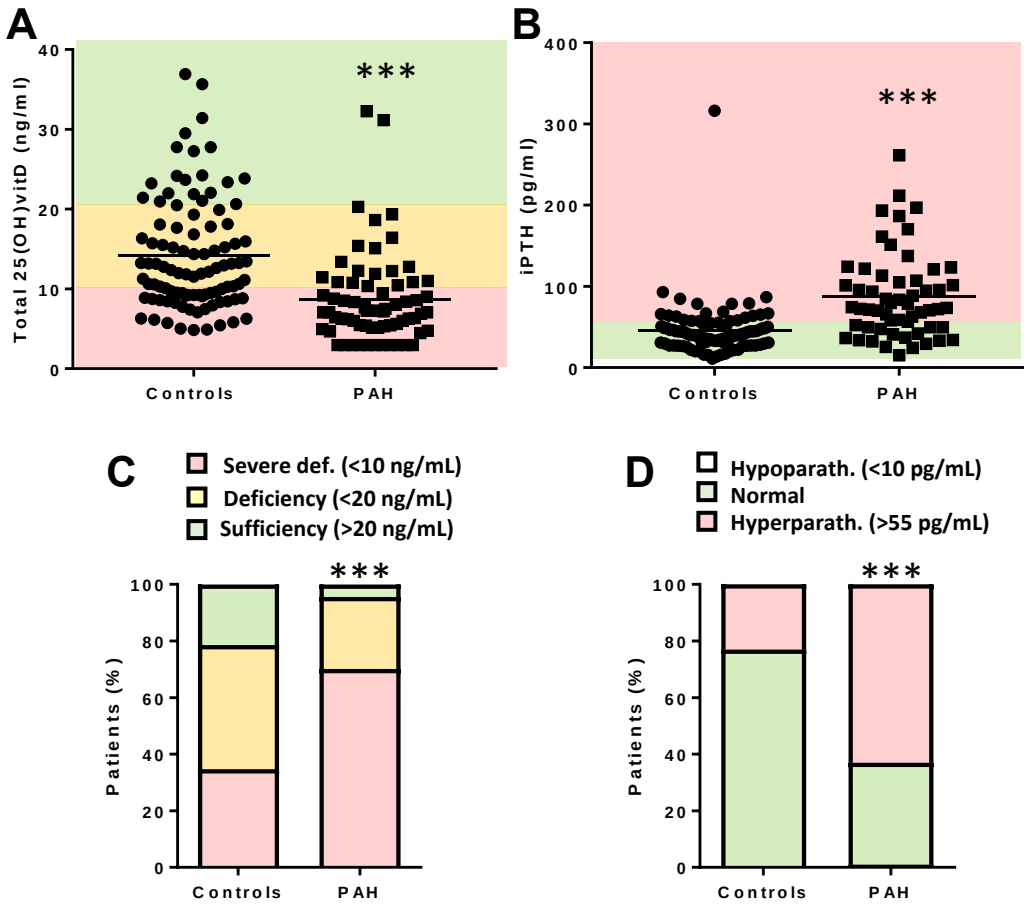

Figure 1. PAH patients present decreased total 25-hydroxy-vitamin D (25(OH)vitD) and increased intact parathyroid hormone (iPTH). (**A**) Total 25(OH)vitD and (**B**) iPTH plasma levels from controls and PAH patients. Colors indicate the ranges of levels as follows: for panels A and C, red encodes severe deficiency (<10 ng/mL), yellow encodes moderate deficiency (10–20 ng/mL), and green encodes sufficiency (>20 ng/mL); for panels B and D, green encodes normal levels (10–55 pg/mL) and red encodes hyperparathyroidism (>55 pg/mL). (**C**) Percentage of patients according to total 25(OH)vitD levels. (**D**) Percentage of patients according to iPTH range. Results in panels A and B are presented as scatter plots and medians; *** indicates $p < 0.001$ vs. controls, Mann–Whitney test. In panels C and D, *** denotes $p < 0.001$ vs. controls, chi-square test for trend.

3.2. Plasma DBP, Albumin, and Calculated Bioavailable and Free 25(OH)vitD

PAH patients showed lower concentration of DBP (median (IQR): 337.3 (258.5–443.8) µg/mL; Figure 2A) than controls (median (IQR): 542.4 (330.9–893) µg/mL; Figure 2A). Plasma albumin concentration was also significantly lower in PAH patients (median (IQR): 6.31 (5.41–7.05) g/dL) than controls (median (IQR): 7.5 (6.51–9.06) g/dL; Figure 2B). Bioavailable and free 25(OH)vitD were calculated based on total 25(OH)vitD, albumin, and DBP levels. We also found that PAH patients presented lower bioavailable 25(OH)vitD (median (IQR): 0.91 (0.64–1.46) ng/mL; $n = 65$) than controls (median (IQR): 1.12 (0.71–2.19) ng/mL; $n = 98$, Figure 2C). Two PAH samples were discarded in this analysis because DBP could not be measured. By contrast, free 25(OH)vitD levels were similar in both groups (Figure 2D).

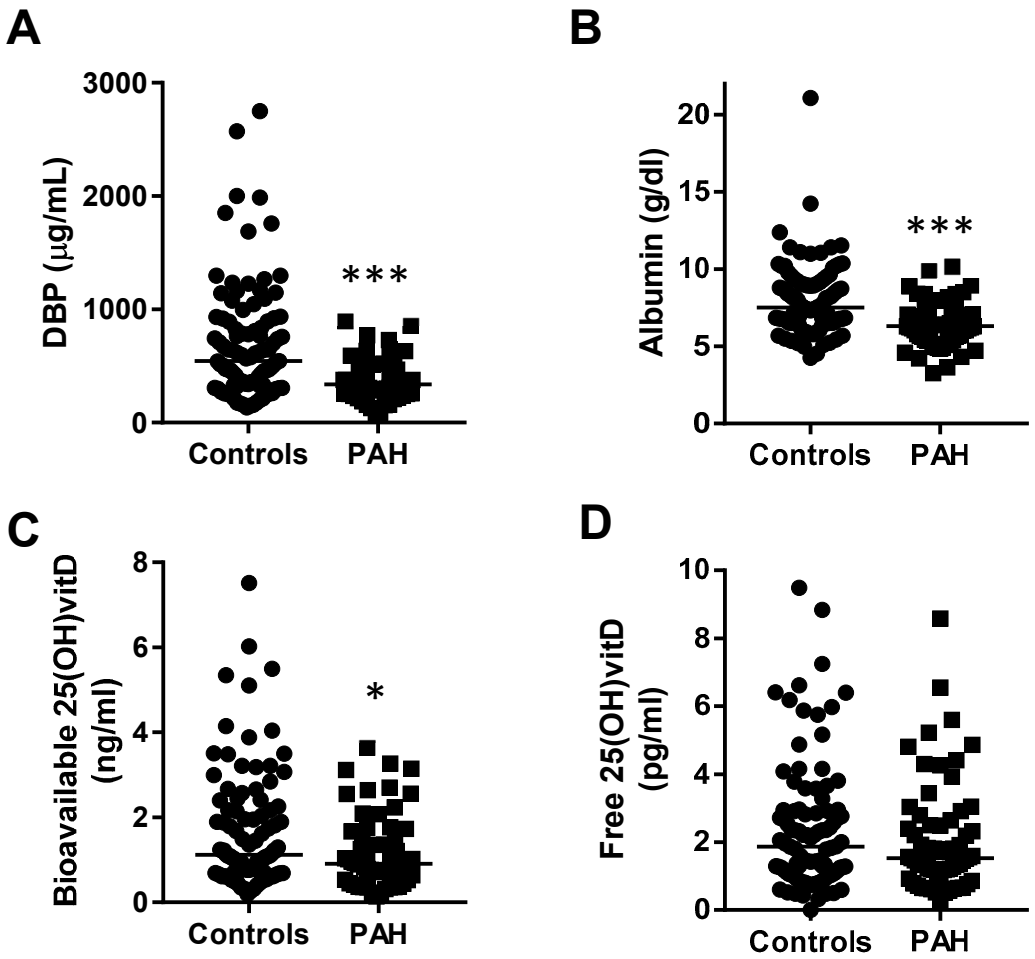

Figure 2. VitD-binding protein (DBP), albumin, and bioavailable but not free 25(OH)vitD are decreased in PAH patients. (**A**) Plasma DBP concentration, (**B**) albumin, (**C**) calculated bioavailable 25(OH)vitD, and (**D**) calculated free 25(OH)vitD from controls and PAH patients. Data are represented as scatter plots and medians. * and *** indicate $p < 0.05$ and $p < 0.001$ vs. controls, respectively, Mann–Whitney test.

We analyzed possible relationships between total 25(OH)vitD, DBP, and albumin. However, we found no correlation among these parameters in either controls or PAH patients, indicating that these three variables were independent (Figure 3).

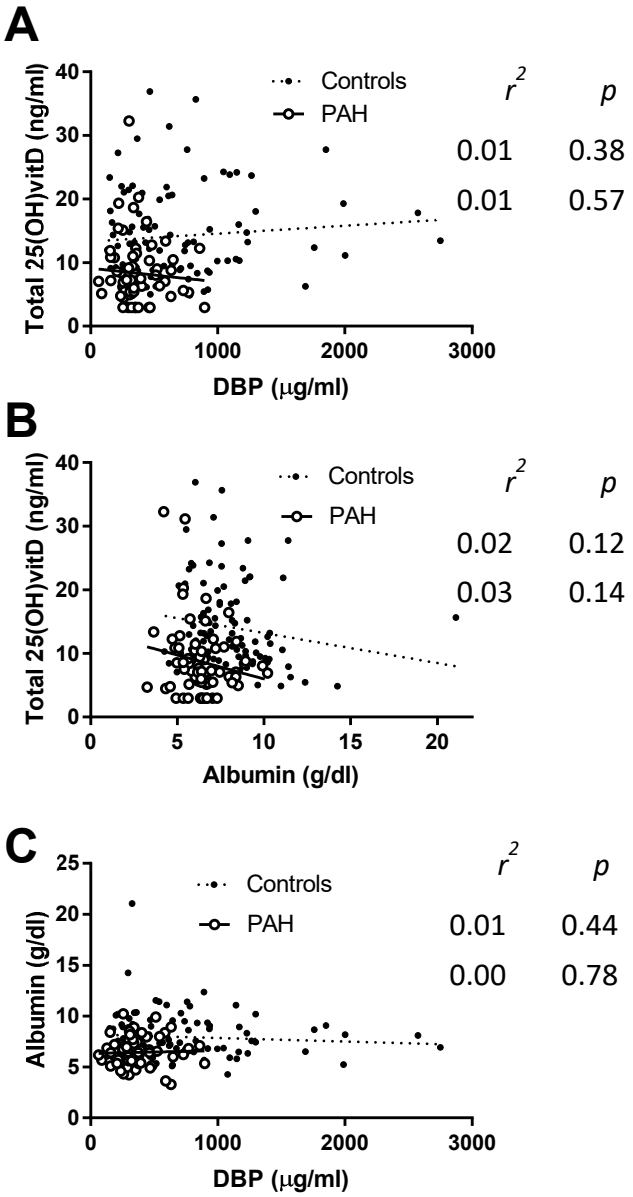

Figure 3. VitD-binding protein (DBP), albumin and total 25(OH)vitD plasma concentrations are independent variables. Correlations between (**A**) DBP and total 25(OH)vitD, (**B**) albumin and total 25(OH)vitD, and (**C**) DBP and albumin in controls and in PAH patients. The calculated Pearson r^2 and p are shown in each panel.

3.3. Total 25(OH)vitD Levels, Clinical Parameters, and Survival

We compared clinical variables (Table S1, Supplementary Materials) and pharmacological treatments (Table S2, Supplementary Materials) in PAH patients with total 25(OH)vitD plasma values below versus patients above the median (7.17 ng/mL) of this cohort. Patients below the median had more advanced functional class (Figure 4A). The exercise capacity, assessed as the 6MWD, and the right-ventricular systolic function, measured at echocardiography (TAPSE), were significantly lower in patients with total 25(OH)vitD levels below the median (Figure 4B,C). BNP levels were available for some patients, while NT-proBNP levels were available for other patients depending on the hospital. Despite these parameters not being significantly different ($p > 0.05$, Mann–Whitney test; Figure 4D), the proportion of patients at high risk according to BNP/NT-proBNP cut off levels as defined by ESC/ERS Guidelines (i.e., BNP > 300 ng/mL or NT-proBNP > 1400 ng/mL) was significantly higher ($p < 0.05$, Fisher chi-square for trend) in patients with total 25(OH)vitD levels below the median. Patients with

higher 25(OH)vitD had significantly lower age and for all other hemodynamical parameters; follow-up data were scarce in the registry within six months of sampling and there were no significant differences (Table S1, Supplementary Materials).

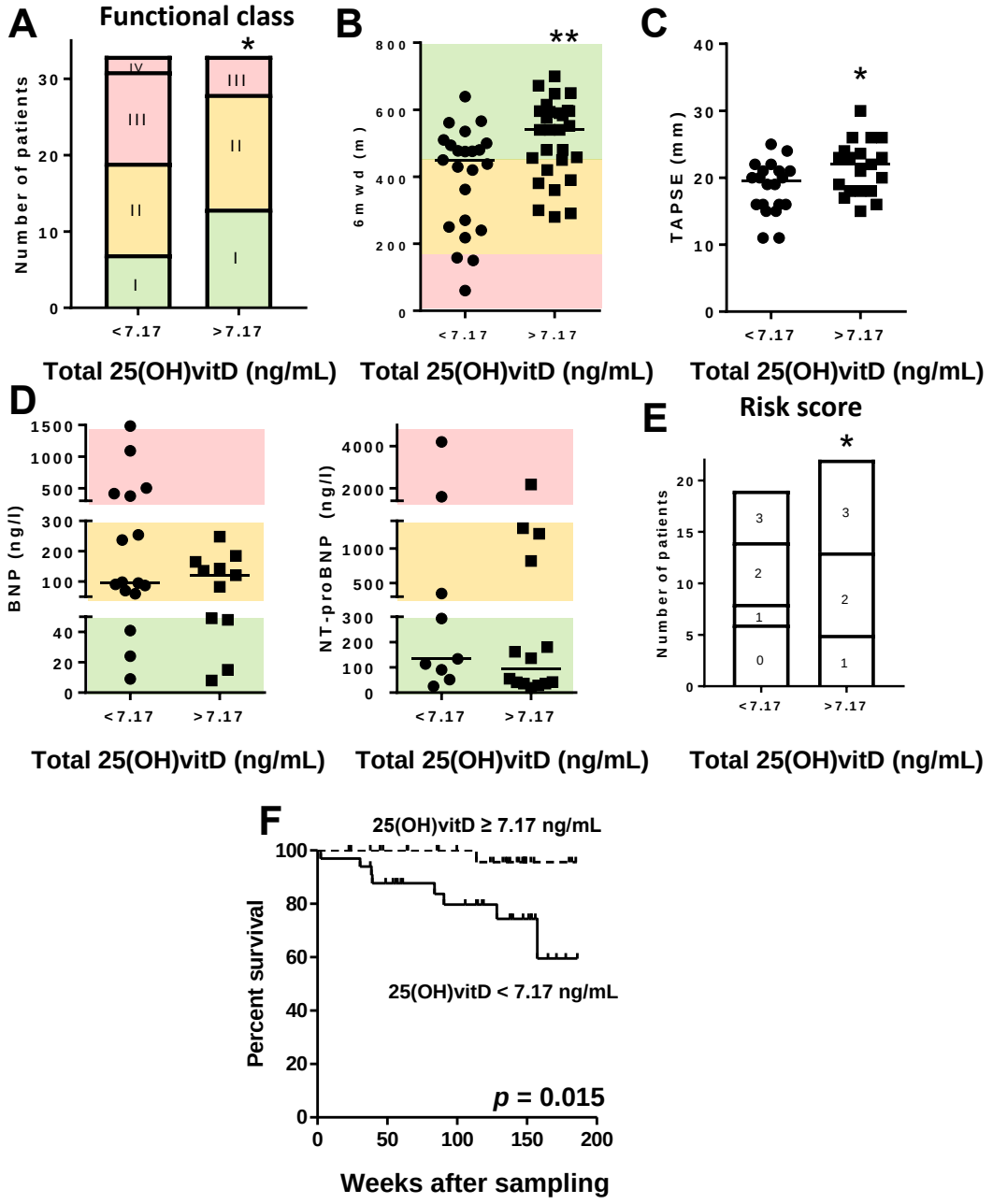

Figure 4. PAH patients with lower total 25(OH)vitD levels present worse prognosis. PAH patients were categorized according to total 25(OH)vitD levels (above vs. below the median (7.17 ng/mL) in the cohort). (**A**) NYHA functional class; (**B**) 6-min walking distance test (6MWD); (**C**) TAPSE; (**D**) BNP (left) and NT-proBNP (right). Colors in panels A, B, and D identify the ranges of these biomarkers considered of low (green), intermediate (yellow), or high (red) risk according to ERS/ESC guidelines for PAH. (**E**) Non-invasive risk score showing the number of patients with zero, one, two, or three low risk factors. In panels A and E, * denotes $p < 0.05$, chi-square test for trend. In panels B and D, data are scatter plots and medians; * $p < 0.05$ and ** $p < 0.01$ Mann–Whitney test. (**F**) Kaplan–Meier analysis of survival in PAH patients with total 25(OH)vitD levels above vs. below the median (7.17 ng/mL); $p < 0.05$, log-rank test.

We also analyzed the non-invasive risk score [31] based on the number of low-risk criteria in all PAH patients with total 25(OH)vitD above versus below the median. PAH patients with total 25(OH)vitD above the median (7.17 ng/mL) presented lower risk of death (Figure 4E). Remarkably, survival after plasma sampling, which was the main outcome measure of the study, was significantly increased ($p < 0.01$) in these patients (hazard ratio: 7.96 (95% confidence interval: 2.99 to 21.16)). Because there was a strong age unbalance between the patients with total 25(OH)vitD levels above versus those below the median, we carried out a Cox proportional hazard model adjusted for age. The age-adjusted hazard ratio was 5.40 (95% confidence interval: 2.88 to 10.12). The Kaplan–Meier analysis (Figure 4F) showed a significant reduced survival ($p = 0.015$) in patients with total 25(OH)vitD levels below the median. Treatments were similar in the two groups except for the use of long-term oxygen therapy, which was more frequent in PAH patients with lower total 25(OH)vitD (Table S2, Supplementary Materials).

3.4. DBP, Bioavailable and Free 25(OH)vitD, and Clinical Outcomes

We also compared the clinical variables (Table S1, Supplementary Materials), the survival, and the pharmacological treatments (Table S2, Supplementary Materials) as a function of the DBP, albumin, and bioavailable and free 25(OH)vitD levels in plasma. For this purpose, we categorized all PAH patients into two halves: those with DBP levels above and those below the median (337.3 μg/mL). A similar categorization was made for albumin (median 6.31 g/dL), as well as for bioavailable (median 0.91 ng/mL) or free 25(OH)Vit (median 1.53 pg/mL). The clinical variables, the survival, and the therapies were similar in the two groups with low vs. high DBP (Tables S1 and S2, Supplementary Materials) However, patients with calculated bioavailable 25(OH)vitD below the median had significantly more advanced functional class (Figure 5A) and significantly lower 6MWD (Figure 5B). Moreover, the proportion of patients at high risk according to BNP/NT-proBNP cut off levels was significantly higher ($p < 0.05$, chi-square for trend) in PAH patients with bioavailable 25(OH)vitD levels below the median (Figure 5D). However, no differences were observed in TAPSE values according to bioavailable 25(OH)vitD levels (Figure 5C). Remarkably, PAH patients with bioavailable 25(OH)vitD levels over 0.91 ng/mL showed lower non-invasive risk score of mortality (Figure 5E), but the analysis of mortality did not show differences ($p = 0.09$, Figure 5F). For all the other clinical variables analyzed in this study, there were no significant differences (Table S1, Supplementary Materials). In addition, similar to total 25(OH)vitD results, long-term oxygen therapy was more frequent in PAH patients with lower bioavailable 25(OH)vitD (Table S2, Supplementary Materials).

Regarding PAH patients with free 25(OH)vitD levels below vs. those above the median, we found that PAH patients with lower free 25(OH)vitD levels had a more advanced functional class (Figure 6A) and higher levels of BNP/NT-proBNP ($p < 0.05$, chi-square for trend, Figure 6D). There was also a significantly smaller proportion of males in the group with lower free 25(OH)vitD levels. However, there was no difference in any of the other clinical parameters analyzed or treatments (Tables S1 and S2, Supplementary Materials; Figure 6), except for a paradoxical borderline decrease in mPAP. However, the non-invasive risk score, which combines several risk factors, was significantly lower in patients with free 25(OH)vitD above the median (Figure 6E). Survival in the Kaplan–Meier analysis was also not significantly different (Figure 6F).

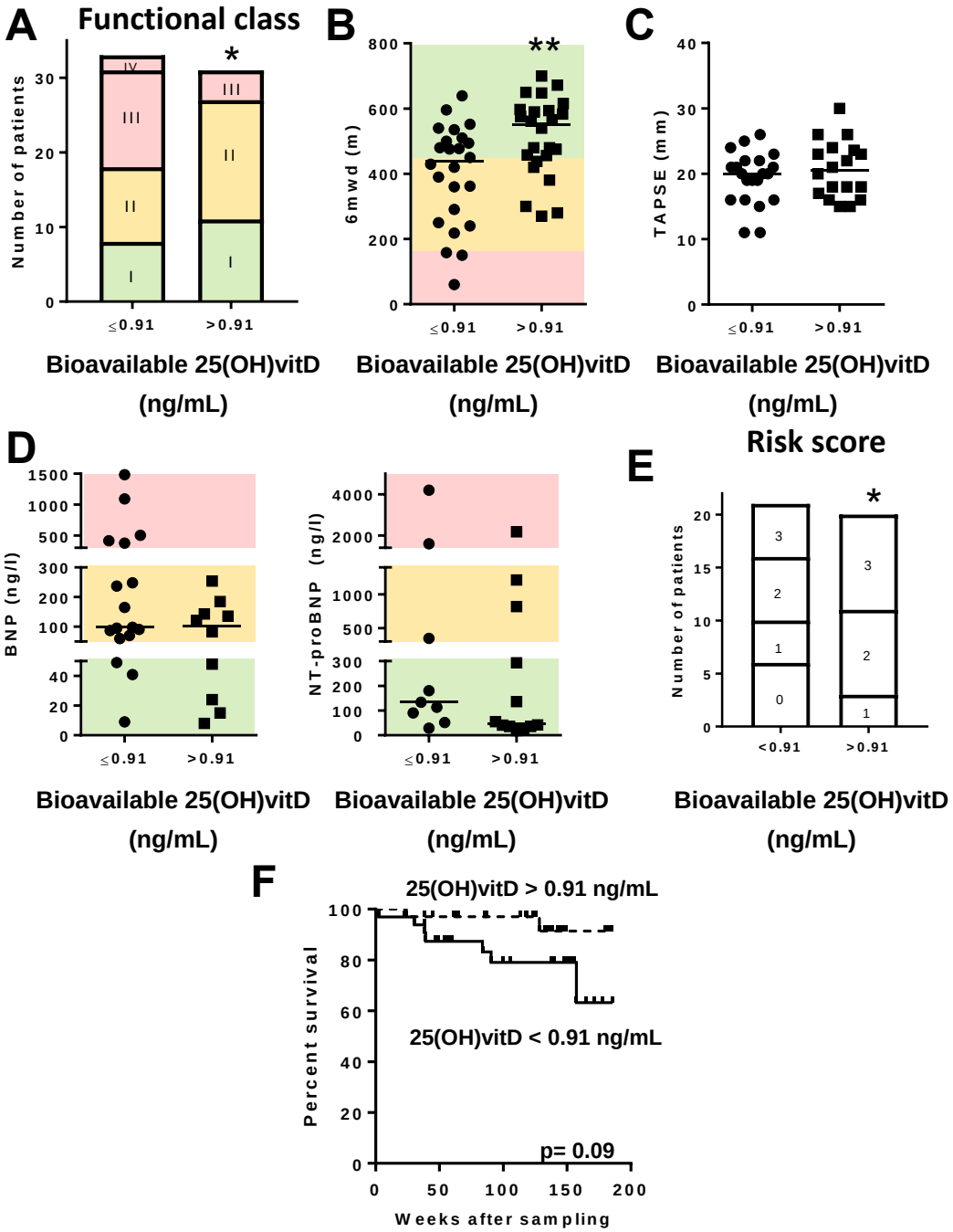

Figure 5. Bioavailable 25(OH)vitD levels in PAH patients and prognosis. PAH patients were categorized according to bioavailable 25(OH)vitD levels (above vs. below the median (0.91 ng/mL) in the cohort). (**A**) NYHA functional class; (**B**) 6-min walking distance test (6MWD); (**C**) TAPSE; (**D**) BNP (left) and NT-proBNP (right). Color codes are as in Figure 4. (**E**) Non-invasive risk score showing the number of patients with zero, one, two, or three low risk factors. In panels A and E, * denotes $p < 0.05$, chi-square test for trend. In panels B and D, data are scatter plots and medians, ** $p < 0.01$ Mann–Whitney test. (**F**) Kaplan–Meier analysis of survival in PAH patients with bioavailable 25(OH)vitD levels above vs. below the median (0.91 ng/mL); $p = 0.09$, log-rank test.

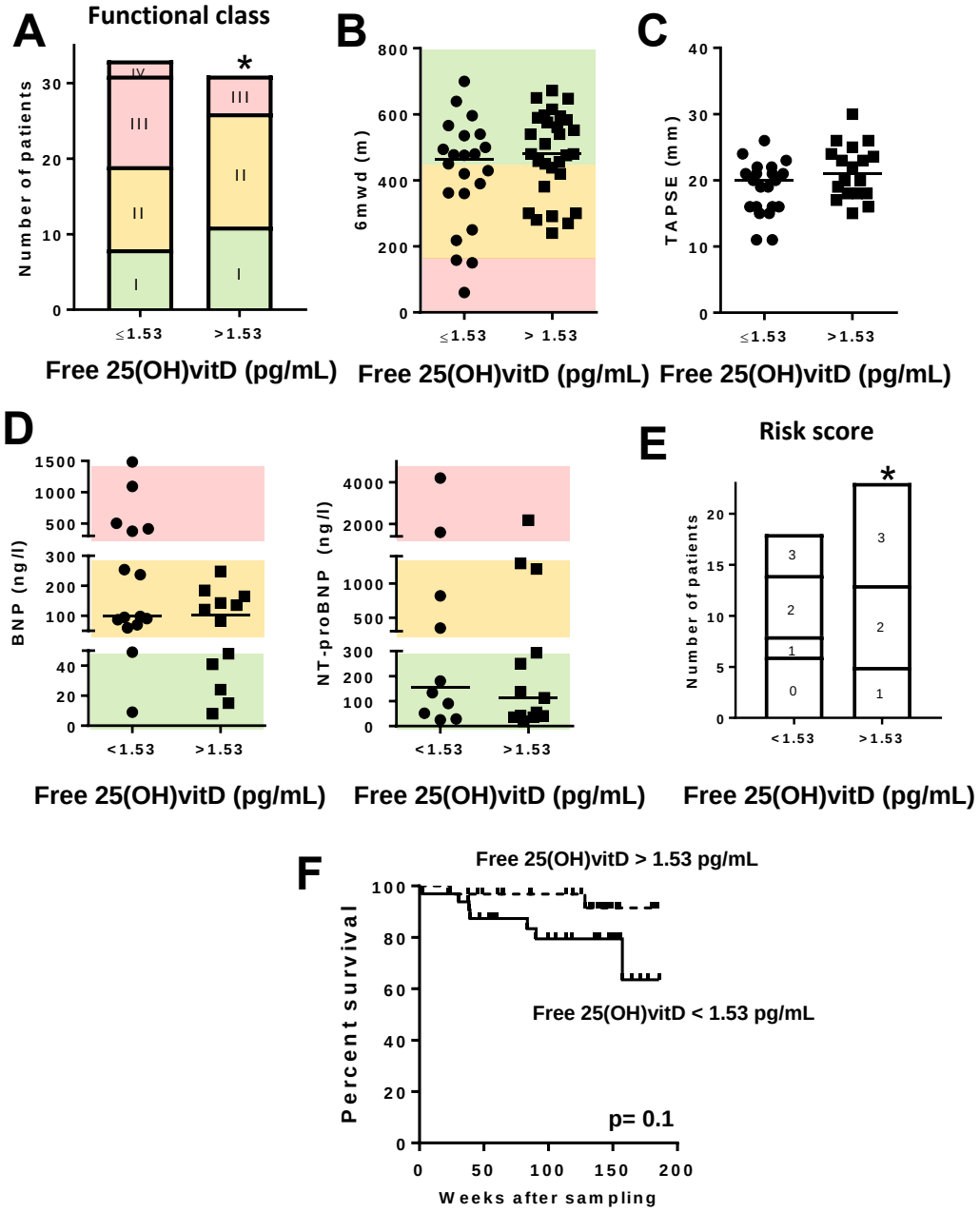

Figure 6. Free 25(OH)vitD levels in PAH patients and prognosis. PAH patients were categorized according to free 25(OH)vitD levels (above vs. below the median (1.53 pg/mL) in the cohort). (**A**) NYHA functional class; (**B**) 6 min walking distance test (6MWD); (**C**) TAPSE; (**D**) BNP (left) and NT-proBNP (right). Color codes are as in Figure 4. (**E**) Non-invasive risk score showing the number of patients with zero, one, two, or three low risk factors. In panels A and E, * denotes $p < 0.05$, chi-square test for trend. In panels B and D, data are scatter plots and medians; ** $p < 0.01$ Mann–Whitney test. (**F**) Kaplan–Meier analysis of survival in PAH patients with bioavailable 25(OH)vitD levels above vs. below the median (0.91 ng/mL); $p = 0.10$, log-rank test.

4. Discussion

The present study shows that up to 95% of patients with PAH have vitD deficiency (25(OH)vitD < 20 ng/mL) with 70% presenting severe deficiency (<10 ng/mL), and hyperparathyroidism (iPTH > 55 pg/mL). Moreover, we found that plasma DBP and bioavailable 25(OH)vitD are decreased in PAH. To our knowledge, this is the largest study analyzing the vitD status in PAH and the first one which

analyzed the association of several plasma vitD biomarkers as total, bioavailable, and free 25(OH)vitD levels and DBP with the prognosis of PAH patients. Our results indicate that total 25(OH)vitD levels may have a prognostic value in PAH. When comparing bioavailable 25(OH)vitD in PAH patients vs. controls subjects, the magnitude of the difference and its statistical significance are smaller than for total 25(OH)vitD, and the significance is lost for free 25(OH)vitD. Likewise, the association of bioavailable 25(OH)vitD with prognosis is weaker than for total 25(OH)vitD, and it was even weaker for free 25(OH)vitD.

In line with the global pandemic of vitD deficiency [32,33], our control subjects present inappropriate vitD levels (median: 12.27 ng/mL) with 35% of them showing severe deficiency. Preliminary epidemiological studies suggested that vitD deficiency is more prevalent in PAH than in the general population. In fact, in our PAH cohort (n = 67), severe vitD deficiency was very common (median 7.17 ng/mL), in concordance with previous reports in small series (n = 22, 19, and 12) [23,24,26]. Values were very similar for idiopathic (class 1.1) and heritable PAH (class 1.2), and the sample of the drug-induced PAH (class 1.3) was too small to draw any conclusion. In a recent published study, 25(OH)vitD levels from PH patients with different etiologies were also analyzed as a single group [34].

Physiologically, decreased 25(OH)vitD levels result in proportionally increased PTH levels in order to maintain adequate serum calcium concentration. Several studies reported that secondary hyperparathyroidism and osteopenia are highly prevalent in PAH patients [24,35]. Therefore, inappropriate vitD status in PAH patients is likely to contribute to the observed elevated iPTH.

Tanaka et al. (23) also reported that total 25(OH)vitD levels negatively correlated with mPAP assessed by right-heart catheterization, with a significant positive correlation with cardiac output. According to the ESC/ERS guidelines for the diagnosis and treatment of PH and the World Health Organization [4], there are several factors that provide prognostic information and may be used to guide therapeutic decisions. Some of them, such as functional class, 6MWD, and BNP or NT-proBNP, are recommended to be assessed at each visit and are useful follow-up criteria. Functional class, despite its interobserver variability, is considered as one of the most powerful predictors of disease progression. In addition, exercise capacity measured by 6MWD test is also a strong predictor of mortality. BNP and NT-proBNP are not specific biomarkers for PAH, but they remain the only plasma biomarkers that are widely used in routine practice and in clinical trials. The non-invasive risk score is a simplified risk assessment tool that combines these three parameters, accurately discriminates prognostic groups, and predicts survival in PAH [31]. Notably, our results show that patients with total 25(OH)vitD levels above the median have better functional class, and they present higher exercise tolerance (around 90 m more) and lower BNP or pro-BNP levels. Likewise, the non-invasive risk score was also significantly different. TAPSE is another non-invasive prognostic factor often used to analyze ventricular function echocardiographically, which was also increased in patients with higher 25(OH)vitD. Other important determinants of prognosis such as right-atrial pressure, right-atrial area, cardiac index, ventilatory equivalents for carbon dioxide, and peak oxygen consumption were only available for few patients at the time of blood sampling and were not significantly different between the groups. Patients with more severe 25(OH)vitD deficiency had also higher requirements for long-term oxygen therapy. Altogether, these data suggest that patients with low 25(OH)vitD present worse prognosis. In fact, we found that these patients followed up after the blood sampling indeed had reduced survival.

VitD status in clinical practice is assessed by determining the total 25(OH)vitD concentration in serum or plasma. It is not the most active metabolite, but it is the major circulating form of vitD [5]. The majority of circulating 25(OH)vitD is tightly bound to DBP [18,19]. Thus, the assessment of total 25(OH)vitD levels principally measures the DBP-bound form, which is not biologically active [19,36,37]. Bioavailable 25(OH)vitD, which includes the free plus the easily released albumin-bound form, may be more representative to determine the vitD status. Diseases or conditions that affect the synthesis of DBP or albumin, thus, have a huge impact on the amount of circulating total 25(OH)vitD [27]. DBP and albumin are synthesized in the liver; hence, all patients with an impairment of liver function have alterations in their total vitD blood concentrations, while free vitD levels may remain mostly

constant. Whether bioavailable or free 25(OH)vitD is a more accurate biomarker of vitD status than total 25(OH)vitD is disputed [18–22]. Remarkably, we found that PAH patients present lower plasma levels of both DBP and albumin than control patients. Since DBP and albumin act as a storage for total vitD, our results indicate that PAH patients show a lower reservoir capacity of vitD. Moreover, we also found that total 25(OH)vitD is not related to either DBP or albumin concentration; they are independent variables. This is consistent with the concept that vitD itself or its metabolites do not regulate DBP production [19,38]. Previous studies also reported lower serum albumin concentrations in PAH patients [39,40] and in PAH animal models [41], suggesting that reduced albumin may be a risk factor for PAH. Following the formulas given above, calculated free 25(OH)vitD is inversely related to DBP and albumin concentrations. Therefore, a decrease in plasma DBP and albumin as observed in PAH results in a relative increase in free 25(OH)vitD levels. Hence, in contrast to the data for total 25(OH)vitD levels, we did not find a significant difference in free 25(OH)vitD levels between controls and PAH patients. On the other hand, bioavailable 25(OH)vitD is inversely related to DBP and has a complex dependence on albumin. Thus, we still found a significant difference in bioavailable 25(OH)vitD levels between controls and PAH patients, but of smaller magnitude than for total 25(OH)vitD.

DBP knock-out mice, in which vitD metabolites are presumably all free and albumin-bound, do not show evidence of vitD deficiency and do not develop rickets. Thus, 25(OH)vitD bound to DBP does not contribute to the biological actions and DBP clearly serves as a critical circulating reservoir of vitD metabolites [19,42]. The mechanism leading to the low DBP concentration in PAH patients remains unknown. Patients with primary hyperparathyroidism have lower concentrations of both DBP and total 25(OH)vitD, without changes in free and bioavailable 25(OH)vitD. It might be possible that higher levels of iPTH inhibit hepatic DBP production [43,44]. Moreover, as noted earlier, albumin concentration does not correlate with total 25(OH)vitD levels.

Our results also show that bioavailable and free 25(OH)vitD were related with some clinical outcomes such as worse functional class and higher risk score. Additionally, bioavailable but not free 25(OH)vitD correlated with lower exercise capacity. By contrast, significant differences in survival were lost for both bioavailable and free 25(OH)vitD levels. Thus, total 25(OH)vitD may be associated more accurately with the progression of PAH, and it could be a more suitable biomarker for vitD status in PAH patients. Moreover, free or bioavailable 25(OH)vitD measurements are highly difficult, and, at this moment, there is only one immunoassay for the direct measurement of free 25(OH)vitD [45]. Some authors suggest that, if there is a correlation between bioavailable and/or free 25(OH)vitD with total 25(OH)vitD, it would only be necessary to measure total 25(OH)vitD [38].

In addition, the lack of activity of DBP-bound 25(OH)vitD was also questioned [36]. DBP can bind megalin, a receptor found in the plasma membrane of many epithelial cells. In the kidney, megalin acts as a cell surface receptor for DBP, and it internalizes DBP-bound 25(OH)vitD, a mechanism which is essential for 25(OH)vitD renal metabolism. Megalin is also highly expressed in the lung, and it is linked to the effects of transforming growth facto-β(TGF-β) [46]; however, it is not yet clear whether it plays a role on the pulmonary actions of vitD.

The reduced 25(OH)vitD plasma values that we found in patients with PAH does not establish a cause–effect relationship with the disease. Due to changes in lifestyle and environment, reduced outdoor activities, and inadequate sun exposure, vitD deficiency is a common phenomenon in the healthy population. Because PAH is a life-limiting disease, associated with weakness, fatigue, and exercise intolerance, PAH patients are more likely to have reduced sunlight exposure and, thus, become more susceptible to vitD deficiency. In fact, critically ill patients show a high prevalence of hypovitaminosis D [47]. Therefore, vitD deficiency may be a consequence of PAH. However, it is also reasonable that vitD-deficient states may worsen existing immune, metabolic, and cardiovascular dysfunctions [5,6], leading to the development of PAH in predisposed patients or to worse outcomes in patients with existing PAH. With the exception of idiopathic PAH, in all other forms of the disease, there is a factor known to be involved in its etiopathogeny, including mutations, systemic diseases, congenital heart

defects, infections, drugs, and toxins. However, none of these factors by itself is able to trigger the disease, and the need for second hit(s) was proposed. vitD is involved in numerous processes of potential relevance in PAH, such as cell proliferation, differentiation, and apoptosis, cell adhesion, oxidative stress, angiogenesis, and immunomodulatory and anti-inflammatory activity [5]. Therefore, vitD deficiency might be one of these second hits. For instance, the prevalence of PAH in patients with systemic sclerosis is around 33%. In a cohort with systemic sclerosis, levels of 25(OH)vitD above 30 ng/mL had an incidence of echocardiographically elevated mPAP of 5% versus 40% in vitD-deficient patients [48]. In another cohort of patients with systemic sclerosis, subjects with vitD levels <30 ng/mL had higher systolic pulmonary arterial pressure than those with lower levels (34 vs. 25 mmHg) [49].

Restoration of vitD status would represent a very feasible health improving therapy for these patients. In fact, vitD supplements should be prescribed in any healthy or sick subject with deficient 25(OH)vitD to prevent osteomalacia [50]. Whether prognosis and symptoms of PAH patients improve after restoring vitD levels remains unclear. Remarkably, in a small uncontrolled cohort of PAH patients, restoring vitD levels by a cholecalciferol treatment at a dose of 50,000 IU weekly for three months improved the 6MWD by around 80 m and right-ventricular size but did not significantly reduce mPAP, measured by echocardiography ($p = 0.07$) and functional class [26]. Further randomized clinical trials are warranted. However, it would be prudent to choose only those patients with severe deficiency for future randomized studies in PAH patients in order to restore 25(OH)vitD values to normal range. Recently, some studies highlighted that an excess of vitD can cause calcified vasculopathy and valvulopathy, increase renin–angiotensin via hypercalciuria, and increase sympathetic activity [51,52]. In fact, vitD supplementation is used as a treatment in several pathologies regardless of baseline serum levels. This mistaken approach rendered dissimilar results; thus, vitD supplements failed to prevent cancer or to reduce cardiovascular events [53–55]. However, in patients with baseline vitD levels less than 25 nmol/L (10 ng/mL), supplements were beneficial in respiratory diseases, such as reducing asthma [16] and chronic obstructive pulmonary disease [17] exacerbations.

Our study presents some limitations. Right-heart catheterization was not available within the predefined six-month period around the moment of blood sampling for the biobank for most patients, and echocardiographic data were available for only 60% of them. Due to the low number of patients in hereditable ($n = 7$) and in drug-induced PAH ($n = 2$), all PAH patients were analyzed as a single group. Moreover, due to the retrospective nature of the trial, 25(OH)vitD levels were only measured once, and it remains unknown whether 25(OH)vitD levels changed over time or whether patients received vitD supplementation thereafter. The differences found cannot be explained by differences in the time of evolution of the disease from the diagnosis to the plasma sampling, i.e., a longer time after diagnosis would predict worse functional class and worse survival expectative. In fact, there was a trend for higher time of evolution in patients with total 25(OH)vitD above the median. However, we found a significantly lower age (median of 49 years) in the group with total 25(OH)vitD levels above the median compared to those with lower levels (58 years). The aging process itself predisposes to vitamin D deficiency due to a progressive decline in the cutaneous capacity to synthetize vitD and reduced exposure to sunlight [56]. This bias may have influenced the results because the five-year survival was reported to be higher in PAH patients aged 18–45 years (88%), while the survival rates were 63%, 56%, and 36% for patients in the groups 46–64, 65–74, and ≥75 years, respectively [57]. However, after adjusting for age, survival still remained significantly different for those with high vs. low levels of total 25(OH)vitD.

5. Conclusions

The present study demonstrates that total 25(OH)vitD, rather than bioavailable or free 25(OH)vitD, is a potential predictor of adverse outcomes in PAH patients. Given the high prevalence of vitD deficiency in PAH population, it seems reasonable that serum total vitD levels should be regularly assessed. Further studies are required to clarify whether our findings have potential clinical implications.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/2/448/s1: Table S1: Exercise capacity, hemodynamic and echocardiographic variables measured within 6 months of the plasma sampling in PAH patients with total, bioavailable and free 25(OH)vitD levels and DBP levels below versus above the median in this cohort, Table S2: Treatments in PAH patients with 25(OH)vitD levels below versus above the median in this cohort.

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Supplementary Tables S1 and S2

Supplementary Table S1. Exercise capacity, hemodynamic and echocardiographic variables measured within 6 months of the plasma sampling in PAH patients with total, bioavailable and free 25(OH)vitD levels and DBP levels below versus above the median in this cohort.

Total 25(OH)VitD					
	25OHVitD <7.17 ng/ml		25OHVitD ≥ 7.17 ng/ml		p
	n	Median (IQR)	n	Median (IQR)	
Days from diagnosis to sampling	33	853 [181-2739]	34	2300 [775-3762]	0.162
Age at plasma sampling	33	59 [43.5-70]	34	48 [34-59.75]	**0.01**
Sex (M/F)	33	10/23	34	5/29	0.15
6MWD (m)	23	450 [250-500]	27	540 [420-596]	**0.009**
BNP	16	97 [62-406]	11	121 [48-165]	0.312
NT-Pro-BNP	9	134 [71-970]	14	96 [36-917]	0.460
mPAP (RHC) (mm Hg)	15	46 [39-54]	11	60 [40-66]	0.073
PCWP (mm Hg)	15	8 [7-10]	11	8 [6-11]	0.812
RAP (mm Hg)	15	6 [4-9]	11	7 [4-11]	0.419
CI ($l/min/m^2$)	15	2.14 [1.74-2.29]	11	2.30 [1.81-3.31]	0.276
TAPSE (mm)	20	19.5 [16-21]	19	22 [18,24]	**0.039**
sPAP (echo) (mm Hg)	22	70 [63.5,84.7]	20	63 [48.5,87.7]	0.290
Functional class (I, II, III, IV)	33	7, 12, 12, 2	33	13,15,5,0	**0.011**[1]
Bioavailable 25(OH)VitD					
	25OHVitD≤0.91 ng/ml		25OHVitD>0.91 ng/ml		p
	n	Median [IQR]	n	Median [IQR]	
Days from diagnosis to sampling	33	1058 [178-2986]	32	2061 [596.5-4366]	0.19

Age at plasma sampling	33	59[41-69]	32	48[37-61]	0.09
Sex (M/F)	33	9/24	32	5/27	0.36
6MWD (m)	24	439.5 [260.3-507.5]	24	550.5 [442.5-596.3]	**0.01**
BNP	17	98 [65-396.5]	10	102 [21.75-153.5]	0.18
NT-Pro-BNP	9	180 [101.5-1057]	12	360 [164.5-751]	0.58
mPAP (RHC) (mm Hg)	14	44 [38-54.5]	11	57 [40-63]	0.16
PCWP (mm Hg)	14	8 [7-10]	11	8 [6-11]	0.99
RAP (mm Hg)	15	7 [5-9]	11	7 [4-10]	0.95
CI ($l/min/m^2$)	15	2.14 [1.74-2.29]	10	2.2 [1.77-3.32]	0.39
TAPSE (mm)	21	20 [16-22]	17	21 [17-23.8]	0.44
sPAP (echo) (mm Hg)	23	65 [55-82]	18	72.5 [56.5-87.75]	0.37
Functional class (I, II, III, IV)	33	8,10,13,2	31	11,16,4,0	**0.01**[1]
Free 25(OH)VitD					
	25OHVitD≤1.53 pg/ml		25OHVitD>1.53 pg/ml		p
	n	Median [IQR]	n	Median [IQR]	
Days from diagnosis to sampling	33	1058 [225.5-3171]	32	2059 [596.5-4032]	0.31
Age at plasma sampling	33	56[38-68]	32	49[38-62]	0.25
Sex (M/F)	33	11/22	32	3/29	**0.03**
6MWD (m)	22	463 [332.5-536.3]	29	480 [400.5-586.5]	0.17
BNP	15	98 [70-416]	12	102 [28.25-159.5]	0.11
NT-Pro-BNP	10	298.5 [123-1012]	11	360 [113-550]	0.9
mPAP (RHC) (mm Hg)	14	43 [38-53]	11	60 [40-63]	**0.04**
PCWP	14	8	11	9	0.56

(mm Hg)		[6.75-10]		[6-11]	
RAP (mm Hg)	15	7 [4-9]	11	7 [4-10]	0.85
CI ($l/min/m^2$)	14	2.145 [1.73-2.29]	11	2.1 [1.81-3.31]	0.46
TAPSE (mm)	21	20 [16-21.5]	17	22 [18-24.5]	0.07
sPAP (echo) (mm Hg)	23	67 [55-83]	18	66.5 [54.5-87]	0.99
Functional class (I, II, III, IV)	33	8,11,12,2	31	11,15,5,0	**0.03**
DBP					
	DBP ≤ 337.3 µg/ml		DBP > 337.3 µg/ml		p
	n	Median [IQR]	n	Median [IQR]	
Days from diagnosis to sampling	34	1533 [301.8-3145]	32	1273 [316-3355]	0.88
Age at plasma sampling	34	53[42-66]	32	54[34-67]	0.51
Sex (M/F)	34	6/28	32	8/24	0.55
6MWD (m)	24	475.5 [300-588.3]	25	480 [371.5-559]	0.91
BNP	10	103 [36.75-284.8]	17	98 [71.5-242.5]	0.64
NT-Pro-BNP	16	326.5 [134.5-542.5]	6	263.5 [74.75-1664]	0.84
mPAP (RHC) (mm Hg)	13	53 [40-61.5]	12	43 [35.5-55.5]	0.15
PCWP (mm Hg)	13	9 [7.5-11]	12	8 [6-9.75]	0.29
RAP (mm Hg)	13	6 [4-13.5]	13	7 [5-8.75]	0.97
CI ($l/min/m^2$)	12	2.22 [1.89-3.16]	13	1.96 [1.67-2.26]	0.11
TAPSE (mm)	22	21.5 [17.5-23.7]	16	19.5 [16.21]	0.26
sPAP (echo) (mm Hg)	20	66.5 [55.25-86.25]	21	67 [55-79.5]	0.85
Functional class (I, II, III, IV)	33	9,16,7,1	32	10,11,10,1	0.76

6MWD: six-minute walk distance; BNP: brain natriuretic peptide; mPAP: mean pulmonary arterial pressure by right heart catheterization; PCWP: pulmonary wedge pressure; RAP: right atrial pressure; CI: cardiac index; TAPSE: tricuspid annular plane systolic excursion; sPAP: systolic pulmonary arterial pressure by echocardiography. RHC right heart catheterization. [1]Chi-square test for trend. Sex (M: male; F: female), Fischer's test.

