Novel mutations and decreased expression of the epigenetic regulator *TET2* in pulmonary arterial hypertension

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Background: Pulmonary arterial hypertension (PAH) is a lethal vasculopathy. Hereditary cases are associated with germline mutations in *BMPR2* and 16 other genes. However, these mutations occur in under 25% of idiopathic PAH patients (IPAH) and are rare in PAH associated with connective tissue diseases (APAH). Preclinical studies suggest epigenetic dysregulation, including altered DNA methylation, promotes PAH. Somatic mutations of Tet-methylcytosine-dioxygenase-2 (*TET2*), a key enzyme in DNA demethylation, occur in cardiovascular disease and are associated with clonal hematopoiesis, inflammation and adverse vascular remodeling. The role of *TET2* in PAH is unknown.

Methods: To test for a role of *TET2*, we utilized a cohort of 2572 cases from the PAH Biobank. Within this cohort, gene-specific rare variant association tests were performed using 1832 unrelated European PAH patients and 7509 non-Finnish European gnomAD subjects as controls. In an independent cohort of 140 patients, we quantified *TET2* expression in peripheral blood mononuclear cells. To assess causality, we investigated hemodynamic and histologic evidence of PAH in hematopoietic *Tet2*-knockout mice.

Results: We observed an increased burden of rare, predicted deleterious, germline variants in *TET2* in PAH patients of European ancestry (9/1832) compared to controls (6/7509; relative risk=6, p=0.00067). Assessing the whole cohort, 0.39% (10/2572) of patients had 12 *TET2* mutations (75% predicted germline and 25% somatic). These patients had no mutations in other PAH-related genes. Patients with *TET2* mutations were older (71±7 years versus 48±19 years, p<0.0001) unresponsive to vasodilator challenge (0/7 vs 140/1055 (13.2%)), had lower PVR (5.2±3.1 versus 10.5±7.0 Woods units, p=0.02) and had increased inflammation (including elevation of IL-1β). Circulating *TET2* expression did not correlate with age and was decreased in >86% of PAH patients. *Tet2*-knockout mice spontaneously developed PAH, adverse pulmonary vascular remodeling and inflammation, with elevated levels of cytokines, including IL-1β. Chronic therapy with an antibody targeting IL-1β blockade regressed PAH.

Conclusions: PAH is the first human disease related to potential *TET2* germline mutations. Inherited and acquired abnormalities of *TET2* occur in 0.39% of PAH cases. Decreased *TET2* expression is ubiquitous and has potential as a PAH biomarker.

Key words: Associated pulmonary arterial hypertension (APAH), epigenetics, scleroderma, connective tissue disease, CREST syndrome, TET methyl-cytosine dioxygenase 2 (*TET2*), clonal hematopoiesis of indeterminate potential (CHIP), myelodysplastic syndrome (MDS), DNA methylation, canakinumab

Clinical Perspective

What is new:

- *TET2*, encoding an epigenetic regulator that demethylates cytosine, is found to be mutated in both idiopathic pulmonary hypertension (IPAH) and associated PAH (APAH)
- *TET2* expression is ubiquitously decreased in peripheral blood cells of both IPAH and APAH patients
- *TET2* depletion creates a pro-inflammatory phenotype
- We present a new preclinical model of PAH, the *Tet2* $^{-/-}$ mouse
- Il-1β blockade improves PAH in mice with *Tet2* depletion

What are the clinical implications:

- *TET2* expression might represent a potential PAH biomarker
- *TET2* mutation(s) are a risk factor for PAH.
- The role of *TET2* mutations in clonal hematopoiesis of indeterminant potential (CHIP) and PAH suggest a relationship between PAH and hematopoietic disorders.
- *TET2* mutation(s) are a potential target for personalized medicine and potential indicator of benefit from anti-inflammatory therapies in PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a lethal vasculopathy hemodynamically characterized by increased mean pulmonary arterial pressure (mPAP >20 mmHg) and pulmonary vascular resistance (PVR >3 Wood units)[1]. Histologically PAH is characterized by obliterative pulmonary vascular remodeling. The current classification system divides Group 1 PAH into idiopathic (IPAH), hereditary (HPAH), and associated PAH (APAH; a category which includes patients with connective tissue diseases (CTD), such as scleroderma). 7-year survival rates of IPAH and CTD-PAH are 56%, and 35%, respectively[2]. High mortality rates in PAH are a consequence of late diagnosis, due to the non-specific clinical manifestations of the disease, the lack of biomarkers, and the absence of a curative treatment[3]. Moreover, the fundamental cause(s) of PAH remain elusive in many patients.

The etiology of PAH is heterogeneous and remains imperfectly understood, although it is characterized by increased inflammation and fibrosis and impaired angiogenesis, mitochondrial metabolism and mitochondrial dynamics[4]. At the genetic level, germline pathologic variants have been reported in 17 genes, by far the most prevalent of which is *BMPR2*[5–7]. Epigenetic dysregulation of genes is also important in PAH, and pathological activation of DNA methyltransferases (DNMT) has been shown to increase DNA methylation of specific genes associated with disease progression[8,9]. DNA methylation is a dynamic process that reflects the balance between the activity of DNMT, which adds methyl groups, and ten-eleven translocation methylcytosine dioxygenase (TET), which removes methyl groups, from cytosine nucleotides in DNA. Excessive methylation generally inhibits gene transcription, although there are also interactions between methylation sites and methyl binding proteins, which can alter the expression of other genes[10]. Somatic inactivating *TET2* mutations have recently been associated with development of inflammation[11,12] and atherosclerosis[13,14]. In addition, acquired mutations in this gene underlie clonal hematopoiesis of indeterminate potential (CHIP), a precursor to myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN) and even acute myeloid leukemia (AML) [15,16]. There is no known germline *TET2* mutation syndrome.