Supplementary Table S2. Treatments in PAH patients with 25(OH)vitD levels below versus above the median in this cohort.

Total 25(OH)vitD			
	25OHvitD <7.17	25OHvitD ≥ 7.17	p
Oxygen	10 (30%)	2 (6%)	**0.01**
Anticoagulants	19 (58%)	24 (71%)	0.31
Diuretics	19 (58)	12 (35%)	0.08
Digoxin	6 (18%)	3 (9%)	0.30
CCBs	9 (27%)	10 (29%)	0.27
PDE5i	24 (73%)	24 (71%)	0.99
ERAs	16 (48%)	21 (62%)	0.33
Prostanoids	7 (21%)	9 (26%)	0.78
Monotherapy	14 (42%)	13 (38%)	0.81
Dual therapy	13 (39%)	11 (32%)	0.62
Triple therapy	4 (12%)	7 (20%)	0.51
Cuadruple therapy	1 (3%)	2 (6%)	0.99
Bioavailable 25(OH)vitD			
	25OHvitD ≤ 0.91 ng/ml	25OHvitD > 0.91 ng/ml	p
Oxygen	10 (30%)	2 (6%)	**0.02**
Anticoagulants	19 (57%)	22 (68%)	0.44
Diuretics	15 (45%)	14 (43%)	0.99
Digoxin	5 (15%)	4 (12%)	0.99
CCBs	9 (27%)	10 (31%)	0.78
PDE5i	22 (66%)	25 (78%)	0.40
ERAs	18 (54%)	17 (53%)	0.99
Prostanoids	5 (15%)	10 (31%)	0.15
Monotherapy	15 (46%)	11 (35%)	0.45
Dual therapy	13 (40%)	11 (35%)	0.80
Triple therapy	3 (9%)	7 (22%)	0.18
Cuadruple therapy	1 (3%)	2 (6%)	0.61

Free 25(OH)vitD			
	25OHvitD ≤ 1.53 pg/ml	25OHvitD > 1.53 pg/ml	p
Oxygen	9 (27%)	3 (9%)	0.11
Anticoagulants	19 (57%)	22 (68%)	0.79
Diuretics	17 (51%)	12 (37%)	0.32
Digoxin	5 (15%)	4 (12%)	0.99
CCBs	9 (27%)	10 (31%)	0.79
PDE5i	24 (72%)	23 (71%)	0.99
ERAs	19 (57%)	16 (50%)	0.62
Prostanoids	6 (18%)	9 (28%)	0.39
Monotherapy	14 (43%)	12 (37%)	0.80
Dual therapy	12 (37%)	12 (37%)	0.99
Triple therapy	5 (15%)	5 (16%)	0.72
Cuadruple therapy	1 (3%)	2 (6%)	0.61
DBP			
	DBP ≤ 337.3 µg/ml	DBP > 337.3 µg/ml	p
Oxygen	8 (23%)	5 (15%)	0.56
Anticoagulants	20 (58%)	22 (68%)	0.45
Diuretics	17 (50%)	12 (37%)	0.33
Digoxin	3 (8%)	6 (18%)	0.30
CCBs	8 (23%)	11 (34%)	0.42
PDE5i	27 (79%)	21 (65%)	0.27
ERAs	19 (55%)	16 (50%)	0.81
Prostanoids	8 (23%)	7 (21%)	0.99
Monotherapy	13 (38%)	14 (46%)	0.61
Dual therapy	15 (44%)	9 (30%)	0.31
Triple therapy	5 (14%)	5 (16%)	0.99
Cuadruple therapy	1 (2%)	2 (6%)	0.60

Chapter 2

Vitamin D deficiency downregulates TASK-1 channels and induces pulmonary vascular dysfunction

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RESEARCH ARTICLE

Vitamin D deficiency downregulates TASK-1 channels and induces pulmonary vascular dysfunction

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Callejo M, Mondejar-Parreño G, Morales-Cano D, Barreira B, Esquivel-Ruiz S, Olivencia MA, Manaud G, Perros F, Duarte J, Moreno L, Cogolludo A, Perez-Vizcaíno F. Vitamin D deficiency downregulates TASK-1 channels and induces pulmonary vascular dysfunction. *Am J Physiol Lung Cell Mol Physiol* 319: L627–L640, 2020. First published July 29, 2020; doi:10.1152/ajplung.00475.2019.—Vitamin D (VitD) receptor regulates the expression of several genes involved in signaling pathways affected in pulmonary hypertension (PH). VitD deficiency is highly prevalent in PH, and low levels are associated with poor prognosis. We investigated if VitD deficiency may predispose to or exacerbate PH. Male Wistar rats were fed with a standard or a VitD-free diet for 5 wk. Next, rats were further divided into controls or PH, which was induced by a single dose of Su-5416 (20 mg/kg) and exposure to hypoxia (10% O_2) for 2 wk. VitD deficiency had no effect on pulmonary pressure in normoxic rats, indicating that, by itself, it does not trigger PH. However, it induced several moderate but significant changes characteristic of PH in the pulmonary arteries, such as increased muscularization, endothelial dysfunction, increased survivin, and reduced *bone morphogenetic protein* (*Bmp*) *4*, *Bmp6*, *DNA damage-inducible transcript 4*, and *K^+ two-pore domain channel subfamily K member 3* (*Kcnk3*) expression. Myocytes isolated from pulmonary arteries from VitD-deficient rats had a reduced whole voltage-dependent potassium current density and acid-sensitive (TASK-like) potassium currents. In rats with PH induced by Su-5416 plus hypoxia, VitD-free diet induced a modest increase in pulmonary pressure, worsened endothelial function, increased the hyperreactivity to serotonin, arterial muscularization, decreased total and TASK-1 potassium currents, and further depolarized the pulmonary artery smooth muscle cell membrane. In human pulmonary artery smooth muscle cells from controls and patients with PH, the active form of VitD calcitriol significantly increased KCNK3 mRNA expression. Altogether, these data strongly suggest that the deficit in VitD induces pulmonary vascular dysfunction.

calcitriol; endothelial dysfunction; K^+ channels; pulmonary artery; vitamin D response element

INTRODUCTION

Pulmonary hypertension (PH) is characterized by an abnormally high mean pulmonary arterial pressure (mPAP) due to distal pulmonary vessel structural remodeling, altered pulmonary arterial tone, and inflammation (12). Several specific signaling proteins have been found to be mutated and/or dysregulated in some forms of this disease (34, 45, 46). Loss of function mutations in K^+ two-pore domain channel subfamily K member 3 (*KCNK3*) and K^+ two-pore domain channel subfamily K member 5 (*KCNA5*), the genes encoding the K^+ channels two-pore domain K^+ channel KCNK3 (TASK-1) and voltage-gated K^+ channel (Kv) 1.5 (Kv1.5), respectively, have been identified in some patients suffering from heritable and idiopathic pulmonary arterial hypertension (PAH) (2). The downregulation of the expression of these channels is also a key event in the pathogenesis of nonheritable PH, both in patients and in experimental models. Reduced activity of TASK-1 contributes to pulmonary artery smooth muscle cell (PASMC) vasoconstriction and proliferation (1). Endothelial dysfunction is another key early event in all forms of PH (16). In fact, an improved understanding of the pathophysiology of the disease has resulted in the development of effective therapies targeting endothelial dysfunction (31).

In addition to the well-known regulatory role in calcium-phosphorus homeostasis, vitamin D (VitD) is also involved in the control of other physiological processes, such as cellular growth, intracellular metabolism, and innate and adaptive immunity. In population surveys, a large proportion of the global population shows VitD deficiency (35). VitD deficiency has been associated to increased all-cause and cardiovascular mortality in large epidemiological studies (37) but its causal relationship and the effects of VitD supplements are unclear. Widespread supplementation with VitD regardless of the baseline plasma VitD has proven to be ineffective to prevent cancer and cardiovascular diseases in the general population (3, 22). However, supplements succeeded in reducing respiratory diseases, including asthma and chronic obstructive pulmonary disease exacerbations, especially in patients with lower baseline levels (17, 23). Recently, we have reported that VitD and VitD-carrier proteins (VitD-binding protein and albumin) are downregulated in PAH patients compared with the control cohort. Lower levels of bioavailable VitD were associated with more advanced functional class, lower exercise capacity, and higher risk of mortality. These data suggest that VitD defi-

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ciency is highly prevalent in PAH, and low levels are associated with poor prognosis (5).

Vitamin D_3 is obtained from dermal synthesis following exposure to sunlight or from the diet. It is converted in the liver into 25-OH-cholecaciferol (25OHVitD), the most abundant circulating form whose measure is commonly used as indicator of VitD status. 25OHVitD is further metabolized in the kidney into the most active form 1-α,25-dihydroxycholecalciferol, called calcitriol. Calcitriol is a steroid hormone. Its receptor, the VitD receptor (VDR), is a member of the nuclear receptor family of transcription factors (32). Upon calcitriol binding, VDR forms a heterodimer with the retinoid X receptor (RXR) and interacts with VitD response elements (VDRE) on the promoter DNA region of specific genes, resulting in changes in gene expression. Notably, several downstream molecular targets of VDR are altered in PAH. Thus, downregulated genes by VDR include vaculoviral IAP repeat containing 5 (*BIRC5*), which encodes for the antiapoptotic factor survivin (21). Upregulated genes by VDR include *KCNK3* (encoding for TASK-1 channels) (26, 38), the bone morphogenetic protein receptor type 2 (BMPR2) ligands bone morphogenetic protein (*BMP*) *4* and *BMP6* (15, 38), rat calcipressin-1 [*RCAN1*, encoding for the nuclear factor of activated T cells (NFAT) inhibitor calcipressin-1] (41), and DNA damage-inducible transcript [*DDIT4*, encoding for the mechanistic target of rapamycin (mTOR) inhibitor DDIT4] (47). The effect of supratherapeutic doses of VitD on PAH has been recently reported (42, 50). However, the impact of VitD deficiency on the pulmonary circulation is unknown.

We hypothesized that VitD deficiency may induce pulmonary vascular dysfunction. The putative mechanism might involve the dysregulation of VDR target genes. The aims of this study were to investigate if VitD deficiency may induce pulmonary vascular dysfunction and/or exacerbate it in an experimental model of PH and to explore the potential mechanisms involved.

METHODS

All animal procedures conform to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. no. PROEX-301/16). All investigators understand the ethical principles.

The experimental design is illustrated in Fig. 1*A*. Male Wistar rats of 180 g body weight from Envigo (Barcelona, Spain) were maintained in the general animal facility of Universidad Complutense. Animals ($n = 32$) were randomly allocated into the following two groups: rats fed with a standard diet ($n = 17$, Teklad Global 18% Protein Rodent Diet; Envigo) and rats fed a VitD-free diet ($n = 15$, Teklad Custom Diet TD.120008; Envigo) for 5 wk. After this period of time, animals from both groups were further randomly assigned to control or PH groups. PH was induced by a single subcutaneous

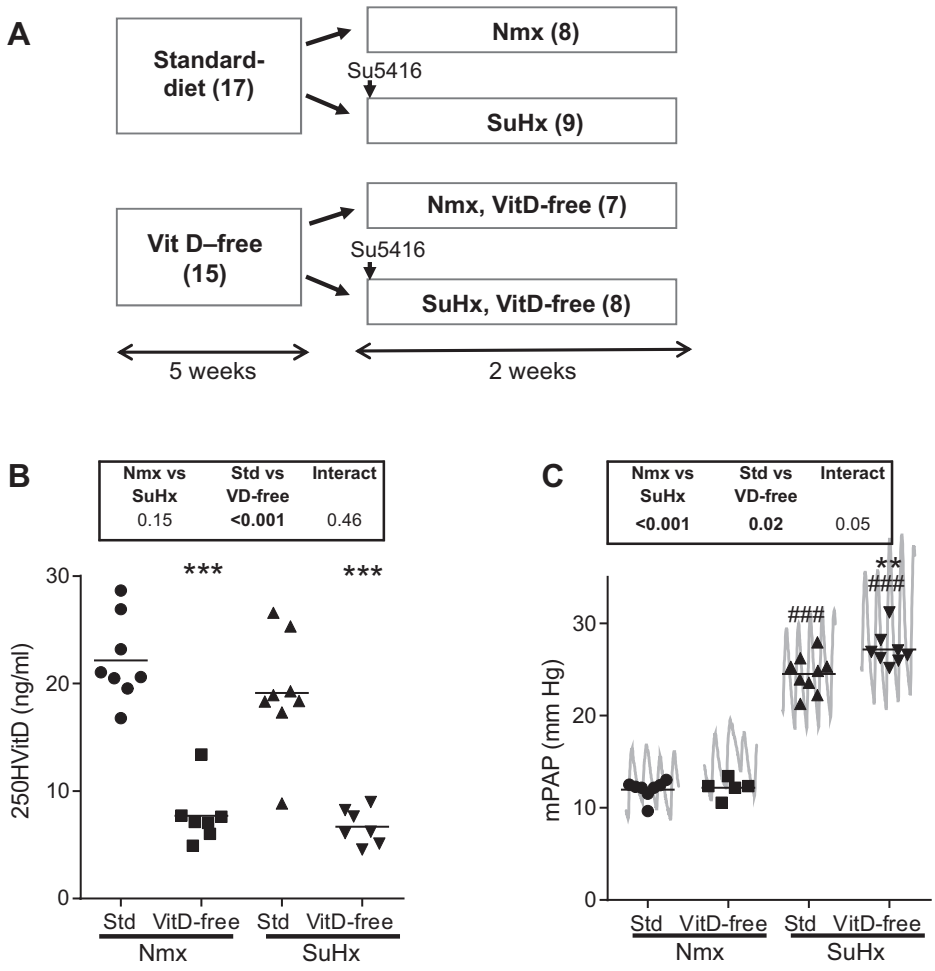

Fig. 1. Vitamin D (VitD) deficiency increases mean pulmonary arterial pressure (mPAP) in rats exposed to SU-5416 and hypoxia (SuHx). *A*: study protocol. Nos. in parentheses indicate the no. of rats in each group. *B*: 25-OH-cholecaciferol (25OHVitD) levels in the groups at the end of the study period. *C*: mPAP. Original pressure recordings for each group are superimposed. Results are scatter plot and means, and *n* is shown in Table 3. Nmx, normoxia; Std, standard diet. Two-way ANOVA results are shown in the boxes. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. standard diet. $^{\#\#\#}P < 0.001$ vs. normoxia Bonferroni test.

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injection of the vascular endothelial growth factor receptor type 2 inhibitor SU-5416 (20 mg/kg; Tocris) or vehicle (28, 43). SU-5416 was suspended in 0.5% carboxymethyl cellulose sodium, 0.9% sodium chloride, 0.4% Tween 80, and 0.9% benzyl alcohol in deionized water. Subsequently, SU-5416-treated animals were placed in glass cages and ventilated continuously with 10% O_2 (hypoxia, SuHx) for 2 wk. CO_2 and water vapor produced by the animals were captured with soda lime and silica gel, respectively. Oxygen was continuously monitored by an oxygen sensor (DrDAQ Oxygen Sensor; Pico Technology) to maintain 10% O_2. The chambers were opened only for 20–30 min daily for regular animal care. Control rats (normoxia) were kept in the same room. Animals were kept under standard conditions of temperature 22 ± 1°C and a 12:12-h dark-light cycle with free access to food and water.

Hemodynamic measurements. At the end of the experimental protocol, rats were anesthetized intraperitoneally with 80 mg/kg ketamine plus 8 mg/kg xylazine and ventilated with room air (tidal volume 9 ml/kg, 60 breaths/min, positive end-expiratory pressure 2 cmH_2O). Right ventricular systolic and diastolic pressure and systolic, diastolic, and mean pulmonary arterial pressures (sPAP, dPAP, and mPAP, respectively) were then measured in open-chest rats with a pressure transducer via a catheter advanced through the right ventricle in the pulmonary artery (PA) (28, 33). It should be noted that open-chest measurements in anaesthetized animals underestimate real PAP.

RV hypertrophy and lung histology. At the end of the hemodynamic measurements, hearts were excised, and the right ventricle (RV) and the left ventricle plus septum (LV+S) were dissected and weighed separately. Fulton index [RV/(LV+S)] was calculated to assess the right ventricular hypertrophy. The left lung was inflated in situ with paraformaldehyde saline solution (4%) through the left bronchus and embedded in paraffin. Lung sections were stained with hematoxylin and eosin techniques and examined by light microscopy. Elastin was visualized by its green autofluorescence. Small arteries (25–75 μm outer diameter) were analyzed in a blinded fashion and categorized as muscular, partially muscular, or nonmuscular as previously described (33). The medial wall thickness was calculated as external elastic lamina diameter minus the internal lamina diameter using ImageJ software.

Electrophysiological studies. PASMC were isolated as previously described (9). Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA) using the whole cell configuration of the patch-clamp technique. Myocytes were superfused with an external Ca^{2+}-free HEPES solution (see above) and a Ca^{2+}-free pipette (internal) solution containing (in mmol/L): 110 KCl, 1.2 $MgCl_2$, 5 Na_2ATP, 10 HEPES, and 10 EGTA (pH adjusted to 7.3 with KOH). Kv currents were evoked following the application of 200-ms depolarizing pulses from −60 to +60 mV in 10-mV increments. To characterize the contribution of Kv1.5 channels to the total Kv current, cells were exposed to the selective inhibitor diphenyl phosphine oxide-1 (DPO-1; 1 μmol/L). To study TASK currents (1, 30), cells were clamped at 0 mV for 3 min, allowing Kv current inactivation. Thereafter, a 1-s voltage ramp from +60 to –100 mV was applied. The ramp was applied again after 5 min perfusion with an external solution buffered at pH 6.3. Noninactivating TASK currents were identified as the current sensitive to pH. Currents were normalized to cell capacitance and expressed in picoamperes per picofarad. Membrane potential was recorded under the current-clamp mode. All experiments were performed at room temperature (22–24°C).

Arterial reactivity. For contractile tension recording, distal resistance intrapulmonary artery (PA) rings (1.7–2 mm long, 200–400 μm resting internal diameter) were mounted in a wire myograph with Krebs solution maintained at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. Vessels were stretched to an internal diameter of 500–800 μm to give an equivalent transmural pressure of 30 mmHg. After equilibration, arterial rings were first stimulated with KCl (80 mmol/L). After that, rings were precontracted with phenylephrine (1 μmol/L) and endothelial function was assessed by cumulative addition of acetylcholine (10 nmol/L to 10 μmol/L). After washing, a dose response curve to serotonin (5-HT, 30 nmol/L to 30 μmol/L) was performed by cumulative drug addition.

In some experiments, PAs from control rats fed with VitD-free diet for five weeks ($n = 7$) were incubated with calcitriol (100 nmol/L) or vehicle (DMSO 0.1%) in DMEM culture medium supplemented with nonessential amino acid solution (1×), penicillin (100 U/mL), streptomycin (0.1 mg/mL), and amphotericin B (0.25 μg/mL) and maintained for 48 h in an incubator with 21% O_2 and 5% CO_2. Next, PAs were mounted in a wire myograph to assess their endothelial function as described above.

Human PASMC. Primary PASMC from controls and patients with PAH were obtained from the French Network on Pulmonary Hypertension, a program approved by the institutional Ethics Committee. Cells were isolated and characterized as described by the expression of muscle-specific contractile and cytoskeletal proteins, including smooth muscle cell α-actin, desmin, and vinculin (11). Control PASMC were obtained during lobectomy or pneumonectomy for localized lung cancer tumor in non-PAH patients (3 males and 3 females, 61 ± 12 yr). PAH PASMC were obtained during transplantation of six idiopathic PAH and two heritable PAH (2 males and 6 females, 37 ± 12 yr). PAH-PASMC were cultured as previously described (11) and used within passages 2–4. Serum-starved cells (0.1% fetal calf serum, for 24 h) were treated with 100 nmol/L of calcitriol (1α,25-dihydroxyvitamin D_3; Sigma-Aldrich) or vehicle (DMSO, 0.1%) in DMEM supplemented with 10% of FBS and 1% antibiotic/antimycotic solution for 48 h.

RNA extraction and quantitative RT-PCR. Total RNA was extracted with the miRNeasy Mini Kit for rat lungs (Qiagen, Hilden, Germany) or the NucleoSpin RNA kit for human PASMC (Macherey-Nagel), according to the supplier's instructions. RNA quantity and quality were assessed with NanoDrop 1000 Spectrophotometers (Thermo Scientific). Lung RNA (1 μg) was reverse transcribed into cDNA using an iScript cDNA Synthesis Kit for rat samples (Bio-Rad) or a High Capacity cDNA Reverse Transcription Kit for human samples (Thermo Fisher Scientific), following the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR with a TaqMan Gene Expression Master Mix (Applied Biosystems, Thermo Fisher Scientific), using specific primers from Applied Biosystems databases (Table 1). Amplifications, detections, and analysis were performed in a 7.900HT Fast Real-time PCR System at Centro de Genomica from Complutense University (Madrid, Spain) or a StepOne Plus Real-Time PCR System (Life Technologies). The $\Delta\Delta C_t$ method was used to quantify relative changes in mRNA expression. Gene expression was normalized to the geometrical mean of β-actin and β_2-microglobulin expression or to the β-actin expression for human PASMC.

Table 1. *Taqman primers used from Applied Biosystems*

Gene	Reference
Rat vaculoviral IAP repeat containing 5 (*Birc5*)	Rn00574012_m1
Rat bone morphogenetic protein 4 (*Bmp4*)	Rn00432087_m1
Rat bone morphogenetic protein 6 (*Bmp6*)	Rn00432095_m1
Rat bone morphogenetic protein receptor type 2 (*Bmpr2*)	Rn01437214_m1
Rat DNA damage-inducible transcript (*Ddit4*)	Rn01433735_g1
Rat K^+ two-pore domain channel subfamily K member 3 (*Kcnk3*)	Rn04223042_m1
Rat calcipressin-1 (*Rcan-1*)	Rn01458494_m1
Rat β-actin (*Actb*)	Rn00667869_m1
Rat β_2-microglobulin (*B2m*)	Rn00560865_m1
Human K^+ two-pore domain channel subfamily K member 3 (*KCNK3*)	Hs00605529_m1
Human β-actin (*ACTB*)	Hs01060665_g1

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Western Blotting analysis. Lungs were homogenized with a lysis buffer containing: Trizma preset crystals pH 7.5, 1 mol/L DL-dithiothreitol, and 1% NP-40 and supplemented with protease (Protease inhibitor cocktail tablets; Roche Diagnostics) and phosphatase inhibitor (PhosSTOP; Roche Diagnostics) cocktail in a Tissuelyser device (Qiagen, Hilden, Germany). After four short pulses (30 s, stopping 15 s between each pulse) of homogenization, the lysates were centrifuged for 10 min at 10,000 revolutions/min. Protein concentration was determined by a colorimetric assay based on the Lowry method (Bio-Rad). Proteins (25 μg) were run on a sodium dodecyl sulfate-polyacrylamide electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were blocked by incubation with 5% of BSA or nonfat milk for 1 h and incubated overnight at 4°C with primary antibodies (Table 2). The specificity of primary antibody used [anti-endothelial NO synthase (eNOS) (8), anti-p-eNOS (25), and anti-survivin (44)] has been tested and reported in previous studies. Membranes were then incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. Antibody binding was detected by an ECL system [Amersham Pharmacia Biotech (Amersham, UK) or Super-Signal West Fento Chemiluminescent Substrate (Thermo Scientific)]. Blots were imaged using an Odyssey Fc System (Li-COR, Biosciences) and were quantified by densitometry using Quantity One software. Results were normalized relative to smooth muscle β-actin signal intensity. Following the new National Institutes of Health guidelines to enhance rigor/reproducibility/transparency within the scientific community, the full-length gels have been deposited in a public repository (Supplemental Fig. S1; Supplemental Material is available at https://doi.org/10.6084/m9.figshare.12323804).

VitD measurement. Plasma 25OHVitD was measured using a chemiluminescence immunoassay (ADVIA Centaur Vitamin D Total assay; Siemens Healthcare Diagnostics) that conforms with the National Institutes of Health CDC Vitamin D Standardization-Certification Program at the Clinical Biochemistry Service at Gregorio Marañon Hospital.

Identification of VDRE in KCNK3 *gene and in silico analysis.* To identify VDREs in the *KCNK3* gene, we searched the database of consensus DR3 and ER6 elements, and DR3 elements containing single nucleotide substitutions (48). The predicted VDRE sequence in *KCNK3* gene promoter conserved between human and mouse was subjected to in silico analysis using 3D-footprint, a database for the structural analysis of protein-DNA complexes (10). This database predicts proteins that could recognize the specific DNA motif and provides a string of concatenated interface residues (proteic interface signature) and the estimated binding specificities in the VDRE sequence.

Statistics. Analysis was performed using GraphPad Software version 7 (GraphPad Software). All data were tested for normal distribution, and parametric statistics were used as appropriate. Data are presented either as scatter plots and means or as means ± SE. Two-way ANOVA analysis and Bonferroni post hoc test were used. Two-sample comparison was analyzed using a Student's *t* test. Differences in pulmonary artery muscularization were analyzed by a Chi square test. *P* values of <0.05 were considered statistically significant.

RESULTS

Effects of VitD-free diet on pulmonary pressure. After 7 wk in VitD-free diet (protocol shown in Fig. 1*A*), rats had a severe reduction in plasma 25OHVitD (Fig. 1*B*). As expected, rats exposed to SuHx under a standard diet showed a marked increase in mPAP, dPAP, and sPAP (Fig. 1*C*, Table 3). The VitD-free diet did not modify PAP in rats breathing room air (Fig. 1*C*), indicating that the VitD-free diet does not directly cause PH. Interestingly, in SuHx rats VitD-free diet induced a significant ($P < 0.01$) albeit modest increase in mPAP.

SuHx led to the expected significant increase in RV weight (Table 3), either referred to body weight, tibial length, or LV+S weight (i.e., the Fulton index). However, the effects of VitD-free diet on the heart were complex (Table 3). It significantly increased LV+S weight but had no significant effect on RV in absolute terms ($P = 0.06$) or after correction for tibial length or body weight.

Effects of VitD-free diet on Kv currents and membrane potential. To study the role of VitD in the regulation of potassium channels, the total Kv currents were recorded in fresh PASMC isolated from the four groups shown in Fig. 1*A*. Representative original traces of the total Kv currents and averaged values (Fig. 2, *A* and *B*) show a very marked decrease of the total current density that was accompanied by membrane depolarization in SuHx rats (Fig. 2*C*). Remarkably, we also found a significant decrease in the current density in the SuHx rats and a depolarization of the membrane by the VitD-free diet.

Kv1.5 channel represents a main component of the total Kv current in the pulmonary vasculature and is involved in the ionic remodeling produced in the SuHx (29). In the presence of the Kv1.5 channel inhibitor DPO-1, i.e., the DPO-1-insensitive current (Fig. 2*D*), differences between the four groups were still evident, suggesting the impairment of K^+ channels different from Kv1.5. The DPO-1-sensitive current, i.e., the current resulting from the subtraction of the total current minus that in the presence of DPO-1, which represents mainly Kv1.5 current, was strongly inhibited by SuHx but not significantly affected by VitD-free diet (Fig. 2, *D* and *E*). However, there was a positive interaction between VitD and SuHx, suggesting that VitD has some effect in SuHx-treated animals.

VDR regulates the expression of the *KCNK3* gene (encoding the pH-sensitive TASK-1 channel), which has been shown to be expressed in PASMC and to contribute significantly to the resting membrane potential (13). To study the activity of TASK-1 channel, we applied a clamping protocol at 0 mV for 3 min to inactivate Kv channels and isolate the noninactivating current (I_{KN}). In addition, to characterize the activity of TASK-1 channels, extracellular pH was changed from 7.3 to 6.3 to inactivate these channels. Figure 3*A* shows representa-

Table 2. *Primary antibodies used*

Primary antibody	Protein	Species	Supplier	Reference	Dilution
Anti-β-actin	β-actin	Mouse	Sigma-Aldrich	A1978	1:10,000
Anti-eNOS	eNOS	Mouse	BD Transduction Laboratories	610296	1:1,000
Anti-p-eNOS (S1170)	p-eNOS (S1170)	Mouse	BD Transduction Laboratories	612393	1:200
Anti-survivin	Survivin	Mouse	Santa Cruz	SC-17779	1:200

eNOS, endothelial NO synthase.

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Table 3. *Changes in VitD status, body weight, RV weight, LV+S weight, TL and their ratios and in hemodynamic variables in rats exposed to normoxia or SU-5416 plus hypoxia with standard or VitD-free chow*

	Normoxia		Hypoxia + SU-5416		2-Way ANOVA		
	Standard	VitD free	Standard	VitD free	N vs. H+S	S vs. VitD free	Interaction
n	8	7[b]	9[c]	8			
25OHVitD, ng/mL	22.2 ± 1.4	7.7 ± 1.0**	19.1 ± 1.9	6.68 ± 0.61**	0.28	<0.001	0.81
Body wt, g	359 ± 12	384 ± 16	311 ± 11	305 ± 16	<0.001	0.51	0.27
RV, mg	168 ± 7	188 ± 7	270 ± 10	280 ± 7	<0.001	0.06	0.50
LV+S, mg	614 ± 14	706 ± 34*	551 ± 22	581 ± 18	<0.001	0.01	0.18
TL,[a] cm	3.99 ± 0.03	4.05 ± 0.03	3.89 ± 0.05	3.93 ± 0.02	0.005	0.17	0.94
RV/body wt, $\times 10^3$	466 ± 10	491 ± 10	871 ± 31	930 ± 41	<0.001	0.14	0.55
RV/TL[a]	43 ± 2	48 ± 2	71 ± 3	73 ± 2	<0.001	0.10	0.51
(LV+S)/body wt, $\times 10^3$	1,716 ± 38	1,840 ± 43	1,768 ± 41	1,917 ± 45*	0.14	<0.001	0.77
(LV+S)/TL[a]	154 ± 3	177 ± 9	129 ± 7	128 ± 5	<0.001	0.09	0.06
RV/(LV+S)	0.272 ± 0.008	0.267 ± 0.004	0.494 ± 0.19	0.484 ± 0.012	<0.001	0.55	0.84
mPAP, mmHg	12.0 ± 0.4	12.2 ± 0.5	24.5 ± 0.7	27.2 ± 0.6**	<0.001	0.02	0.05
sPAP, mmHg	16.4 ± 0.5	16.4 ± 0.5	30.9 ± 1.6	34.2 ± 1.2	<0.001	0.18	0.17
dPAP, mmHg	7.9 ± 0.4	8.2 ± 0.7	18.2 ± 0.2	20.2 ± 0.4**	<0.001	0.02	0.09
HR, beats/min	216 ± 10	240 ± 14	277 ± 6	281 ± 17	<0.001	0.25	0.42
RV dP/dt_{max}	476 ± 15	479 ± 22	795 ± 56	832 ± 87	<0.001	0.74	0.77
RV dP/dt_{max}/mPAP	29.5 ± 1.3	30.2 ± 1.4	27.4 ± 0.6	26.0 ± 1.4	0.02	0.76	0.41

Results are means ± SE. *n*, No. of animals. All parameters were determined at the end of the experimental period. VitD, vitamin D; RV, right ventricle; LV+S, left ventricle + septum; TL, tibia length; N, normoxia; H+S, Hypoxia + SU-5416; S, standard; 25OHVitD, 25-OH-cholecaciferol; mPAP, mean pulmonary arterial pressure; sPAP, systolic pulmonary arterial pressure; dPAP, diastolic pulmonary arterial pressure; HR, heart rate. [a]*n* for TL were 7, 6, 7, and 6, respectively. [b]*n* for hemodynamic variables was 5 in the VitD-free normoxic group. [c]*n* for plasma VitD was 8. *$P < 0.05$ and **$P < 0.01$ standard diet vs. VitD-free Bonferroni test.

tive original I_{KN} ramps recorded from +60 to –100 mV measured at pH 7.3 and 6.3 in freshly isolated PASMC. The pH-sensitive currents obtained by measuring the difference in K^+ current at external pH values of 7.3 and 6.3 are shown in Fig. 3*B*. The results showed a significant decrease of the TASK current (~3-fold) in SuHx rats compared with normoxic rats with a standard diet. Moreover, in normoxic rats, we observed a reduction of the TASK-1 current by the VitD-free diet. Expression of *Kcnk3* mRNA, gene encoding TASK-1, is shown in Fig. 3*C*. VitD-free diet caused a reduction in the lung expression of *Kcnk3* mRNA compared with the control group. The expression of TASK-1 at the protein level cannot be performed because the available commercial antibodies lack specificity (19).

VDRE in the KCNK3 *gene and KCNK3 expression induced by VDR in PASMC.* We searched the database of the possible DNA motifs contained within the *KCNK3* gene promoter comprising the consensus sequence formed by direct or everted repeats of PuG(G/T)TCA motifs separated by 3 or 6 bp (DR3 or ER6), including those that differed by a single nucleotide substitution to the consensus sequence (48). Three DR3 elements were found in the human *KCNK3* gene with a single nucleotide substitution, but only one of them (AGGTCAGCAGGTTCT) was conserved between human and mouse (Fig. 4*A*). We also analyzed this predicted VDRE in the *KCNK3* gene in 3D-footprint software to seek for the VDR-VDRE interaction. This analysis showed that among the predicted proteins that could recognize and bind to this human and mice VDRE sequences were VDR and its coreceptor RXR-α (Fig. 4*B*). We found that VDR has a DNA-binding domain for the VDRE-KCNK3 sequence composed of the residues chain EKRRK, which recognize a DR3 sequence. On the other hand, RXR has two other DNA-binding domains composed by the residues (EKRR) that can bind to two different five-nucleotide sequences.

To confirm that KCNK3 is regulated by VDR in PASMC, we analyzed whether VDR stimulation with calcitriol (100 nM, for 48 h) could induce KCNK3 expression in vitro in human PASMC from controls and patients with PAH (Fig. 4*C*). We found that calcitriol significantly increased the KCNK3 mRNA expression in PASMC compared with PASMC treated with vehicle.

Endothelial function. The endothelium-dependent relaxation induced by acetylcholine in isolated PA was strongly reduced in the SuHx group. VitD-free diet also reduced endothelial function in both normoxic and SuHx-treated rats (Fig. 5*A*). Because endothelium-dependent relaxation in pulmonary arteries is strongly dependent on NO release from endothelial cells, we analyzed eNOS expression and phosphorylation. However, VitD-free diet had no effect on eNOS expression or its phosphorylation at the activator site S-1177 (Fig. 4*B*). SuHx augmented the contractile response to 5-HT, and this effect was further potentiated in rats with VitD-free diet ($P < 0.05$, Fig. 5*C*).

To test whether VitD-free diet-induced endothelial dysfunction could be reversible, we incubated isolated PA from VitD-deficient rats (5 wk in VitD-free diet) in vitro in the presence or absence of calcitriol (100 nmol/L) for 48 h. Figure 5*D* shows that calcitriol significantly increased the relaxation induced by acetylcholine, i.e., reversed the endothelial dysfunction. However, acute calcitriol had no effect on endothelial function in PA from rats fed a standard diet (data not shown).

PA remodeling. As expected, SuHx induced a strong increase in the muscularization of small rat PA (Fig. 6, *A* and *B*). Remarkably, VitD-free diet also significantly increased the percentage of muscular arteries in both normoxic and SuHx-treated animals. An increase in PA wall thickness was observed for both SuHx and VitD-free diet (Fig. 6*C*). We also analyzed the expression of some genes potentially involved in PASMC proliferation. The expression of the antiapoptotic protein survivin was increased by both SuHx and by VitD-free diet (Fig. 7*A*). The expression of BMPR2 was significantly

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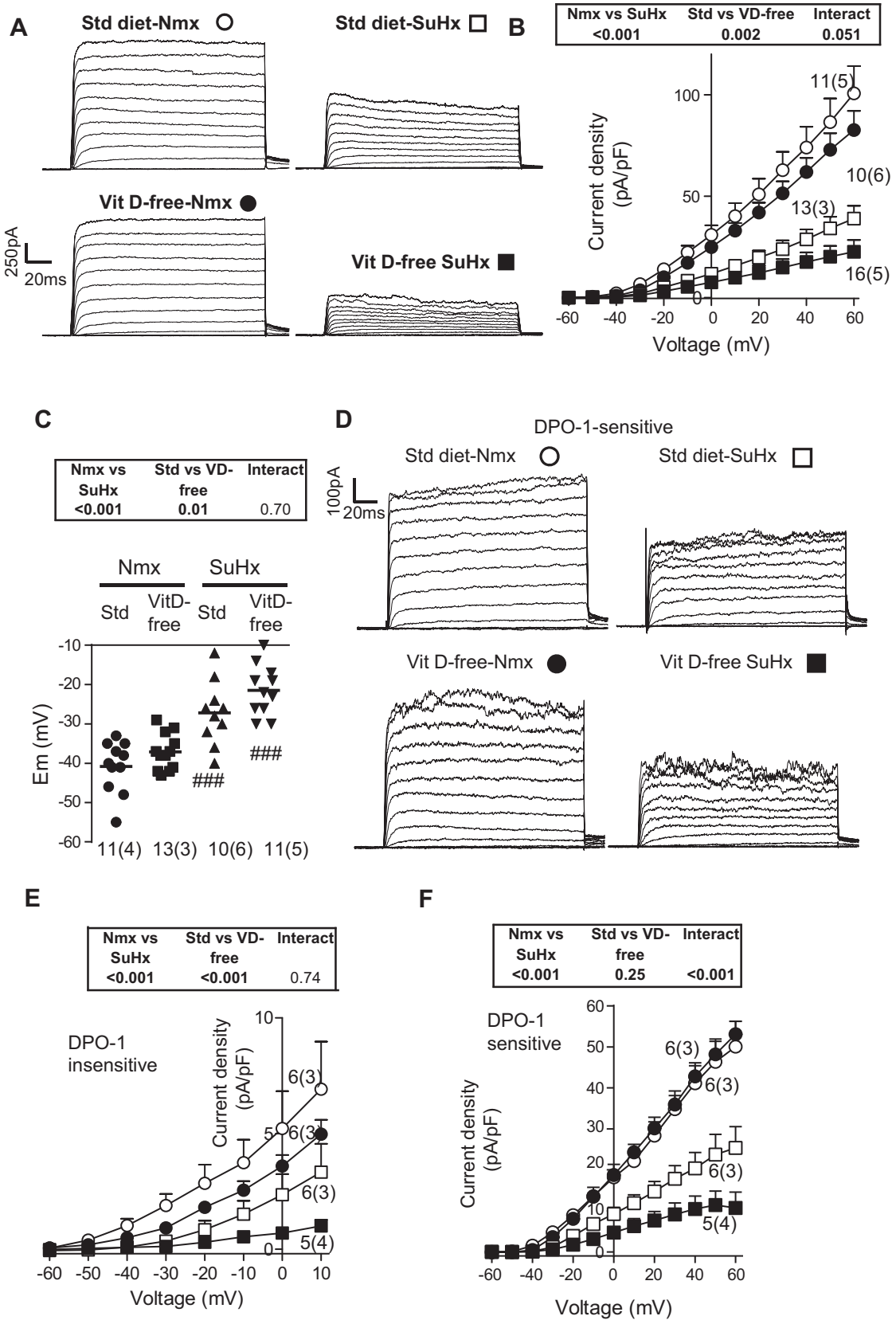

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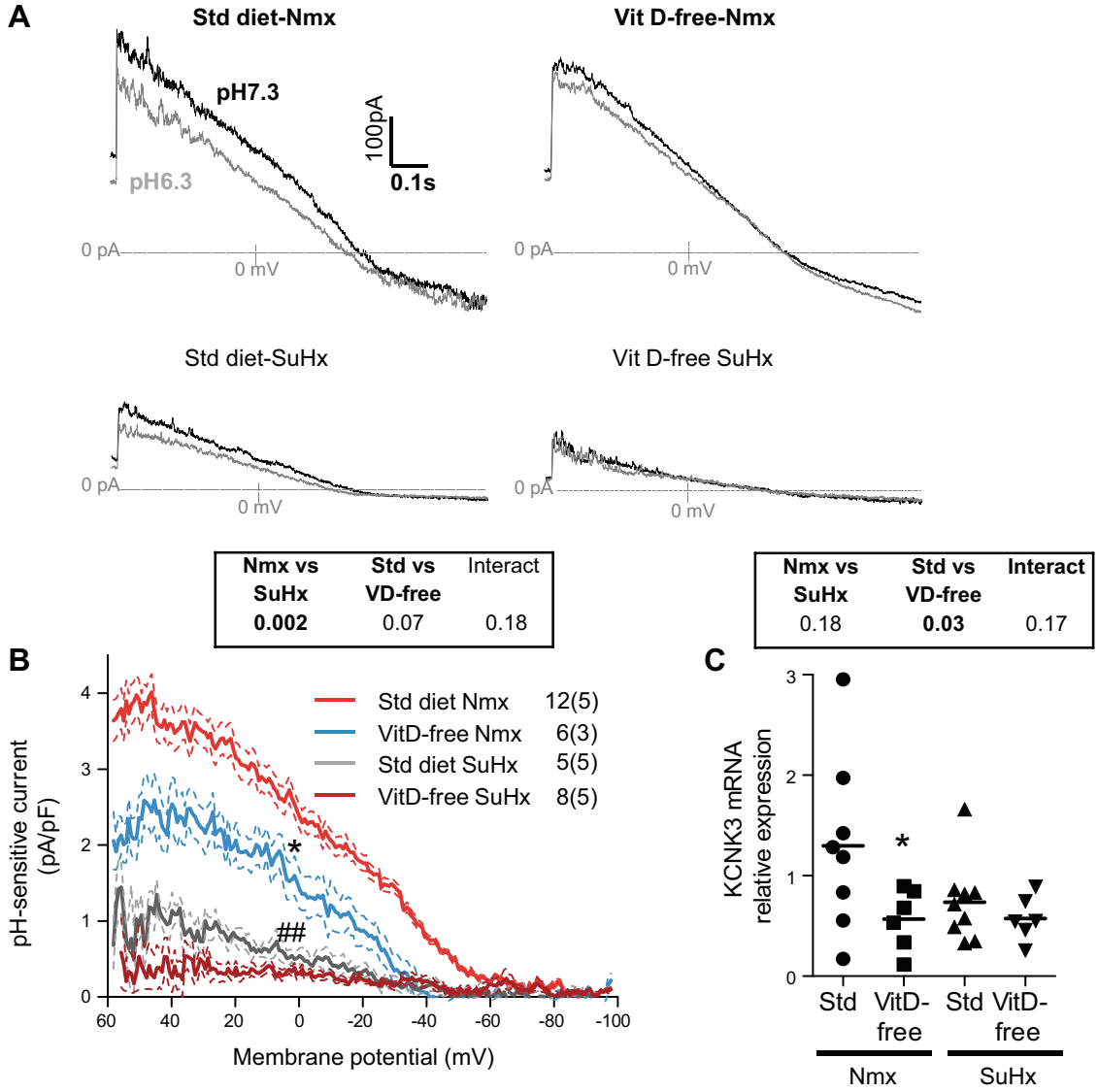

Fig. 3. Vitamin D (VitD and VD) deficiency downregulates two-pore domain K^+ channel KCNK3 (TASK-1) channels. *A*: representative noninactivating current (I_{KN}) traces recorded at pH 7.3 (black) and after changing to pH 6.3 (gray) in pulmonary artery smooth muscle cells (PASMCs) isolated from the four groups. The broken lines represent zero current level. *B*: pH-sensitive currents obtained by measuring the difference in K^+ current at external pH values of 7.3 and 6.3. Data are averaged traces ± SE shown as discontinuous lines. Statistics are calculated at 0 mV. *C*: K^+ two-pore domain channel subfamily K member 3 (KCNK3) mRNA expression (which encodes for TASK-1) by RT-PCR. Nmx, normoxia; SuHx SU-5416 plus hypoxia; Std, and standard diet. No. of cells is indicated in the panel, and the no. of animals is in parentheses. Data in *C* are scatter plots and means; each symbol represents one animal. Two-way ANOVA results are shown in the box. *$P < 0.05$ vs. standard diet. ##$P < 0.01$ vs. normoxia.

reduced by SuHx but not by VitD-free diet (Fig. 7*B*). In contrast, VitD-free diet reduced the BMPR2 ligands BMP4 and BMP6. VitD-free diet also downregulated DDIT4, an inhibitor of mTOR, but we found no differences in the expression of RCAN, an NFAT inhibitor (Fig. 7*B*).

DISCUSSION

In the present study we exposed Wistar rats to a VitD-free diet for 7 wk to reduce plasma 25OHVitD levels to an average value of 7.19 ng/mL, which corresponds to a severe VitD

Fig. 2. Vitamin D (VitD and VD) deficiency induces ionic remodeling. *A*: representative current traces for 200-ms depolarization pulses from −60 to +60 mV in 10-mV increments from a holding potential of −60 mV in pulmonary artery smooth muscle cells (PASMCs). *B*: current-voltage relationships of K^+ currents measured at the end of the pulse. *C*: membrane potential (Em) values in PASMC measured in mV. *D*: representative diphenyl phosphine oxide-1 (DPO-1)-sensitive current traces obtained by digital subtraction, measuring the difference in K^+ current in the presence and in the absence of the voltage-gated K^+ channel (Kv) 1.5 inhibitor DPO-1 (1 μmol/L). *E* and *F*: current-voltage relationships of DPO-1-insensitive (*E*) and -sensitive (*F*) currents. Nmx, normoxia; SuHx, SU-5416 plus hypoxia; Std, standard diet. Results in *B*, *E*, and *F* are means ± SE. Results in *C* are shown as scatter plot and means. No. of cells is indicated in the panel, and the no. of animals is in parentheses. A 3-way ANOVA was performed, and values shown in the boxes only refer to SuHx and VitD factors. ###$P < 0.001$ vs. normoxia.

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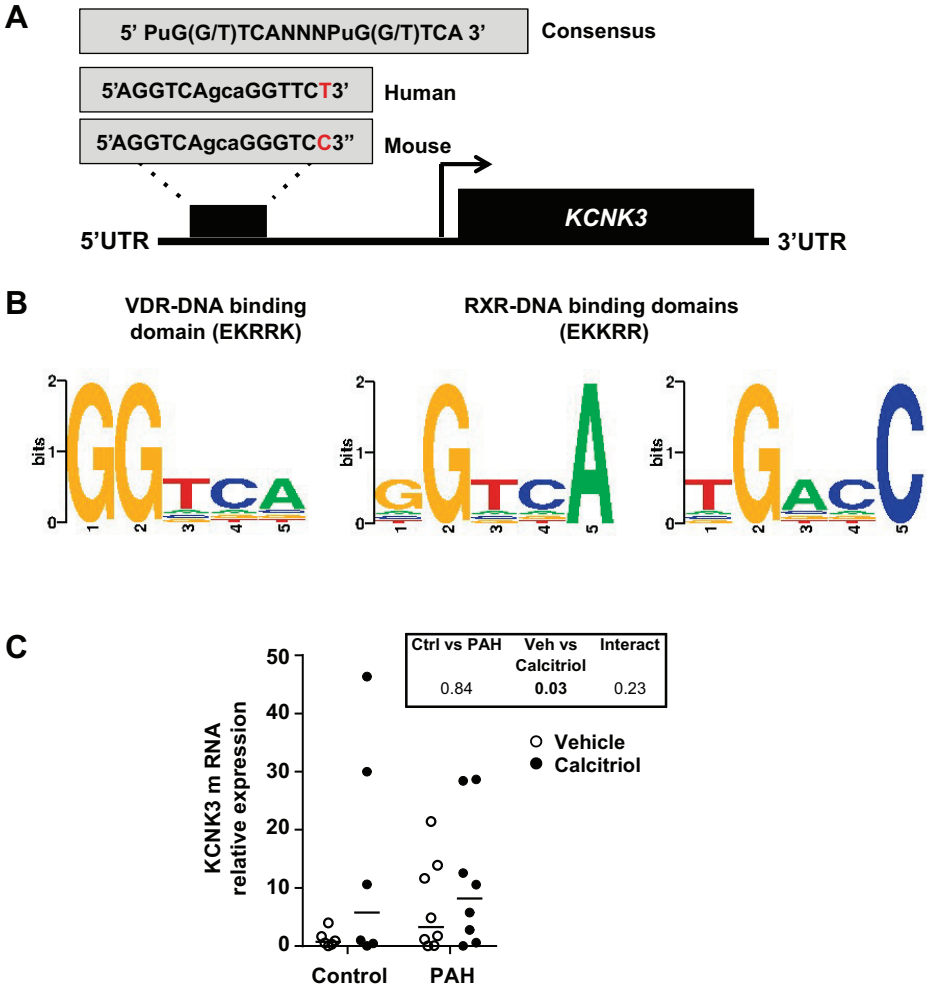

Fig. 4. Vitamin D response element (VDRE) and K^+ two-pore domain channel subfamily K member 3 (KCNK3). *A*: VDRE sequence in the promoting region of *KCNK3* gene conserved between human and mouse predicted using large-scale in silico analysis. UTR, untranslated region. *B*: vitamin D receptor (VDR) and retinoid X receptor (RXR) proteic interface signatures and the estimated binding specificities in the VDRE-*KCNK3* gene as sequence logo provided by 3D-footprint. Larger letters indicate greater affinity and smaller letters indicate the allowable variability. *C*: KCNK3 mRNA expression measured using RT-PCR in human pulmonary artery smooth muscle cells (PASMCs) from controls or pulmonary arterial hypertension (PAH) patients treated with calcitriol (100 nM) or vehicle (Veh) for 48 h. Two-way ANOVA results are shown in the box.

deficiency, a widespread clinical condition. We found that VitD-free diet had no effect on mPAP in normoxic rats and did not increase RV weight, indicating that, by itself, a deficit of VitD does not trigger PH. However, it induced several moderate but significant changes characteristic of PH in the PAs, such as increased muscularization, endothelial dysfunction, increased survivin, reduced *Bmp4*, *Bmp6* and *Kcnk3*, and reduced TASK-1 currents. We also analyzed the effects of a VitD-free diet in animals exposed to SU-5416 and chronic hypoxia (SuHx). In these animals, VitD-free diet further induced a modest but significant increase in mPAP, worsened endothelial function, increased the hyperreactivity to 5-HT, PA muscularization, and decreased TASK-1 currents. Altogether, these data strongly suggest that VitD deficiency induces pulmonary vascular dysfunction and aggravates that induced by SuHx . We also identified a VDRE sequence in KCNK3 promoter conserved in human and mouse and VDR stimulation with calcitriol increased KCNK3 expression in human PASMC.

A recent study in Fisher 344 rats has analyzed the effects of a high VitD diet compared with a standard VitD-containing diet in rats exposed to SuHx (42). In this report, RV systolic pressure and arterial structure were unchanged, but high VitD diet reduced RV hypertrophy and increased survival, suggesting that supratherapeutic doses of VitD may be beneficial for the RV. In Sprague-Dawley rats fed a standard VitD-containing diet and exposed to hypoxia, a single intraperitoneal injection (20 mg/kg) of calcitriol reduced PAP and RV hypertrophy compared with vehicle (50). Our study shows that VitD deficiency affects the vessel structure and function, aggravating PAH. The effects on the heart in the present study are complex. There was a highly significant left ventricular hypertrophy but no significant change in RV weight. This is consistent with reports in both VDR knockout mice and VitD-deficient humans, probably secondary to increased intact parathormone (36, 40). It could be argued that increased mPAP in our study could be secondary to left heart disease. We cannot rule out this possibility, but we think it is unlikely because of the change in the vascular phenotype.

Mutations in the *KCNK3* gene have been identified in some patients suffering from heritable PAH (2). *KCNK3* encodes TASK-1, an important outward-rectifier K^+ channel for PA function (13). Downregulation of these channels is a key event in PAH pathogenesis (1). We found that TASK-1 currents, measured by the pH-sensitive noninactivating background current, were strongly reduced in the PASMC from SuHx-treated rats, as previously reported in right ventricular cardiomyocytes in a PAH model (18). Likewise, in $BMPR2^{+/-}$ knockout rats, TASK-1 currents were also almost abolished while KCNK3 expression was not affected at the mRNA level (14). This may

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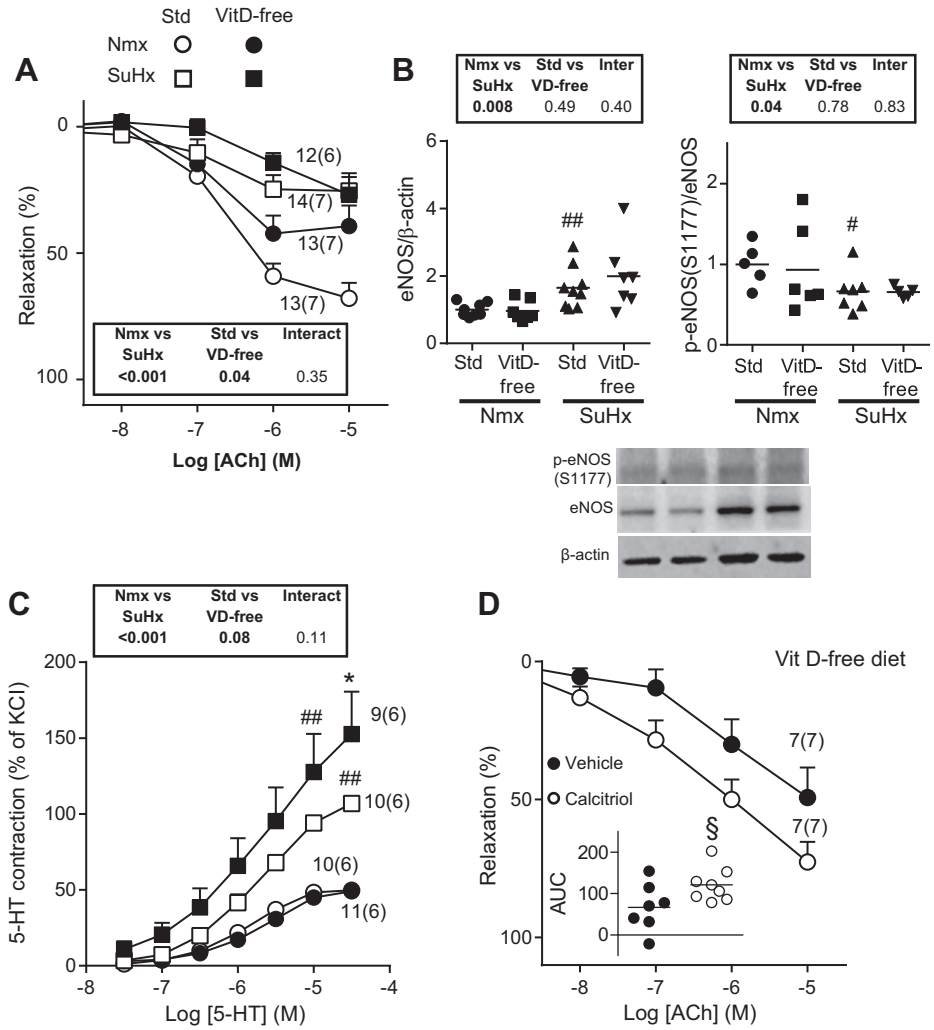

Fig. 5. Vitamin D (VitD and VD) deficiency induces endothelial dysfunction and hyperresponsiveness to serotonin (5-HT) in pulmonary arteries (PA). *A*: relaxant effects of the endothelium-dependent vasodilator acetylcholine (ACh) in PA stimulated with phenylephrine. *B*: lung endothelial NO synthase (eNOS) and p-eNOS (S1177) protein expression. *C*: contractile responses to 5-HT. *D*: calcitriol rescues endothelial dysfunction in vitro. PA from VitD-deficient rats (5 wk in VitD-free chow) were incubated with or without calcitriol (100 nmol/L) for 48 h in culture. Results in *A*, *C*, and *D* are means ± SE, and statistics are calculated for the area under the curve (AUC). No. of arteries is indicated in the panel, and the no. of animals is in parentheses. Data in *B* are presented as scatter plot and means; each symbol represents one animal. Two-way ANOVA results are shown in the boxes. #$P < 0.05$ and ##$P < 0.01$ vs. normoxia (Nmx). *$P < 0.05$ vs. standard diet (Bonferroni test). §$P < 0.05$ in calcitriol vs. vehicle treated, Student's *t* test.

be explained because TASK-1 protein was not present at the cell surface of PASMCs and was abnormally retained in the intracellular compartments (14). We found that VitD deficit also reduced TASK-1 currents in isolated PASMC and KCNK3 mRNA expression in the whole lung. TASK-1 channels are known to be expressed in the lung at least in PASMC and endothelial and epithelial cells (1, 4). Despite that there were not linear changes in mRNA expression and activity for VitD deficiency, SuHx, and its combination, it is clear that both VitD deficiency and SuHx treatment have a strong impact on the function of TASK-1, which is thought to be an essential player in some forms of heritable and in idiopathic PAH. Previous studies have suggested that *KCNK3* is a VDR-targeted gene. Calcitriol upregulated the expression of this channel in human coronary artery smooth cells and in fresh slices from breast cancer (26, 38). Notably, we found that VitD-free diet downregulated *Kcnk3* gene and the TASK-1 current, which suggests that this may be a key mechanistic factor in the effects of VitD-free diet. Therefore, TASK-1 downregulation may account for the VitD-induced depolarization (13), changes in pulmonary arterial tone, and distal neomuscularization of PA (1). We used an in silico analysis that allows to identify potential target genes independent of their tissue of expression. We identified a putative VDRE in the *KCNK3* gene, conserved in humans and mice that is recognized by VDR and its coreceptor, the RXR. Moreover, we also confirmed that VDR stimulates the upregulation of KCNK3 in human PASMC from either control or PAH patients. Other K^+ channels, particularly within the Kv subfamily, have also been found to be downregulated in PAH (20). Using the Kv1.5 channel blocker DPO-1, we could isolate by digital subtraction the Kv1.5-sensitive current, a major contributor to the whole cell Kv current. This current was strongly reduced by SuHx but not significantly affected by VitD.

We have found that chronic VitD deficiency induced vascular dysfunction with reduced NO-dependent relaxation to acetylcholine and increased contractile response to 5-HT. Calcitriol has been reported to increase eNOS expression and activity in cultured endothelial cells and in vivo after 48 h (24). However, in our experiments, the opposite approach, i.e., chronic VitD depletion, had no effect on eNOS expression or eNOS phosphorylation at the activator site S1177. Thus, the mechanism involved in the VitD deficiency-induced endothelial dysfunction is unclear. Unpaired NO activity may be dependent on multiple factors, including abundance of substrate and cofactors, presence of endogenous inhibitors and chaperones, inactivation by superoxide or changes in the soluble guanylyl cyclase activity. However, reduced *Kcnk3* ex-

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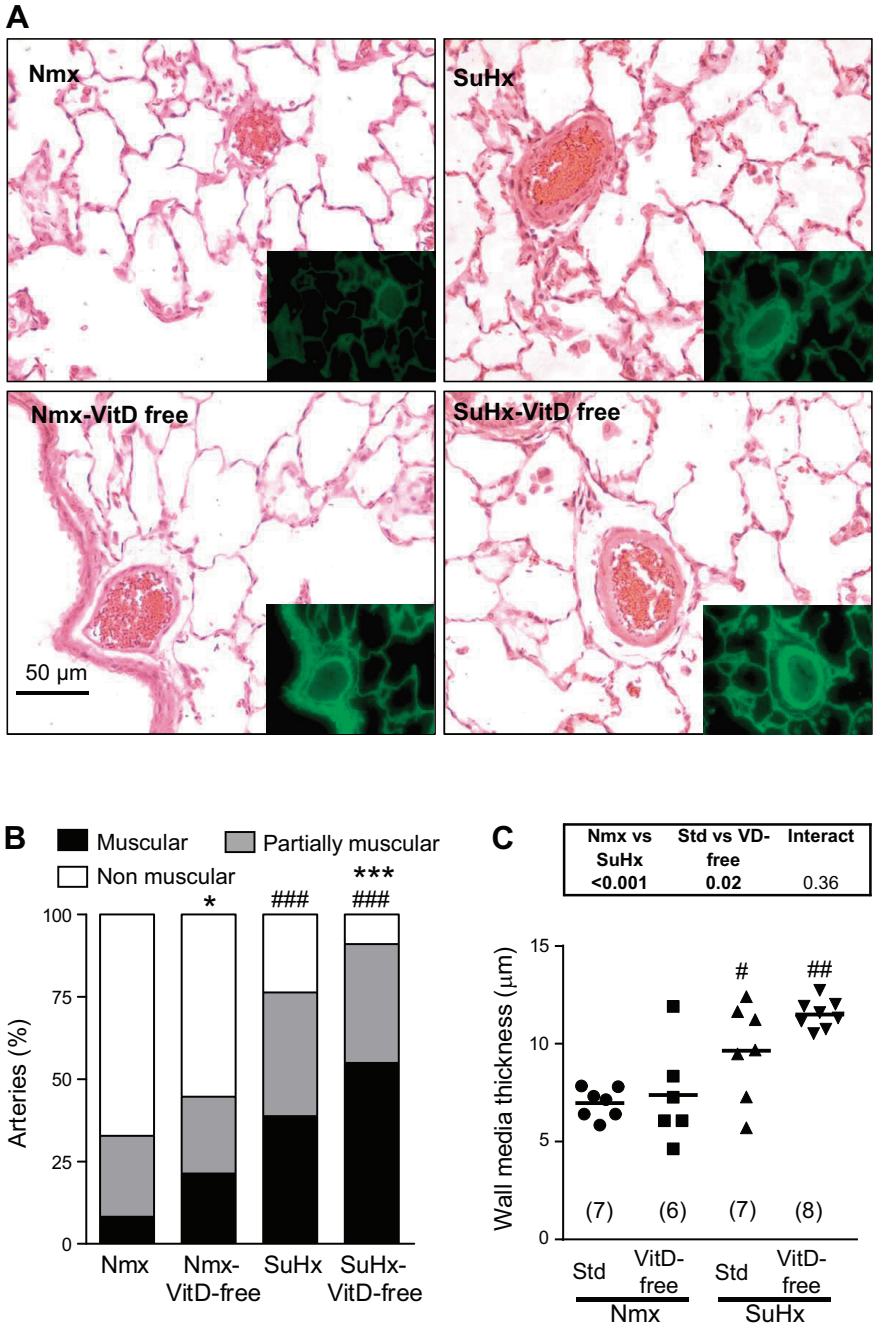

Fig. 6. Vitamin D (VitD) deficiency induces pulmonary arterial remodeling. *A*: representative images of cross sections of lungs stained with hematoxylin and eosin and the elastin autofluorescence in the *insets*. *B*: percentage of muscular, partially muscular, and nonmuscular pulmonary arteries (PA). $*P < 0.05$ and $***P < 0.001$ vs. standard diet. $\#\#\#P < 0.001$ vs. normoxia, Chi square test. *C*: wall thickness in PA <75 μm is presented as scatter plot and means. Two-way ANOVA results are shown in the box. No. of animals analyzed is shown in *C*. $\#P < 0.05$, $\#\#P < 0.01$ vs. normoxia, Chi square test.

pression might also be involved in this effect because PA from Kcnk3 knockout rats also show reduced acetylcholine-induced relaxation (19).

We also wondered whether the changes induced by VitD-free diet are reversible. As a preliminary approach to answer this question, we exposed PA rings from VitD-deficient rats to calcitriol in culture. After 48 h, calcitriol increased the relaxant response to acetylcholine, and this effect was specific for VitD-deficient rats. These data indicate that at least some of the VitD-free diet-induced deficiencies may be rescued by restoring VDR activity.

Calcitriol is a known inhibitor of the growth of proliferating vascular smooth muscle cells from systemic arteries (7, 27). Herein we confirmed these effects in PA in vivo. Thus, PA from VitD-deficient rats showed increased percentage of muscularized arteries and increased wall media thickness, and these effects were additive to those of SuHx. Several VDR-targeted genes may be responsible for the antiproliferative effects. We have focused on some of them, such as the BMPR2 ligands BMP4 and BMP6, well known antiproliferative factors in PASMC (49), the antiapoptotic factor survivin (encoded by the *Birc5* gene), the mTOR inhibitor DDIT4, and the NFAT inhibitor calcipressin-1 (encoded by *Rcan1* gene). We confirmed that VitD deficiency upregulated *Birc5* and its encoded protein survivin and downregulated *Bmp4*, *Bmp6*, and *Ddit4* expression in the rat lungs, which may explain the observed VitD-free diet-induced PA muscularization. We found that survivin protein expression was increased in the lungs of the

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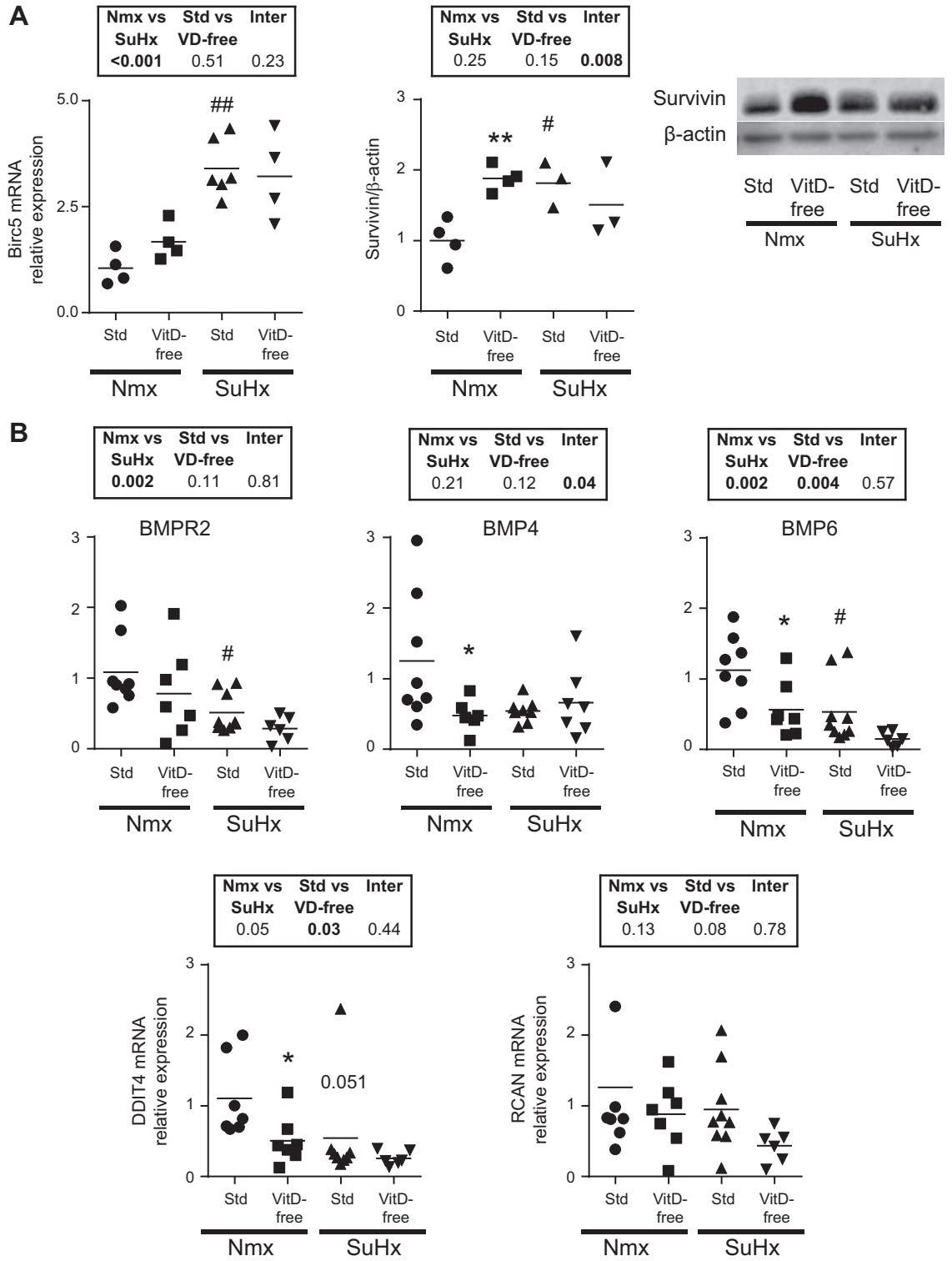

Fig. 7. Vitamin D (VitD and VD) deficiency induced changes in gene expression. *A*: vaculoviral IAP repeat containing 5 (*Birc5*) mRNA (gene encoding survivin protein) and survivin protein expression. *B*: bone morphogenetic protein receptor type 2 (*Bmpr2*), bone morphogenetic protein (*Bmp*) *4*, *Bmp6*, DNA damage-inducible transcript (*Ddit4*), and rat calcipressin-1 (*Rcan*) mRNA expression. Two-way ANOVA results are shown in the boxes. $*P < 0.05$ and $**P < 0.01$ vs. standard diet. $\#P < 0.05$ and $\#\#P < 0.01$ vs. normoxia, Bonferroni test. Each symbol represents one animal.

SuHx group. This upregulation mediates resistance to apoptosis in this model (39). VitD deficiency upregulated survivin to values similar to those induced by SuHx, suggesting that, as reported in other tissues and cell types (21), VitD may increase survivin-dependent apoptosis in the lung. However, we did not analyze specifically this issue in the present study.

The classical SuHx model is a model of PAH in which after 3 wk of hypoxia and SU-5416 there are irreversible changes in

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the lung structure. We have used the model at 2 wk to analyze early changes that might have a higher pathophysiological importance while the late changes might be just aggravating nonspecifically the poor health condition associated to this severe cardiovascular disease. We found that these animals showed increased mPAP, RV hypertrophy, endothelial dysfunction, hyperreactivity to 5-HT, increased PA muscularization, reduced Kv and TASK-1-like currents, and membrane depolarization as described for the 3-wk model (43). However, at this early time point the model may be behaving more similarly to the chronic hypoxia model of PH.

The epidemiological evidence indicates that there is a clear association between low levels of VitD and PAH. There are several possibilities to explain this relationship. It may be that *1*) PAH induces VitD deficiency, *2*) VitD deficiency induces or aggravates PAH, or *3*) there is no direct cause-effect relationship and there are confounding factors in this association. Our current data indicate that PH does not induce VitD deficiency, at least in an animal model, ruling out the first possibility. Regarding the third one, clinical PAH reduces exercise capacity, and it seems very likely that it induces changes in lifestyle with reduced time outdoors and hence sunlight exposure and VitD levels, a possibility that may partly explain the association but does not apply to the animal model. Our study specifically addresses the second hypothesis. These data indicate that VitD deficiency per se does not induce PH. That makes sense because one-fifth to one-third of the world population is VitD deficient, and PAH is a rare disease. However, our data strongly indicate that VitD deficiency induces many changes associated with pulmonary vascular disease with a modest, but significant, mPAP increase. Taken together we speculate that VitD deficiency, which is very common worldwide, may trigger/accelerate/worsen PAH in predisposed patients. Reduced time outdoors associated with PAH may further decrease VitD levels in a positive feedback loop.

In summary, our results indicate that VitD deficiency induces pulmonary vascular dysfunction. Dysregulation of the expression of several VDR-targeted genes may account for its molecular mechanism, including *Kcnk3*, the BMPR2 ligands *Bmp4* and *Bmp6*, *Ddit4*, and *Birc5*. Treatment with VitD might simultaneously restore these and possibly other VDR-targeted genes by a direct interaction of the activated VDR-RXR complex with the VDRE sequence in the promoter of these genes.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

F.P.-V. conceived and designed research; M.C., G.M.-P., D.M.-C., B.B., S.E.-R., M.A.O., and G.M. performed experiments; M.C., G.M.-P., D.M.-C., B.B., S.E.-R., and M.A.O. analyzed data; F.P., J.D., L.M., A.C., and F.P.-V. interpreted results of experiments; M.C., G.M.-P., and F.P.-V. prepared figures; M.C. and F.P.-V. drafted manuscript; J.D., L.M., A.C., and F.P.-V. edited and revised manuscript; M.C., G.M.-P., D.M.-C., B.B., S.E.-R., M.A.O., J.D., L.M., A.C., and F.P.-V. approved final version of manuscript.

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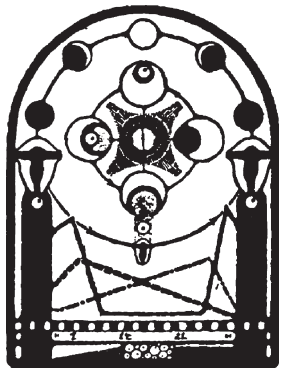

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Chapter 3

Restoration of vitamin D levels improves endothelial function and increases TASK-Like K^+ currents in pulmonary arterial hypertension associated with vitamin D deficiency

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Restoration of Vitamin D Levels Improves Endothelial Function and Increases TASK-Like K^+ Currents in Pulmonary Arterial Hypertension Associated with Vitamin D Deficiency

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Abstract: Background: Vitamin D (vitD) deficiency is highly prevalent in patients with pulmonary arterial hypertension (PAH). Moreover, PAH-patients with lower levels of vitD have worse prognosis. We hypothesize that recovering optimal levels of vitD in an animal model of PAH previously depleted of vitD improves the hemodynamics, the endothelial dysfunction and the ionic remodeling. **Methods:** Male Wistar rats were fed a vitD-free diet for five weeks and then received a single dose of Su5416 (20 mg/Kg) and were exposed to vitD-free diet and chronic hypoxia (10% O_2) for three weeks to induce PAH. Following this, vitD deficient rats with PAH were housed in room air and randomly divided into two groups: (a) continued on vitD-free diet or (b) received an oral dose of 100,000 IU/Kg of vitD plus standard diet for three weeks. Hemodynamics, pulmonary vascular remodeling, pulmonary arterial contractility, and K^+ currents were analyzed. **Results:** Recovering optimal levels of vitD improved endothelial function, measured by an increase in the endothelium-dependent vasodilator response to acetylcholine. It also increased the activity of TASK-1 potassium channels. However, vitD supplementation did not reduce pulmonary pressure and did not ameliorate pulmonary vascular remodeling and right ventricle hypertrophy. **Conclusions:** Altogether, these data suggest that in animals with PAH and severe deficit of vitD, restoring vitD levels to an optimal range partially improves some pathophysiological features of PAH.

Keywords: vitamin D supplementation; pulmonary hypertension; TASK-1 channel; vascular function

1. Introduction

Pulmonary arterial hypertension (PAH) is a progressive disease affecting the lung vasculature, characterized by sustained vasoconstriction and remodeling of distal pulmonary arteries (PA), resulting in abnormal elevated pulmonary vascular resistance, mean pulmonary arterial pressure (mPAP) and right ventricular failure [1]. Altered pulmonary arterial tone due to endothelial dysfunction and ionic remodeling are key features in the pathogenesis of PAH both in patients as well as in experimental models [2]. Endothelial dysfunction occurs due to an altered production of endothelial vasoactive mediators, i.e., decreased vasodilator and antiplatelet factors such as NO and prostacyclin (PGI_2), and increased vasoconstrictors and prothrombotic factors such as endothelin-1 (ET-1), serotonin (5-HT) and thromboxane (TXA_2) among others [3]. Reduced activity and/or expression of K^+ channels, notably Kv1.5 [4,5] and TASK-1 [6], in pulmonary artery smooth muscle

cells (PASMC) results in a more depolarized membrane potential (Em), leading to PASMC vasoconstriction and proliferation [5–7].

In recent years, several studies have reported an association between nutritional factors and PAH [8]. Moreover, important nutritional deficiencies have been consistently described in PAH patients for iron and Vitamin D (vitD) [9,10]. Currently, the ESC/ERS Guidelines recommend regular monitoring the iron status and use supplemental intravenous iron treatment in patients with low ferritin plasma levels but the analysis of vitD levels is not considered for the time being [1].

VitD regulates the expression of multiple genes involved in many regulatory processes as cell growth, innate and adaptive immunity, oxidative stress, angiogenesis and intracellular metabolism. Since the discovery of the vitD receptor (VDR) in extrarenal tissues, vitD deficiency has been related with a large number of pathologies in addition to bone diseases [11]. VitD status is assessed according to the circulating metabolite 25-hydroxyvitamin D, 25(OH)vitD. Despite there is an ongoing debate about the definition of the ranges for insufficient, optimum and potentially harmful levels of 25(OH)vitD, there is increasing agreement that the adequate levels should be above 20 ng/mL (50 nmol/L) [12,13]. The former strategy of widespread vitD supplementation without considering baseline vitD levels has been questioned [14]. In fact, vitD supplementation was mainly effective in patients who are vitD deficient [15–17].

Recently, we [18] and others [19–23] have demonstrated that vitD deficiency is much more frequent in PAH than in the general population. Insufficient levels, i.e., 25(OH)vitD < 20 ng/mL, are present in up to 95% of the patients and a severe deficit, i.e., 25(OH)vitD < 10 ng/mL, with subsequent secondary hyperparathyroidism is observed in 70% of them. Furthermore, lower levels of vitD are associated with worse functional class, higher levels of BNP/proBNP, reduced 6-min-walking-distance (6MWD), increased mPAP, increased pulmonary vascular resistance, decreased cardiac output and/or reduced survival [18,19,21,22].

We have recently evaluated the impact of vitD deficiency on a rat model of PAH [24]. VitD-free diet decreased the expression and the activity of the two-pore domain K^+ channel TASK-1, which is known to be mutated in some patients with familial PAH and downregulated in idiopathic PAH. It also depolarized PASMC and induced pulmonary endothelial dysfunction. In animals with PAH these effects were exacerbated and accompanied by an increase in mPAP and pulmonary arterial muscularization. Interestingly, we have also identified a vitD response element (VDRE) in the promoter of *KCNK3* gene, that encodes TASK-1 channel, conserved in human and mice. Moreover, we also tested in vitro that VDR stimulation with calcitriol (the active form of vitD) upregulates *KCNK3* gene in human PASMC from either control or PAH patients, as previously demonstrated in human coronary artery smooth muscle cells and in fresh slices from breast cancer [25,26].

PAH patients, as any other subjects with severe vitD deficiency, must be treated with vitD to prevent bone fractures. Based on the above clinical and preclinical data, we hypothesize that restoration of vitD status in patients with PAH may have an additional benefit on the evolution of their disease. In this translational study, to mimic the conditions of most PAH patients, rats with PAH were depleted of vitD, and then we analyzed the effects of vitD supplementation to recover optimal vitD levels.

2. Materials and Methods

2.1. Vitamin D Replacement in an Animal Model of PAH Associated with Vitamin D Deficiency

All animal procedures conform to the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-301/16). All investigators understand the ethical principles.

The experimental protocol is illustrated in Figure 1A. Male Wistar rats of 180 g body weight were obtained from Envigo (Barcelona, Spain) and maintained in the general

animal facilities at the School of Medicine of Universidad Complutense. All animals (n = 17) were fed with a vitD-free diet (Teklad Custom Diet TD.120008, Envigo) for five weeks [24]. Then, all animals received a single subcutaneous injection of Su5416 (Tocris, Bristol, UK; 20 mg/kg) and they were exposed to chronic hypoxia (10% O_2) for 3 weeks to cause PAH [5]. After this period, animals were randomized into two groups: they either continued on vitD-free diet (SuHx-Deficit, n = 8) or received a single loading dose of oral vitD (100,000 UI vitD/Kg; 47763, Sigma-Aldrich Merck KGaA, Darmstadt, Germany) and switched to a standard diet (Teklad Global 18% Protein Rodent Diet with 1500 IU/Kg Vitamin D3, Envigo; SuHx-Restored, n = 9) for 3 weeks in a normoxic room.

2.2. Hemodynamic Measurements

At the end of the experimental protocol, rats were anaesthetized i.p. with 80 mg/kg ketamine plus 8 mg/kg xylazine and ventilated with room air (tidal volume 9 mL/kg, 60 breaths/min, positive end-expiratory pressure of 2 cm H_2O). Systolic, diastolic and mean pulmonary arterial pressures (sPAP, dPAP and mPAP) were measured in open-chest rats with a pressure transducer via a catheter advanced through the right ventricle into the PA [5,24].

2.3. RV Hypertrophy and Lung Histology

At the end of the hemodynamic measurements, hearts were excised and the right ventricle (RV) and the left ventricle plus septum (LV + S) were dissected and weighed separately. Fulton index, [RV/(LV + S)] was calculated to assess the right ventricular hypertrophy. The left lung was inflated in situ with paraformaldehyde saline solution (4%) through the left bronchus and embedded in paraffin. Lung sections were stained with hematoxylin and eosin techniques and examined by light microscopy. Elastin was visualized by its green autofluorescence. PA (26–100 μm outer diameter) were analyzed in a blinded fashion and categorized as muscular, partially muscular or non-muscular as previously described [5,24]. The medial wall thickness was calculated as external elastic lamina diameter minus the internal lamina diameter using ImageJ software.

2.4. Electrophysiological Studies

PASMC were isolated as previously described [4,5]. Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA, USA) using the whole-cell configuration of the patch-clamp technique. Myocytes were superfused with an external Ca^{2+}-free Hepes solution containing (in mM): NaCl 130, KCl 5, HEPES 10, $MgCl_2$ 1.2 and glucose 10 (pH adjusted to 7.3 with NaOH) and a Ca^{2+}-free pipette (internal) solution containing (in mmol/L): KCl 110, $MgCl_2$ 1.2, Na_2ATP 5, HEPES 10, EGTA 10 (pH adjusted to 7.3 with KOH). Total K^+ current was evoked following the application of 250 ms depolarizing pulses from −60 mV to +60 mV in 10 mV increments. To characterize TASK currents, cells were clamped at 0 mV for 3 min, allowing Kv current inactivation and isolating the non-inactivating current, I_{KN}. Thereafter, a 1 s voltage ramp from +60 to −100 mV was applied. The ramp was applied again after 5 min perfusion with an external solution buffered at pH 6.3. Non-inactivating TASK currents were identified as the current sensitive to pH [24]. Currents were normalized to cell capacitance and expressed in pA/pF. Membrane potential was recorded under the current-clamp mode. All experiments were performed at room temperature (22–24 °C).

2.5. Arterial Reactivity

Intrapulmonary arteries (2–3 mm long, ~0.5 mm internal diameter) were mounted in a wire myograph with Krebs buffer solution maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2. Vessels were stretched to give an equivalent transmural pressure of 30 mmHg. After equilibration, PA rings were sequentially exposed to different vasoconstrictor agents to test the contractile capacity of the vessel, raising the K^+ concentration of the buffer to 80 mM, and a dose-response curve to serotonin (5-HT, 30 nmol/L to 30 μmol/L) by

cumulative drug addition. The endothelial function was estimated by the analysis of the relaxant response to the cumulative addition of acetylcholine (ACh, 1 nmol/L to 10 µmol/L) after precontraction with the vasoconstrictor drug phenylephrine (Phe, 1 µmol/L), to induce a contraction of ~75% of the response to KCl. Relaxation was expressed as a percentage of the reduction in 5-HT or Phe-induced contraction.

2.6. Vitamin D Measurement

Plasma from PAH-animals was collected using heparin as an anticoagulant followed by centrifugation at 1000 rpm for 15 min at room temperature and samples were then frozen at −80 °C until analysis. Plasma 25(OH)vitD was measured using a chemiluminescence monoclonal immunoassay (ADVIA Centaur® Vitamin D Total Assay, Siemens Healthcare Diagnostics) a certified procedure of the Vitamin D Standardization-Certification Program [27], at the Clinical Biochemistry Service at Gregorio Marañon Hospital (Madrid, Spain) as previously described [24].

2.7. Western Blotting Analysis

Lungs were homogenized with a lysis buffer containing Trizma pre-set crystals pH 7.5, DL-dithiothreitol (DTT) 1 mol/L, NP40 1% and supplemented with protease (protease inhibitor cocktail tablets, Roche Diagnostics GmbH, San Cugat, Spain) and phosphatase inhibitor (PhosSTOP, Roche Diagnostics GmbH) cocktail in a TissueLyser device (Qiagen, Hilden, Germany), as described [24,28]. Homogenates were run in a sodium dodecyl sulphate-polyacrilamide electrophoresis and proteins were transferred to polyvinylidene difluoride membranes. Membranes were incubated with specific primary antibodies against PCNA (dilution 1:200; sc-56; Santa-Cruz Biotechnology, Heidelberg, Germany) overnight at 4 °C and then with the appropriate secondary peroxidase conjugated antibodies. Antibody biding was detected by an ECL system (SuperSignal West Fento Chemiluminescent Substrate, Thermo Scientific, Madrid, Spain). Blots were imaged using an Odissey Fc System (Li-COR Biosciences, Bad Homburg, Germany) and were quantified by densitometry using Quantity One software. Samples were normalized through expression of smooth muscle β-actin (dilution 1:10,000; A1978; Sigma-Aldrich).

2.8. Statistics

Analysis was performed using GraphPad Software v7 (GraphPad Software Inc., San Diego, CA, USA). Data are presented either as scatter plots and bars ± SEM or as means ± SEM. Statistical analysis were performed using *t*-tests for two sample comparison (vitD deficient vs. restored). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of Vitamin D Supplementation on Pulmonary Pressure

Rats followed the protocol shown in Figure 1A. As expected, after 11 weeks in vitD-free diet, rats from the SuHx-Deficit group presented a severe reduction in plasma 25(OH)vitD levels (mean ± SEM; 4.31 ± 0.24 ng/mL), while a single oral dose of vitD plus standard diet significantly increased plasma 25(OH)vitD concentration to values within an optimal range (70.19 ± 7.27 ng/mL; Figure 1B). Hypoxia plus Su5416 induced weight loss (Figure 1C) and the recovery of optimal vitD levels was accompanied by a higher increase in body weight (344.8 ± 12.99 in deficient rats vs. 401.3 ± 11.5 g in the vitD restored group; Figure 1C).

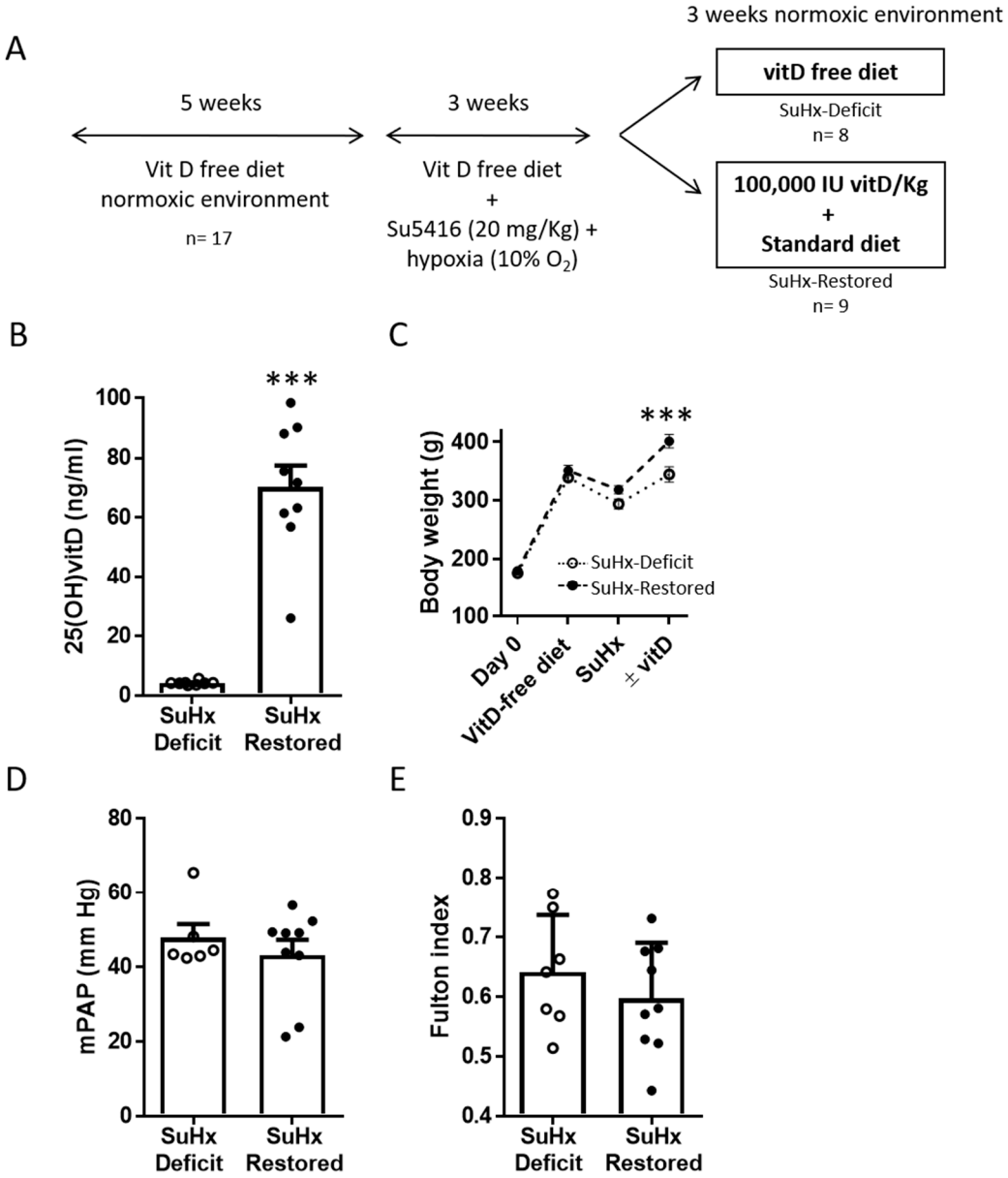

Figure 1. VitD treatment does not reduce mPAP in PAH rats with vitD deficiency. (**A**) Study protocol. (**B**) 25(OH)vitD levels; (**C**) evolution of body weight; (**D**) mPAP and (**E**) Fulton index in the groups at the end of the study period. Results are represented as scatter plots and bars with means ± SEM. SuHx-deficit and SuHx-restored indicate rats exposed to Su5416 and hypoxia (SuHx) with vitD-free diet (SuHx-Deficit) and rats with vitD treatment (SuHx-restored), respectively. *** $p < 0.001$ vs. SuHx-deficit, unpaired *t*-test.

Hemodynamic measurements revealed that after restoration of vitD levels only two animals (out of nine) were within normal mPAP values, so that mean mPAP were not statistically different in the two groups (47.87 ± 3.63 vs. 43.21 ± 4.12 mmHg) (Figure 1D and Table 1). Moreover, vitD treatment did not reduce the right ventricular hypertrophy, measured by the Fulton index, a main characteristic of PAH (Figure 1E) or measured as the right ventricular weight relative to body weight (1.38 ± 0.08 vs. 1.21 ± 0.07 g/kg, $p = 0.12$). As a reference, in our historical controls in healthy rats under normoxic conditions and with standard diet, 25(OH)vitD levels were 22.2 ng/mL, mPAP was 12.0 mmHg and the Fulton index was 0.27 [24].

Table 1. Pulmonary arterial pressure values. All parameters were determined at the end of the experimental period.

	SuHx-Deficit	SuHx-Restored	Student's' *t* Test
n	6 *	9	
sPAP (mmHg)	81.19 ± 7.75	72.38 ± 9.54	0.48
dPAP (mmHg)	29.83 ± 1.29	25.45 ± 2.67	0.18
mPAP (mmHg)	47.86 ± 3.63	43.21 ± 4.67	0.44

Results are means ± SEM. sPAP (systolic pulmonary arterial pressure); dPAP (diastolic pulmonary arterial pressure); mPAP (mean pulmonary arterial pressure). * PAP measurements could not be performed in two animals.

3.2. Effects of Vitamin D Supplementation on Pulmonary Reactivity Endothelial Function

The responses to vasoconstrictors and vasodilators in isolated PA from the two groups of animals were studied in a wire myograph (Figure 2). The responses to the vasoconstrictors KCl (80 mM), which is regarded as an index of the contractile capacity of the vessel (Figure 2A), and serotonin (5-HT; Figure 2B) were unaffected by vitD supplementation. As expected, SuHx caused pulmonary endothelial dysfunction, as observed by the attenuated endothelium-dependent relaxation induced by ACh (Figure 2C). Notably, recovery of vitD levels in PAH animals significantly improved the endothelial function (Figure 2C). As a reference, in our historical controls in healthy rats under normoxic conditions and with standard diet, the maximal ACh-induced relaxation in pulmonary arteries was 68% and the maximal contraction induced by 5-HT was 52% of the response to KCl [24].

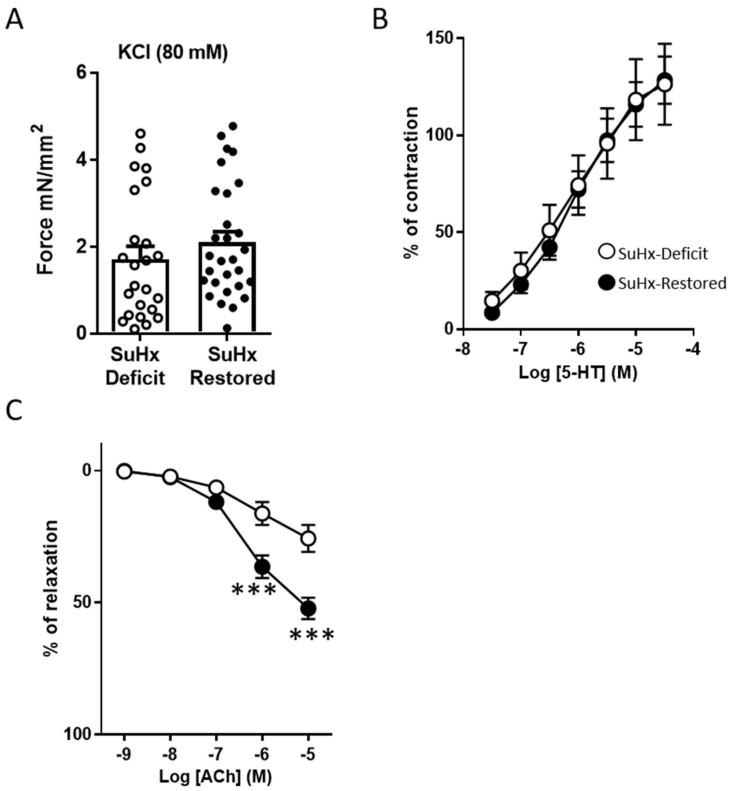

Figure 2. VitD treatment improves endothelial function in isolated PA. (**A**) Contractile responses to KCl (80 mM). Each dot represents the response of a single artery (2–3 arterial rings per animal). Cumulative concentration-responses curves to (**B**) serotonin (5-HT); (**C**) acetylcholine (ACh). Results in Panel A are scatter plots and bars with means ± SEM. Data in Panels B and C are means ± SEM. *** $p < 0.001$ vs. SuHx-deficit, unpaired *t*-test.

3.3. Restoration of Vitamin D Levels Improved TASK Like-Current

We analyzed if vitD treatment might restore TASK-like currents in freshly isolated PASMC. Compared to the values generally observed in cells from healthy animals, PASMC from SuHx showed reduced total K^+ current amplitude (Figure 3A), depolarized membrane potential (Figure 3B) and increased membrane capacitance (Figure 3C), an electrophysiological estimate of membrane surface. Restoring vitD induced significant increase on total K^+ current amplitude compared to PASMC from SuHx-deficit animals (Figure 3A). However, this was not accompanied by changes in PASMC membrane potential (Figure 3B) or capacitance (Figure 3C).

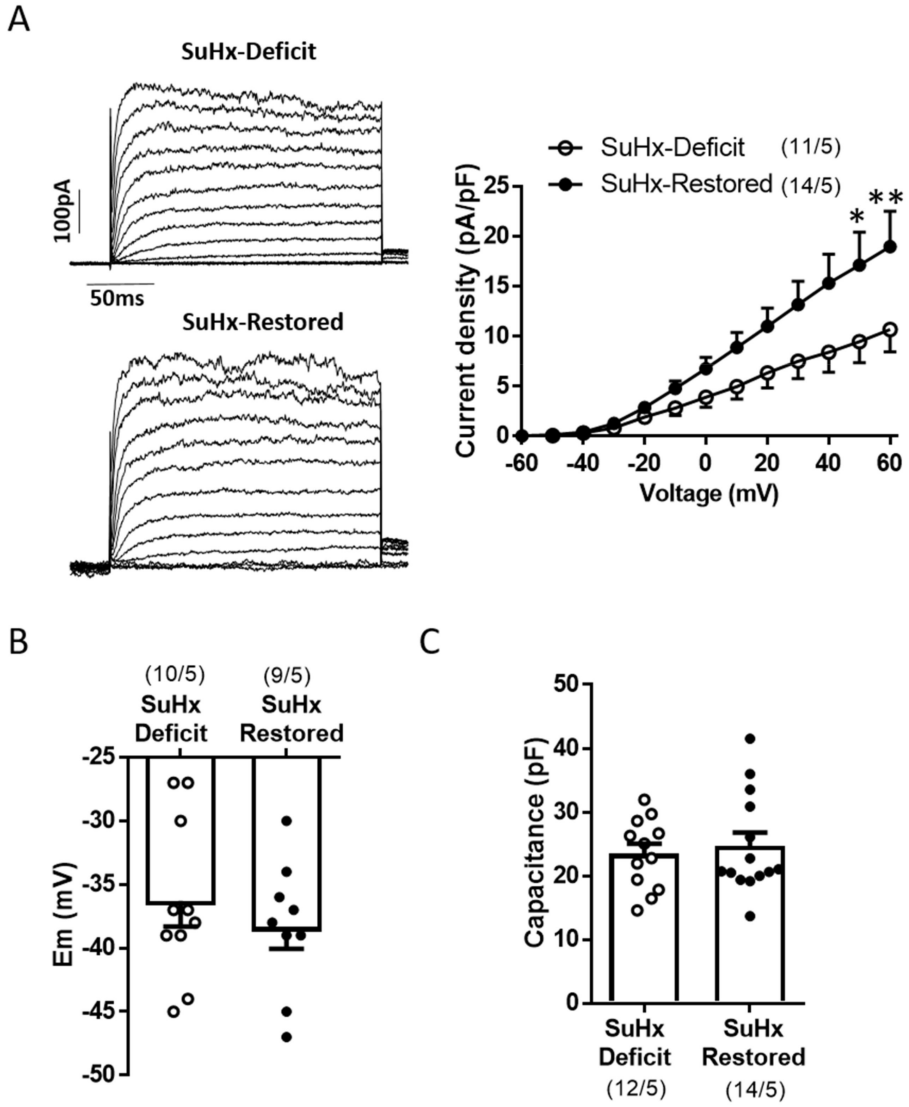

Figure 3. VitD restores Kv currents. (**A**) Representative currents traces (left) for 250 ms depolarization pulses from −60 mV to +60 mV in 10 mV increments and mean current-voltage relationship of K^+ currents measured at the end of the pulse (right). (**B**) Membrane potential (Em) values in freshly PASMC measured in mV and (**C**) average cell capacitance from freshly isolated PASMC measured in pF. The parentheses indicate the number of cells and the number of animals from whom these cells were obtained. Results are means ± SEM. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, vs. SuHx-deficit group, unpaired *t*-test.

VDR can regulate the expression of *KCNK3*, the TASK-1 channel encoding gene, and whose activity and expression were reduced in animals with vitD deficiency and PAH [24]. We tested if vitD replacement improves the activity of TASK-1 channels. To characterize

the TASK-1 activity, freshly isolated PASMC from these animals were held at 0 mV for 3 min to inactivate Kv channels, and to isolate the non-inactivating current, I_{KN}. TASK-1 activity, assessed as the pH-sensitive currents obtained by measuring the difference in K^+ currents at external pH values of 7.3 and 6.3, is shown in Figure 4A [24]. The results showed a significant increase of TASK-like current in SuHx-Restored vs. SuHx-Deficit. The difference at 0 mV is shown in Figure 4B.

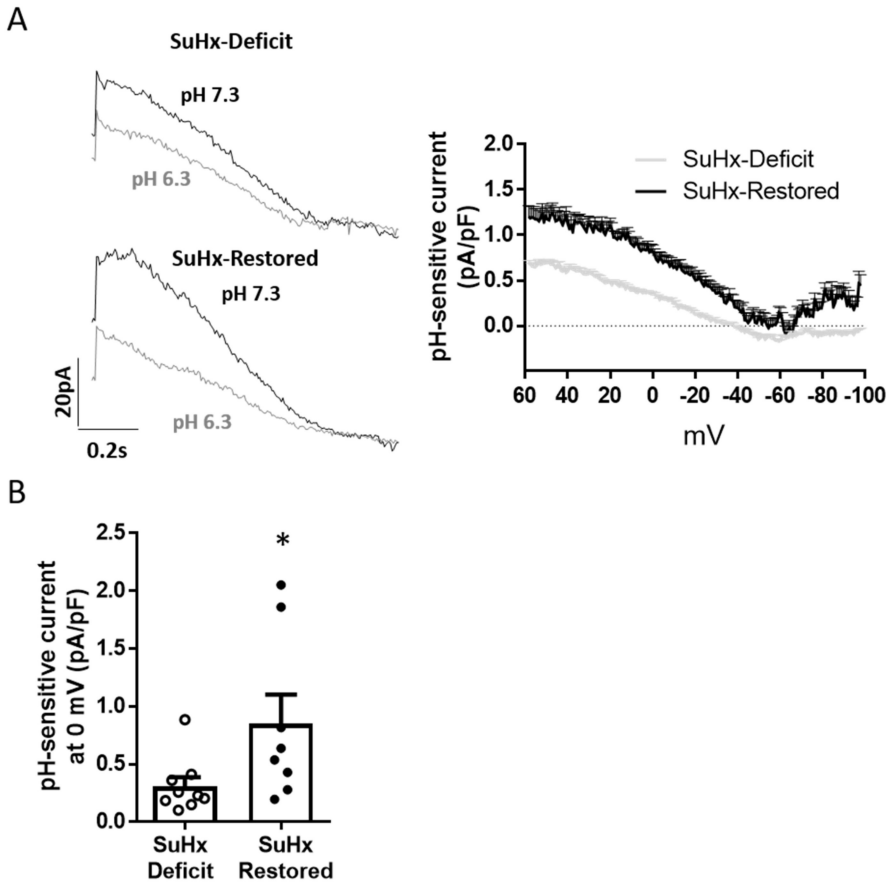

Figure 4. Restoring vitD levels increases TASK-1 activity. (**A**) Representative current traces (left) and average data of pH-sensitive currents obtained by measuring the difference in K^+ current at external pH values of 7.3 and 6.3 (right). Data are averaged traces (mean ± SEM). (**B**) Mean values of the pH-sensitive current recorded at 0 mV. Results are expressed as scatter plots and bars; * $p < 0.05$, unpaired *t*-test.

3.4. Effects of Vitamin D Supplementation on Pulmonary Arterial Remodeling

Resistance PA (25–100 μm) from lung sections (Figure 5A) were classified in a blinded fashion as muscular, partially muscular, and non-muscular arteries. Muscularization of resistance PA induced by SuHx was not reversed after vitD treatment. The percentage of muscularized arteries was not significantly different in SuHx-restored group vs. SuHx-deficit (Figure 5B). Consistently, the protein expression of PCNA (proliferating cell nuclear antigen), a marker of cell proliferation, was also similar in whole lungs from SuHx-deficit and SuHx-restored (Figure 5C).

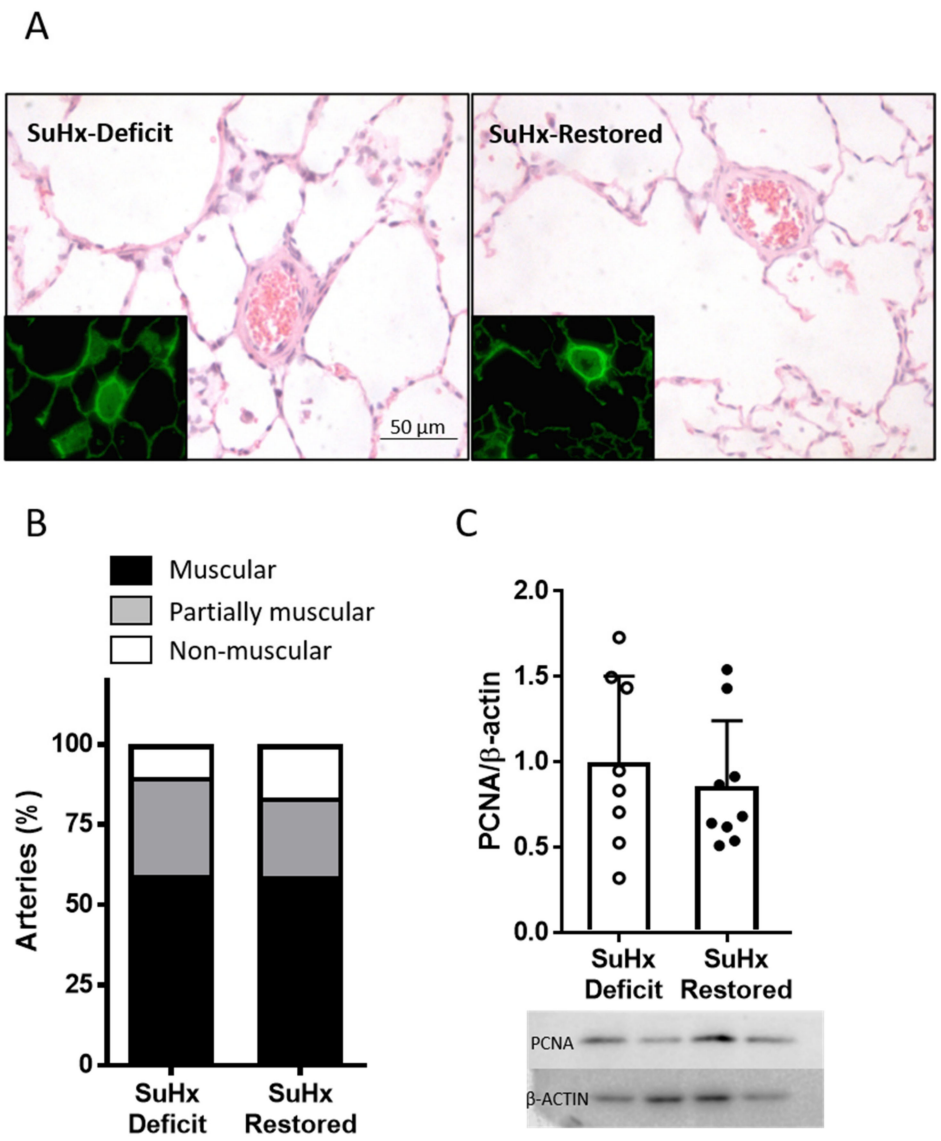

Figure 5. Lung histology. (**A**) Representative images of cross-sections of lungs of SuHx-deficit rats (left) and SuHx-restored (right) stained with hematoxylin and eosin. The elastin autofluorescence is shown in the insets. (**B**) Percentage of muscular, partially muscular, and non-muscular PA. (**C**) Protein expression of PCNA (proliferating cell nuclear antigen; 29 KDa) analyzed by Western blot, typical blots (bottom panel) and densitometric values and normalized by β-actin (top panel); data are expressed as scatter plots and bars with means ± SEM.

4. Discussion

In the present study, we analyzed for the first time the role of vitD supplementation as a treatment for PAH associated to severe deficit of vitD. Restoring vitD levels improved several pathophysiological characteristics of PAH, such as endothelial function, total Kv currents and the activity of TASK-1 potassium channel. In contrast, vitD supplementation did not reduce pulmonary arterial pressure, right ventricular hypertrophy, and vascular remodeling.

As much as 95% of PAH patients show deficit of vitD and 70% of them show severe deficiency associated to secondary hyperparathyroidism [18,21]. Likewise, reduced bone mineral density is present in 61 to 72% of these patients [29,30]. To prevent bone fractures, treatment of vitD deficiency is mandatory to restore calcium and bone homeostasis. Because lower 25(OH)vitD levels vitD are associated with worse prognosis in

PAH patients [18,20–23], we speculate that restoring vitD status may improve their lung pathology, besides improving their bone and muscular health.

All previous published results in PAH-experimental models have explored the role of vitD supplementation (supratherapeutic doses of vitD) as a preventive therapy, i.e., prior to inducing PAH [21,31] and reported divergent effects on mPAP. A vitD enriched diet (10-fold the standard diet) as a preventive therapy in SuHx Sprague–Dawley rats did not affect right ventricular systolic pressure (RVSP), but attenuated RV hypertrophy [21]. Moreover, vitD diet prior to exposure to SuHx in Fisher344 rats, a rat strain which exhibit greater mortality, increased survival [21]. In a model of milder and reversible pulmonary hypertension in Sprague–Dawley rats, i.e., hypoxia without SU5416, a single exceptionally high dose of $1,25(OH)_2D_3$ (20 mg/Kg, equivalent to 800,000 IU/kg) on the first day partly blocked the increase in mPAP and RV hypertrophy [31].

In the present study, we mimicked in the SuHx PAH animal model the conditions of severe deficit of vitD observed in most PAH patients [18] and investigated whether vitD supplementation to recover 25(OH)vitD levels can positively impact on their pulmonary vascular disease. Only one prospective, longitudinal study analyzed vitD replacement as therapy in PAH patients with vitD deficiency [20]. This study showed clear benefits of vitD on right ventricular size and 6MWD and a borderline significant effect on echocardiographycally estimated mPAP, but because this was a small and uncontrolled study with no placebo arm, the results are not conclusive.

Although currently, there is no clear consensus to define vitD status, it is widely accepted that sufficient levels of vitD should be above 20 ng/mL—with some experts recommending levels above 50 ng/mL—and below 100 ng/mL to prevent hypercalcemia [32]. In clinical practice, it is widely established to administer a high dose of Vitamin D in order to recover rapidly 25(OH)vitD levels, followed by maintenance doses. After vitD supplementation (a bolus of 100,000 IU vitD/Kg followed by standard vitD containing diet for 3 weeks) we achieved 25(OH)vitD levels of ~70 ng/mL while animals that continued on vitD free diet had a very severe deficit with ~4 ng/mL. Therefore, the rats achieved an increase in 25(OH) VitD after the supplementation higher than we expected but still below the toxic range. VitD deficient rats exposed to hypoxia exhibit a marked weight loss which is partially recovered after returning to normoxia. However, the recovery of adequate 25(OH)vitD levels was accompanied with an increase in body weight, possibly indicating a better general health.

Our previous results [24] showed that vitD deficiency aggravates the main features of the early stages of PAH in Wistar rats: moderately increased mPAP, worsened vascular function and structure and induced ionic remodeling with a decrease of TASK-1 channel activity but did not change right ventricular weight. Nevertheless, herein, restoring vitD levels in Wistar rats with PAH did not result in a decrease in pulmonary pressure, the main endpoint of the present study. Notably, two out of nine animals from the SuHx-restored group had normal mPAP values of 24 and 21 mmHg.

Calcitriol has been described to have antiproliferative properties in vascular SMC [11,33]. We have found that vitD deficiency worsened PA remodeling in SuHx rats with increased PA muscularization [24]. However, in the present study, restoration of vitD did not ameliorate vascular remodeling of distal PA. Similarly, Tanaka et al. showed that medial wall thickness of PA of SuHx-rats was not affected by supratherapeutic doses of vitD [21]. In the study of Yu et al. [31], the reported histological changes induced by vitD are unclear, probably reflecting changes in the airways.

The active form of vitD, calcitriol has been shown to control vascular tone, enhance NO release and reduce contractile responses to vasoconstrictors in systemic arteries [11,33,34]. Aortic vascular function is also altered in endothelial specific VDR gene knockout mice ($Vdr^{-/-}$) [34]. However, vitD supplementation has rendered dissimilar results on systemic endothelial function in humans [35–37]. Endothelial dysfunction, due to an altered production of endothelial vasoactive mediators, is an early event in all forms of PAH [3]. Severe deficit of vitD impaired endothelial function in PA as we have previously observed [24].

Likewise, present data show that restoring 25(OH)vitD levels enhance ACh-induced NO-dependent PA relaxation, indicating an improved endothelial function. The dissociation between the effects on endothelial function and the lack of significant effects on mPAP is not unexpected. In fact, pharmacologic inhibition of eNOS has no effect on mPAP in rats despite the inhibitory effect on endothelial function [38].

K^+ channels play a fundamental role in controlling PASMC membrane potential and pulmonary arterial tone [39]. Ionic channel remodeling due to a decrease in the activity and expression of potassium channels, mainly Kv1.5 and TASK-1, which result in PA vasoconstriction, hypertrophy and proliferation, is considered an early event in the pathobiology of PAH [7,40–42]. Recently, it has been reported that dysfunctional KCNK3 leads to the activation of several signaling pathways favoring proliferation, migration, and alteration of PA tone, highlighting the role of this channel in PAH [41].

Previously, we demonstrated that vitD-free diet in control rats impairs TASK-1 current in freshly isolated PASMC and downregulates *Kcnk3* gene [24]. In PAH-rats, vitD deficiency further diminished TASK-1 activity, suggesting that low levels of vitD may be implicated in the inhibition of TASK-1 channel and contribute to the pathology of PAH [24]. We also found a VDR response element in *KCNK3* gene, which may account for the stimulatory effect of calcitriol, the active form of vitD, on Kcnk3 mRNA expression in isolated PASMC [24]. Therefore, we wondered if vitD treatment in PAH-rats with severe vitD deficit could restore ionic remodeling in our model. We found that total Kv current density was strongly increased (~2 fold) after recovering optimal vitD levels. In addition, vitD also increased ~2 fold the TASK-1 current, identified as the pH-sensitive current. To date, some drugs have been identified to rescue dysfunctional TASK-1 currents, i.e., the phospholipase inhibitor ONO-RS-082 [43] and prostacyclin via protein kinase A (PKA) [44]. In the present study we have also identified vitD as a novel factor enhancing TASK-1 currents in PASMC.

A loss of function mutation in *Kcnk3* gene in rats impairs acetylcholine-induced PA relaxation [45] indicating that functional TASK-1 channel may be essential for NO-induced PA relaxation. Therefore, the recovery of TASK-1 currents observed herein may explain the enhanced response to NO following repletion of vitD.

We suggest that in addition to iron and ferritin, monitoring vitD levels should be also addressed in the ESC/ERS Guidelines.

The main limitation of the present study is that we followed the animals after vitD restoration for only three weeks. Whether a longer time may exert additional benefits and reverse an established PA remodeling and to lower mPAP is unknown.

5. Conclusions

In conclusion, although vitD treatment did not reduce mPAP and pulmonary vascular remodeling, it did improve endothelial function, and enhanced TASK-1 currents. Overall, vitD supplements to recover optimal levels is a required intervention to prevent bone fractures for at least 70% of PAH patients who show severe deficiency. Besides improving calcium and phosphate homeostasis and bone health, vitD supplementation to patients with PAH might help to improve the symptoms and prevent the progression of the disease.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Ethical Committees of the Universi-

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Informed Consent Statement: Not applicable.

Data Availability Statement: All data is already reported in the manuscript. Individual data of datasets expressed as averaged values is available on request.

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Chapter 4

Vitamin D deficiency, as a potential cause for insufficient response to sildenafil in pulmonary arterial hypertension

Vitamin D deficiency, a potential cause for insufficient response to sildenafil in pulmonary arterial hypertension

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To the Editor:

Phosphodiesterase 5 inhibitors (PDE5i), such as sildenafil and tadalafil, are frequently used to treat pulmonary arterial hypertension (PAH) [1]. Combined therapy of PDE5i with drugs acting *via* other signalling mechanisms, *i.e.* the endothelin pathway, is currently recommended as it provides better clinical outcomes than monotherapy with PDE5i [1, 2]. The soluble guanylyl cyclase (sGC) stimulator riociguat is an interesting alternative to PDE5i, with a different mode of action but on the same signalling pathway. Recently, the results of the REPLACE study show that, in patients remaining at intermediate risk of 1-year mortality after PDE5i treatment, switching to riociguat is beneficial in terms of clinical improvement and risk status as compared with PDE5i maintenance therapy [3].

There are theoretical reasons and empirical evidence to suggest that some patients with PAH may have a treatment response suboptimal to PDE5i but preserved to riociguat. Phosphodiesterase 5 degrades cyclic guanosine monophosphate (cGMP), which mediates the vasodilator and antiproliferative effects of nitric oxide (NO). Thus, PDE5i inhibits cGMP degradation and hence potentiates the effects of endogenous NO. Failure of PDE5i is presumed to be the consequence of low basal NO–sGC–cGMP activity, which is known to occur in PAH [4]. In contrast, riociguat increases intracellular cGMP levels by directly stimulating sGC, independent of available NO, but also sensitises sGC to NO. We have recently reported in an animal model that severe deficiency of vitamin D (vitD) further impaired endothelial-derived NO activity in PAH, and that calcitriol, the active form of vitD, can restore endothelial function [5]. Likewise, several reports have shown that vitD regulates NO activity in systemic arteries in both human and animal models [6].

Herein, we compared the responses to sildenafil and riociguat in isolated pulmonary arteries from rats with PAH that had been exposed to a vitD-free diet for 8 weeks with those after restoring vitD status for the last 3 weeks.

VitD deficiency led to a poor vasodilator response to sildenafil *ex vivo*, which can be reverted by restoring vitD levels (figure 1a). Remarkably, the response to riociguat is unaffected by the vitD status (figure 1b). Thus, these data indicate that vitD deficiency reduces the apparent basal NO-dependent cGMP production, decreasing the response to sildenafil in rats with PAH.

In Spanish and Japanese cohorts of PAH patients, 95% and 92% of them, respectively, showed vitD deficiency (serum 25(OH)vitD <20 $ng \cdot mL^{-1}$) and 70% and 42%, respectively, had severely decreased levels (<10 $ng \cdot mL^{-1}$) [7, 8]. 70% of the patients in the Spanish cohort also showed subsequent secondary hyperparathyroidism. Moreover, reduced bone mineral density and secondary hyperparathyroidism was also found in 80% and 55% of patients, respectively, in a Swiss PAH cohort [9]. We speculate that reduced vitD levels in PAH might partially account for the limited efficacy of sildenafil in some patients. Thus, we retrospectively compared the 25(OH)vitD levels in serum samples of PAH patients that responded *versus* those who did not respond to PDEi. Serum samples were obtained from the Spanish National PH Biobank and clinical data from the REHAP registry. Among the 113 PAH patients with available 25(OH)vitD levels, 67 had idiopathic, hereditary or drug-induced PAH [7], and 46 had associated PAH. 30 of them were on PDE5i as monotherapy and could be classified into responders or

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Vitamin D deficiency causes a reduced response to sildenafil but not to riociguat in rats with PAH. Lower levels of vitamin D were also retrospectively associated to insufficient response to PDE5 inhibitors in PAH patients. https://bit.ly/3rZDcTF

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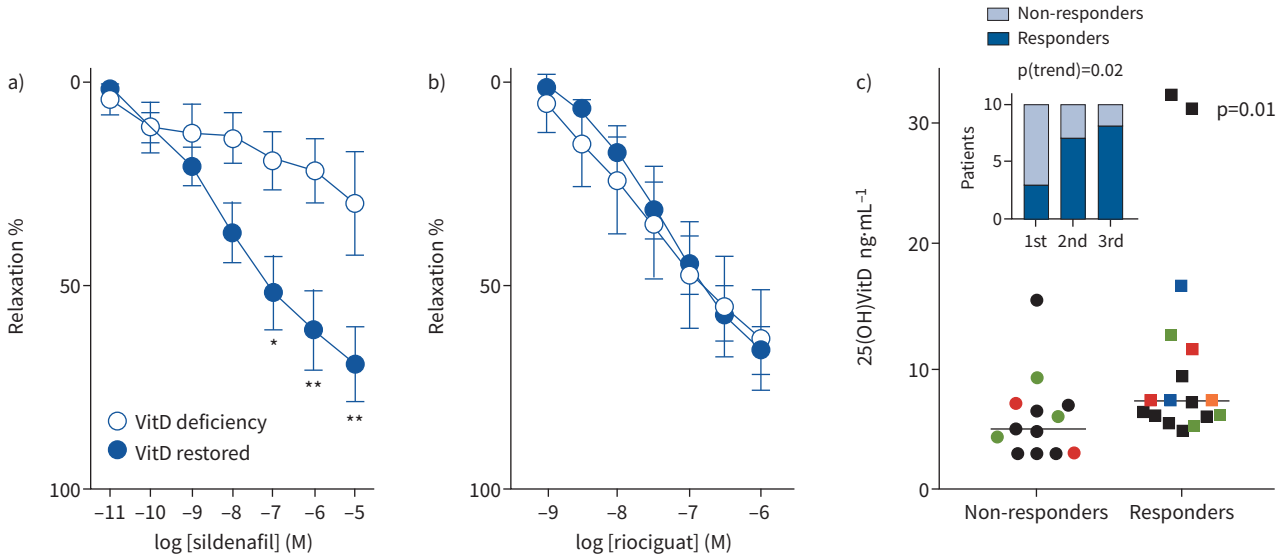

FIGURE 1 a, b) Wistar rats on vitamin D (vitD)-free diet for 5 weeks and further exposed to Su5416 (20 mg·kg^{-1}) and hypoxia (10% O_2) for an additional 3 weeks were returned to normoxia and randomised to either maintenance on vitD-free diet (vitD deficiency; n=8) or a single oral dose of 100 000 UI·kg^{-1} vitD_3 plus standard diet (vitD restored; n=9) for 3 weeks (final 25(OH)vitD serum levels were mean±SEM 4.3±0.3 and 70.2±7.3 ng·mL^{-1}, respectively) [10]. Pulmonary arteries from these animals were isolated, contracted with 5-HT (30 µmol·L^{-1}), and cumulative concentration–response curves to sildenafil (a) and riociguat (b) were performed in a wire myograph [5]. Results (presented as mean±SEM) indicate percent reversal of 5-HT-induced contraction. *: p<0.05; **: p<0.01 (t-test). c) Pulmonary arterial hypertension (PAH) patients treated with phosphodiesterase 5 inhibitor as monotherapy were classified into responders (n=17) and non-responders (n=13). 25(OH)vitD serum levels (scatter plot and median) were compared between the two groups with a Mann–Whitney test. PAH subtype is identified by colors: idiopathic (black), familial (red), portal hypertension (green), connective tissue disease (blue) and congenital heart disease (orange). The inset shows the number of non-responders and responders in the first, second and third tertile of 25(OH)vitD levels.

non-responders. Responders (n=17) were those meeting the three following criteria 12 months after PDE5i treatment initiation: 1) alive and free of lung transplant, 2) without clinical worsening (*i.e.* without treatment modification) and 3) improved risk score or remaining in low-risk profile. All others (n=13) were considered non-responders. Interestingly, responders to sildenafil had significantly higher 25(OH)vitD levels than non-responders (figure 1c). We also analysed the distribution of responders across tertiles of 25 (OH)vitD levels. Similarly, there was a significant increase in the percentage of responders with increasing 25(OH)vitD (inset in figure 1c).

In conclusion, vitD deficiency causes a poor vasodilator response to PDE5i but preserved response to riociguat in rats with PAH. The high prevalence of vitD deficiency worldwide, which seems to be even higher in at least some patients with PAH, and the lower levels of 25(OH)vitD in non-responders to PDE5i compared to responders, suggest that this deficiency may cause insufficient response to PDE5i in some patients with PAH, a possibility that remains to be tested. Therefore, in addition to recovery of optimal vitD status being indicated to restore calcium homeostasis and prevent bone fractures in those PAH patients with severe deficiency, it might help to improve responsiveness to PDE5i.

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All individual participant data that underlie the results reported in this article will be available on request.

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Chapter 5

Vitamin D receptor and its antiproliferative effect in pulmonary arterial hypertension

Vitamin D receptor and its antiproliferative effect in human pulmonary arterial hypertension

ABSTRACT

Background and objective: Vitamin D (vitD) deficiency is frequently observed in patients with pulmonary arterial hypertension (PAH) and, in these patients, low levels of vitD correlate with worse prognosis. The aim of this study is to examine the localization, expression and the antiproliferative role of vitD receptor (VDR) and its signalling pathway in the human pulmonary vasculature from controls and PAH-patients

Methods: VDR presence and expression was analysed in lungs, pulmonary artery smooth muscle cells (PASMC) and endothelial cells (PAEC). VDR expression and localization and VDR-target genes (*KNCK3, BIRC5* and BMP signalling pathway) were examined in PASMC treated with calcitriol, the active form of vitD. The antiproliferative effect of 48h-calcitriol was studied in PASMC by MTT and BrdU assays.

Results: VDR is expressed in the nucleus and in the cytoplasm of PASMC. It is downregulated in lungs and PASMC from PAH patients, without changes in PAEC. Calcitriol strongly upregulated VDR expression in PASMC from controls and PAH patients. Calcitriol also induced the translocation of VDR from the cytosol to the nucleus. The antiproliferative effect of VDR stimulation was similar in PASMC from controls and PAH patients and was inhibited by silencing or pharmacological inhibition of survivin or BMPR2, but not of KCNK3.

Conclusions: The expression of VDR is low in PAH patients which may contribute to the pathogenesis of PAH. Low VDR expression can be rescued by calcitriol. VDR exerts an antiproliferative effect in PASMC by opposing the actions of survivin and partially by modulation the BMP signalling pathway.

Keywords: pulmonary hypertension, vitamin D receptor, proliferation

INTRODUCTION

Pulmonary arterial hypertension (PAH) is a multifactorial, chronic disorder characterized by pulmonary vascular remodelling and pulmonary artery (PA) vasoconstriction [1]. A key hallmark is the medial hypertrophy due to an excessive and uncontrolled proliferation and resistance to apoptosis of PA smooth muscle cells (PASMC), which lead to intimal thickening and PA obliteration [2, 3]. As a consequence, the right ventricle (RV) tends to adapt to afterload with compensatory RV hypertrophy, which may follow with RV failure and premature death [4]. Several signalling pathways and many factors have been identified to contribute to this dysregulated PASMC proliferation. The impairment of canonical and non-canonical BMP signalling pathway, or mutations, upregulation of anti-apoptotic and pro-survival proteins, dysregulated K^+ channels like TASK-1 and Kv1.5, vasoconstrictors such as serotonin (5-HT), growth factors, tyrosine kinase activation or hypoxia are some of the potentially factors triggering PASMC proliferation [2, 5].

In recent years, we [6] and others [7-11] have demonstrated that vitamin D (vitD) deficiency is much more frequent in PAH patients than in the general population or even compared to patients with other cardiovascular diseases. In both, PAH patients and in animal models, lower levels of vitD are associated with worse functional class, reduced 6-min-walking-distance, increased mPAP, increased pulmonary vascular resistance, higher levels of BNP/pro-BNP, decreased cardiac output and/or reduced survival [6-9, 12].

The active form of vitD $1,25(OH)_2$vitD or calcitriol activates vitD receptor (VDR), a transcriptional factor that regulates the expression of specific target genes[13]. VDR is expressed in many tissues and cell types, including endothelial and smooth muscle cells. It exerts a wide variety of actions unrelated to its well-known effects on calcium and phosphorus homeostasis, i.e. the so-called non-calcemic actions. These effects have potential relevance in key processes within the cardiovascular and respiratory systems, such as cell proliferation, differentiation and migration, control of vascular tone, immunomodulation, regulation of metabolism, among others [14]. Previously, we have identified a vitD response element (VDRE) in the promoter of *KCNK3* gene, which encodes TASK-1 channel, whose downregulation is an early hallmark in PAH. Moreover, VDR stimulation with the active form of vitD, calcitriol, upregulated the expression of *KCNK3* gene in human PASMC from control and PAH patients [12].

Previous studies have demonstrated the presence and functionality of VDR in human bronchial epithelial cells and alveolar macrophages [15, 16]. For instance, Vdr $^{-/-}$ mice present alterations in adherent junctions in the lung, suggesting that VDR may play an important role in maintaining pulmonary barrier integrity [17]. Remarkably, decreased lung VDR levels have been observed in

various pulmonary diseases and correlates with worse prognosis; i.e. pulmonary VDR expression is decreased in patients with COPD [18], in idiopathic pulmonary fibrosis [19] or acute lung injury [20, 21].

The role of VDR in pulmonary vasculature and PAH has not been explored. The aim of the present study is to characterize the presence, localization, expression profile and effects mediated by VDR in the pulmonary vasculature and its potential antiproliferative role in PAH.

METHODS

Ethics statements in human samples

Research using human samples were approved by the ethics committee of the Getafe Hospital (Madrid, Spain), Ciberes Biobank (Barcelona, Spain) and French institutional Ethics Committee (Protocol N8CO-08-003, ID RCB: 2008-A00485-50). Informed consent was obtained in all cases. Human explanted lung tissues from non-PAH subjects were obtained from non-tumour lung areas of the resection specimens from patients undergoing surgery for lung carcinoma or discarded for lung transplantation. The donor lungs that were included in this study were thoroughly reviewed by a pathologist. PAH samples were obtained from lung transplantation.

Human pulmonary artery smooth muscle cell culture

From donor subjects, PA explants were isolated from non-tumour lung areas from patients undergoing surgery for lung carcinoma. Primary human PASMC cultures were derived from small PA (< 1mm internal diameter). Briefly, human PA were dissected in physiological Krebs solution, cut longitudinally into small fragments and endothelium was removed. PA explants were placed in 25cm^2 cultured flask and maintained in Smooth Muscle Cell Growth Medium supplemented with Smooth Muscle Cell Growth Supplement (310-500, Cell Applications). Around two weeks, PASMC reach 80-90% confluence and they were subcultured.

Human PASMC from patients with idiopathic PAH were used in this study. Research with these PAH-patient samples was part of the French Network on Pulmonary Hypertension, a programme approved by the institutional Ethics Committee. PAH-PASMC were cultured as previously described [22].

PASMC were seeded at 3.000 cells per well for proliferation assays in 96-well plate and 100.000 cells per well in 6-well plate. After adhesion, cells were starved of serum (0.1% of

FBS) for 24 hours. Then, PASMC were treated with different concentrations (1-100 nmol/l) of calcitriol (1α,25-Dihydroxyvitamin D_3, D1530, Sigma-Aldrich) or vehicle (DMSO, 0.1%).

PASMC were also incubated with DMH-1 (5 µmol/l, Tocris), SB203580 (10 µmol/l, Sigma-Aldrich) and PD98059 (5 µmol/l, Merck Biosciences), p38 and ERK MAPK inhibitors, respectively, and YM155 (20 nmol/l, Sigma-Aldrich) a survivin inhibitor.

All PASMC were used within passages 2-4 and were cultured in a humidified atmosphere of 5% CO_2 in air at 37 ºC. Treated cells were used to analyse gene expression, immunocytochemistry, and cell viability and proliferation assays.

Human pulmonary artery endothelial cells

Primary human PAEC were isolated from PA from controls subjects (n= 9) and PAH patients (n=13), as previously described [22]. Research PAH-patient samples was part of the French Network on Pulmonary Hypertension, a programme approved by the institutional Ethics Committee. These cells were used for gene expression experiments.

Moreover, commercial control PAEC (kindly provided by Professor Maria Jose Calzada from Hospital La Princesa (Madrid, Spain)) and PAEC from patients with chronic thromboembolic PH (CTEPH) [23] were used in this study for proliferation assays. PAEC were seeded at a density of 5.000 cells per-96-well and treated with calcitriol (1-100 nmol/l) or vehicle (DMSO, 0.1%). All human PAEC were maintained with EGM-2 Endothelial Cell Growth Medium (CC-4176, Lonza) and were used for this study between passages 3 and 4.

Immunofluorescence staining

Immunostaining was performed in frozen lung slices from control subjects as described previously [22] and in human PASMC from patients with idiopathic PAH (n=3) and healthy donors (n=3). Culture human PASMC were fixed with 4% paraformaldehyde/PBS for 10 min at room temperature and blocked and permeabilized with PBS containing 0.4% Triton X-100 and 3% BSA for 1 h at room temperature. Samples were subsequently incubated with the primary antibody mouse anti-VDR (1:100, SC-13133, Santa Cruz) at 4 ºC overnight and then 15 min at 37 ºC. After that, PASMC were incubated with the secondary antibody goat anti-mouse Alexa Fluor 594 (1:200, A11032, Thermo Fisher Scientific) for 1 h. Nuclei were stained with DAPI. All images were taken by confocal fluorescence microscope.

siRNA transfection

Human control PASMC were transfected with specific siRNA against *BMPR2* (SR300456B, Origene), *KCNK3* (gene encoding TASK-1, SASI_Hs01_00108786, Sigma-Aldrich), *BIRC5 (*gene encoding survivin, SASI_Hs01_00052229, Sigma-Aldrich) and control non-targeting siRNA (SIC001 scramble siRNA, Sigma-Aldrich) using LipofectamineTM RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's instructions and previously reported [25]. Briefly, PASMC were seeded in a 96-well-plate or 6-well-plate at a density of 3.000 cells/well and 100.000 cells/well, respectively, with complete medium Smooth Muscle Cell Growth Medium supplemented with Smooth Muscle Cell Growth Supplement (310-500, Cell Applications). After 24 h of serum starvation, the complex of siRNA with a final concentration of 10 nmol/l and lipofectamine were mixed with Opti-MEM Reduced Serum Media (Thermo Fisher Scientific) supplemented with 2% FBS and 1% antibiotic/antimycotic solution and transfected into PASMC. Twenty-four hours after transfection, the medium was replaced with Smooth Muscle Cell Growth Medium supplemented with 5% FBS, non-essential amino acid solution (1x), pyruvate solution (1x), penicillin (100 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 µg/ml) plus calcitriol (1-100 nmol/l) or vehicle (DMSO, 0.1%). After 48 h, PASMC were collected for RNA isolation and to perform cell viability and proliferation assays.

Cell viability and cell proliferation assays

Cell viability was assessed by the colorimetric MTT assay (1 mg/ml; Sigma-Aldrich). After one hour of incubation at 37 ºC, absorbance was recorded at a wavelength of 540 nm by a colorimetric plate reader (EZ Read 400 Microplate Reader, Biochrom). Data were expressed as percentage of absorbance values at baseline time or vehicle. In addition, cell proliferation was also analysed by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, following the manufacturer's protocol (Roche Applied Science). The absorbance was measured at dual wavelength of 450-620 nm. Results were normalized as a percentage of the vehicle's values. Each experimental condition was performed at least in triplicate.

Gene expression by qRT-PCR

Total RNA from human lung and PASMC were extracted with the NucleoSpin RNA kit (Macherey-Nagel), Absolutely RNA Microprep kit (Stratagene Cloning Systems, Agilent Technologies), depending on the sample, according to the supplier's instructions in all cases and including DNAse digestion. RNA quantity and quality were assessed with NanoDropTM 1000 Spectrophotometers (Thermo Fisher Scientific). One microgram of total RNA extracted was reverse transcribed into cDNA using iScriptTM cDNA Synthesis Kit (Biorad) or High Capacity

cDNA Reverse Transcription Kit (Thermo Fisher Scientific), following manufacturer's instructions. Gene expression was determined by quantitative real-time PCR (qRT-PCR) with a TaqMan Gene Expression Master Mix (Thermo Fisher Scientific), using specific primers from Applied Biosystems databases (Thermo Fisher Scientific) listed in table 1. Amplifications, detections and analyses were performed in a 7.900HT Fast Real-time OCR System (Centro de Genomica, Universidad Complutense, Madrid, Spain). The delta-delta Ct method was used to quantify relative changes in mRNA expression. Gene expression was normalized against *18S*, *β-ACTIN* or *GNB2L1*.

Table 1. List of human primers used in the study

Gen	*Reference*
Activin A receptor type 2A (*ACVR2A*)	Hs00155658_m1
Activin A receptor type 2B (*ACVR2B*)	Hs00609603_m1
Baculoviral IAP repeat containing 5 (*BIRC5*)	Hs04194392_s1
Bone morphogenetic protein 2 (*BMP2*)	Hs00154192_m1
Bone morphogenetic protein 4 (*BMP4*)	Hs00370078_m1
Bone morphogenetic protein 6 (*BMP6*)	Hs01099594_m1
Bone morphogenetic protein receptor type 2 (*BMPR2*)	Hs00176148_m1
Potassium two pore domain channel subfamily K member 3 (*KCNK3*)	Hs00605529_m1
Smad family member 1 (*SMAD 1*)	Hs00195432_m1
Smad family member 3 (*SMAD 3*)	Hs00969210_m1
Smad family member 5 (*SMAD 5*)	Hs00195437_m1
Smad family member 6 (*SMAD 6*)	Hs00178579_m1
Smad family member 7 (*SMAD 7*)	Hs00998193_m1
Smad family member 9 (*SMAD 9*)	Hs00195441_m1
Transforming growth factor beta 1 (*TGFb1*)	Hs00998133_m1
Vitamin D receptor (*VDR*)	Hs01045843_m1
Actin beta (*ACTB*)	Hs01060665_g1
Eukaryotic 18S rRNA (*18S*)	Hs03003631_g1
Guanine nucleotide binding protein beta polypeptide 2 like 1 (*GNB2L1*)	Hs00272002_m1

Western Blot

Human lungs were homogenized with a lyses buffer containing Trizma Pre-set crystals pH 7.5, DL-dithiothreitol (DTT) 1M, NP40 1% and supplemented with protease and phosphatise inhibitor cocktail (Roche Diagnosis GmbH, Mannheim, Germany) in a TissueLyser device (Qiagen, Hilden, Germany). Twenty-five µg from lung homogenates were run on a sodium dodecyl sulphate-polyacrylamide electrophoresis and proteins were transferred to polyvinylidene difluoride membranes. After blocking membranes, they were incubated with the

primary antibody against VDR (1:200, SC-13133, Santa Cruz) overnight at 4 ºC and then with the appropriate secondary horseradish peroxidise conjugated antibody. Results were normalized by the relative signal of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH; 1:10.000, Sigma-Aldrich). Antibody binding was detected by an ECL system (SuperSignal West Fento Chemiluminescent Substrate, Thermo Fisher Scientific). Blots were imaged using an Odissey Fc System (Li-COR, Biosciences) and were quantified by densitometry using the Quantity One software (Bio-Rad laboratories).

Vascular reactivity

For contractile tension recording, human donor PA rings (1.7-2 mm long, ~0.8 mm internal diameter) were mounted in a wire myograph with Krebs solution maintained at 37 ºC and bubbled with 95% O_2 and 5% CO_2. Pulmonary arteries were stretched to give an equivalent transmural pressure of 30 mmHg. After equilibration, the possible vasodilator effect of the active form of vitD was assessed by cumulative doses of calcitriol (0.01 – 10 nmol/l) in pre-contracted PA rings with a cocktail of pulmonary vasoconstrictors: endothelin-1 (ET-1, 3 nmol/l), thromboxane A2 mimetic U46619 (30 nmol/l) and serotonin (5-HT, 3 µmol/l).

VDR analysis in transcriptomic databases

Tissue distribution of VDR expression was analysed using RNA-Seq data from 27 human healthy tissues provided by the National Center for Biotechnology Information (NCBI) portal (bioproject PRJEB4337, ID: 231263). To compare normal *vs* PAH VDR lung expression, RNA-Seq data was downloaded from the publically available Gene Expression Omnibus (GEO) dataset GSE117261 (https://www.ncbi.nlm.nih.gov/gds/). This dataset is the largest published trascriptome in PAH, containing the gene expression of 58 patients with PAH who underwent lung transplantation [26]. It included explanted lungs from 32 patients with idiopathic PAH (IPAH), 17 patients with associated PAH (APAH), 5 patients with familial PAH (FPAH) and 4 with other or unknown forms of PAH. Control lung samples were obtained from 25 transplant failed donors, i.e. those who did not find an appropriate recipient, but still meeting physiologic standards. The patient demographics and the detailed methods for RNA extraction and analysis using Affymetrix human HuGene1.0-ST microarray technology are described in detail in the original paper and its supplement [26].

Statistics

Statistical analyses were performed using GraphPad Software v7 (GraphPad Software Inc., USA). All data were tested for normal distribution (D'Agostino-Pearson normality test) and parametric or non-parametric statistics were used as appropriate. Multiple samples comparisons

were analysed by one-way or two-way ANOVA following by Bonferroni post hoc test. Data are presented either as scatter plots and medians or as means ± sem. P-values less than 0.05 were considered statistically significant.

RESULTS

Lung VDR gene expression is downregulated in PAH

We searched the VDR expression in a public transcriptome comparing 27 different organs. VDR expression in the lungs was lower than in gastrointestinal organs, the kidneys and the skin but higher than in most other organs (Figure 1A). In another transcriptomic database (GSE11726126), VDR lung expression was downregulated in PAH-patients compared to controls (Figure 1B, control *vs* all-PAH, $p<0.05$, Mann-Whitney test). VDR lung expression was not significantly different among the different PAH subgroups (One-way ANOVA, Fig 1B). We also analysed *VDR* mRNA expression by qRT-PCR and VDR protein expression by Western blot in lungs from PAH-patients after lung transplantation and in control human lungs from transplant donors (Figure 1C and 1D). We confirmed that there was a significant VDR protein downregulation (Figure 1D), and a borderline significant reduction ($p=0.08$) in the *VDR* mRNA from PAH patients (Figure 1C).

VDR gene expression within pulmonary arteries

Immunofluorescent staining in healthy human lung sections indicated that VDR was expressed *in situ* in PA, delineated by elastin green autofluorescence in Figure 2A. VDR in these PA was mainly visualized in the nucleus as shown in pink by the co-localization with the blue nuclear stain DAPI. In non-vascular cells, VDR staining showed a preferential extra-nuclear distribution. Moreover, VDR expression, analysed by immunocytochemistry, was maintained in cultured PASMC from control subjects (Figure 2B). In these cells, VDR was also located mainly in the nucleus with lower expression in the cytosol. We compared *VDR* mRNA expression in cultured control human PASMC *vs* PAEC by qRT-PCR. Figure 2C shows that VDR *mRNA* was much higher in PASMC compared to cultured human control PAEC. PASMC from PAH-patients in culture also showed lower VDR expression compared to those from healthy donors (Figure 2D). In contrast, VDR expression in PAECs was similar in controls and PAH patients (Figure 2E).

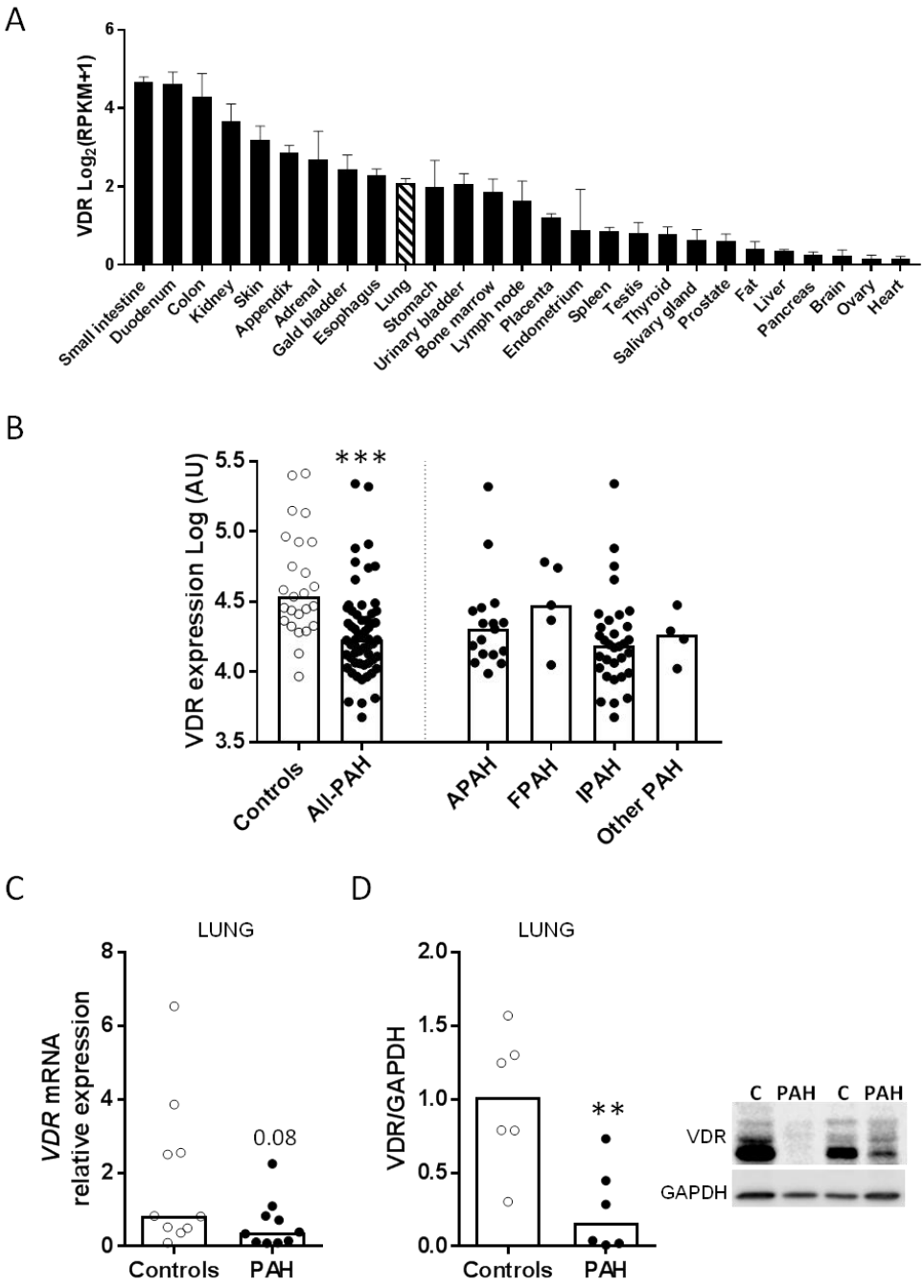

Figure 1. VDR is relatively highly expressed in human lungs and it is downregulated in PAH. *(A) VDR gene expression pattern in different tissues. Data from the NCBI database, project PRJEB4337. Data is log-transformed into Log_2(RPKM+1) and represented as mean±SD. (B) Analysis of VDR mRNA expression in transcriptome database GSE117261 in controls and PAH-patients (All PAH). PAH patients included associated (APAH), familial PAH (FPAH), idiopathic PAH (IPAH) or other or unknown causes. (C) VDR mRNA expression by qRT-PCR and (D) VDR protein normalized by GAPDH expression by Western Blot in lungs samples from controls and PAH-patients. Results are represented as scatter plots and bars with medians. ** p<0.01 and *** p<0.001, non-parametric Mann Whitney test.*

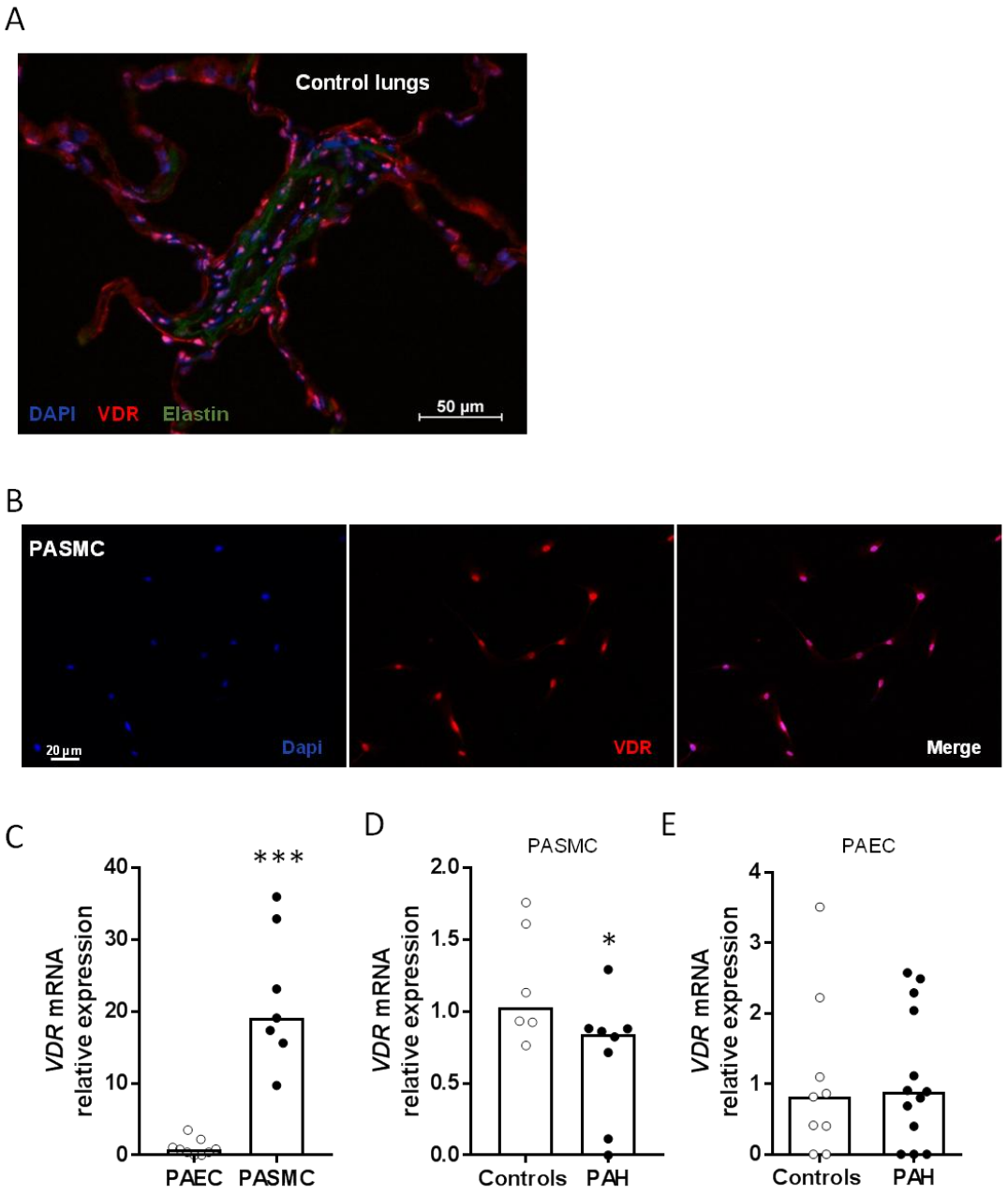

Figure 2. VDR is expressed in human PA and in cultured human PASMC in vitro. *(A) VDR localization by immunofluorescence labelling and confocal imaging in control human lungs. VDR expression is shown in red, elastin in green and nuclei are shown in blue (DAPI); pink indicates co-localization of VDR and DAPI. (B) Localization of VDR by immunofluorescent labelling in cultured control human PASMC. VDR expression is shown in red and nuclei are shown in blue (DAPI). Scale bar, 20 µm. (C, D, E) VDR mRNA expression by qRT-PCR in (C) PAEC vs PASMC from control donors, (D) PASMC from control donors vs PASMC from PAH patients, and (E) PAEC from control donors vs PAEC from PAH patients. Results are represented as scatter plots and bars with medians. *** $p<0.001$ vs PAEC, * $p<0.05$ vs controls, non-parametric Mann Whitney test.*

VDR expression and localization after calcitriol treatment

We treated human PASMC from controls subjects and PAH-patients with calcitriol for 24 and 48 h. *VDR* mRNA expression was strongly and time-dependently upregulated as analysed by RT-qPCR (Figure 3A). Notably, *VDR* mRNA expression was significantly lower in PASMC from PAH patients at 24 h after calcitriol treatment. However, at 48 h, *VDR* mRNA expression in PAH-PASMC was similar to control-PASMC. Cellular VDR localization was also studied in PASMC from controls and PAH patients treated with calcitriol for 48 hours. After calcitriol treatment for 48 hours, VDR is mainly translocated to the cell nucleus (Figure 3B).

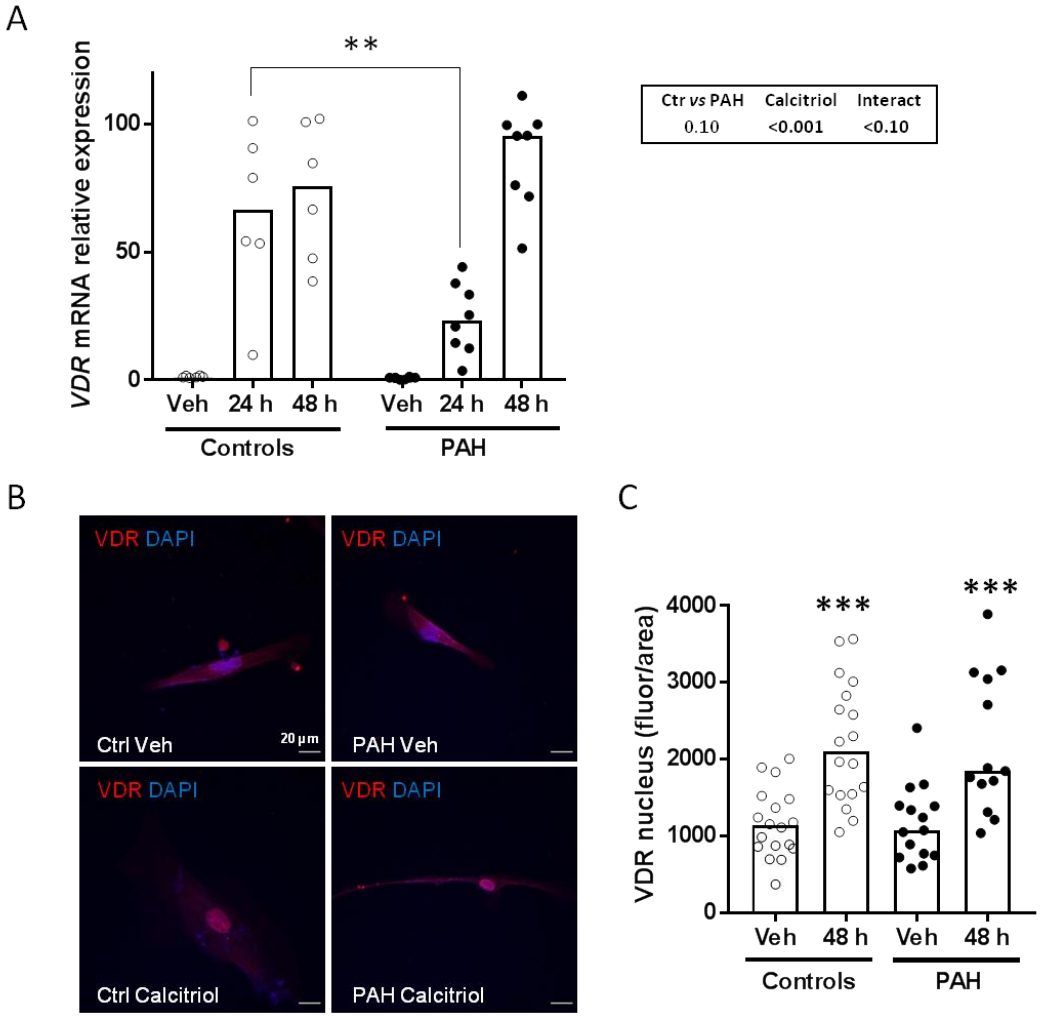

Figure 3. VDR upregulation and nuclear translocation by calcitriol in PASMC. *(A) VDR mRNA expression (at 24 and 48 hours) after calcitriol treatment (100 nmol/l). (B) Representative confocal images of VDR immunocytochemistry labelling in the presence or absence of calcitriol for 48 h in cultured PASMC from controls and PAH-patients. Merged VDR expression (red) and DAPI (blue). Scale bar, 20 µm. (C) VDR located in the nucleus (red fluorescence located in the nucleus referred to whole cell surface, arbitrary units). Data in panels A and C are represented as scatter plots and bars with medians. ** p<0.01 and *** p<0.001, two-way ANOVA, following Bonferroni's multiple comparisons test.*

VDR inhibits PASMC proliferation

We then examined the antiproliferative effects of VDR stimulation with calcitriol in PASMC from PAH and controls. In the presence of vehicle, PASMC exhibited a proliferative response with a 50-100% increase in viable cells at 48 h as measured by the MTT assay (Fig 4A and 4C). The proliferation was strongly inhibited in a concentration-dependent manner by calcitriol (1-100 nmol/l) as measured by either the MTT or the BrdU assay (Figures 4A and 4B, respectively). The inhibitory effect of calcitriol on proliferation was also observed in PASMC from PAH-patients (Figure 4C).

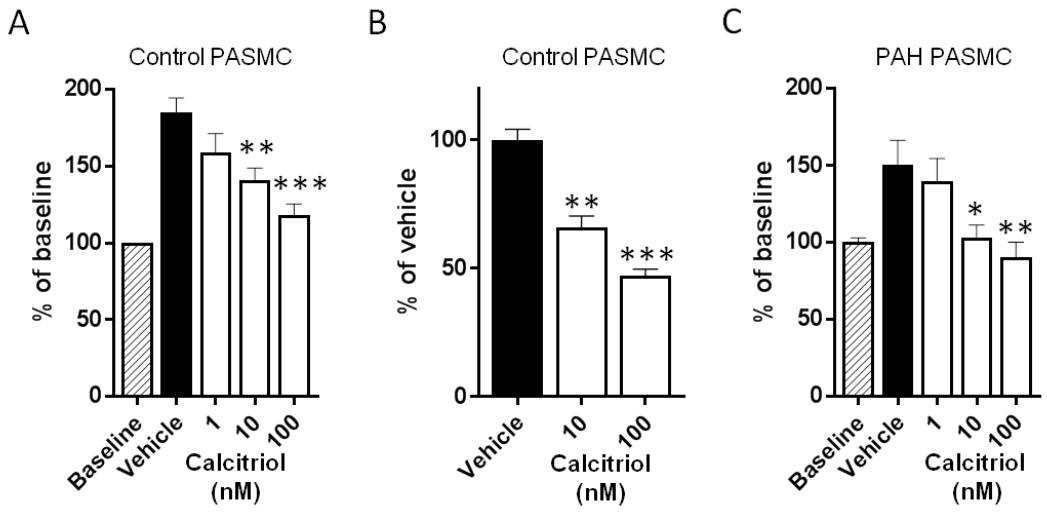

Figure 4. Calcitriol exerts antiproliferative effects in human PASMC. *(A) and (B) Proliferation in human PASMC from 5 controls measured by MTT assay and BrdU incorporation, respectively, and (C) proliferation in PASMC from 4 PAH-patients by MTT assay, after exposure to calcitriol (1-100 nmol/l) for 48 h. Results are expressed as mean±SEM. *, **, *** indicates p<0.05, p<0.01 and p<0.001 vs vehicle, one-way ANOVA, Bonferroni test.*

Calcitriol modulates several genes of interest in PAH

Next, we analyse the expression of several potential genes modulated by VDR and whose dysregulation is known to be involved in PASMC proliferation in PAH. Interestingly, in human control PASMC, calcitriol treatment for 48h increased the expression of *KCNK3* (Figure 5A), the gene encoding TASK-1 potassium channel, as well as decreased the expression of *BIRC5*, gene encoding survivin protein (Figure 5B). We also analysed its effects on genes of the BMPR2 and TGFβ signalling pathway. Calcitriol significantly enhanced the expression of *BMP4* and caused a borderline increase in *BMP6* (p=0.06), ligands of BMPR2, but had no effects on BMPR2 itself, or in genes related to its canonical, Smad-dependent, signalling pathway (Figure 5C).

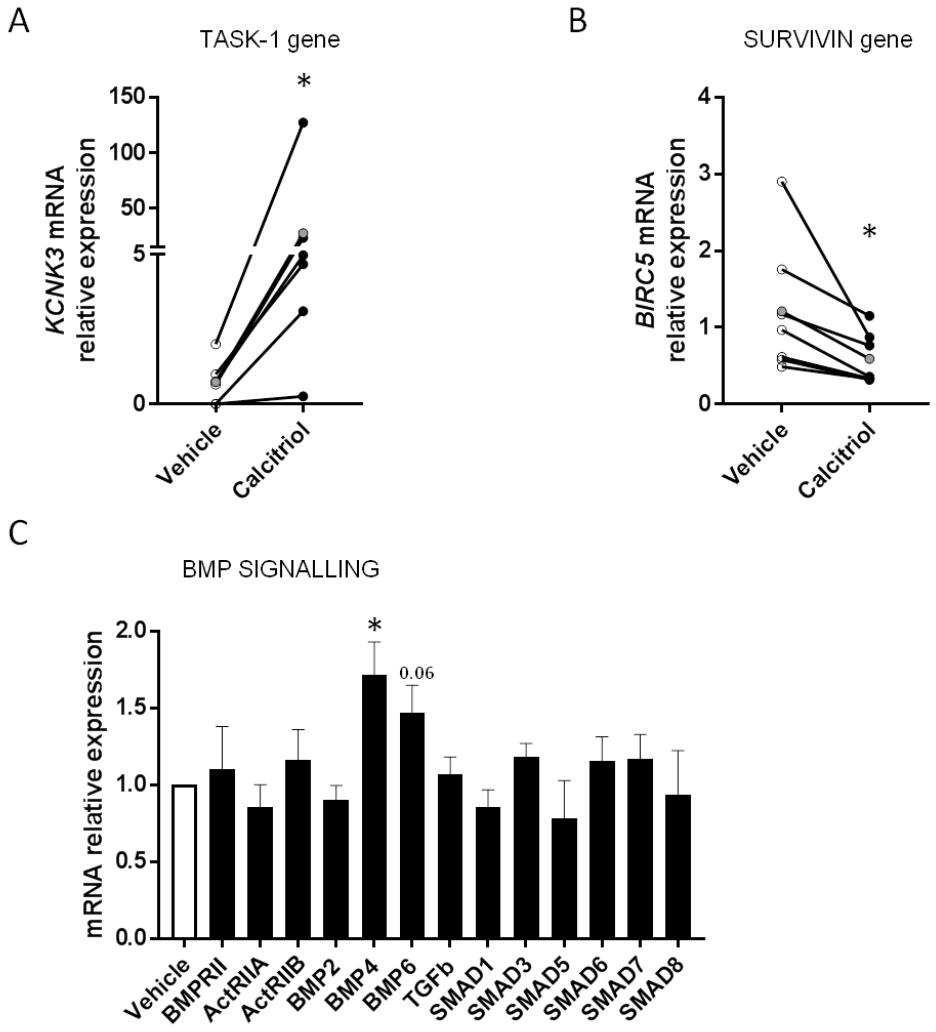

Figure 5. Calcitriol modulates the expression of genes of interest in PAH. *Expression of (A) KCNK3 (gene encoding TASK1, n=6), (B) BIRC5 (gene encoding survivin, n=7) and (C) Genes involved in the of BMP signalling pathway (n=3-6) after exposure to vehicle o calcitriol (100 nmol/l) for 48 h. Data in panels A and B are represented as plot before-after. The grey point represents the mean. *p<0.05, non-parametric paired test vs vehicle. Results in panel C are expressed as mean±SEM.*

Calcitriol inhibits PASMC proliferation partly via BMPR2 pathway

We analysed whether the BMPR2 pathway was involved in the antiproliferative effect of calcitriol in PASMC by using a gene silencing and a pharmacological approach. Forty-eight hours after targeted knockdown (siBMPR2), *BMPR2* mRNA expression was reduced by~65% as compared to a scramble siRNA (Figure 6A) leading to a significant increase in cell proliferation measured by the MTT assay (Figure 6B). Notably, loss of *BMPR2* gene partially decreased the antiproliferative effect of vitD in human cultured PASMC, analysed both by MTT (Figure 6C) and BrdU assays (Figure 6D). To confirm this result, in other set of experiments, we treated human control PASMC with the BMPR2 signalling inhibitor [27], DMH1(5 µmol/l).

We did not find that the inhibition of BMPR2 signalling by DMH1 increased PASMC proliferation (Figure 6E). However, and consistent with the *BMPR2* silencing experiments, the antiproliferative effect of calcitriol was also partly inhibited by DMH1 as measured by either MTT or BrdU assays (Figure 6E). Because BMPR2 can also signal via a noncanonical Smad-independent pathway involving MAP kinases, we also analysed the effects of calcitriol in the presence of MAP kinases inhibitors. The antiproliferative effect of calcitriol was not modified in the presence of the p38 MAPK inhibitor, SB203580 (10 µmol/l) or the ERK MAPK selective inhibitor, PD98059 (5 µmol/l) as analysed by either BrdU and MTT assays (supplementary figure 1A and 1B).

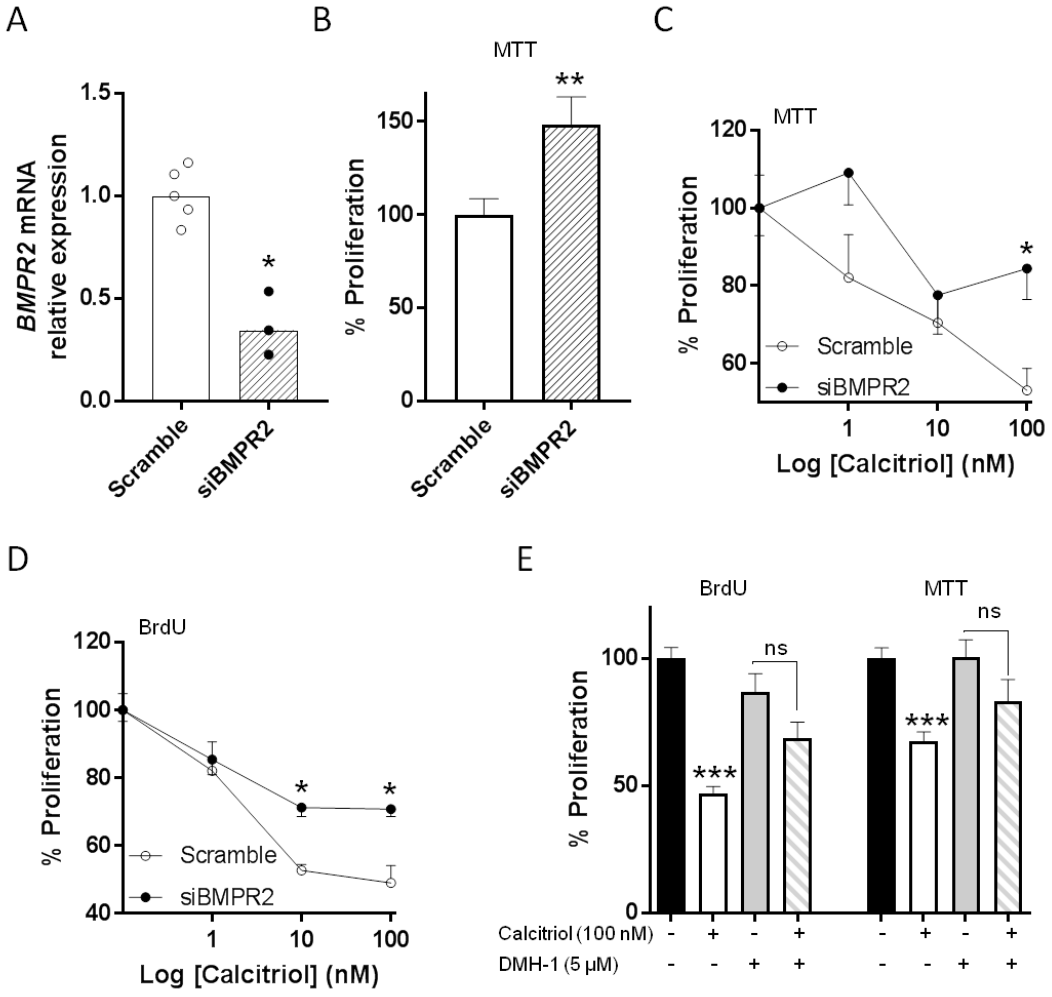

Figure 6. Calcitriol inhibits PASMC proliferation via BMPR2. *Human control PASMC were transfected with siRNA for BMPR2 (siBMPR2) or control siRNA (scramble). (A) BMPR2 mRNA expression assessed by qRT-PCR after 48 h post-transfection. Data is expressed as scatter plots and bars with medians, *p<0.05, Mann-Whitney test. (B) % of proliferation in silenced BMPR2 PASMC measured by MTT assay. Results are expressed as mean±SEM. *p<0.05, t-test vs scramble. (C) and (D) Effects of calcitriol (1-100 nmol/l) on proliferation in PASMC transfected with siBMPR2 or scramble, measured by MTT and BrdU assays, respectively. * p<0.05, two-way ANOVA, Bonferroni post hoc test, vs scramble. (E) % of proliferation in PASMC treated with calcitriol (100 nmol/l) and in the presence or absence of DMH1 (5 µmol/l), by MTT and BrdU assay; ***p<0.001 calcitriol vs vehicle (black column), two way-ANOVA.*

Calcitriol inhibits PASMC proliferation partly by via survivin downregulation

The antiproliferative effect-induced by calcitriol was also evaluated in human PASMC transfected with siRNA against *BIRC5*, the gene encoding survivin protein, or were incubated with the survivin inhibitor YM155. Forty-eight hours after targeted knockdown, *BIRC5* mRNA expression was reduced by~80% (Figure 7A) leading to a moderate decrease in cell proliferation measured by the MTT assay and a stronger effect as measured by the BrdU assay (Figure 7B). The MTT assay (Figure 7C) revealed that survivin silencing did not affect the antiproliferative effect of calcitriol. However, the results obtained from the BrdU cell proliferation assay (Figure 7D) showed that *BIRC5* knockdown markedly abolished the calcitriol induced-growth inhibitory effect of calcitriol in human PASMC. To confirm these results, we tested the growth inhibitory effect of survivin inhibitor, YM155. PASMC proliferation was blocked in a concentration-dependent manner by YM155 (supplementary figure 2A), with a half-maximal inhibitory concentration (IC_{50}) of approximately 20 nmol/l, assessed by MTT assay (left panel) and BrdU incorporation assay (right panel). In the presence of the YM155 at 20 nmol/l, the antiproliferative effect of 100 nmol/l of calcitriol was abolished (supplementary figure 2A). Likewise, we analysed the effect of 20 nmol/l of YM155 plus three different doses of calcitriol (1-100 nmol/l) on PASMC proliferation. Remarkably, the dose-dependent antiproliferative effect of calcitriol was strongly inhibited by the presence of survivin inhibitor, analysed by either the MTT (Figure 7E) or the BrdU assay (Figure 7F).

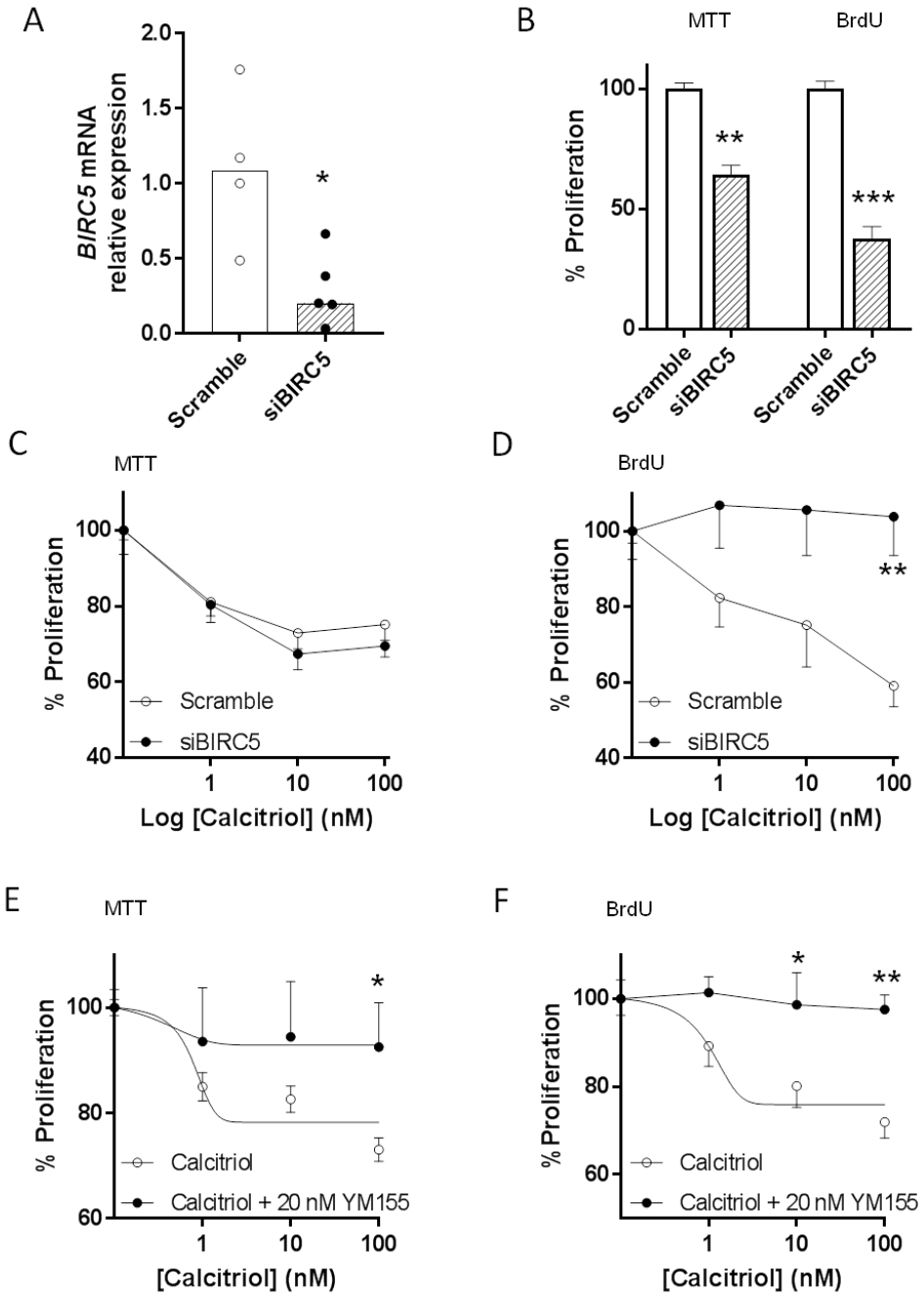

Figure 7. The antiproliferative effect of calcitriol is supressed in the presence of survivin inhibition. *(A) BIRC5 (gene encoding survivin) mRNA expression assessed by qRT-PCR after 48 h post-transfection with siRNA against BIRC5 (siBIRC5) or scramble siRNA. Data is expressed as scatter plots and bars, *p<0.05, Mann-Whitney test. (B) Proliferation of PASMC transfected with siBIRC5 or scramble, measured by MTT and BrdU assays. (C) Effects of calcitriol (1-100 nmol/l) on proliferation in PASMC transfected with siBIRC5 or scramble, measured by MTT and BrdU assays, respectively. (E) and (F) Effects of calcitriol (1-100 nmol/l) on proliferation in human control PASMC (n=3, in triplicate) and in combination with YM155 (20 nmol/l) for 48 hours, measured by MTT assay BrdU incorporation, respectively. Data are expressed as mean±SEM. *p<0.05 and **p<0.01 vs scramble or calcitriol, two-way ANOVA, Bonferroni test.*

The antiproliferative effects of calcitriol are preserved in *KCNK3* silenced PASMC

To analyse the role of *KCNK3* in the antiproliferative effect of calcitriol we transfected control human PASMC with *KCNK3* siRNA (n=5; supplementary figure 3A). This resulted in an increase of proliferation, measured by either MTT or BrdU assays, respectively (supplementary figure 3B). Notably, *KCNK3* silencing did not affect the antiproliferative effect of calcitriol, analysed by MTT and BrdU (supplementary figures 3C and 3D).

Effects of calcitriol on PAEC proliferation

We studied the effects of calcitriol (10-100 nmol/l) on proliferation of control PAEC and PAEC derived from patients with CTEPH, measured by MTT and BrdU incorporation assays. Calcitriol at 10 nmol/l increased cell growth in PAEC measured by MTT (supplementary figure 4A) and borderline by BrdU assay (One-way ANOVA, p=0.07, supplementary figure 4B) and in PAEC from CTEPH patients, measured by BrdU (supplementary 4C). By contrast, 100 nmol/l of calcitriol had no effect on cell growth.

Calcitriol has no relaxant effect in PA

We further evaluated the direct vasodilator effect of calcitriol in PA from control subjects were mounted in a wire myograph, pre-contracted with a cocktail of pulmonary vasoconstrictors: endothelin-1 (ET-1, 3 nmol/l), thromboxane A2 mimetic U46619 (30 nmol/l) and serotonin (5-HT, 3 µmol/l). Under these conditions, calcitriol (0.01-10 nmol/l) had no acute vasodilator effect (supplementary figure 5A).

DISCUSSION

In the present study we have studied the expression and location of VDR and the effects mediated by VDR in the pulmonary vasculature and its potential role in PAH. For the first time, we have provided evidence that: 1) VDR is expressed *in situ* in PA, mainly in PASMC, 2) it is downregulated in lungs and PASMC from PAH patients, 3) calcitriol treatment can rescue the expression of VDR, 4) it upregulates *KCNK3* and *BMP4* and downregulates survivin gene expression in PASMC and finally, 5) it exerts an antiproliferative effect in PASMC by modulating the survivin and the BMP signalling pathways. However, calcitriol had no antiproliferative effect in PAECs and had no direct vasodilator effect in human PA.

VDR is widely distributed throughout the small intestine from the duodenum to the ileum and the large intestine, specifically in intestinal epithelial cells to promote calcium absorption; and

in distal renal tubules to regulate calcium re-absorption[28]. VDR gene expression pattern in different tissues in the NCBI database is consistent with the fact that tissues with the highest VDR content are those associated with maintenance of calcium homeostasis. Apart from this group of organs, there is another group of tissues with lower, but considerable expression of VDR, including the lung. Previous studies have demonstrated that VDR is expressed in human bronchial epithelial cells [15, 16]. Immunofluorescence assay in control human lungs yielded the first evidence that VDR is also expressed in the PA. In addition, VDR is highly expressed in human cultured PASMC as compared to PAEC.

Previous studies from our group [6] and others [7-11] have demonstrated that vitD deficiency is much more frequent in PAH than in general population and, remarkably, low levels of total 25(OH)vitD are associated with poor prognosis. However, to date, none of these studies have explored the role of VDR in PAH. First, we analysed VDR in the publicly available whole transcriptome database GSE117261[26]. The analysis showed that VDR expression is downregulated in lungs from PAH patients. We also confirmed by PCR and Western blot that VDR expression is decreased in lungs from a cohort of Spanish PAH patients compared to lungs discarded from transplant donors. Because VDR is apparently highly expressed in pulmonary epithelial cells [28], we also examined VDR expression in human cultured PASMC and PAEC. *VDR* mRNA expression was significantly downregulated in cultured PASMC from PAH patients, but unchanged in PAEC. Notably, low levels of lung VDR is associated with several pulmonary diseases, including COPD [18] and idiopathic pulmonary fibrosis [19], and correlates with worse prognosis. Moreover, a lack of VDR in the pulmonary epithelial barrier increases the severity of acute lung injury (ALI) [20, 21]. Homozygous VDR-deficient mice present alterations in adherent junctions in the lung, suggesting that VDR may play an important role in maintaining pulmonary barrier integrity [17].

VDR gene can be regulated in a tissue-specific manner by a variety of factors, which include its own most active ligand, calcitriol [29]. Thus, there is a physiological positive feedback loop between the levels of calcitriol and VDR expression [30]. This autoregulation has been observed in a wide variety of cells [29, 31, 32]. It can be caused by increased VDR gene transcription, concordant with the presence of VDRE in the promoter region of VDR gene, and by stabilization of VDR, decreasing the rate of receptor degradation [30, 33]. Consistent with this view, our previous results showed that PAH patients presented severe deficit of vitD and we now show decreased lung VDR expression in these patients. At this point, the following step was whether calcitriol treatment would rescue the downregulated VDR expression in PAH-PASMC. Our results showed that VDR mRNA expression was strongly upregulated by calcitriol. Remarkably, VDR expression in PAH-PASMC reached similar levels compared to those in control PASMC after

48 h of calcitriol treatment. In the absence of calcitriol, VDR is uniformly located in the cytosol and nucleus, while after 48 h of calcitriol treatment, VDR is mainly translocated into the PASMC nucleus. Previous studies have observed in cancerous and vascular cells [34-36] that there is an equilibrium between nuclear and cytoplasmatic VDR [37, 38]. In the absence of ligand, there is a substantial proportion of VDR in the cytoplasm, and calcitriol induces gradually VDR translocation to the nucleus [37, 39]. In human cancer cells, a decrease in nuclear VDR expression may be an adaptive mechanism for cells that are tending toward aberrant growth [16, 40], while cytoplasmatic VDR in cardiomyocytes is closely associated to the t-tubules to promote rapid nongenomic effects such as maintenance of contraction and relaxation [35]. Our data is consistent with VDR translocation to the nucleus where it is expected to interact with DNA via specific VDRE in order to modulate gene expression. In fact, as discussed below, translocation is associated with changes in gene expression and reduced proliferation. Thus, the nuclear to cytoplasmic VDR ratio can be a potential marker of calcitriol activity [41].

The discovery of VDR in many tissues that do not participate in calcium and phosphorous homeostasis led to identify a wide variety of functions mediated by VDR of potential relevance in cardiovascular diseases, such as cell proliferation, differentiation and apoptosis, cell adhesion, oxidative stress, angiogenesis and anti-inflammatory activity [42]. Therefore, we studied some potential VDR-mediated effects of interest in PAH, including those on cell proliferation.

VitD metabolites have been demonstrated to alter cellular proliferation through multiple mechanisms. Calcitriol was shown to elicit antiproliferative effects in both normal and in pathologic situations, including cancer-derived cell lines and vascular smooth muscle cells (VSMC) [43-46]. Accordingly, we have found that calcitriol exerts antiproliferative effects in human PASMC *in vitro*. Notably, the inhibitory effect of calcitriol on cellular growth was also observed in PASMC from PAH patients. In fact, calcitriol treatment attenuated pulmonary vascular remodelling in an animal model of PAH [47]. Our results revealed that calcitriol rescues the expression of downregulated VDR in PASMC from PAH patients and induces its translocation into the nucleus to exert antiproliferative effects. Nuclear localization of VDR seems to be necessary to exert antiproliferative actions as reported in other cell types. For instance, in breast cancer, in the absence of ligand, accumulation of VDR in the cytoplasm promotes cell growth, in contrast to the antiproliferative nuclear action of the calcitriol-VDR complex [40].

Next, we analysed several possible targets of VDR, whose dysregulation are involved in PASMC proliferation in PAH. Loss of function mutations and downregulation of *KCNK3* (gene encoding TASK-1 potassium channel) are present in hereditable and idiopathic PAH and contribute to the increased pulmonary arterial vasoconstriction and PASMC and PAEC

proliferation, leading to pulmonary arterial remodelling [23, 48]. Few reports have demonstrated that *KCNK3* may be a target of VDR [49-51] and we have also identified a VDRE in the *KCNK3* gene promoter [12]. It must be highlighted that *KCNK3* expression and activity in VSMC seems to be almost lost under culture conditions (~75% reduction) [52]. Despite this, we found that calcitriol is able to significantly upregulate *KCNK3* mRNA expression in cultured PASMC from controls as well as from PAH patients. However, the antiproliferative effects of calcitriol were not affected by *KCNK3* inhibition suggesting that the upregulation of *KCNK3* does not contribute to the calcitriol-induced antiproliferative effect. Nevertheless, it is expected that the increase in *KCNK3* expression may enhance and improve TASK-1 function and thereby limiting PA vasoconstriction in PAH patients.

A misbalance between BMPRII and transforming growth factor-β (TGF-β) pathways is a well-known hallmark of PAH [53]. Loss of function mutation of *BMPR2* gene or downregulated or dysfunctional BMPRII signalling leads to aberrant PASMC proliferation [54, 55]. Accordingly, we found increased proliferation after *BMPR2* silencing in PASMC. Interestingly, our results indicate that loss of *BMPR2* gene or pharmacological inhibition of the canonical BMPRII pathway partially decreased the antiproliferative effect of calcitriol in human cultured PASMC, suggesting that the BMPRII pathway plays a role on the inhibition of cellular growth in PASMC induced by VDR. However, the inhibition of the non-canonical Smad-independent pathway of BMPR2 signalling with p38 or ERK MAPK inhibitors, did not affect the antiproliferative effect of calcitriol. The activation of BMPR2 signalling pathway may be mediated, at least in part, by the ligand BMP4, whose expression is increased after calcitriol treatment in human PASMC. Accordingly, some previous studies have identified that *BMP4* may be a target of VDR in VSMC [50, 56] and in other cell types [57, 58]. In the context of PAH, *BMP4* [59] and its antiproliferative effects via a canonical Smad-dependent pathway are decreased [60-62]. Therefore, those agents enhancing BMP/Smad signalling in PASMC can restore the growth-suppressive effects of BMP4. In vitD deficient rats we also found reduced *BMP4* and *BMP6* expression in the lung [12]. In addition, calcitriol treatment attenuated the upregulation of *Tgfβ2* in PAH-animals [47]. However, our *in vitro* data in control PASMC showed that calcitriol did not reduce *TGFβ* expression.

Survivin protein, encoded by the *BIRC5* gene, belongs to the inhibitor of apoptosis family. In the context of PAH, survivin is upregulated in PA from PAH patients and in experimental models of PAH [63-66]. Both *in vitro* and *in vivo*, inhibition of survivin induces PASMC apoptosis, decreases proliferation and increases Kv channel activity [63, 65]. Previous published studies have reported that calcitriol negatively regulates survivin, and this downregulation is essential to the antiproliferative property of calcitriol in several types of cancer cells [67-69]. Likewise, we have

found that calcitriol decreases *BIRC5* gene expression in control human PASMC. In addition, silencing *BIRC5* supressed the antiproliferative effect of calcitriol measured by BrdU incorporation, despite it did not affect viability measured by MTT. We confirmed these results using the YM155 compound [70], which blocks the expression of this protein via inhibition its promoter. Altogether the data indicate that calcitriol inhibits PASMC proliferation at least partly by the suppression of survivin.

Finally, we found that calcitriol did not induce an acute vasodilation in precontracted human PA, ruling out a regulation of pulmonary vascular tone by VDR though non-genomic mechanisms. However, this does not exclude long term effects of VDR controlling vessel tone. In fact, mice with endothelial specific *Vdr* gene deletion show endothelial dysfunction [71]. Moreover, calcitriol did not elicit an antiproliferative effect in PAEC.

In conclusion, PAH patients not only present severe deficit of vitD, but also a reduction in lung VDR. Calcitriol rescues VDR expression and induces an antiproliferative effect in PASMC. These data reinforce the view that vitD deficiency may contribute to the pathogenesis of PAH. From a mechanistic point of view, the antiproliferative effect of calcitriol involves an upregulation of the BMPRII pathway and an inhibitory effect on survivin pathway.

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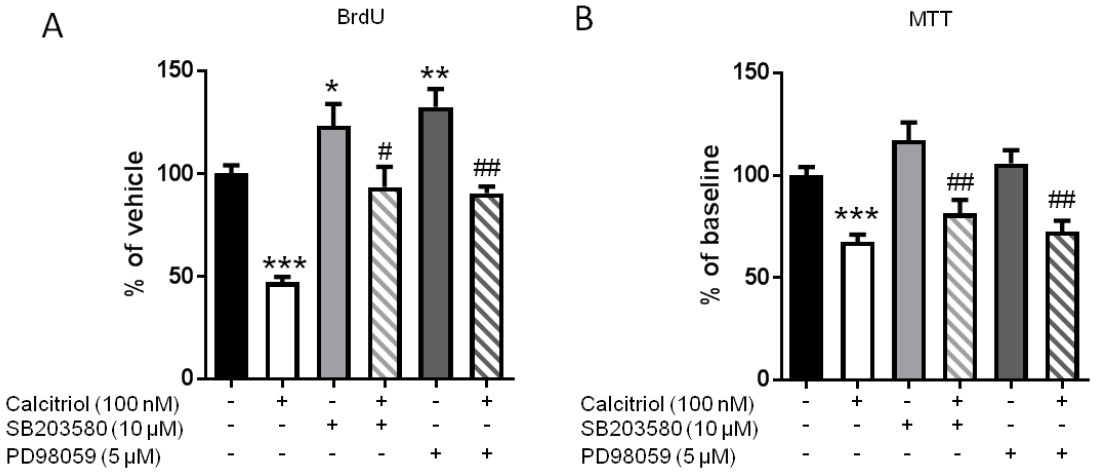
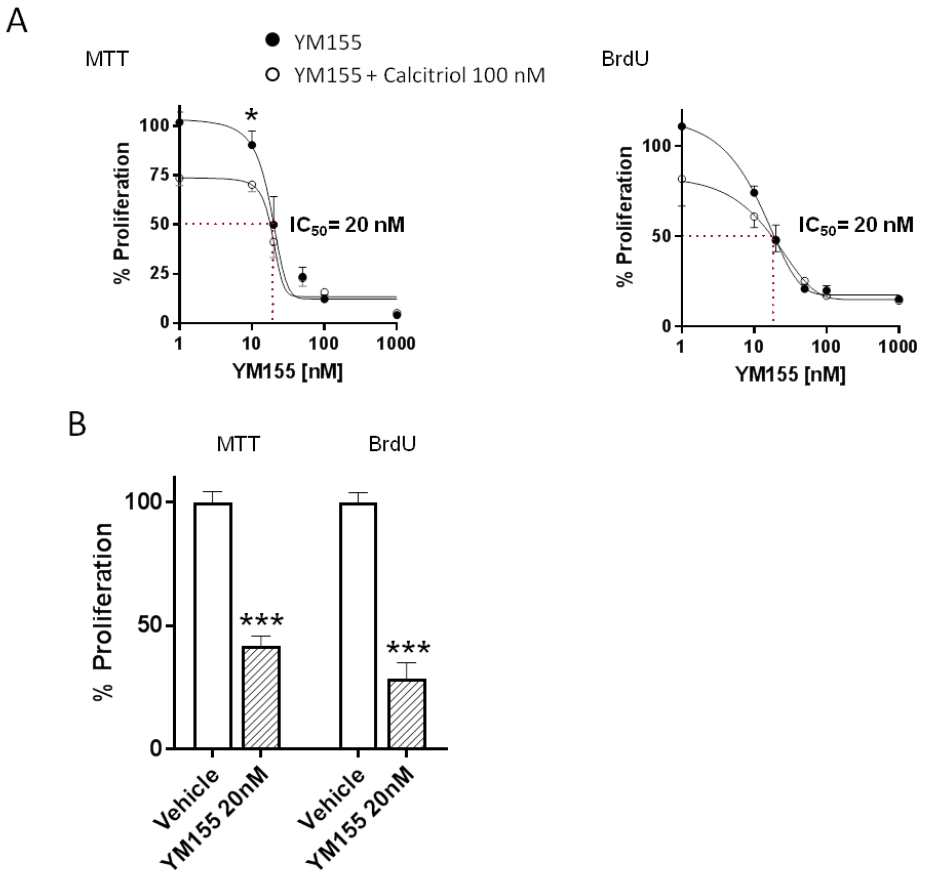
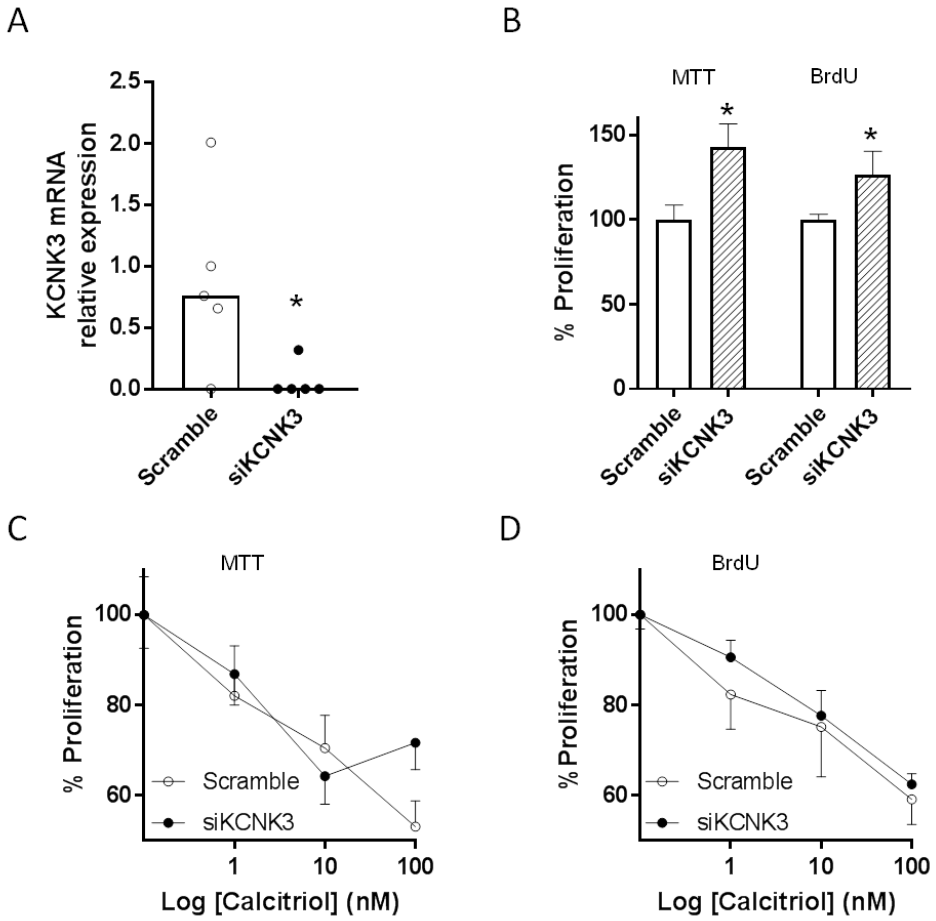
DATA SUPPLEMENT



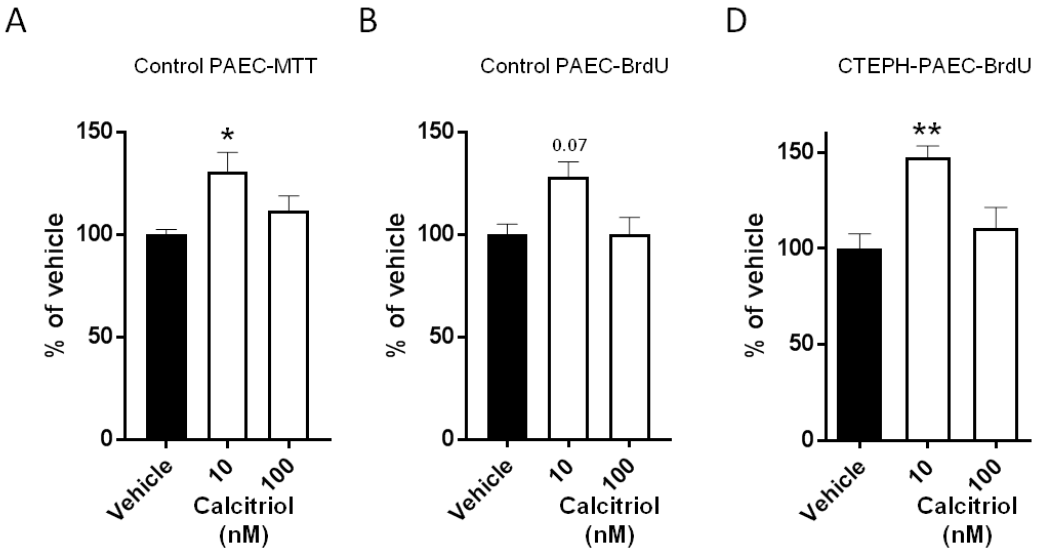
Supplementary figure 1. The antiproliferative effect of calcitriol is preserved in the presence of MAP kinase inhibitors. *Proliferation measured by BrdU (A) and MTT (B) in PASMC treated with calcitriol (100 nmol/l) and p38 inhibitor (SB203580, 10 µmol/l) or ERK inhibitor (PD98059, 5 µmol/l). *p<0.05; **p<0.01; p<0.001 vs vehicle (black column). #p<0.05 and ##p<0.01 vs SB203580 or PD98059; two-way ANOVA, Bonferroni test.*



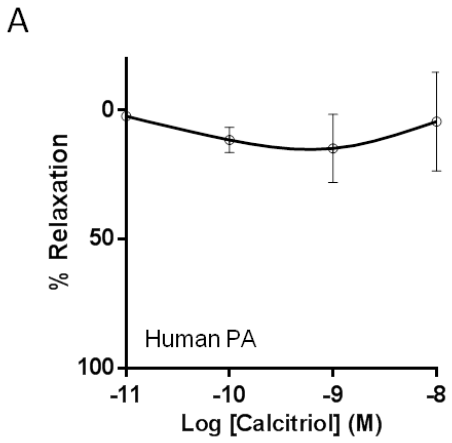
Supplementary figure 2. Inhibitory effect of YM155 on proliferation in combination with calcitriol on human PASMC. *A) PASMC treated with different doses of YM155 and calcitriol (100 nmol/l), assessed by MTT (left) and BrdU assays (right). Cell proliferation was calculated as a % of vehicle (DMSO), with IC50 values obtained from a logarithmic curve. *p<0.05 YM155 (1 nmol/l) vs YM155+calcitriol (100 nmol/l), one-way ANOVA. B) % PASMC proliferation treated with 20 nmol/l of YM155 measured by MTT and BrdU assays. Data are expressed as mean±SEM, ***p<0.001 vs vehicle.*



Supplementary figure 3. The antiproliferative effect of calcitriol is preserved after loss of KCNK3. *(A) KCNK3 gene expression in human control PASMC transfected with siRNA for KCNK3 (siKCNK3) or control nontargeting siRNA (scramble), assessed by qRT-PCR after 48 h. Data is represented as scatter plots and bars with medians, *p<0.05, Mann-Whitney test. (B) Effect on proliferation in human control PASMC of transfection with scramble or siKCNK3, by MTT (left) and BrdU assays (right). Results are expressed as mean±SEM. *p<0.05, t-test. (C) Calcitriol concentration-response curve (1-100 nmol/l) on proliferation in PASMC transfected with siKCNK3 or scramble, measured by MTT and by (D) BrdU incorporation assays.*



Supplementary figure 4. Calcitriol at 10 nmol/l increases human PAEC growth. *Proliferation in human control PAEC measured by MTT (B) and BrdU incorporation (C) and in PAEC from CTEPH patients by BrdU, after exposure to calcitriol (10-100 nmol/l) for 48 h. Results are expressed as mean±SEM. *, ** indicates p<0.05 and p<0.01 vs vehicle, One-way ANOVA, Bonferroni test.*



Supplementary figure 5. Calcitriol has no acute relaxant effects in PA. *Human control PA were mounted in a myograph and stimulated with a cocktail of submaximal concentrations of vasoconstrictors (3 nmol/l ET-1 + 30 nmol/l U46619 3µmol/l 5-HT) and calcitriol was added in a cumulative fashion. Data are means ± SEM of 4 arteries).*

Global overview

Vitamin D in pulmonary arterial hypertension

Throughout this Doctoral Thesis, we have deepened our understanding of the role of vitD in PAH. We have investigated, first, the vitD status in a cohort of PAH patients and its prognostic value in PAH. Secondly, we analysed the role of vitD deficiency as an aggravating factor at an early stage in an animal model of PAH. Thirdly, we tested the effects of vitD supplementation to optimal vitD levels as a treatment in an animal model of PAH associated with severe deficit of vitD. Fourthly, we examined the effect of vitD deficiency on the efficacy of sildenafil. And finally, we studied the location, expression, modulation and the antiproliferative effect of VDR in PASMC from controls and PAH patients.

Our data indicate that vitD deficiency is very prevalent in PAH patients. Furthermore, they show a decrease in various metabolites and/or proteins related to vitD metabolism, such as bioavailable 25(OH)vitD, DBP and VDR, i.e., lower reservoir capacity for vitD and reduced physiological activity and functionality. Moreover, we found that total 25(OH)vitD, rather than bioavailable or free 25(OH)vitD, is a potential biomarker of adverse outcomes in PAH patients. Severe deficit of total 25(OH)vitD in patients with PAH is associated with more advanced functional class, lower exercise capacity, higher risk of mortality, reduced survival, and limited efficacy to sildenafil. In fact, our results also support that severe deficit of vitD aggravates the progression of PAH in an animal model, exacerbating some of the hallmarks of the disease, such as pulmonary pressure, vascular remodelling, endothelial dysfunction and impairing the activity and expression of potassium channels, like TASK-1, strongly suggesting that vitD deficiency induces pulmonary vascular dysfunction.

The results of this Doctoral Thesis also support that, at least *in vitro*, treatment with calcitriol, the active form of vitD, restores VDR levels, enhances its nuclear localization and consequently its antiproliferative activity in PASMC. In addition, the recovery of vitD levels to an optimal range improves some of the pathological characteristics of the disease, such as enhanced pulmonary endothelial function, the response to the vasodilator sildenafil and the activity of TASK-1 potassium channel.

Loss of function mutations or downregulation of the *KCNK3* gene (encoding TASK-1 potassium channel) have been identified in hereditable and idiopathic PAH and this impairment is a key event in PAH pathogenesis, contributing to increased PA vasoconstriction and PASMC proliferation [22, 23]. We have demonstrated that vitD deficiency in control rats reduced TASK-1 currents in PASMC and downregulated *Kcnk3* gene expression. Moreover, in PAH rats, vitD deficiency further impaired TASK-1 activity. With the opposite approach, TASK-1 current was increased after recovering optimal vitD levels in vitD deficiency-PAH animals. In addition, we

have also identified a putative VDRE in the *KCNK3* promoter gene, which may account for the stimulatory effect of calcitriol on *KCNK3* expression in human PASMC. However, this *KCNK3* upregulation does not contribute to the calcitriol-induced antiproliferative effects.

Altogether, these results indicate that: 1) low levels of vitD may be implicated in the inhibition of TASK-1 activity; 2) vitD may be a novel factor enhancing TASK-1 currents in PASMC and 3) it is expected that the increase in the activity and expression of *KCNK3* may enhance and improve TASK-1 function and thereby limiting PA vasoconstriction in PAH.

Pulmonary endothelial dysfunction is a key early event in all forms of PAH. In fact, current effective pharmacological therapies in PAH patients target the three main dysfunctional endothelial pathways (NO, prostacyclin and endothelin-1) [3, 30-32]. First, we have found that chronic vitD deficiency in control animals reduced NO-dependent relaxation to acetylcholine and further worsened endothelial function in PAH animals. On the other hand, both calcitriol *in vitro* and chronic vitD treatment in vitD deficiency-PAH animals improved endothelial function, observed through an increase in the vasodilator response to acetylcholine and sildenafil, without changes in the response to riociguat. Contrary, calcitriol did not induce an acute vasodilation in precontracted human PA, suggesting that the regulation of pulmonary vascular tone by vitD-VDR is mediated through genomic mechanisms and ruling out possible non-genomics effects.

Our results do not allow us to explain the mechanism involved in the vitD deficiency-induced endothelial dysfunction or how vitD supplementation reverted this specific disturbance. Since the vasodilator effect of the sGC stimulator riociguat is independent of the NO availability, while the PDE5 inhibitor sildenafil potentiates the action of endogenous NO [60, 61], the increase in the relaxant responses to sildenafil and acetylcholine, but not to riociguat, suggest that vitD treatment is likely to raise the production and/or bioavailability of NO . Additionally, and in concordance with our results, *Kcnk3*-mutated rats show an impairment in acetylcholine- and sildenafil-PA relaxation, indicating that functional TASK-1 channel may be essential for NO- and sildenafil-induced vasodilation [35] . Therefore, the recovery of TASK-1 currents by restoring vitD levels in PAH animals might also explain the enhanced response to NO and sildenafil.

Another key hallmark of PAH is the thickening and PA obliteration due to an excessive and uncontrolled PASMC proliferation [3, 30]. In this Doctoral Thesis we have analysed several potential genes modulated by vitD and VDR and whose dysregulation are known to be involved in PASMC proliferation in PAH. First, we found that vitD deficiency increased PA muscularization and wall media thickness, and these effects were additive to those of PAH. However, restoration of vitD levels did not ameliorate vascular remodelling of distal PA in PAH rats.

The BMPR2 ligands BMP4 and BMP6, and the antiapoptotic factor survivin are well-known regulators of cell proliferation in PASMC [42, 43]. Chronic vitD deficiency upregulated lung survivin and downregulated *Bmp4* and *Bmp6*, which may explain the vitD-deficiency-induced PA muscularization. Accordingly, we have also found that calcitriol exerts antiproliferative effects in human PASMC *in vitro* by modulating the downregulation of survivin and the upregulation of BMPR2 signalling pathways. Altogether, these findings support the idea that vitD deficiency contributes to the exacerbated and uncontrolled PASMC proliferation, leading to pulmonary vascular remodelling.

In view of these results, vitD deficiency *per se* does not cause PAH, but it does cause some moderate pulmonary vascular disturbances. This is consistent with the fact that vitD deficiency is very prevalent worldwide while PAH is considered as a rare disease. On the contrary, we must also consider whether vitD deficiency might be a consequence of PAH. Because this disorder is a life-limiting disease, associated with fatigue and weakness, it is quite likely that PAH patients are less exposed to sunlight, and therefore, more susceptible to vitD deficiency. Nevertheless, it is also known that insufficient vitD levels lead to major physiological disturbances, such as vascular, respiratory, cardiovascular, immunological and metabolic alterations [124-126], which could contribute to the development and/or progression of PAH in predisposed patients or to worse outcomes in patients with existing PAH. Hence, it is plausible that vitD deficiency in combination with others risk factors could aggravate PAH. Except for idiopathic PAH, in all other forms of the disease, etiopathogenic factors are known to be involved, including mutations, systemic disorders, congenital heart defects, infections, drugs and toxins [42, 43]. Nevertheless, none of these factors by itself can trigger the disease, and a second hit(s) is considered necessary for PAH development. Therefore, considering our data, vitD deficiency may be one of these predisposing second hits.

Due to the high prevalence of vitD deficiency in PAH population, and that total 25(OH)vitD levels may be a prognostic factor, it may be appropriate to measure 25(OH)vitD regularly in these patients. In addition, it would be necessary to correct the insufficient 25(OH)vitD levels. Ethically, any individual with vitD deficiency, regardless of other comorbidities, should be treated with supplements. Currently, vitD supplements are indicated to prevent and treat bone diseases in any healthy or sick subject with moderate or severe vitD deficiency [158-160]. Thus, restoration of optimal vitD status would represent a very feasible, known, easy in clinical practice and economic strategy to improve the general health of these patients. We suggest that it may have additional benefits on the symptoms and the prognosis of PAH. However, whether the symptoms, quality of life and prognosis of PAH patients improve after restoring 25(OH)vitD levels remains to be analysed. To date, there is only a small prospective, uncontrolled trial, which enrolled PAH patients with vitD deficiency. Restoring vitD levels

improved 6MWD and right ventricular size but did not reduce mPAP and functional class [196]. The therapeutic use of vitD in PAH should be validated in randomized, controlled clinical trials. In recent years whether vitD supplementation provides a beneficial effect in cardiovascular diseases has been a matter of controversy. In most studies, baseline 25(OH)vitD levels were not considered, and patients were given the supplements even when they had normal levels. Therefore, most studies have missed important requisites for an intervention trial: the absence of the problem to be solved; in this case, vitD deficiency. New studies should recruit only patients with severe deficit of vitD, and thus also avoid the undesirable effects of over-supplementation with vitD, mainly hypercalcemia. VitD supplementation is most likely efficient only in deficient patients.

In addition to vitD deficiency, a high percentage of PAH patients show low levels of iron and ferritin. Currently, despite recovering optimal levels of iron, it has not been fully demonstrated clinical benefits in PAH [75-77]. The ESC/ERC Guidelines consider monitoring iron status regularly, at least once a year, and administrate iron treatment in patients with low ferritin plasma levels [4]. So far, vitD levels are not specifically addressed in these clinical guidelines.

As in other conditions, vitD may not be a panacea in PAH, but given its relatively wide safety margin, it may be a feasible, inexpensive and safe coadjuvant therapy. The findings of this doctoral thesis will open a new line of research with relevancy in the prognosis, prevention and treatment of this devastating disease. Further studies are required to clarify whether our findings have true clinical implications.

Vitamin D deficiency in PAH

↓ Total 25(OH)vitD

↓ Bioavailable 25(OH)vitD

↑ iPTH ↓ DBP ↓ VDR

Lower 25(OH)vitDlevels are associated with:

↓ Worse functional class

↓ 6MWD

↑ TAPSE

↑ BNP/NT-proBNP

↓ Response to sildenafil

↑ Risk of mortality ↓ Survival

VitD deficiency in PAH rats induce pulmonary vascular dysfunction:

↓ TASK-1 currents
↓ Response to ACh
↑ Response to 5-HT
→ ↑ Pulmonary vascular tone

↑ Wall media thickness
↑ % muscular arteries
↑ Survivin
↓ *Bmp4 / Bmp6*
→ Pulmonary vascular remodelling

↑ mPAP

Vitamin D/calcitriol treatment in PAH

Calcitriol treatment *in vitro* induces:

↑ Response to ACh in PA from vitD deficiency control rats

PASMC:
↑ *VDR* Nuclear translocation
↓
↑ *BMP4*
↓ *Survivin*
→ Antiproliferative effect
↑ *KCNK3* → VDRE in *KCNK3*

≠ No antiproliferative effect in PAEC

Recovering optimal 25(OH)vitD levels in vitD deficiency-PAH rats:

↑ Response to ACh
↑ Response to sildenafil
↑ Total K^+ currents
↑ TASK-1 currents
↑ Body weight
→ ↓ Pulmonary vascular tone

≠ Response to riociguat
Vascular remodelling
RV hypertrophy
mPAP

Conclusions

1. Vitamin D deficiency is very prevalent in PAH-patients and it is accompanied by secondary hyperparathyroidism. Total 25(OH)vitD, rather than bioavailable or free 25(OH)vitD, is a potential predictor of adverse outcomes in PAH patients. Low levels of total 25(OH)vitD are associated with worse prognosis, as indicated by a more advanced functional class, lower exercise capacity and higher risk of mortality. Moreover, plasma vitamin D-binding protein levels are decreased in PAH-patients, hence PAH patients show a lower reservoir capacity for vitamin D.

2. Vitamin D deficiency induces pulmonary vascular dysfunction. Vitamin D deficiency does induce several moderate but significant changes in pulmonary arteries characteristics of PAH such as increased muscularization, endothelial dysfunction, reduced TASK-1 activity and expression, increased Survivin protein and reduced Bmp4 and Bmp6 expression. Vitamin D deficiency in PAH-rats further increased mean pulmonary arterial pressure, worsened endothelial function, increased the hyperreactivity to the vasoconstrictor 5-HT, pulmonary artery muscularization, decreased TASK-1 current and further depolarized the PASMC membrane. Therefore, vitamin D deficiency aggravates most pathological features of PAH and strongly suggest that severe deficit of vitamin D may play a pathophysiological role in clinical PAH.

3. Restoring optimal vitamin D levels in vitD-deficiency-PAH rats, mimicking the condition of most PAH patients, improves endothelial function and the activity of TASK-1 channels, main hallmarks in PAH. Therefore, vitamin D may be a novel factor limiting the sustained pulmonary artery vasoconstriction in PAH. This positive impact of vitamin D was not sufficient to reduce mPAP and pulmonary vascular remodelling.

4. Vitamin D deficiency causes a poor vasodilator response to sildenafil, which can be reverted by restoring optimal vitamin D levels, but preserved response to riociguat in rats with PAH. The lower levels of 25(OH)vitD in non-responders to PDEi compared to responders suggest that vitamin D deficiency may cause insufficient response to PDEi in some patients with PAH.

5. Vitamin D receptor is expressed in the nucleus and the cytosol of human PASMC. Vitamin D receptor is downregulated in the lungs and PASMC from PAH patients. Calcitriol rescues vitamin D receptor expression, drives its translocation to the nucleus and induces an antiproliferative effect in PASMC by opposing the actions of survivin

and by the upregulation of BMPR2 signalling pathway. These data reinforce that vitD deficiency and reduced vitamin D receptor may contribute to the pathogenesis of PAH.

6. Taken together, the data suggest that vitamin D deficiency may be a predisposing factor leading to the development of PAH or to worse outcomes in patients with existing PAH. Therefore, it would be appropriate to measure 25(OH)vitD levels regularly in PAH patients and to correct it. Restoring optimal vitamin D levels in patients with severe deficiency would represent a very feasible, safe, easy, economic and promising coadjuvant therapy to improve the symptoms, quality of life and prognosis of PAH patients.

Future perspectives

The most remarkable findings of this Doctoral Thesis are that vitD deficiency is very prevalent in PAH patients and that vitD deficiency is a prognostic factor in PAH and a biomarker of poor response to sildenafil. The detrimental effect of vitD deficiency is also supported by studies in animal models. Based on our findings, we postulate that: 1) vitD deficiency plays a pathophysiological role in many patients with PAH, and 2) restoring vitD levels may be a therapeutic approach to improve the outcome of these patients. However, randomized clinical trials in vitD deficient PAH patients with vitD supplements compared with placebo are required to demonstrate or refute these two issues.

In the absence of a definitive evidence, from a clinical and ethical point of view we propose: 1) to monitor vitD status regularly in patients with PAH; 2) to correct vitD levels in those patients who do not have optimal levels, in order to improve their general musculoskeletal health with possible additional benefits on the symptoms and progression of PAH.

Related to the above mentioned, the results of this Doctoral Thesis also indicate that vitD deficiency leads to a reduced response to sildenafil *in vitro*. This suggest that restoring vitD levels should be an effective adjuvant therapy to PDE5 inhibitors in deficient patients, a possibility that remains to be analysed. This possibility could also be tested in animal models *in vivo*. Thus, an interesting translational study will be to analyse the combination of vitD plus sildenafil. For this purpose, rats with PAH with a severe deficit of vitD could be randomized into four groups: 1) continue on vitD-free diet; 2) receive a vitD treatment; 3) continue on vitD-free diet plus sildenafil administration and 4) receive a vitD treatment plus sildenafil administration and followed for at least 3 weeks.

The known risk factors for the development of PAH including mutations, drugs, toxins, infections or associated morbidities, by themselves are unable to induce the disease. In fact, only 0.5 to 30% of the patients carrying these factors do develop the disease. This suggest that other unknown factor(s), i.e. a second hit, might trigger the pathological condition. We speculate that vitD deficiency may be a novel predisposing second hit for PAH. Future research could address, for instance, the effect of the combination of vitD deficiency with other predisposing factors for PAH such as *BMPR2* or *TASK-1* mutations, congenital heart diseases, metabolic pathologies as diabetes, or HIV infections, among others.

All these perspectives would be aimed at deepening, providing answers and making progress in the prevention and treatment of patients with pulmonary hypertension and improving their quality of life.

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Annexes

Annex 1

Impact of nutrition on pulmonary arterial hypertension

nutrients

MDPI

Review

Impact of Nutrition on Pulmonary Arterial Hypertension

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Abstract: Pulmonary arterial hypertension (PAH) is characterized by sustained vasoconstriction, vascular remodeling, inflammation, and in situ thrombosis. Although there have been important advances in the knowledge of the pathophysiology of PAH, it remains a debilitating, limiting, and rapidly progressive disease. Vitamin D and iron deficiency are worldwide health problems of pandemic proportions. Notably, these nutritional alterations are largely more prevalent in PAH patients than in the general population and there are several pieces of evidence suggesting that they may trigger or aggravate disease progression. There are also several case reports associating scurvy, due to severe vitamin C deficiency, with PAH. Flavonoids such as quercetin, isoflavonoids such as genistein, and other dietary polyphenols including resveratrol slow the progression of the disease in animal models of PAH. Finally, the role of the gut microbiota and its interplay with the diet, host immune system, and energy metabolism is emerging in multiple cardiovascular diseases. The alteration of the gut microbiota has also been reported in animal models of PAH. It is thus possible that in the near future interventions targeting the nutritional status and the gut dysbiosis will improve the outcome of these patients.

Keywords: pulmonary hypertension; microbiota; vitamin C; vitamin D; iron; diet

1. Pulmonary Hypertension

The pulmonary circulation in healthy individuals is a high flow, low resistance circuit. It accommodates a similar cardiac output as the systemic circulation but with one sixth of its pressure. Normal mean pulmonary arterial pressure (mPAP) at rest is 14.0 ± 3.3 mmHg, with an upper limit of normal of 20 mmHg [1]. Pulmonary hypertension (PH) is due to a rise in pulmonary vascular resistance and mPAP. It is a chronic vascular disorder resulting in progressive right heart failure and eventually death [2,3]. A clinical classification categorizes PH into five groups according to their pathophysiological mechanisms, clinical presentation, hemodynamic characteristics, and treatment strategy [1,3]. Group 1, Pulmonary Arterial Hypertension (PAH) is also subclassified into idiopathic, familial, associated with other disorders or infections, or resulting from drug or toxin exposure [3,4]. The definition of PAH has been revised in the 6th World Symposium on Pulmonary Hypertension. PAH is now defined as a mPAP > 20 mmHg at right heart catheterization, normal left atrial pressure, and pulmonary vascular

resistance ≥ 3 Wood units [1]. In Europe, PAH prevalence is in the range of 15–60 subjects per million population and an incidence of 5–10 cases per million per year [5,6]. In addition to poor prognosis, with one- and three-year survival rates around 87% and 67%, respectively, limitations in functional status affect the patient's quality of life, daily life activities, and employment [7,8].

1.1. Etiology

Several genetic and environmental factors for the development and progression of PAH have been identified [3,4,9]. In the West, idiopathic PAH, i.e., without any familial history or known triggering factor, is the most common subtype (30–50% of all cases of PAH), followed by connective tissue disease-associated PAH, congenital heart disease-associated PAH, and heritable PAH [5]. Mutations in *BMPR2* (bone morphogenetic protein receptors type II) can be detected in approximately 70% of cases of heritable PAH and they are also identified in 10–20% of IPAH [10]. In addition, mutations in other genes related to BMPR2 signaling axis have been discovered [9]: *ACVRL1/ALK1* (Activin receptor-like kinase 1), *ENG* (endoglin), and *SMAD9* (decapentaplegic homolog 9) [9,11]. Mutations in the *KCNK3* gene, which encodes the potassium channel TASK-1 [12], and in *KCNA5,* which encodes the voltage-dependent potassium channel Kv1.5, have also been identified in PAH patients [13]. Numerous drugs and substances have been involved in the development of PAH, including anorexigens, selective serotonin reuptake inhibitors, interferons, antiviral therapies, chemotherapeutic agents, and tyrosine kinase inhibitors such as dasatinib [3,14]. Finally, PAH is also associated with other systemic disorders, such as connective tissue diseases and portal hypertension, and infections, such as HIV and schistosomiasis [3]. In summary, with the exception of idiopathic PAH, in all forms of the disease, there is a factor known to be involved in its etiopathogeny, including mutations, systemic diseases, congenital heart defects, infections, drugs, and toxins. However, none of them by itself can trigger the disease and the need for a second hit has been proposed. For instance, *BMPR2* mutations present low penetrance: only 42% of the women and 14% of the men carrying the mutation develop the disease [11,15]. Similarly, about 30% of patients with scleroderma and 0.5% of HIV patients develop it [16,17].

1.2. Pathophysiology

The main pathophysiological mechanisms of PAH are sustained vasoconstriction, endothelial dysfunction, pulmonary vascular remodeling, in situ thrombosis, and inflammation [2,18,19]. Sustained vasoconstriction and endothelial dysfunction are due to an altered production of endothelial vasoactive mediators. These include decreased vasodilator and antiplatelet factors such as nitric oxide (NO) and prostacyclin (PGI_2), and increased vasoconstrictors and/or prothrombotic factors such as endothelin-1 (ET-1), serotonin (5-HT), thromboxane (TXA_2), angiotensin II (Ang II), and diverse growth factors, which also contribute to a hyperproliferative and procoagulant state. Ionic remodeling is also a key feature of PAH. The downregulation of voltage potassium channels, notably Kv1.5 [20,21] and TASK-1 [22,23], results in a more depolarized membrane potential in pulmonary arterial smooth muscle cells (PASMC) in PAH patients, leading to increased intracellular calcium and consequently PASMC vasoconstriction and also PASMC proliferation. Excessive smooth muscle proliferation and resistance to apoptosis due to paracrine growth factors, dysregulation of BMPR2 signaling pathway, dysfunctional potassium channels, and rise of anti-apoptotic proteins, among other factors, lead to smooth muscle hyperplasia. These deranged processes culminate in the obliteration of the pulmonary artery by enlarged intima and media layers [18,24] and the formation of proliferating vascular structures called plexiform lesions [24,25]. Thrombotic events in situ are frequent in PAH and contribute to the narrowing of pulmonary arteries too [19]. Altered immune mechanisms also play a significant role in the pathogenesis of PAH. Pulmonary vascular lesions in PAH patients and animal models reveal a recruitment of inflammatory cells as T- and B-lymphocytes, macrophages, dendritic cells, and mast cells [2,18]. In addition, there is an abnormal circulating level of certain cytokines, such as IL-1β, IL-6, IL-17, TNF-α, and CCL5. Notably, some of these cytokines correlate with a worse prognosis in PAH patients [26].

1.3. Current Pharmacological Therapies

Over the last decades, intensive research on the cellular and molecular mechanisms and signaling pathways has provided a better understanding of the pathophysiology of PAH and consequently the identification of different pharmacological treatments. Unfortunately, a definitive cure does not exist for PAH. Currently, the five classes of therapies approved for PAH target the Ca^{2+} entry and the three main dysfunctional endothelial pathways: NO, prostacyclin, and endothelin-1 pathways [27,28]. Inhibitors of cyclic nucleotide phosphodiesterase type 5 (PDE-5), sildenafil and tadalafil, potentiate the action of endogenous NO and promote vasodilation [5,27,28]. Soluble guanylate cyclase (sGC) also acts in the NO signaling pathway catalyzing the transformation of GTP to cGMP. The sGC stimulator riociguat promotes the synthesis of cGMP favoring vasodilation and inhibiting cell proliferation. The action of riociguat is independent of the NO availability. Available prostacyclin-related therapies include synthetic (epoprostenol), prostacyclin analogs (treprostinil and iloprost) and the prostacyclin receptor agonist selexipag [27,28]. Endothelin-1 receptor antagonists (ERAs) include bosentan, macitentan, and ambrisentan [5,27,28].

Despite the current approved drugs as monotherapy have shown a favorable impact on clinical, functional, and hemodynamic outcomes, disease progression is frequently observed. At the 5th World Symposium of PH and based on the high level of evidence gathered from numerous randomized, controlled trials, the use of sequential combination therapy was proposed, at least in PAH patients with inadequate response to monotherapy, and possible first-line therapy in patients with advanced disease (New York Heart Association Functional Class III/IV). In addition, to achieve greater therapeutic response, currently, initial combination therapy at the time of diagnosis is recommended. Moreover, triple combination regimens are also considered in severe PAH, when double therapy fails [27,29].

1.4. Non-Pharmacological Therapies

In randomized controlled trials, exercise therapy improves exercise tolerance, functional capacity, and quality of life, with a positive impact on social, emotional, and psychological aspects [3,30]. Therefore, supervised exercise rehabilitation programs are recommended [3]. In addition, it is recommended that patients should avoid excessive physical activity that leads to distressing symptoms such as due to poor gas exchange or improper ventilation. Moreover, exercise programs are not well-stablished and present several limitations based on the gaps in the knowledge of the optimal method, intensity, and duration of the training [31].

Dietary modification is one of the first steps in the treatment of cardiovascular diseases. The routine treatment of systemic arterial hypertension involves dietary interventions for all patients including salt and alcohol restriction; increased consumption of vegetables, fresh fruits, whole grains, soluble fiber, fish, nuts, and olive oil; low consumption of red meat; and consumption of low-fat dairy products [32]. However, the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) Guidelines [3] have not established specific recommendations for dietary habits or nutrient supplementation for PAH.

Interestingly, associations between nutritional factors and PAH have recently been reported in both human epidemiological studies and animal models. Recently, it has been reported that multiple-target nutritional intervention with extra protein, leucine, fish oil, and oligosaccharides can be a new strategy to prevent the pathophysiological alterations such as cardiac and skeletal muscle hypertrophy in PAH [33].

Herein, we focus on the scientific evidence on how the deficit in iron and vitamins C and D as well as other dietary components such as flavonoids may affect the progression of PAH. Finally, the role of the gut microbiota and its interplay with the diet and the host immune system is emerging in multiple cardiovascular and respiratory diseases including PAH. Other dietary factors such as n-3 polyunsaturated fatty acids (PUFAs), vitamin E, melatonin, and coenzyme Q10 may theoretically have an effect in PAH but there is no experimental or clinical evidence to support it and they are not discussed herein.

2. Dietary Components with an Impact on PAH

2.1. Vitamin C

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin found in several fruits and vegetables. It is required for the activity of several enzymes, involved in tissue repair, important for the immune system function, and functions as an antioxidant. Severe deficit of vitamin C leads to scurvy, causing general weakness, anemia, skin hemorrhages, gum disease, and teeth loss [34,35].

Many studies have shown that oxidative stress is involved in cardiovascular disease [36]. Nitric oxide inactivation by reactive oxygen species is a key event in endothelial dysfunction associated to hypertension and atherosclerosis and other vascular pathologies [37]. On the other hand, oxidation of LDL in the endothelial wall makes these particles more atherogenic and allows them to accumulate in the artery walls [36]. This has led to the wide use of antioxidants including vitamin C to slow the progression of atherosclerosis. However, the meta-analysis of pooled data from randomized controlled trials have concluded that antioxidant vitamin supplementation has no effect on the incidence of major cardiovascular events, myocardial infarction, stroke, total death, and cardiac death [38].

Several case reports have shown that pulmonary hypertension is a complication of scurvy [39–42]. Elevated mPAP was reversible after the administration of ascorbate. Two possible mechanisms for the involvement of vitamin C deficiency in PAH have been proposed [41]. First, vitamin C increases the availability of endothelial NO that has vasodilatory and antiproliferative capacity [43]. Second, a deficiency of vitamin C can inactivate prolyl hydroxylases, the cellular oxygen sensors, uncoupling hypoxia-inducible factor (HIF) from oxygen control [44]. Uncontrolled HIF activity may lead to activation of pulmonary hypertensive mechanisms [45].

Whether moderate vitamin C deficiency rather than clinical scurvy, which is rare in Western societies, plays a role in PAH is unknown. Moreover, the effect of vitamin C supplements on PAH patients has not been well-addressed yet and there is only preliminary experimental evidence of its effectiveness. For example, a study in broiler chickens has shown that vitamin C reduced the incidence of PAH and the associated muscularization of pulmonary arterioles [46].

2.2. Vitamin D

Vitamin D is a fat-soluble vitamin that acts as a steroid hormone. It was discovered as an essential nutrient for the prevention of rickets. Although vitamin D may be obtained from diet, the main source is derived from endogenous synthesis in the skin under the influence of solar ultraviolet B radiation [47]. The inactive precursor synthetized in the skin or diet undergoes a two-step activation process to become biologically active. The first step is the 25-hydroxylation in the liver by CYP2R1 resulting in 25-hydroxyvitamin D_3 (25(OH)D_3), also named calcidiol, which has partial activity. The second hydroxylated metabolite is the active 1α, 25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), also called calcitriol, by the 1α-hydroxylase enzyme or CYP27B1 mainly in the kidney [47,48]. Although calcitriol is the active metabolite of vitamin D, calcidiol is the best circulating biomarker of vitamin D status because the calcitriol half-life is shorter than that of calcidiol [47,48]. Calcitriol exerts its functions through the vitamin D receptor (VDR). Similar to other steroid receptor family members, VDR acts as a transcription factor [49]. VDR binds calcitriol with high affinity and specificity and then heterodimerizes with the retinoid-X receptor (RXR). After that, the VDR–RXR complex interacts with the vitamin D response elements on the promoter DNA region of target genes, resulting in changes in gene expression [48,50]. VDR regulates the expression of mRNAs as well as several miRNAs, indirectly regulating the expression of other genes [51].

There is no clear consensus on the definition of vitamin D deficiency; the optimum levels and the dietary requirements are uncertain [52,53]. However, even using conservative thresholds, nowadays, there is a pandemic of vitamin D deficiency [54]. The principal causes of low 25(OH)D_3 levels are inadequate sun exposure and/or reduced dietary intake [54].

Classically, vitamin D deficiency was related to bone diseases. Currently, because of VDR is found is many tissues, such as immune and cardiovascular cells, vitamin D deficiency has also been related to infection, cancer, and respiratory and cardiovascular diseases [53,55,56]. In fact, vitamin D deficiency has been associated with increased all-cause and cardiovascular mortality [57,58]. The discovery of VDR in many tissues that do not participate in calcium and phosphorous homeostasis led to identify a great variety of functions mediated by VDR, such as cell proliferation and differentiation, immunomodulation, and intracellular metabolism, among others [48].

In the context of PAH, there is some basic and clinical evidence suggesting a role for vitamin D in the pathophysiology of the disease. VDR was identified in vascular cells, including endothelial and smooth muscle cells. It is involved in numerous processes of potential relevance in cardiovascular diseases, such as cell proliferation, differentiation, and apoptosis; cell adhesion; oxidative stress; angiogenesis; and immunomodulatory and anti-inflammatory activity [53]. Therefore, it is assumed that vitamin D levels may affect the development of PAH.

To clarify whether vitamin D levels could be involved in PAH progression, Tanaka et al. treated PAH rats with a diet containing 10;000 UI/kg of cholecalciferol [59]. Notably, in this study, they found that vitamin D supplementation in PAH rats improved survival and attenuated some typical features in PAH such as right ventricle remodeling, assessed by Fulton index (ratio of right ventricle weight to left ventricle plus septum weight), and medial thickness of muscular pulmonary arteries. Despite these benefits of vitamin D, cholecalciferol treatment did not decrease pulmonary artery pressure [59]. Moreover, in an in vitro setup, calcitriol treatment inhibited the hypoxia-induced proliferation and migration in rat pulmonary artery endothelial cells (PAEC) via miR-204/TGFβ/Smad signaling pathway. Specifically, calcitriol suppressed the expression of Tgfbr2, α-SMA, and Smad7 and induced miR-204, p21, and Smad2 expression [60]. In the same study, similar results were found in an in vivo rat model. Remarkably, intraperitoneal calcitriol administration (20 mg/kg) partly reversed the rise in mPAP and Fulton index induced by three weeks of hypoxia [60].

In the clinical arena, Ulrich et al. showed that secondary hyperparathyroidism is highly prevalent in PAH patients [61]. Physiologically, decreased serum 25(OH)D_3 results in increased parathyroid hormone (PTH) levels in order to maintain adequate serum calcium concentrations. Therefore, low vitamin D status in PAH patients could be the reason for the elevated PTH. Later, epidemiological studies demonstrated that vitamin D deficiency is quite prevalent in PAH patients [59,62,63]. In the prospective study carried out by Demir et al., PAH patients presented much lower vitamin D levels (median of 6.79 ng/mL), considered as severe deficit of vitamin D (<10 ng/mL of serum 25(OH)D_3), than controls (18.76 ng/mL) [62]. In line with this result, Tanaka et al. found that, in a cohort of PAH patients, 39 out of 41 (95.1%) presented vitamin D insufficient and 25 patients (61%) showed deficient levels [59].

The relationship between vitamin D deficiency and PAH prognosis was evaluated. Serum 25(OH)D_3 levels were negatively correlated with mPAP assessed by right heart catheterization, and a significant positive correlation with cardiac output was found [59]. The potential benefits of vitamin D replacement on clinical outcomes has been also studied [63]. Twenty-two PAH patients were enrolled in a prospective uncontrolled longitudinal study. All PAH patients received cholecalciferol at a dose of 50,000 IU weekly for three months. In addition to the rise of serum 25(OH)D_3 levels from 14 ± 9 to 69 ± 31 ng/mL, remarkably, vitamin D supplements improved the 6-min-walk-distance (6MWD) test by around 80 m and right ventricle size. Mean PAP estimated by echocardiography was reduced from 79 ± 25 to 69 ± 23 mmHg but this effect did not reach statistical significance. Pro-BNP (pro-Brain Natriuretic Peptide) and functional class were also unchanged after vitamin D therapy [63].

All these data point to beneficial effects of vitamin D in PAH. However, the therapeutic use of vitamin D in this context has not been validated in randomized clinical trials. Nevertheless, given the high prevalence of vitamin D deficiency associated to PAH, it seems reasonable that serum vitamin D levels should be regularly assessed in these patients. Vitamin D supplements should be used to prevent bone diseases in any subject showing moderate or severe deficiency. Whether the symptoms,

quality of life, and prognosis of patients with PAH improve after restoring vitamin D levels is unclear. Vitamin D supplementation has been used in other conditions. For instance, vitamin D supplements succeeded in respiratory diseases, decreasing the incidence of asthma [64] and chronic obstructive pulmonary disease (COPD) [65] exacerbations in patients with baseline 25(OH)D_3 levels lower than 25 nmol/L [65]. On the contrary, vitamin D supplementation has failed in other pathologies. In several of these latter studies, baseline vitamin D levels have not been taken into account [66–68].

In view of these results, it is plausible that vitamin D deficiency in combination with others risk factors could aggravate PAH. Vitamin D deficiency per se does not cause PAH. This is consistent with the fact that vitamin D deficiency is very prevalent in the population [54] while PAH is a rare disease. Therefore, further research is necessary to investigate the harmful effects of vitamin D deficiency in the pathogenesis of PAH and the efficacy and safety of vitamin D treatments.

2.3. Iron

Iron is essential in several physiological processes, including oxygen delivery and energy metabolism. In fact, about 70% of iron is bound to hemoglobin and around 5–10% is found in myoglobin. Serum ferritin is the most specific indicator used in laboratories for evaluating iron stores. Ferritin levels below 30 ng/mL are considered iron deficiency with or without anemia [69,70]. Circulating soluble transferrin receptor levels is another biomarker of iron deficiency. Iron deficiency is the most common cause of anemia worldwide and it is particularly common in specific chronic diseases such as heart failure or chronic renal diseases [71].

Recent data indicate that iron deficiency is also prevalent in patients with idiopathic PAH (IPAH) and it correlates with disease severity [72–74]. In fact, anemia is also an indicative of poor prognosis [75,76]. For the first time, Ruiter et al. [73] reported that around of 40% IPAH patients present iron deficiency and it is associated with decreased exercise capacity, assessed by the 6MWD test without anemia. Similar results were found by Yu in patients with PAH associated with congenital heart disease [77]. The significantly decreased 6MWD suggests that iron is essential in maintaining exercise performance. The authors speculated that iron deficiency might impair oxygen transport and delivery and finally disturb muscle oxygen homeostasis. Consequently, the clinical manifestation is shorter 6MWD. Interestingly, restoring iron levels in patients with chronic left heart failure significantly improves 6MWD and New York Heart Association (NYHA)-Functional Class [78,79].

Although epidemiological data show iron deficiency in PAH, the physiological contribution of iron in PAH is unknown. Few studies have been carried out in this context [80–83]. Variation in iron availability without anemia can affect pulmonary vascular tone. Intravenous infusion of iron attenuated the increased in mPAP in response to sustained hypoxia in 16 healthy volunteers with normal iron levels [83]. Likewise, acute iron depletion exacerbates PAP and pulmonary vasoconstrictive response to hypoxia condition [83]. In line with these results, in individuals exposed to high altitude, PAH may be attenuated by iron supplementation [84].

After four weeks of iron deficient diet, rats present vascular remodeling in resistance pulmonary arteries and PAH. These vascular changes were accompanied by activation of HIF, STAT3, and mitochondrial dysfunction. In addition, in this study, mPAP and pulmonary vascular muscularization was reversed by intravenous iron therapy [80]. Transferrin-1 receptor (TfR1) knock-out mice show protection against the development of hypoxia-induced PAH. Similarly, downregulation of TfR1 in vitro also inhibits human PASMC proliferation [85]. Moreover, recently, Lakhal-Littleton et al. demonstrated that intracellular iron deficient in PASMC induces PAH in mice via increasing expression of ET-1 [86].

The cause of the increased prevalence of iron deficiency in PAH is not completely clear. Iron deficiency can be related to reduced intake, impaired uptake, or increased loss of iron. Some authors postulated that the predominance of PAH in women vs. men could be due to a higher prevalence of iron deficiency in premenopausal women compared to postmenopausal women and men [77,87,88]. However, ferritin levels and circulating soluble transferrin receptor levels did not differ with gender or

age in a large cohort of IPAH patients [72,73]. On the other hand, it is interesting that only a small proportion of IPAH responded to oral iron therapy, suggesting that, at least in these group of patients, a disturbance in iron absorption could be responsible for iron deficiency [73,89]. In line with this theory, elevated hepcidin levels were found in IPAH patients [72]. Hepcidin is a hormonal inhibitor of the intestinal absorption of dietary iron synthesized by the liver, which is elevated in inflammation [90]. However, plasma hepcidin concentration did not correlate with IL-6 levels, suggesting that, at least in these cohorts of IPAH patients, raised hepcidin levels were not due to inflammation. Of particular interest is BMP signaling. In vitro, *BMPR2* downregulation by a short interfering RNA increased hepcidin production. Rhodes et al. speculated that BMPR2-heritable PAH might be associated with more severe iron deficiency due to increased hepcidin levels [72].

All this evidence suggests that intravenous iron replacement could be a potential treatment in PAH patients [91,92], improving hemodynamic and clinical outcomes.

2.4. Flavonoids and Other Polyphenols

Polyphenols are a large group of plants metabolites commonly present in the human diet, specifically in vegetables, fruits, and beverages. Flavonoids comprise the major group of polyphenolic compounds. They are chemically characterized, sensu stricto, by the presence of a skeleton of 2-phenyl-4H-1-benzopyrane [93]. Isoflavonoids, neoflavonoids, chalcones, and aurones are related compounds often considered flavonoids as well. Other important polyphenols include stilbenoids.

Many studies have analyzed the influence of polyphenols in human heath [94], and more especially its positive role against cardiovascular diseases [95,96]. In addition to their antioxidant action, they also present vasodilator, antithrombotic, antiapoptotic, anti-inflammatory, hypolipidemic, and antiatherogenic effects, associated with decreased cardiovascular risk [96]. The flavonoids present in fresh fruits, vegetables, and wine are considered major contributors to the antihypertensive effects of these foodstuffs. In particular, the effects of the flavonoid quercetin, the isoflavonoid genistein, and the stilbenoid resveratrol have been studied in animal models of PAH and are reviewed herein.

Resveratrol is found in red wine, grapes, and berries. This polyphenol has been shown to attenuate right ventricular systolic pressure and pulmonary artery remodeling in monocrotaline-induced PAH in rats [97]. Moreover, this study demonstrated that resveratrol treatment (25 mg/kg per day) improved pulmonary endothelial function, assessed by increased eNOS expression, and decreased oxidative stress due to decreased of NADPH oxidase activity. Resveratrol also reduced the inflammatory cytokines IL-1β, IL-6, and TNFα, and inhibited PASMC proliferation [97]. Therefore, resveratrol exerted anti-oxidant, anti-inflammatory, and anti-proliferative effects, reducing the main hallmarks of PAH. It was speculated that ROS scavenging mediated by resveratrol may be the central process of these pleiotropic actions [98]. Several experiments have been performed to elucidate the underlying mechanisms. Chen et al. found that in vitro resveratrol treatment attenuated the hypoxia-induced proliferation in human PASMC by the inhibition of arginase II. The inhibitory effect of resveratrol on arginase II was PI3K-Akt signaling pathway-dependent [99]. Similar results were found in rat PASMC [100], in hypoxic pulmonary hypertension rats [101], and in monocrotaline-induced PAH [102].

Quercetin is probably the most widely distributed in foods and best studied flavonoid. Multiple studies have highlighted its biological activity to reduce arterial blood pressure in both human and experimental systemic hypertension [103,104]. Several animal models have also been used to examine the protective effect of quercetin in PAH. The first report analyzed the effects of quercetin as a preventive strategy for PAH (100 mg/kg from the day after monocrotaline infusion) [105]. Consecutively, our group investigated the therapeutic role of quercetin in PAH induced by monocrotaline in rats (10 mg/kg once daily from Day 21 after PAH was established) [106]. In both studies, the authors found that quercetin administration significantly alleviated mPAP, right ventricular hypertrophy, and pulmonary artery remodeling. Furthermore, quercetin treatment significantly increased survival in monocrotaline rats. However, classic biomarkers of PAH, such as endothelial dysfunction, pulmonary artery hyperresponsiveness to 5-HT, and downregulation of BMPR2 and Kv1.5, were unaffected by

quercetin [106]. These were unexpected results because quercetin has been widely reported to improve endothelial function in systemic arteries in in vivo and in vitro experiments [104,107]. Our group also demonstrated that quercetin exerted vasodilator effect in isolated pulmonary arteries, induced apoptosis and inhibited cell proliferation in PASMC [106]. The mechanism involved in the antiproliferative effects in both PASMC and endothelial cells seem to involve AKT [106,108,109], FOXO1-mTOR [110], and altered Bax/Bcl-2 ratio [108,111].

Genistein is an isoflavone abundant in soybeans. It has been widely used as a phytoestrogen substitute for hormone replacement therapy in postmenopausal women [112]. Genistein consumption is thought to reduce the incidence or severity of cardiovascular disease and of some forms of cancers [113]. It is a wide spectrum tyrosine kinase inhibitor (TKI) and it is well-known that tyrosine kinase inhibitors play an important role in the control of pulmonary vascular tone. Genistein can behave as an antioxidant and improves endothelial function in systemic and pulmonary arteries from several models of cardiovascular disease, through increasing endothelial NO synthase levels, restoring NO-mediated PA relaxation, reducing vascular superoxide production or decreasing angiotensin II receptor [114–116]. The vasodilator effect of genistein has also been studied in isolated pulmonary arteries precontracted by 5-HT [21] and ET-1 [117]. The activation of 5-HT_{2A} receptors inhibits K_V currents, and genistein treatment prevented this effect in rat PASMC [21]. In PA from chronic hypoxia rats, the contraction induced by ET-1 appeared to be mediated by the activation of tyrosine kinase, and genistein reduced the ET-1-induced response [117]. All these effects make genistein a potential therapy for PAH. In the rat model of PAH induced by monocrotaline, genistein both prevents [118] and reverses [116] the increased PAP. Moreover, genistein significantly improved pulmonary vascular remodeling, right ventricular function, and survival. It also inhibited human PASMC proliferation in vitro [116]. In addition, genistein also ameliorated pulmonary hemodynamics and vascular remodeling in a rat model of hypobaric hypoxia [119]. Some authors suggested that the mechanism underlying genistein-improved main characteristics of PAH is mediated through the improvement of PI3K/Akt/eNOS signaling pathway [119,120]. In addition, genistein also potently attenuates hypoxia-induced hypertrophy of PASMC through estrogen receptor and β-adrenoreceptor signaling [121].

2.5. Microbiota

The human gut is a bacterial ecosystem that harbors >100 trillion microbial cells and presents a symbiotic relationship with the host. Gut microbes provide help with digestion, promote gut immunity, and prevent the colonization of pathogens, while the host supplies them with a favorable environment for survival. A healthy gut microbiome is characterized in terms of diversity and richness as well as its stability and resistance to any perturbation. In contrast, gut dysbiosis is any disruption of the normal balance between the gut microbial community and the host, which can result in several diseases [122]. Gut dysbiosis is typically characterized by a lower diversity and richness of the microbial communities, an increase in Firmicutes to Bacteroidetes ratio (F/B), and altered short chain fatty acids (SCFA) producing bacteria, with an increase in lactate-producing bacteria and a decrease in acetate- and butyrate-producing bacteria [123,124]. In recent years, a growing body of evidence points to a relationship between gut dysbiosis and many diseases, including essential hypertension [124,125], obesity [126,127], inflammation [128] neurologic disorders [129], and pulmonary hypertension [130].

The diet is a critical regulator of the composition and function of the microbiota [131]. Multiple studies have focused on the effects of macronutrients (fat, carbohydrate, and protein) on the gut microbiome. Other dietary components such as soluble or insoluble fibers may be important as well [132,133]. Moreover, several food components are substrates for bacterial enzymes. These enzymatic processes lead to the production of other byproducts which can be absorbed in the gut. Importantly, SCFAs, particularly butyric and acetic acid, which derive mainly from the bacterial fermentation of fiber, are considered to promote cardiovascular health. In contrast, trimethylamine-N-oxide (TMAO), a metabolite produced by the gut microbiota from choline, betaine, and carnitine, which are abundant in meat, eggs, and fish, is associated with excess risk of heart disease [134].

In addition, it has been reported that some dietary components such as sweeteners, minerals, and vitamins can modify the microbiota. Remarkably, some of the nutrients with an impact on PAH progression, as described above, such as iron and vitamin D deficiency as well as quercetin and resveratrol significantly affect the intestinal microbiota [135–137]. Therefore, besides the aforementioned mechanisms of action of these dietary components, the changes in the gut microbiota may also be responsible of the actions of iron, vitamin D, or polyphenols. On the contrary, the composition of microbiota may affect the absorption of calcium, phosphate, iron, and zinc. Moreover, in addition to dietary sources of water-soluble vitamins, the microbiota can also synthetize some of these vitamins [132].

The role of the diet on the microbiome in the context of PAH is not known. However, it could be speculated that part of the effects of the dietary factors mentioned above in PAH might be due to changes in the microbiota. We demonstrated for the first time that there are several changes in the gut microbiota in PAH [130]. In a rat model of PAH induced by a single dose of Sugen5416 plus chronic hypoxia for two weeks, we found two main hallmarks of gut dysbiosis: a three-fold increase in F/B ratio, driven by a decrease in all Bacteroidetes families in PAH animals (2–10-fold decrease) and no changes in Firmicutes abundance. Furthermore, feces from PAH rats present a decreased in acetate-producing bacteria, accompanied by a reduced serum acetate, without changes in butyrate and lactate producing bacteria [130]. In contrast, we did not find global differences in microbial diversity and richness, as happened in other diseases [124]. Although this study is preliminary, it indicates that the abnormalities in the gut microbiota observed might play a pathophysiological role in the development and/or progression of PAH, rather than being a consequence. Likewise, Wedgwood et al. also suggested that intestinal dysbiosis may impact on distal organs including the lung, contributing to the development of PH [138]. In this study, rat pups with PH induced by postnatal growth restriction (PNGR) present gut dysbiosis and the probiotic treatment attenuates PNGR-induced PH. Considering these results, the authors suggested that PH is in part driven by the alteration of the gut microbiome [138].

It is tempting to speculate that changes in intestinal microbiota and circulating microbial products can contribute to PAH. Thenappan et al. suggested that gut dysbiosis might be involved in perivascular inflammation in the early development of PAH [139]. Gut dysbiosis can result in increased gut permeability, allowing bacteria and/or bacterial products translocation, with an increase in plasma bacterial lipopolysaccharide (LPS), the main ligand for toll-like receptor 4 (TLR4). TLR4 activation has been implicated in the pathogenesis of PAH [140]. Ranchoux et al. demonstrated that bacterial translocation occurs in PAH, suggesting a gut-lung cross-talk, in which TLR4 antagonists are plausible to be effective at disrupting this circle [141]. Gut dysbiosis also produces a pro-inflammatory environment, increasing IL-17 secretion and a downregulation of Treg cells [142]. Likewise, an increase in Th17 cells and a deficiency in normal Treg cells are observed in PAH patients, promoting vascular remodeling [26,143]. In addition to platelets, serotonin (5-HT) is also stored and produced in enterochromaffin cells. Thus, gut microbiota plays a key role in regulating 5-HT levels at colon and serum. Notably, clinical and experimental PAH showed elevated serum 5-HT levels. It is well-known that 5-HT promotes pulmonary artery remodeling, PASMC proliferation, and constriction of pulmonary arteries through the 5-HT_{1B} receptor [21,144].

3. Conclusions

Although there have been important advances in the knowledge of the pathophysiology of PAH, it remains a debilitating, limiting, and rapidly progressive disease. Targeted nutritional and lifestyle interventions could have a great clinical importance (Figure 1). Vitamin D and iron deficiency are worldwide health problems of pandemic proportions. Notably, these nutritional alterations are largely more prevalent in PAH patients than in the general population and there are several pieces of evidence suggesting that they may trigger or aggravate the disease progression. However, to date, most of this evidence is based on observational studies, animal models, and small series of uncontrolled trials.

Therefore, robust randomized clinical trials are required to establish cause–effect relationships. In the meantime, it seems reasonable to study the nutritional status of all PAH patients with particular emphasis on vitamins C and D and iron. Severe nutritional deficiencies leading to scurvy, osteoporosis, or ferropenic anemia must be corrected using the appropriate supplements. Based on the above discussed evidence, the correction of these nutritional defects may be expected to have additional positive impact on the severity of the disease, the quality of life, and the prognosis of the patients.

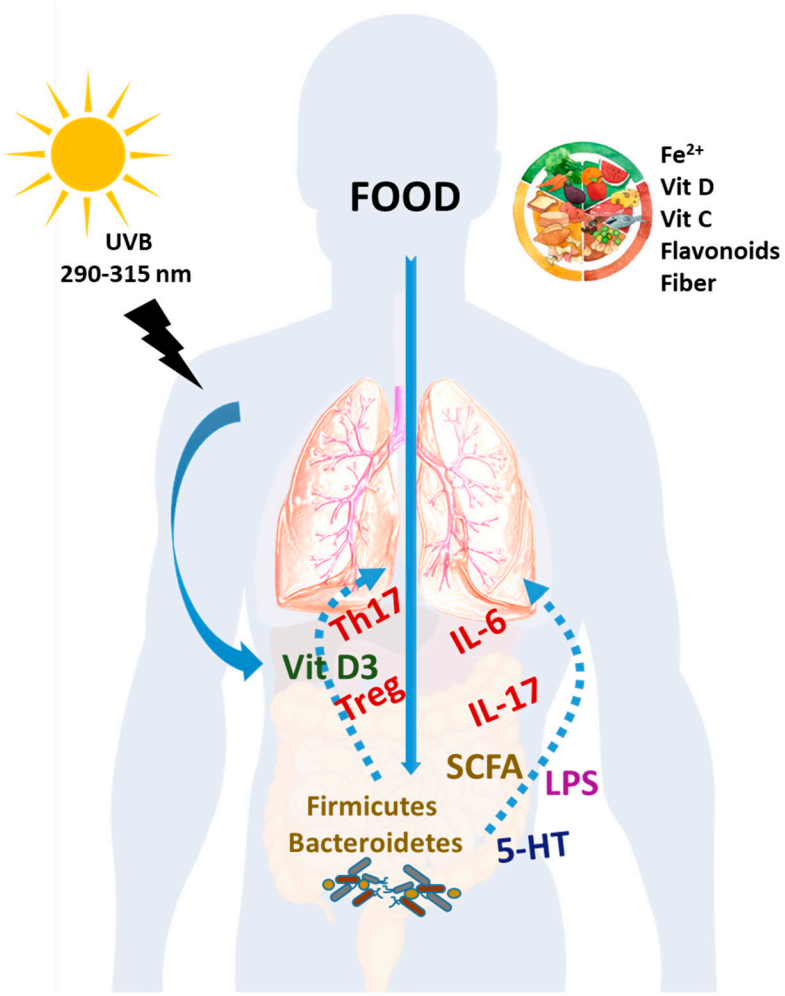

Figure 1. Impact of nutrition in PAH. Dietary components such as Fe^{2+}, vitamins C and D, flavonoids and other related polyphenols, and fiber as well as vitamin D obtained from the exposure to sunlight may have a positive impact the quality of life and prognosis of PAH patients. Each dietary factor may have its own mechanism of action. However, part of the effects of these nutrients may be related to their effect on the immune system with restoration of T cells and cytokines, changes in the microbiota and their bacterial products, and bacterial translocation.

The possible positive effects of the polyphenols quercetin, resveratrol, and genistein in PAH remain to be determined in clinical trials. The use of supplements containing these polyphenols cannot be recommended at this stage. However, given the encouraging effects of fruits and vegetables on cardiovascular health with particular impact on systemic hypertension, it seems reasonable to stimulate PAH patients to adhere to diets rich in these foods.

The role of gut dysbiosis in the pathogenesis of PAH has not been firmly established. At present, no recommendations directed to modify the gut or the lung microbiota can be established. However, if the role of dysbiosis is confirmed, several interventions may be implemented to correct or compensate the altered microbial ecosystem including the use of specific bacterial strains (probiotics), fiber and

dietary polyphenols (i.e., prebiotics), fecal transplantation, antibiotics, and beta-adrenergic antagonists or replacing the deficit in specific SCFAs (e.g., acetate).

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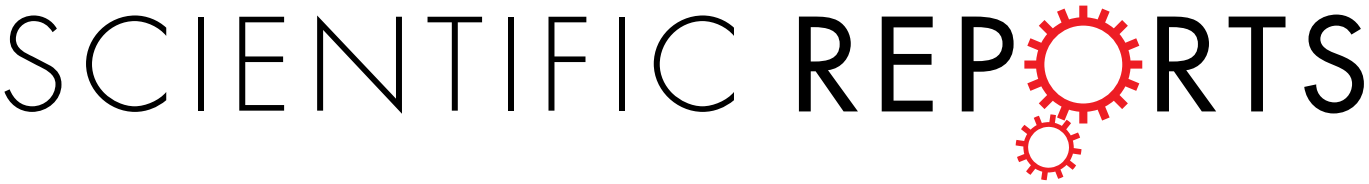
Annex 2

Pulmonary arterial hypertension affects the rat gut microbiome



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Pulmonary Arterial Hypertension Affects the Rat Gut Microbiome

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We have analysed whether pulmonary arterial hypertension (PAH) alters the rat faecal microbiota. Wistar rats were injected with the VEGF receptor antagonist SU5416 (20 mg/kg s.c.) and followed for 2 weeks kept in hypoxia (10% O_2, PAH) or injected with vehicle and kept in normoxia (controls). Faecal samples were obtained and microbiome composition was determined by 16S rRNA gene sequencing and bioinformatic analysis. No effect of PAH on the global microbiome was found (α- or β-diversity). However, PAH-exposed rats showed gut dysbiosis as indicated by a taxonomy-based analysis. Specifically, PAH rats had a three-fold increase in Firmicutes-to-Bacteroidetes ratio. Within the Firmicutes phylum, there were no large changes in the relative abundance of the bacterial families in PAH. Among Bacteroidetes, all families were less abundant in PAH. A clear separation was observed between the control and PAH clusters based on short chain fatty acid producing bacterial genera. Moreover, acetate was reduced in the serum of PAH rats. In conclusion, faecal microbiota composition is altered as a result of PAH. This misbalanced bacterial ecosystem might in turn play a pathophysiological role in PAH by altering the immunologic, hormonal and metabolic homeostasis.

Pulmonary arterial hypertension (PAH) is a progressive disease affecting the lung vasculature that is characterized by sustained vasoconstriction, vascular remodelling and *in situ* thrombosis[1]. It evolves into an occlusive arteriopathy with high resistance to blood flow, leading to right heart failure and premature death[2,3]. In recent years, altered immune and inflammatory processes are being considered as pathological hallmarks of the disease[2,3]. In addition, altered metabolism involving a switch to glycolysis, fatty acid oxidation, and production of reactive oxygen species are also being currently recognized in the pathogenesis of PAH[4].

The human gut is colonized by a huge number of bacteria, archaea, protists, fungi and viruses, forming an ecological community known as the gut microbiota. The gut microbiota communicates with distal organs by producing numerous metabolites that may be absorbed into the systemic circulation and exert biological effects[5]. The microbiota is also responsible for the integrity of the gut barrier function. Low-grade bacterial translocation from the intestines into the circulation with increased plasma bacterial endotoxins (lipopolysaccharides, LPS) may also result from gut barrier dysfunction[6]. In recent years, multiple evidences point to a relationship between the composition of the gut microbiota and an appropriate immunologic, hormonal and metabolic homeostasis[7–9]. The changes in the composition of gut microbiota associated with disease are referred to as dysbiosis. This misbalanced bacterial ecosystem may be therapeutically targeted using probiotics -live strains of selected bacteria- or prebiotics -food components modulating the microbiota[10,11].

Multiple cardiovascular, metabolic and respiratory diseases such as atherosclerosis, hypertension, heart failure, chronic kidney disease, obesity, type 2 diabetes mellitus and sleep apnoea have been linked to gut dysbiosis[12–15]. This is characterized by a microbial flora that is less diverse and less rich with an increased Firmicutes to Bacteroidetes ratio (F/B)[6,16]. Changes in short chain fatty acids (SCFA) producing bacteria are also characteristic of gut dysbiosis with a decrease in acetate- and butyrate-producing bacteria and an increase in lactate-producing bacterial populations[6,8,16]. Moreover, the meta-analysis of the human studies supports that supplementation with probiotics in disease restores the proper gut microbiota and improves disease biomarkers. For instance, probiotics reduce blood pressure in essential hypertensives[17,18].

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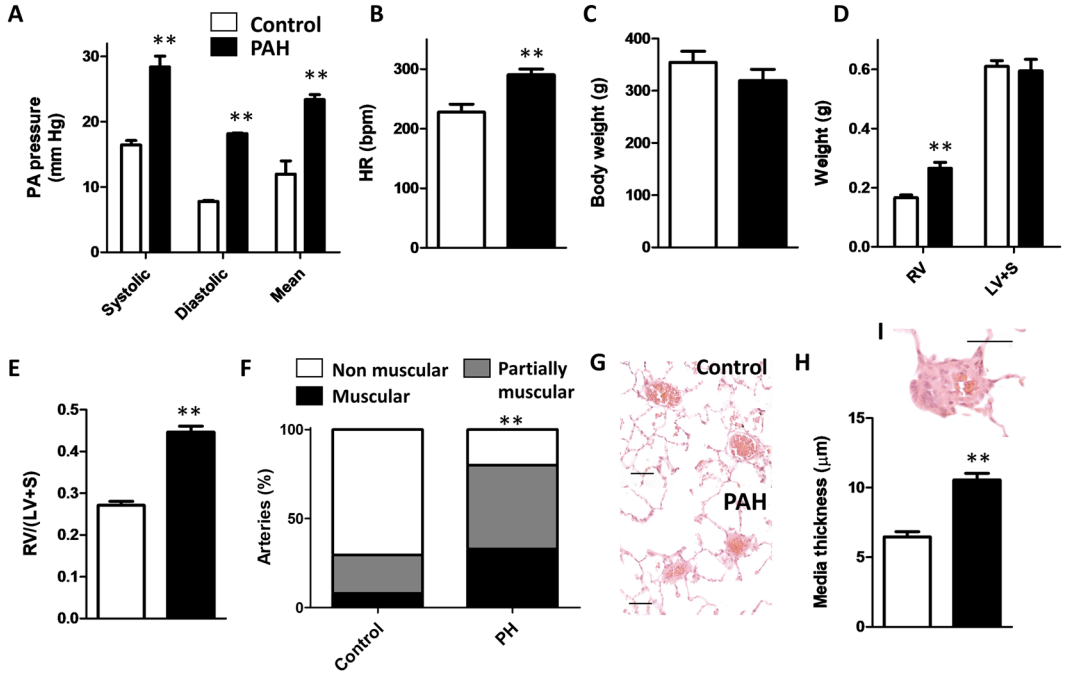

Figure 1. Hemodynamic and histological changes. (**A**) Systolic, diastolic and mean PAP, (**B**) Heart rate, (**C**) Body weight, (**D**) RV and LV+S weight, (**E**) Fulton index [RV/(LV+S)] and (**F**) percentage of arterial muscularization. (**G**) Typical hematoxilin-eosin staining of arterial sections (scale bar 50 µm), (**H**) Medial thickness, (**I**) An early obliterated lesion (scale bar 50 µm). Results are means ± s.e.m. of 4 animals, $^{**}p < 0.05$ versus control (Students' t test for panels A–E and Square Chi test for panel F).

Despite the gut microbiota has been suggested to affect the development of pulmonary vascular disease on a theoretical basis[19], the microbiome has not been studied so far in the context of preclinical or clinical PAH. We hypothesized that the development of PAH may be associated to changes in the intestinal bacterial composition. Therefore, we investigated the effect of PAH on the faecal microbiome in rats using 16S rRNA metagenomics[20]. We have used a representative animal model of PAH, consisting in the combination of hypoxia plus the VEGF antagonist SU5416[21]. Herein, we report that PAH is associated to gut dysbiosis, namely an increased F/B ratio. This represents the first, but still preliminary, evidence suggesting a possible pathophysiological role of intestinal bacteria in the disease.

Results

Hemodynamics and vascular remodelling. SU5416 plus hypoxia for two weeks produced the expected increases in systolic, diastolic and mean pulmonary arterial pressure (PAP) characteristic of PAH (Fig. 1A). This was associated with an increased heart rate, and a trend for reduced body weight (Fig. 1B,C). PAH animals developed a marked right ventricular hypertrophy as shown by the increased RV weight in either absolute values or referred to LV + S (Fig. 1D,E). In addition, animals with PAH showed arterial wall remodelling with an increased muscularization of the small resistance arteries (Fig. 1F,G) with increased wall thickness (Fig. 1H) and occasionally early obliterated lessions were observed (Fig. 1I).

Bacterial α- and β-diversity. The number of species identified was similar in the control and PAH group (Fig. 2A). Shannon, Chao, Simpson and PD whole tree indexes, which represent both the richness and evenness of its species diversity within each sample, i.e. α-diversity, were also similar in both groups (Fig. 2B). We performed a tridimensional principal component analysis (PCA) of the bacterial community, which measures microorganism diversity between samples, i.e. β-diversity, at the level of the different taxa (phylum, class, order, family, genus and species), in an unsupervised manner. This analysis showed no perfect clustering of the animals into the control and PAH groups; e.g. Fig. 2C shows the analysis at the species level.

Taxa composition. The analysis of the phyla composition showed that Firmicutes was the most abundant phylum in the rat faeces, followed by Verrucomicrobia, Bacteroidetes, Proteobacteria, Tenericutes and Actinobacteria (Fig. 3A). Each of these taxa represented above 0.5% of total bacteria and altogether accounted for 98.7% and 98.8% of total bacteria in control and PAH groups, respectively. A nearly four-fold relative decrease in the Bacteroidetes phylum (5.7 vs 1.5%) with lower relative changes in the other most abundant phyla was found in the animals treated with hypoxia plus SU5416. Among the less abundant phyla, there was a ≈three-fold decrease in Cyanobacteria-related bacteria and Thermotogae and a ten-fold decrease in Acidobacteria. The Partial Least Square (PLS) loadings which indicate both the magnitude of the change and the statistical probability of the difference are shown in Fig. 3B for phyla representing >0.1% of total bacteria. The most relevant differences were found in the Bacteroidetes phyla. A 3-dimensional scatterplot was generated by PLSR to visualize the differences

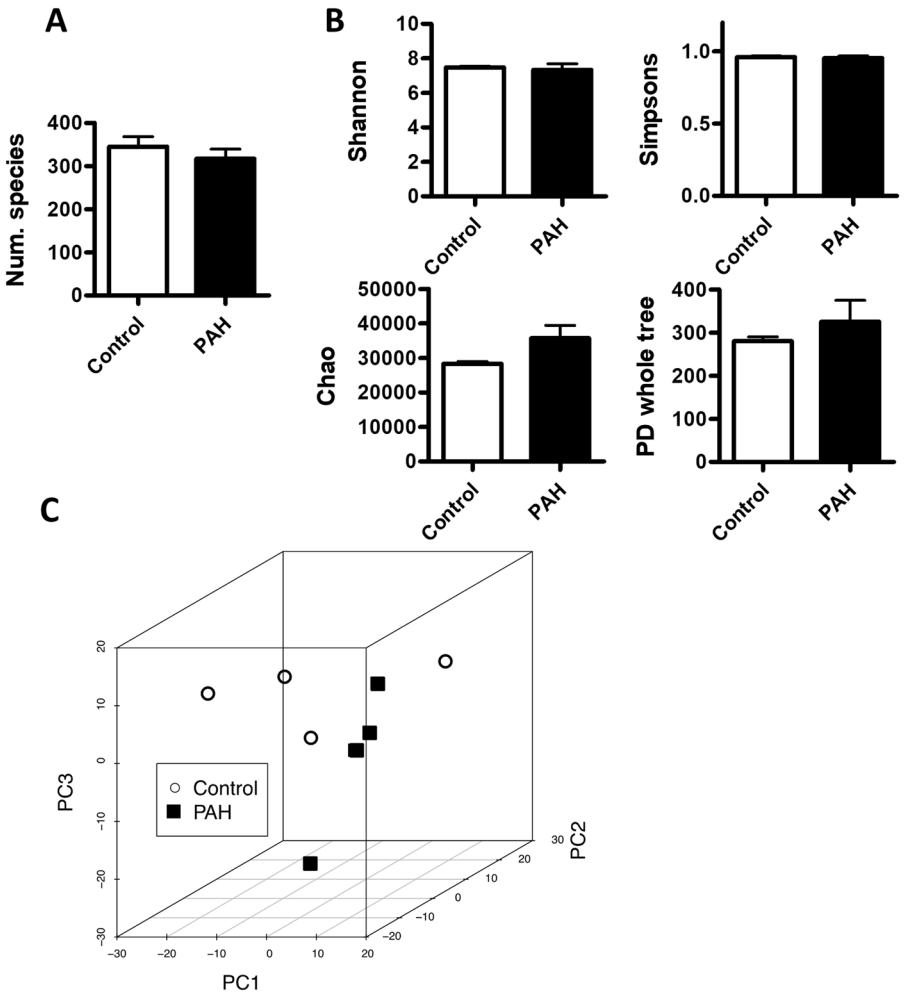

Figure 2. Microbial richness and diversity in PH and Principal Coordinate analysis (PCA). (**A**) Number of species identified. (**B**) α-diversity in rats in control and PAH rats measured by the Shannon, Chao, Simpsons and PD whole tree indexes. Results are means ± s.e.m. of 4 animals. (**C**) Unsupervised PCA were carried out to analyse the differences between control and PAH groups. Each principal component describes most of the variation between samples.

in composition of the faecal microbial communities (Fig. 3C). A clear separation was observed between the control and PAH clusters. Notably, the calculated F/B, a hallmark of gut dysbiosis, was significantly increased in PAH (Fig. 3C).

Given the altered F/B ratio, we analysed which families of bacteria contributed to this imbalance. Regarding the most common families of the Firmicutes phylum, in general, there were no large changes in their relative abundance in the PAH compared to the control group (Fig. 4A,B) with the exception of Peptostreptococcaceae, which suffered a seven-fold increase (0.5 to 3.7%). In the less abundant families, there was also trend for a decrease (≈three-fold) in Aerococcaceae and Pasteurellaceae and a five-fold decrease in Syntrophomonadaceae.

Among the Bacteroidetes phylum, all families were decreased in PAH (from 2- to 20-fold decrease, Fig. 5A). Figure 5B shows the PLS loadings. The most relevant decreases at the genus level were observed in *Butyricimonas* and *Odoribacter* among Odoribacteraceae and *Porphyromonas* in Porphyromonadaceae (Fig. 5D). PLS analysis of the families within Bacteroidetes (Fig. 5C) clearly separated the control and PAH clusters. *Bifidobacterium*, a commonly considered beneficial genus[22] that belongs to the Actinobacteria phylum, was not significant different (0.094 ± 0.039% in control and 0.072 ± 0.005% of total reads in PAH).

SCFA-producing bacteria and SCFA in serum. We analysed the changes in the relative abundance of SCFA-producing bacteria as another hallmark of gut dysbiosis (Fig. 6A) and the SCFA levels in serum (Fig. 6B).

We found a trend for reduced acetate-producing bacteria that was reproduced for all individual acetate-producing genera and also for most butyrate-producing bacteria (based on PLS loadings as shown in Fig. 6F). A statistically significant reduction was only observed for some acetate-producing and butyrate-producing genera (Student t test, Fig. 6C,D). However, an overall trend for increased butyrate-producing bacteria was driven by the changes in the most abundant genus Anaerostipes (Fig. 6D). Lactate-producing bacteria were essentially unchanged (Fig. 6A,E and F). We also analysed the serum levels of SCFA from the NMR spectra (Fig. 6B). Acetate was significantly decreased in serum while butyrate levels were not detected in the NMR

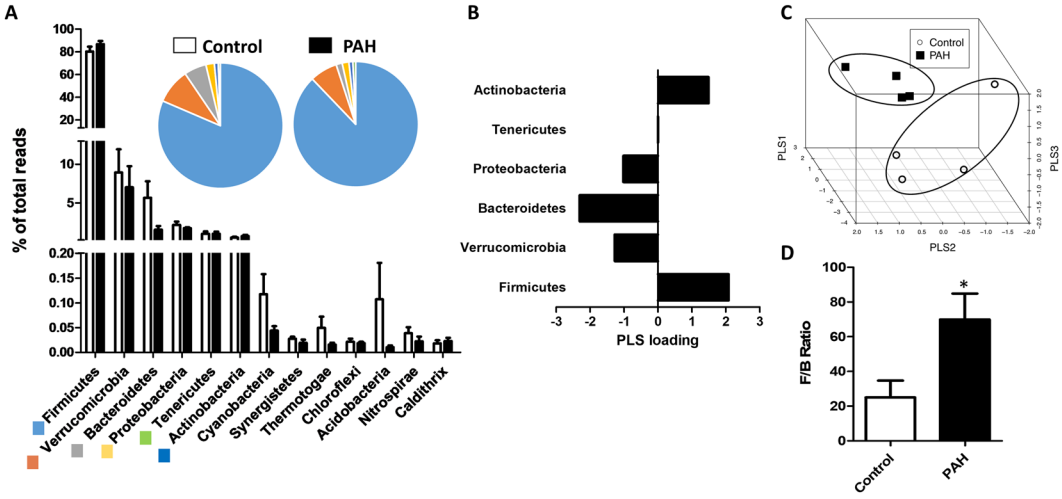

Figure 3. Phyla composition. (**A**) Composition of the most abundant bacterial phyla (>0.01%) expressed as a percent of total bacteria (means ± s.e.m. of 4 animals). The inset shows the pie charts for control and PAH. (**B**) PLS loadings (data shown for phyla representing >0.1% of total bacteria) highlight variable significance to discriminate between PAH and control samples in PLS scores. (**C**) Tridimensional PLS scores plot. (**D**) The Firmicutes to Bacteroidetes ratio (F/B ratio) was calculated as a biomarker of gut dysbiosis (means ± s.e.m., n = 4, *p = 0.04 vs control with student's t-test).

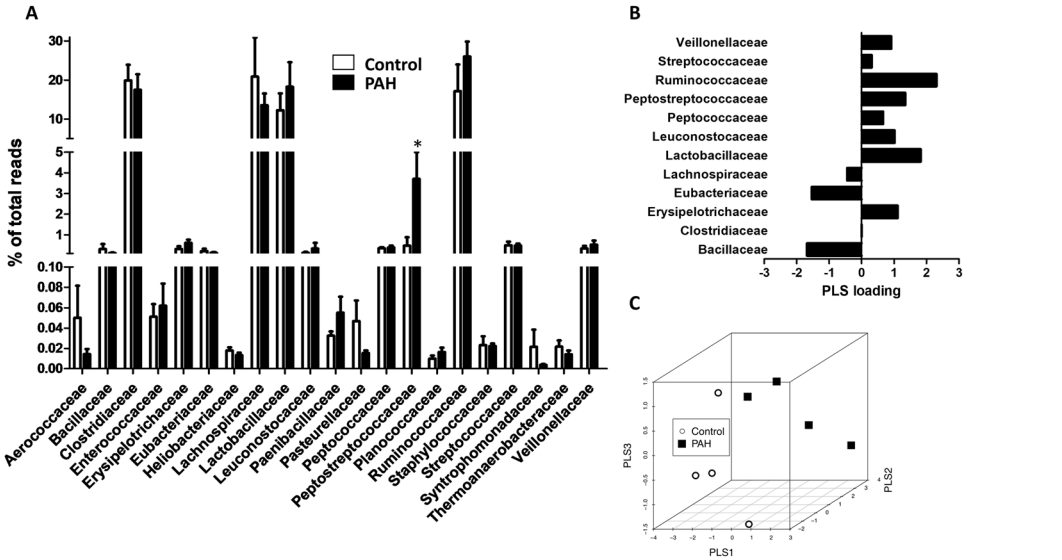

Figure 4. Bacterial families within the Firmicutes phylum. (**A**) Composition of the most abundant bacterial families (>0.01%) expressed as a percent of total bacteria in control and PAH rats (means ± s.e.m. of 4 animals, *p < 0.05 vs control with student's t-test). (**B**) PLS loadings (data shown for phyla representing >0.1% of total bacteria) highlight variable significance to discriminate between PAH and control samples in PLS scores. (**C**) Tridimensional PLS scores plot.

spectra. In contrast, there was increased serum levels of lactate (P < 0.01) in PAH vs control animals (Fig. 6B). The PLS analysis clearly separated the control and PAH clusters based on SCFA-producing bacteria (Fig. 6G).

Discussion

The role of gut dysbiosis in the pathogenesis of many diseases, including diabetes mellitus, obesity, cancer, psychiatric, respiratory and cardiovascular disorders is rapidly emerging. In this study, we present the first evidence of changes in the microbiota in a small sample of rats during the early phases of PAH. Notably, we found an increased F/B that is considered the hallmark of gut dysbiosis, in a rat model of PAH. We also found some specific changes in several taxa, which reproduce the changes previously observed associated to other cardiovascular and metabolic diseases.

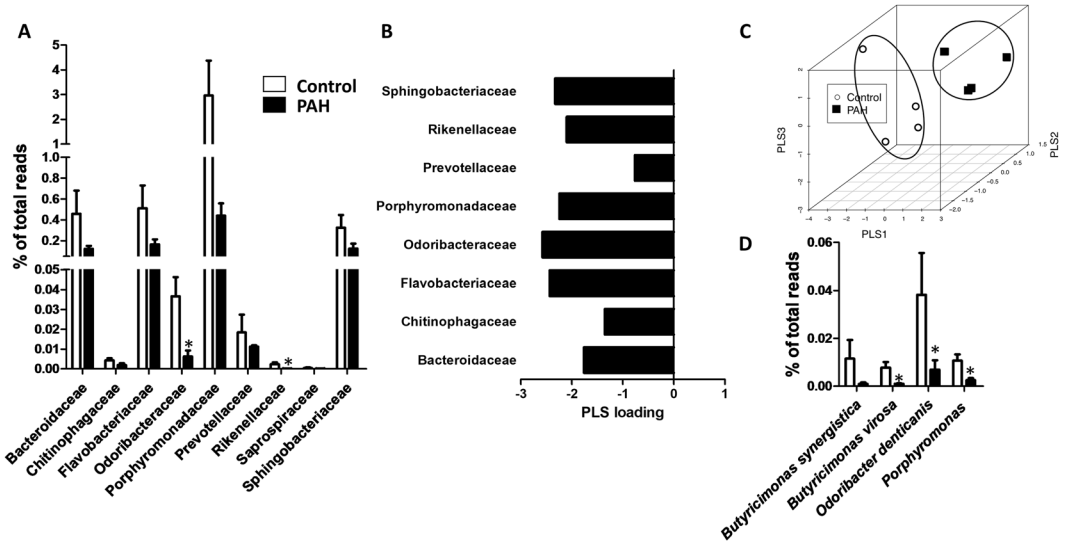

Figure 5. Bacterial families within the Bacteroidetes phylum. (**A**) Composition of the bacterial families expressed as a percent of total bacteria in control and PAH rats (means ± s.e.m. of 4 animals, *$p < 0.05$ vs control with student's t-test) (**B**) PLS loadings (data shown for phyla representing >0.1% of total bacteria) highlight variable significance to discriminate between PAH and control samples in PLS scores. (**C**) Tridimensional PLS scores plot. (**D**) Composition of the species within the Odoribacteraceae family and *Porphyromonas* (means ± s.e.m. of 4 animals, *$p < 0.05$ vs control with student's t-test).

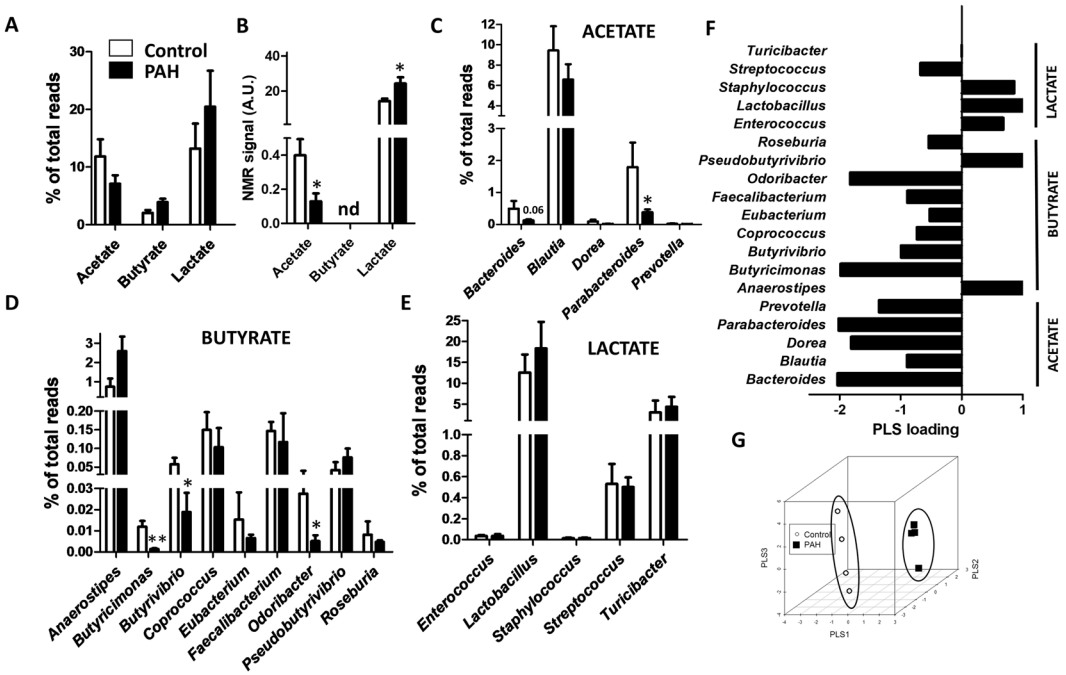

Figure 6. SCFA and SCFA-producing bacteria. (**A**) Composition of the acetate-, butyrate- and lactate-producing bacteria in control and PAH rats. Data is the sum of all SCFA-producing genera expressed as a percent of total bacteria (means ± SEM of 4 animals). (**B**) Acetate, butyrate and lactate in rat serum (AU = arbitrary units, nd = not detected, n = 4, *$p < 0.05$ vs control student's t-test). (**C**–**E**) Most abundant acetate-, butyrate- and lactate-producing genera (means ± s.e.m. of 4 animals, *$p < 0.05$ vs control with student's t-test). (**F**) PLS loadings (data shown for phyla representing >0.1% of total bacteria) highlight variable significance to discriminate between PAH and control samples in PLS scores. (**G**) Tridimensional PLS scores plot.

We have used the animal model of PAH of hypoxia plus SU5416, which best conforms to human PAH[21]. At two weeks, it develops a clear increase in PAP at the threshold values for clinical diagnosis and strong right ventricular hypertrophy and arterial remodelling. We deliberately chose this time to analyse the early changes in the

microbiota, which might play a pathophysiological role in the development of the pathology rather than being a consequence of the long-term disease. Longer exposure leads to further disease worsening. For instance, at baseline and after the second and the third week of treatment, mPAP increases from ≈13 to 24 (present data) and 45 mm Hg (authors unpublished data), respectively. This time-course is similar to the one shown in the original report of the model by Taraseviciene-Stewart *et al.*[23] and consistent with RV pressure values of ≈30, 60 and 90 mm Hg, respectively, in Oka *et al.*[24]. Likewise, the Fulton indexes as a measure of RV hypertrophy were ≈0.25, 0.45 and 0.65, respectively, in our hands and ≈0.3, 0.5 and 0.7, respectively, in Oka *et al.*[24].

The Shannon, Simpsons, Chao and PD whole tree indexes and the PCA plot showed no apparent change in α- and β-diversity in controls and PAH rats. This indicates that there is no global differences in the microbiota, most taxa were unchanged. In contrast, other diseases have found reduced richness and diversity[6,16]. However, the most important and recognized biomarker of dysbiosis, the F/B, was significantly increased in PAH. This ratio has been reported to be modified in multiple pathological conditions in both human and animal models. In systemic hypertension increased F/B has been found in animal models of disease, including spontaneously hypertensive rats, deoxycorticosterone-salt- and angiotensin II-induced hypertension, as well as in essential hypertensive patients[16,25].

We also evaluated which subtaxa contributed to the alteration of Firmicutes and Bacteroidetes. Notably, all families from the Bacteroidetes phylum were decreased (from 2 to 10-fold decrease). Odoribacteraceae may be of special interest because several species within this family belonging to the genera *Odoribacter* and *Butyricimonas* have been reported to be depleted in overweight and obese pregnant women with high blood pressure, in sedentary mice, liver injury and multiple sclerosis[26–28]. In contrast to the present report, *Odoribacter* was found to be increased in mice with intermittent hypoxia[29]. Among Firmicutes, we found minor absolute changes, with an overall trend for an increase in bacterial reads. Peptostreptococcaceae, which suffered the largest absolute increase, is a family of Gram-positive bacteria that is over-represented in the guts of patients and mice with colorectal and oral cancer[30,31].

Dietary fibre is fermented in the colon by commensal bacteria, leading to the release of the SCFAs, acetate, butyrate, and lactate, which may be absorbed into the circulation and interact with G protein-coupled olfactory receptors in the gut epithelium and immune cells[32]. Besides the changes in taxonomic categories, gut dysbiosis associated to cardiovascular disease is characterized by a decrease in acetate- and butyrate-producing bacteria and an increase in lactate-producing bacterial populations[6,8,16]. We found no significant changes in the sum of butyrate- or lactate-producing bacteria but a trend for reduced acetate-producing bacteria was found in PAH. A clear separation was observed between the control and PAH clusters based on short chain fatty acid producing bacterial genera. We also analyzed the levels of the three SCFA in serum of the PAH rats and controls by quantifying the NMR spectra. We found that acetate was reduced in the serum of PAH rats. This change parallels the observed differences in acetate-producing genera. It is therefore tempting to speculate that the observed serum changes are secondary to the different bacterial composition. However, our experiments cannot rule out that the observed changes in serum SCFA are generated by the host metabolism. In fact, lactate was increased in the serum of PAH rats, which is expected as a result of the hypoxic environment in the host cells. Interestingly, acetate supplementation or an intervention with fibre to restore acetate production in mice with mineralocorticoid-dependent hypertension significantly reduced systolic and diastolic blood pressures, cardiac fibrosis, and left ventricular hypertrophy[25]. These protective effects seem to be related to the regulation of key pathways and genes involved in cardiovascular health, including the transcription factor Egr1, a master regulator of cardiovascular disease[33,34] which has also been reported to play a role in PAH[35].

Interestingly, there is certain parallelism between factors affected by gut dysbiosis and those involved in the pathophysiology of PAH. First, it is now well established that Th17 cell development in the gut is specifically impacted by commensal bacteria[36]. Th17 cell expansion originated in the intestine is associated with gut dysbiosis (higher F/B ratio) in several pathologies such as multiple sclerosis[37] and lupus erythematosus[38]. A characteristic increase in peripheral Th17 cells and the Th17-produced cytokine IL-17 and a decrease in Treg cells is common to all forms of PAH and contributes to the development and the progression of the disease[39,40]. Second, gut microbiota is also a key regulator of Tph1 transcription (the gene encoding for the rate limiting enzyme in serotonin synthesis) in enterochromaffin cells which supplies the platelets of serotonin[41]. On the other hand, clinical and experimental PAH is associated with up-regulation of Tph1 gene transcripts as well as a rise in platelet-rich serotonin[21,42]. Third, gut dysbiosis leads to low grade commensal bacterial translocation[6,7] with increased plasma bacterial LPS, the main ligand for toll-like receptor 4 (TLR4). This innate immune receptor has been reported to play a key role in the pathogenesis of pulmonary hypertension[43]. Therefore, there might be a pathophysiological link between gut dysbiosis and PAH that involves the upregulation of Egr1, Th17 polarization, elevation of plasma serotonin and TLR4 activation.

The mechanism of how PAH induces gut dysbiosis remains to be determined. It has been reported that the sympathetic nervous system, via beta-adrenoceptor activation, in the gut compromises its barrier function, and it is capable of altering the microbiota[44,45]. Interestingly, PAH patients and animal models (including chronic hypoxia plus SU5416-induced PAH) have high sympathetic activity and circulating catecholamine levels[46,47], which is strongly related to mortality[48]. Therefore, it seems reasonable to tentatively propose sympathetic overstimulation as a mechanism for PAH-induced gut dysbiosis. If this is the case, neurohumoral activation might exert deleterious effects in PAH not only by adrenergic receptor stimulation on the heart and pulmonary vessels but also on the splachnic circulation. However, we cannot rule out that the effects of hypoxia and SU5416 may not be limited to the pulmonary vasculature and could impact directly on other tissues, including the gut epithelium and/or the mesenteric vasculature, leading to gut dysbiosis. Moreover, we cannot exclude that the gut is a primary target of hypoxia and/or SU5416 and that the subsequent changes in the microbiota secondarily trigger or potentiate PAH. In addition, our experiments do not clarify whether the changes in the microbiota are induced by hypoxia, SU5416 or the combination of both. SU5416 itself induces mild pulmonary hypertension[23] and lung

cell apoptosis and emphysema[49]. A possible strategy to address these issues could be to treat animals with SU5416 by inhalation to minimize the direct systemic effects. However, to our knowledge, there are no reports using this administration route for this drug.

In conclusion, the present study is the first one showing that PAH affects the gut microbiota. Further research is required to determine whether dysbiosis plays a pathophysiological role in the development of PAH or if it is just an epiphenomenon. If the former is true, a new therapeutic window will be opened in PAH. Several therapeutic strategies can be used to restore the microbiota in disease[10,11,17,25], including specific bacterial strains (probiotics), fibre and dietary polyphenols (i.e. prebiotics), faecal transplantation, antibiotics, beta-adrenergic antagonists[45] or to replace the deficit in specific SCFAs (e.g. acetate)[25].

Material and Methods

Animals. Pathogen-free male Wistar rats (300 g, 11–12 weeks of age) were obtained from Envigo (Barcelona, Spain). All experimental procedures utilizing animals were carried out according to the Spanish Royal Decree 1201/2005 and 53/2013 on the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEXO-301/16).

Model of PAH. PAH was induced in rats by a single subcutaneous injection of SU5416 (20 mg/kg; Tocris, UK) and then maintained in hypoxia for two weeks[21]. Hypoxic animals (n = 4) breathed a gas mixture (N_2 and room air) in a semi-closed chamber where oxygen was continuously monitored by an oxygen sensor (DrDAQ, PicoTechnology, UK) to maintain 10% O_2. Control animals (n = 4) were exposed to rom air (21% O_2, normoxia) in another chamber. CO_2 and water vapour produced by the animals were captured with soda lime and silica gel, respectively. Animals were fed normal rat chow.

Hemodynamic measurements. At the end of two weeks, rats were anesthetized (80 mg/kg ketamine and 8 mg/kg xylacine i.p.), tracheostomyzed and ventilated with room air (tidal volume 9 mL/kg, 60 breaths/min, and a positive end-expiratory pressure of 2 cm H_2O, Nemi Scientific Inc, Medway, USA). After sternotomy, a catheter was placed in the pulmonary artery (PA) through the right ventricle for systolic, diastolic and mean PA pressure (sPAP, dPAP and mPAP) recording[50]. It should be noted that open-chest measurements in anaesthetized animals underestimate real PAP. At the end of the experiment, the right ventricle (RV) and the left ventricle plus the septum (LV + S) were dissected and weighed.

Lung histology. The left lung was inflated *in situ* with formol saline through the left bronchus and embedded in paraffin. Lung sections were stained with haematoxylin and eosin and examined by light microscopy, and elastin was visualized by its green auto-fluorescence. Small arteries (25–100 mm outer diameter) were analysed in a blinded fashion and categorized as muscular, partially muscular or non-muscular as previously described[50].

DNA Extraction, 16S rRNA Gene Amplification, Bioinformatics. For the analysis of the bacterial population present in the gut, faecal samples were collected from four individual animals at the end (day 14) of the experimental period. Bacterial genomic DNA was extracted from faecal samples using G-spin columns (INTRON Biotechnology) starting from 30 mg of samples resuspended in PBS and treated with proteinase K and RNAses. DNA concentration was determined in the samples using Quant-IT PicoGreen reagent (Thermo Fischer) and DNA samples (about 3 ng) were used to amplify the V3-V4 region of 16S rRNA gene[51]. PCR products (approx. 450 pb) included extension tails which allowed sample barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR. Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent) and a pool of samples was made in equimolar amounts. The pool was further cleaned, quantified and the concentration estimated by real time PCR (Kapa Biosystems). Finally, DNA samples were sequenced on an Illumina MiSeq instrument with 2 × 300 paired-end read sequencing at the Unidad de Genómica (Parque Científico de Madrid). Negative controls included from the beginning of the procedure were completely negative and therefore not included in the sequencing run. We did not carry positive controls in our experiments since these primers have been extensively used[51]. The two-step PCR amplification that we have used[52] allows the successful recovery of mock community species. Our approach to increase diversity included: (a) running different projects in the same run so that proximal clusters can easily start with different sequences; (b) increasing the percentage of PhiX174 DNA, to further increase diversity with an equilibrated shotGun DNA; and (c) diluting cluster density to suboptimal concentration for Miseq v3 runs. DNA reads were quality filtered according to MiSeq standard parameters (Illumina) resulting in a final output of around 150 K reads on average per rat (range: 90–220 K). Operational taxonomic units (OTUs) were assigned using the 16S-metagenomics workflow (1.0.1) associated to the Base Space Hub (Illumina). Classification was based on an Illumina-curated version of the GreenGenes taxonomic database which implements the Ribosomal Database Project (RDP) Classifier[53]. The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature. Bacteria were classified based on the SCFA end product as previously described[54,55].

Serum SCFA measurements. Serum samples (40 µL) were examined by 500 MHz High-Resolution Magic Angle Spinning Nuclear Magnetic Resonance Bruker AMX500 spectrometer at CIC Biomagune (Donostia, Spain). Samples were placed into a 50-µl zirconium oxide rotor using a rinsed cylindrical insert, together with 15 µl 0.1 mM solution Trimethylsilyl propanoic acid (TSP) in deuterium water (D2O). Standard solvent-suppressed spectra were acquired using a sequence based on the first increment of the nuclear Overhauser effect spectroscopy (NOESY) pulse sequence. A number of bidimensional homonuclear and heteronuclear experiments such as standard gradient-enhanced correlation spectroscopy (COSY), ^1H–^1H total correlated spectroscopy (TOCSY), and gradient-selected heteronuclear single quantum correlation (HSQC) protocols were performed to carry out

metabolites assignments. Spectral processing was performed using the "Metabonomic" R package[56]. 1H NMR spectra were referenced to the TSP signal at 0 ppm chemical shift and normalized to total sum of the spectral regions. Two-dimensional spectral processing and editing was performed using MestRenova v. 11.0.3 (Mestrelab Research S.L., Santiago de Compostela, Spain).

Statistical analysis. The Shannon, Chao, Simpsons and PD whole tree indexes were calculated to analyse α-diversity using QIIME. Reads in each OUT were normalized to total reads in each sample. Only taxa with a percentage of reads $>0.001\%$ were used for the analysis. Data are expressed as means $\pm$ s.e.m. Statistical comparisons were performed using two-tailed unpaired *t* tests at $\alpha < 0.05$ where appropriate. Unsupervised classification studies with Principal Components Analysis (PCA)[57] were carried out to analyse the differences between groups for each taxonomic level. Partial Least Square (PLS) analysis was also applied to these data to identify significant differences between groups. PLS analysis[58] is a commonly used supervised multivariate method for analysing high-dimensional data where PLS loadings highlight the most significant variables from the total pool. The PLS components are composed of so-called scores and loadings. PLS loadings contain information about the variables in the dataset highlighting the most significant variables from the total pool. PLS scores hold information on samples in the dataset highlighting the differences between groups. PLS analyses were performed with the Metabonomic package (rel.3.3.1)[56] using the algorithm proposed by Ding and Gentleman[59]. Three PLS components were chosen to build the model based on the percentage of variance explained, the R2, and the mean squared error of cross-validation graphics.

Data availability. All data of the present study are available on request.

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Author Contributions

M.C., J.L.I.-G. and F.P.-V. analysed the data, J.L.I.-G. did the metabolomics analysis, G.M.-P., M.C., S.E.-R. B.B. and D.M.-C. made the animal model, B.B. made the histological analysis, D.M.-C. made the hemodynamic measurements, F.P.-V. designed the study and wrote the manuscript with important contributions from A.C., L.M., M.C. and J.D.

Additional Information

Competing Interests: The authors declare no competing interests.

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Annex 3

Impact of vitamin D deficit on the rat gut microbiome

nutrients

MDPI

Article

Impact of Vitamin D Deficit on the Rat Gut Microbiome

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Abstract: Inadequate immunologic, metabolic and cardiovascular homeostasis has been related to either an alteration of the gut microbiota or to vitamin D deficiency. We analyzed whether vitamin D deficiency alters rat gut microbiota. Male Wistar rats were fed a standard or a vitamin D-free diet for seven weeks. The microbiome composition was determined in fecal samples by 16S rRNA gene sequencing. The vitamin D-free diet produced mild changes on α- diversity but no effect on β-diversity in the global microbiome. Markers of gut dysbiosis like *Firmicutes*-to-*Bacteroidetes* ratio or the short chain fatty acid producing bacterial genera were not significantly affected by vitamin D deficiency. Notably, there was an increase in the relative abundance of the *Enterobacteriaceae*, with significant rises in its associated genera *Escherichia*, *Candidatus blochmannia* and *Enterobacter* in vitamin D deficient rats. *Prevotella* and *Actinomyces* were also increased and *Odoribacteraceae* and its genus *Butyricimonas* were decreased in rats with vitamin D-free diet. In conclusion, vitamin D deficit does not induce gut dysbiosis but produces some specific changes in bacterial taxa, which may play a pathophysiological role in the immunologic dysregulation associated with this hypovitaminosis.

Keywords: microbiota; 16S rRNA sequencing; vitamin D deficit

1. Introduction

The gut microbiota is an ecological community constituted by a large number of bacteria, archaea, protists, fungi and viruses. The microbiota produces multiple metabolites that may cross the intestinal barrier and exert biological effects [1]. The microbiota also impacts on the integrity of the gut barrier. Gut barrier dysfunction may result in bacterial translocation from the intestines with increased plasma lipopolysaccharides (LPS) [2]. Multiple studies have found a relationship between the microbiota and adequate metabolic, hormonal and immunologic homeostasis [3–5]. Gut dysbiosis, i.e., an altered composition of the intestinal microbiota in disease is associated with poor health outcomes. Dysbiosis may be treated with probiotics, i.e., live strains of selected bacteria, or prebiotics, food components that modulate the microbiota [6,7]. Multiple metabolic, cardiovascular, and respiratory diseases including type 2 diabetes mellitus, obesity, systemic and pulmonary hypertension, atherosclerosis, heart failure and chronic respiratory diseases have been linked to impaired gut microbiota [8–13]. Dysbiosis is

frequently characterized by: (a) lower microbial diversity and richness, (b) changes in the relative abundance of phyla with an increased *Firmicutes* to *Bacteroidetes* ratio (F/B), and (c) changes in short chain fatty acids (SCFA) producing bacteria with a relative increase in lactate-producing bacteria and a relative decrease in acetate- and butyrate-producing bacteria [2,4,14,15].

Vitamin D is produced from dermal exposure to sunlight or from the diet and is converted in the liver into 25-OH-cholecaciferol or calcifediol (25OHVitD), whose measure serves as an indicator of the vitamin D status. This is further metabolized mainly in the kidney into 1-α,25-dihydroxycholecalciferol, the most active form, also named calcitriol, which activates the vitamin D receptor (VDR). Vitamin D deficiency, which causes rickets and osteomalacia, is very common worldwide due to insufficient solar light exposure and/or a reduced dietary intake [16]. Besides this well-known regulatory role in the calcium-phosphorus and bone homeostasis, vitamin D is also involved in the control of other physiological processes, such as cellular growth, intracellular metabolism and innate and adaptive immunity. Vitamin D deficiency has also been related to infection, cancer and respiratory and cardiovascular diseases [17–21]. Immune cells and peripheral tissues can also synthetize calcitriol and express VDR. Thus, vitamin D has been proposed to have immunomodulatory properties and insufficient vitamin D levels may lead to dysregulation of immune responses [22]. Moreover, vitamin D is able to induce the expression of antibacterial proteins and exert antibiotic effects in a variety of cell types [23].

We hypothesized that a deficiency in vitamin D induces changes in the gut microbiota.

2. Materials and Methods

The procedures involving animals were carried out according to the Spanish Royal Decree 1201/2005 and 53/2013 on the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-301/16).

2.1. Model of Vitamin D Deficiency

Twenty male Wistar rats of 180 g body weight (BW) from Envigo (Barcelona, Spain) were maintained in the general animal facility of Universidad Complutense. Animals were randomly allocated into two groups; rats fed with a standard diet (n = 10), which contained 1500 IU/kg cholecalciferol (Teklad Global 18% Protein Rodent Diet, Envigo) and rats fed a vitamin D-free diet (n = 10) (VitD-free, Teklad Custom Diet TD.120008, Envigo, n = 10) for seven weeks. Rats were housed, two per box, with food and water ad libitum under standard conditions (22 ± 1 °C and 12:12 h dark/light cycle). At week 7, animals were euthanized, plasma and feces were collected and the right and left ventricle plus septum were weighed. Plasma 25OHVitD was measured using a chemiluminescence immunoassay (ADVIA Centaur® Vitamin D Total assay, Siemens Healthcare Diagnostics) at the Clinical Biochemistry Service, Gregorio Marañón Hospital.

2.2. DNA Extraction, 16S rRNA Gene Amplification, Bioinformatics

The gut microbiome was analyzed as previously reported [13]. Briefly, the feces were collected at week 7 from each individual rat. We used G-spin columns (INTRON Biotechnology) to extract DNA and treated the samples with proteinase K and RNAses. The V3–V4 region of the 16S rRNA gene was amplified for 20–22 cycles from 3 ng of DNA [24]. PCR products (approx. 450 bp) included extension tails, which allowed sample barcoding and the addition of specific Illumina sequences in a second PCR for 10–12 cycles. Individual amplicon libraries were analyzed using a Bioanalyzer 2100 (Agilent) and a pool of samples was made in equimolar amounts. DNA samples were sequenced at the Unidad de Genómica (Parque Científico de Madrid) on an Illumina MiSeq instrument with 2 × 300 paired-end read sequencing. DNA reads were quality filtered according to MiSeq standard parameters (Illumina). We discarded all reads whose corrected brightest intensity in any of the first 25 sequencing cycles was less than 60% of the sum of brightest intensity and the next brightest. We find

that this criterion provides reasonable discrimination between good and bad data [25]. Depending on the loading density, we typically kept between 50% and 70% of the raw reads. The final output was around 150 K reads on average per rat (range: 90–220 K). Operational taxonomic units (OTUs) were assigned using the 16S-metagenomics workflow (1.0.1) associated with the Base Space Hub (Illumina, 2013 version [26]). An Illumina-curated version of the GreenGenes taxonomic database, which implements the Ribosomal Database Project (RDP) Classifier was used to classify the OTUs [27]. The rarefaction curves (Supplementary Materials, Figure S1) show that the main OTUs are effectively detected. The Taxonomy Database (National Center for Biotechnology Information) was used for classification and nomenclature. Bacteria were classified based on the SCFA end product as previously described [28,29] (Supplementary Materials, Table S1).

2.3. Statistical Analysis

Reads in each operational taxonomic unit (OTU) were normalized to total reads in each sample. Only taxa with a percentage of reads >0.001% were used for the analysis. The Shannon, Chao1, Simpsons and Pielou indices were calculated to analyze α-diversity using Past software (ver3.21, Oslo, Norway) [30]. Principal components analysis (PCA) [31] was also carried out with Past software [32]. Statistical taxonomic comparisons were performed using the linear discriminant analysis (LDA) effect size (LEfSe) using the Galaxy Hutlab online platform [33,34], considering alpha values of 0.05 for the factorial Kruskal-Wallis test among classes and for the pairwise Wilcoxon test between subclasses. Values > 2 or < −2 for the logarithmic LDA score were considered significant [35]. The Galaxy Hutlab web was also used to plot the cladogram. All other analysis and plots were carried out using Prism software (Prism version 7.04 for Windows, GraphPad Software, La Jolla California USA [36]). Normally distributed variables were compared using a two-tailed unpaired *t* test, otherwise data was analyzed using the Mann Whitney test.

3. Results

3.1. Model of Vitamin D Deficit

The exposure to a vitamin D-free diet for 7 weeks induced a marked decrease in 25OHVitD plasma values from 22.2 ± 1.4 to 7.7 ± 1.0 ng/mL (Figure 1A). There was no change in body weight and a modest increase in the weight of the left ventricle plus septum but not in the right ventricle (Figure 1B,C).

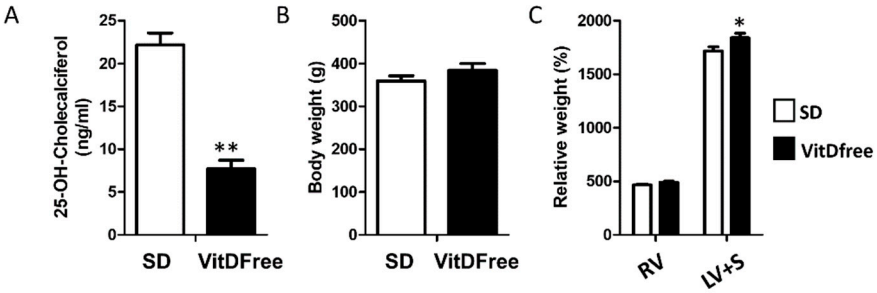

Figure 1. Vitamin D status, and body and heart weight. (**A**) Plasma 25OHVitD. (**B**) Body weight and (**C**) right ventricular (RV) and left ventricular + septum (LV + S) weights. Results are mean ± s.e.m. for the standard diet (SD, $n = 10$) and vitamin D-free diet group ($n = 10$). Results were compared by student's t-test * $p < 0.05$ and ** $p < 0.01$ vs. SD.

3.2. Bacterial α- and β-Diversity

Between 250 and 450 species were identified in each sample. The number of species identified was similar in the rats under the standard diet and those with vitamin D-free diet (Figure 2A). The parameters

indicating α-diversity such as the Pielou, Shannon and Simpson indices were significantly increased in vitamin D-free diet group but the Chao1 index was not significantly affected (Figure 2).

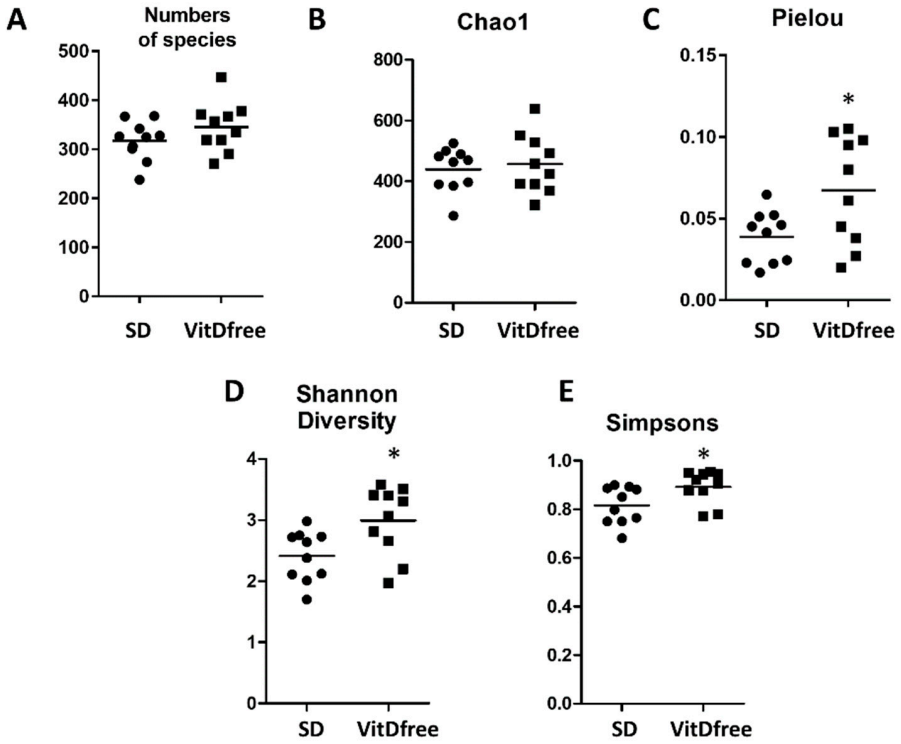

Figure 2. Effects of vitamin D-free diet on ecological parameters of the bacterial communities. Fecal samples were collected from standard diet (SD, $n = 10$) and vitamin D-free diet group ($n = 10$) rats. The microbial alpha diversity was analyzed using (**A**) numbers of species, (**B**) Chao richness, (**C**) Pielou, (**D**) Shannon and (**E**) Simpsons indices. Results are expressed as a scatterplot and means and were compared by a student's t-test; * $p < 0.05$ vs. SD.

A bidimensional principal component analysis (PCA) of the microbiome was performed in an unsupervised manner, which measures the diversity of microorganisms among samples, i.e., β-diversity. This analysis showed no clear clustering of the animals into the control and vitamin D-free diet groups (Figure 3).

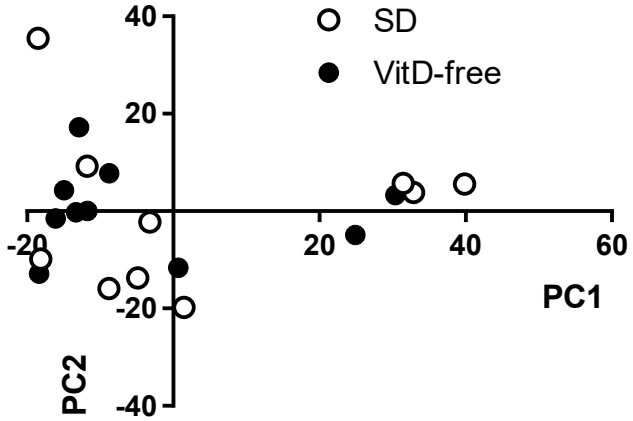

Figure 3. Effects of vitamin D-free diet on bacterial β-diversity. β-diversity was analyzed using the principal coordinate analysis (PCA) of the standard diet group using PAST3.0. The principal components PC1 and PC2 are plotted in a bidimensional figure. The white spheres represent rats fed a standard diet (SD, $n = 10$) and black spheres rats fed a vitamin D-free diet ($n = 10$).

3.3. Taxa Composition

We performed an LDA effect size (LEfSe) analysis for the whole bacterial taxa identified in the feces to support high-dimensional class comparisons. The results of this analysis are shown in Supplementary Materials, Figure S2. Forty-one taxa were significantly increased in rats exposed to a VitD-free diet and five taxa were decreased. A cladogram summarizing the phylogenetic relationship between these changes is shown in Supplementary Materials, Figure S3.

Firmicutes was the most abundant phylum in the rat feces, followed by *Verrucomicrobia*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Tenericutes*, in both groups (Figure 4A). Among these most abundant phyla there was no significant differences in their relative abundance when comparing animals with the standard diet and those with the vitamin D-free diet. We found a large variability in the *Verrucomicrobia* and to a lesser extent in the *Bacteroidetes* phylum. The ratio of *Firmicutes* to *Bacteroidetes* (F/B), which is a widely used measure of bacterial dysbiosis was highly variable (Figure 4B). There was an apparent trend for an increased F/B ratio in the Vit D-free group, the median increased from 9.3 to 19.7, but the difference was not statistically significant ($p > 0.05$, Mann Whitney test). Among the less abundant phyla, there was a highly significant increase in *Synergistetes* (Figure 4A).

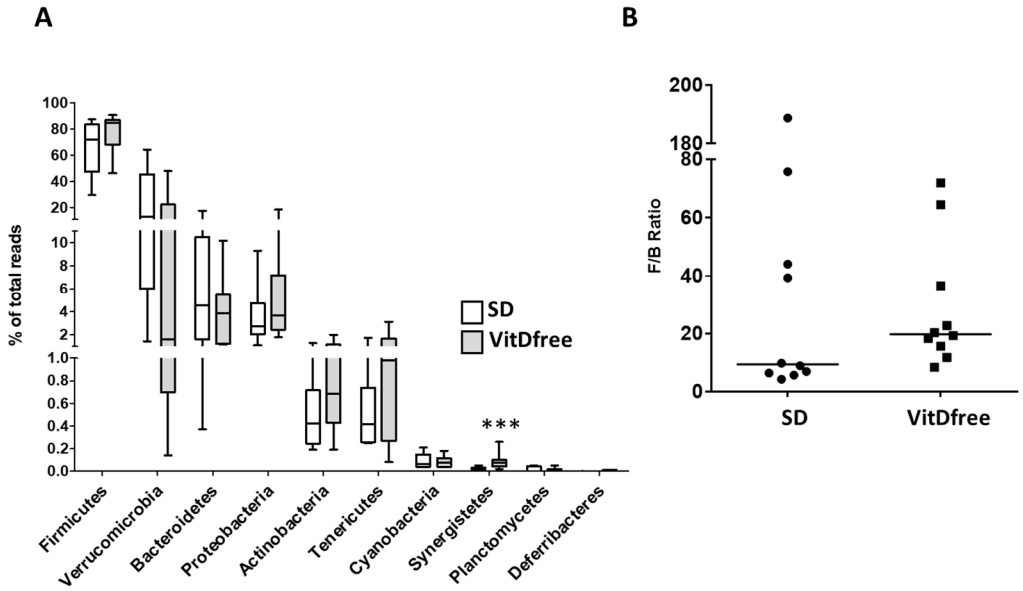

Figure 4. Effects of the vitamin D-free diet on the relative phylum abundance. The most abundant phyla for the standard diet (SD, $n = 10$) and vitamin D-free diet group ($n = 10$) were expressed as a percent of total reads and represented in (**A**) as a box and whiskers plot, *** $p < 0.001$ vs. SD, student's t-test. (**B**) Firmicutes to Bacteroidetes ratio (F/B ratio), as a biomarker of gut dysbiosis, is represented as a scatterplot and median.

The significant changes induced by vitamin D-free diet in bacterial families and genera are shown in Figures 5 and 6A, respectively. Notably, there was an increase in the *Enterobacteriaceae* family with significant rises in its associated genera *Escherichia*, *Candidatus blochmannia and Enterobacter*, an increase in Prevotellaceae and its genus *Prevotella* and a decrease in the family Odoribacteraceae and its genus *Butyricimonas* in rats with vitamin D-free diet.

3.4. SCFA-Producing Bacteria

We analyzed the changes in the relative abundance of SCFA-producing bacteria as an additional measure of gut dysbiosis (Figure 6B). Some specific acetate-producing genera such as *Blautia*, *Lachnospira*, *Actinomyces* and *Prevotella* were increased by vitamin D-free diet as mentioned above. Similarly, some butyrate-producing genera such as *Faecalibacterium* and *Roseburia* were relatively more abundant in

rats with VitD-free diet. However, we found no significant changes in the sum of all bacterial reads for acetate-, butyrate-, lactate- or propionate-producing bacteria.

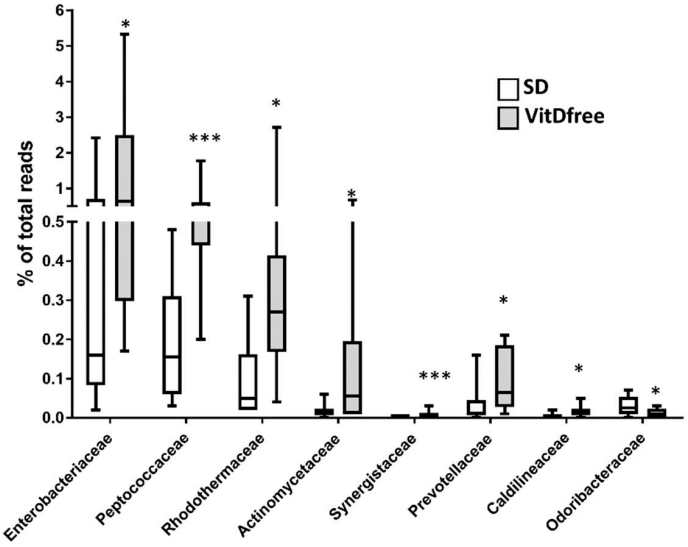

Figure 5. Effects of vitamin D-free diet on the relative family abundance. The most significant changes at the family level are shown as a percent of total reads and represented in a box and whiskers plot for the standard diet (SD, n = 10) and vitamin D-free diet group (n = 10). * $p < 0.05$ and *** $p < 0.001$ vs. SD, student's t-test.

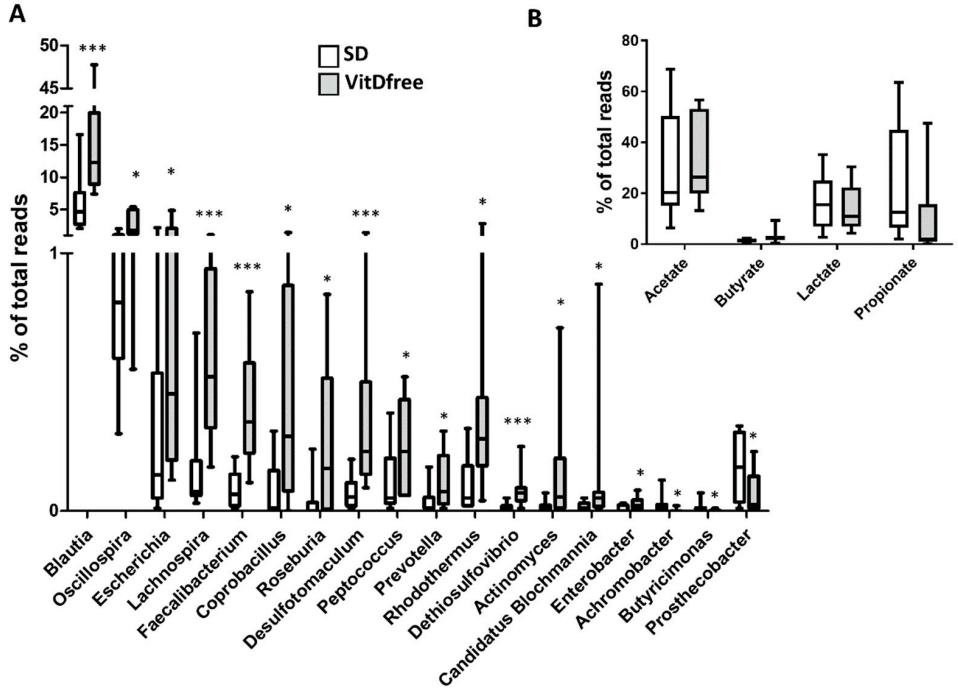

Figure 6. Effects of vitamin D-free diet on the relative genera abundance. (**A**) The most significant changes at the genus level are shown as a percent of total reads and represented in a box and whiskers plot for the standard diet (SD, n = 10) and vitamin D-free diet group (n = 10). (**B**). The relative proportions of acetate, butyrate, lactate and propionate producing bacteria in the gut microbiota. Sequence reads were classified according to the primary end product of the assigned bacterial genera. Genera were classified into more than one group correspondingly if they were defined as producers of multiple metabolites. Results were compared by student's t-test; * $p < 0.05$ and *** $p < 0.001$ vs. SD.

4. Discussion

The role of gut dysbiosis in the pathogenesis of many diseases, including diabetes mellitus, obesity, cancer, and psychiatric, respiratory and cardiovascular disorders is rapidly emerging. Similarly, vitamin D deficiency, which is the most common nutritional deficiency, has been related to a number of metabolic, cardiovascular, respiratory and psychiatric disorders. The primary end point of the present study was to analyze whether vitamin D deficiency induces gut dysbiosis. This might help to explain some of the health-related disorders associated with this hypovitaminosis.

Determination of 25OHVitD plasma levels are the most reliable indicator of vitamin D storage. Values below 20 ng/mL are usually considered deficient [37,38] and are associated with secondary hyperparathyroidism, muscle weakness, osteomalacia or osteoporosis. We fed the rats for seven weeks with a VitD-free diet, leading to a severe vitamin D deficiency (mean plasma 25OHVitD <10 ng/mL). Animal studies of the microbiota, like the present one, can be performed in strictly controlled conditions of diet and environment. However, extrapolation of rodent models to human beings should be done with caution. For the first time we report vitamin D-free diet-induced changes in the microbiota in the rat, which is more representative of the human gut microbiota than mice [39]. For obvious reasons, a randomized controlled study inducing vitamin D deficiency in humans cannot be performed. To our knowledge, only a small controlled human study in patients with cystic fibrosis [40] has been carried out to study the opposite approach, i.e., to analyze the impact on the microbiota of restoring vitamin D levels in patients with vitamin D deficiency. The cutoff value of 25OHVitD used in this study to classify patients as deficient was 30 ng/mL and the post intervention mean values in the placebo arm was ≈ 25 ng/mL, which is often considered within the normal range, compared to the active VitD arm ≈ 46 ng/mL. Several non-controlled or observational human studies and some studies in mice have also been published [41].

Our results show that a VitD-free diet did not produce large changes in the gut microbiota characteristics of dysbiosis, i.e., it did not lower microbial α-diversity and richness, it did not increase the Firmicutes to Bacteroidetes ratio, and it did not change the relative abundance of SCFA-producing bacteria. Moreover, principal component analysis of the bacterial community could not discriminate VitD-free diet treated from control animals in an unsupervised manner. α-diversity represents both the richness and evenness of species bacterial diversity within each sample and it is usually analyzed using the Chao1, Pielou, Shannon and/or Simpsons indices and the number of species. They are commonly used as indirect indicators of a healthy bacterial community and associated with a better health status. However, paradoxically, these indices, with the exception of the Chao1 index, were modestly but significantly increased by the vitamin D-free diet. This reduced diversity may be associated with the antibacterial effects of vitamin D [23]. A similar trend was observed both in mice with colitis and in healthy mice exposed to a vitamin D-free diet compared to a standard diet [42]. Other studies analyzing the association between vitamin D and alpha diversity in human studies were not consistent [41].

Despite the lack of effect on the α- and β-diversity, vitamin D-free diet produced significant changes in the relative abundance of forty-nine taxa, with fourty-four increased and five decreased. The present study does not establish a causal relationship between the specific bacterial changes described herein and the potential health deleterious effect of vitamin D deficiency or the putative mechanisms involved. However, these changes resemble those found in other pathological conditions. Notably, with vitamin D-free diet we found significant rises in typical members of the oral and gut microbiota, which are often responsible for enteral, urinary and respiratory tract infections, such as Enterobacteriaceae and its genera *Escherichia* and *Enterobacter*. These genera belong to the class Gammaproteobacteria, which was significantly more abundant in the stool samples of vitamin D-insufficient subjects compared with vitamin D-sufficient subjects [42]. Other opportunistic bacteria, *Prevotella* and *Actinomyces*, commonly found in multiple types of infection, were also increased in vitamin D deficient rats. These data suggest that vitamin D deficiency increases the relative abundance of opportunistic pathogens which, in the context of intestinal barrier dysfunction, may favour pathogen bacterial translocation and systemic infection and inflammation. In fact, vitamin D preserves the intestinal epithelial barrier

function [43]. Because the composition of the microbiota is essential for the intestinal barrier integrity, we speculate that the changes in the microbiota described herein may also be responsible for the vitamin D deficiency-induced alteration of the gut barrier. We also found a highly significant increase in bacteria from the phylum Synergistetes, which are considered as opportunistic pathogens involved in periodontitis [44], and its genus *Dethiosulfovibrio*, but its significance is unknown. Synergistetes and *Dethiosulfovibrionaceae* have also been reported in a rat model of acute myocardial infarction [45].

The family Odoribacteraceae and its genera *Odoribacter* and *Butyricimonas* have been reported to be depleted in several pathological conditions and animal models of disease such as overweight and obese pregnant women with high blood pressure, in sedentary mice, liver injury and pulmonary hypertension [13,46,47]. We found that this family and *Butyricimonas*, were decreased in vitamin D deficient rats. Likewise, this family was increased in response to vitamin D supplementation in vitamin D-insufficient subjects with cystic fibrosis [40]. Interestingly, *Odoribacter* was also depleted in vitamin D receptor knockout mice suggesting that this change is a VDR-mediated effect [48]. The potential protective effect of these genera is unclear. However, the abundance of *Butyricimonas*, which was decreased after high fat diet and reversed by statins or fecal transplantation, was correlated with the inflammatory cytokines IL-1β and TGFβ1 in the ileum, suggesting an anti-inflammatory effect [49].

5. Conclusions

In conclusion, vitamin D deficiency does not induce gut dysbiosis in the rat but produced specific changes in bacterial taxa, which may play a pathophysiological role in the immunologic dysregulation associated with this hypovitaminosis.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/11/2564/s1.

Author Contributions: Conceptualization, J.D. and F.P.-V.; Formal analysis, R.R., I.R.-V. and M.C.; Investigation, I.R.-V. and M.C.; Supervision, J.D. and F.P.-V.; Writing—original draft, I.R.-V. and M.C.; Writing—& editing, F.P.-V.

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Annex 4

Curriculum vitae

MARÍA CALLEJO ARRANZ

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2009-2013 Grado en Biología Sanitaria. Facultad de Biología, Universidad Complutense de Madrid.

2013-2014 Máster en Investigación Farmacológica. Facultad de Medicina, Universidad Autónoma de Madrid.

EXPERIENCIA CIENTÍFICA

- Mayo 2016-2021. *Personal Investigador Predoctoral en Formación de la Universidad Complutense de Madrid* (2015-CT45/15). Dpto. de Farmacología y Toxicología, Facultad de Medicina, Universidad Complutense de Madrid. Tesis doctoral: Vitamina D en hipertensión pulmonar. Directores: Prof. Francisco Pérez Vizcaíno y Prof. Ángel Cogolludo Torralba.
- Febrero 2016-Mayo 2016. *Ayudante de investigación* del Programa Operativo de Empleo Juvenil (YEI). Facultad de Medicina, Universidad Complutense de Madrid. Tutor: Prof. Francisco Pérez Vizcaíno.
- Julio 2015-2016. *Investigador colaborador*. Dpto. de Farmacología, Facultad de Medicina, Universidad Complutense de Madrid. Tutor: Prof. Francisco Pérez Vizcaíno.
- 2014-2015. *Colaboración en tareas de investigación*. Dpto. de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid. Tutor: Prof. Gloria Balfagón Calvo.
- 2012-2013. *Beca de colaboración* Ministerio de Educación, Cultura y Deporte. Dpto. de Fisiología Animal II, Facultad de Biología, Universidad Complutense de Madrid. Tutor: Prof. Esther Isorna Alonso.

ESTANCIAS DE INVESTIGACIÓN

- Septiembre-Diciembre 2018. *Centre Chirurgical Marie Lannelongue, INSERM U999*, Hypertension Artérielle Pulmonaire: Physiopathologie et Innovation Thérapeutique. Le Plessis Robinson, France. Supervisor: Prof. Frédéric Perros.
- Marzo-Abril 2016. *Dpto. de Farmacología*, Facultad de Farmacia, Universidad de Granada. Supervisor: Prof. Juan Manuel Duarte Pérez.

CONGRESOS, SEMINARIOS

- Más de 40 contribuciones a congresos nacionales e internacionales. Comunicaciones orales o póster; castellano o inglés.
- Impartición Seminario en el Instituto de Investigación Sanitaria del Gregorio Marañón, 2019.

DOCENCIA

- Prácticas de Farmacología. Grado en Medicina, Facultad de Medicina, UCM. Cursos 2016-2017; 2017-2018; 2018-2019; 2019-2020.
- Supervisión estudiantes prácticas externas Grado Nutrición (UCM), TFM Investigación Medicina Traslacional (UCM)

PROYECTOS DE INVESTIGACIÓN

- *MicroRNAs implicados en disfunción vascular pulmonar: implicaciones fisiopatológicas y terapéuticas.* Ministerio de Economía, Industria y Competitividad. SAF2014-55399-R.
- *Vitamina D en la hipertensión pulmonar*. Ministerio de Economía, Industria y Competitividad. SAF2016-77222-R.
- *Déficit de vitamina D en los pacientes con hipertensión pulmonar arterial y potencial valor terapéutico de la vitamina D como inhibidor de la proliferación de las células de músculo liso vascular arterial pulmonar*. Beca Actelion 2016. Fundación Contra la Hipertensión Pulmonar.
- *New markets and Therapeutic Targets for the Diagnosis and Treatment for Pulmonary Hypertension (EMPATHY).* Centro de Investigación Biomédica en Red de Enfermedades Respiratorias y Fundación Contra la Hipertensión Pulmonar.
- *Coinfection of HIV and schistosomiasis on the pulmonary vascular bed*. Cardiovascular Medical Research and Education Fund. FIBHGM-CCA028-2017.
- *Déficit de vitamina D y diabetes como factores predisponentes de hipertensión pulmonar*. Línea de Hipertensión Pulmonar CIBERES. Proyecto intramural.
- *Plan de formación docente de jóvenes investigadores pre- y postdoctorales del Departamento de Farmacología y Toxicología*. Proyectos de Innova-Docencia año 2018, Universidad Complutense de Madrid.

PREMIOS, RECONOCIMIENTOS

- 2019 Early Investigator Prize Journal of Physiology, Respiratory category.
- Segundo puesto mejor comunicación oral de la 3ª Jornada del PhDay de la Facultad de Medicina, UCM, año 2019.
- Primer puesto fase previa del II Concurso Tesis en 3 minutos ámbito Ciencias de la Salud, UCM, año 2018.
- Tercer premio en la categoría póster en la 2ª Jornada del PhDay de la Facultad de Medicina, UCM, año 2018.

DIVULGACIÓN CIENTÍFICA

- Organización Pint of Science en Madrid, año 2019.
- Noticia de prensa: Nuevos avances en el abordaje de la hipertensión arterial pulmonar a través de la microbiota intestinal.
- Participación Semana de la Ciencia en Madrid, año 2017 y 2015

Callejo M, Blanco I, Barbera JA and Perez-Vizcaino F. Vitamin D deficiency, a potential cause for insufficient response to sildenafil in pulmonary arterial hypertension. Eur Respir J. 2021; 58(5):2101204.

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Mondejar-Parreno G, **Callejo M**, Barreira B, Morales-Cano D, Esquivel-Ruiz S, Filice M, Moreno L, Cogolludo A and Perez-Vizcaino F. miR-1 induces endothelial dysfunction in rat pulmonary arteries. J Physiol Biochem. 2019;75(4):519-529.

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Morales-Cano D, Moreno L, Barreira B, Briones AM, Pandolfi R, Moral-Sanz J, **Callejo M**, Mondejar-Parreno G, Cortijo J, Salaices M, Duarte J, Perez-Vizcaino F and Cogolludo A. Activation of PPARbeta/delta prevents hyperglycaemia-induced impairment of Kv7 channels and cAMP-mediated relaxation in rat coronary arteries. Clin Sci (Lond). 2016;130:1823-36.