In this study we evaluated gene specific *TET2* exome sequencing data from the largest PAH cohort assembled to date, including 2572 patients in the PAH Biobank. Unlike prior genetic studies, the biobank includes subjects with APAH and non-European ancestry. We performed gene-specific rare variant association analyses using up to 1832 cases of European origin from the PAH Biobank and transcriptomic analysis in an independent cohort to assess *TET2* expression. In the entire cohort, we identified 12 predicted deleterious variants in *TET2* (75% predicted germline and 25% somatic), novel to PAH. None of the variant carriers were responsive to acute vasodilator challenge. This is the first time that putative germline *TET2* mutation has been associated with a human disease. We also identified ubiquitous downregulation of the expression of *TET2* in the peripheral blood mononuclear cells (PBMC) of IPAH and APAH patients. Finally, we evaluated *Tet2* depleted mice and demonstrated that they spontaneously develop inflammation, pulmonary vascular obliteration, and pulmonary hypertension, providing biological plausibility that disorders in this pathway can cause PAH.

Methods

The data, analytic methods, and study materials for the purposes of reproducing the results or replicating procedures can be made available on request to the corresponding author who manages the information.

Subject study

All patient samples were obtained following written informed consent and approval from local Institutional Review Boards. The study was approved by an institutional review committee and the subjects gave informed consent. Clinical characteristics of the PAH Biobank and John Hopkins cohorts are described in Table-S1. Additional methodologies are available in online supplements.

Exome sequencing, bioinformatics and statistical analyses for genetic studies

DNA was extracted from whole blood from participants in the National Biological Sample and Data Repository for PAH (PAH Biobank), and exome sequencing was carried out in

collaboration with the Regeneron Genetics Center or at the Cincinnati Children's Hospital Medical Center DNA Sequencing and Genotyping Core, as described previously[17,18]. We used Burrows-Wheeler Aligner (BWA-MEM)[19] to map and align paired-end reads to human reference genome (GRCh38), Picard MarkDuplicates to identify and flag PCR duplicates and GATK[20] HaplotypeCaller to call genetic variants. We used ANNOVAR[21] to aggregate variant annotations, including population allele frequency (gnomAD[22] exome/genome, ExAC, and 1000 genomes), predicted functional effects based on RefSeq, and predicted deleterious scores by methods such as REVEL[23].

For case-control comparisons, we performed principle components and relatedness analyses for the whole PAH cohort and identified 1832 unrelated European cases. We defined rare variants as variants with an allele frequency <0.01% across all gnomAD exome sequencing samples (European and non-European). We used heuristic filters to minimize technical artifacts between cases and controls, excluding variants that met any of the following criteria: missingness >10%, minimum alternate allele read depth ≤4 reads, alternative allele fraction ≤25%, or genotype quality <90. We used 7509 European gnomAD whole genome sequence (WGS) data as the control set. Only variants with FILTER "PASS" in gnomAD WGS (data release v 2.02) and located in the IDT xGen captured protein coding region were included in the analysis. Finally, we observed that the frequency of rare synonymous variants in cases and controls was virtually identical, indicating that the data sets are comparable (enrichment rate=1.0, p=0.11) (Table-S2). Assuming linkage disequilibrium is negligible among ultra-rare variants, we performed gene-wise burden tests of rare variants for *TET2* using Fisher's exact test to compare carrier frequency between cases and controls. We tested the association in three groups of variants between cases and controls: (a) rare deleterious missense variants ("D-MIS", defined as REVEL score >0.5); (b) likely-gene-disrupting (LGD) variants; and (c) D-MIS+LGD. We tested 3 genes *TET1, TET2, TET3*. We also tested the association in IPAH alone and APAH alone. Thus, we performed 11 tests (3 genes x 3 sets of variants and 1 gene x 2 PAH subclasses) and set the Bonferroni-corrected threshold for significance at p<0.0045. We then assessed the entire PAH Biobank (n=2572 cases) for rare (AF<0.0001), deleterious (LGD or D-MIS) variants in *TET2* using Integrative Genome Viewer (Illumina, San Diego, CA). Nearly 100% of both cases and controls had >10X sequencing coverage across the gene, and 99.6% of the targeted regions had >15X coverage in cases and

100% in controls (Figure-S1A,B), indicating that there was no systematic bias in the detection of *TET2* variants in cases vs controls.

To detect likely somatic variants (mosaicism) in *TET2*, we first used SAMtools (version 1.3.1-42)[24] to improve calling of genetic variants with low allele fraction. We then processed SAMtools calls using a set of heuristic filters to remove variants located in repeat regions (mapability, segmental duplication) or showing evidence of strand bias, and screened all variants using Integrative Genome Viewer. We then took the union of variants called by GATK HaplotypeCaller and SAMtools and considered variants with alternative allele fraction less than 25% as likely somatic mutations. Mosaic mutations were confirmed by TA cloning (Thermo Fisher, Waltham, MA) of exonic PCR products followed by Sanger sequencing of individual clones (Figure-S1C). Details are available in the online supplements.

Microarray and gene expression

A microarray assay was performed on RNA extracted from peripheral blood mononuclear cells (PBMC) of 50 scleroderma-associated PAH (SScPAH), 30 IPAH, 19 scleroderma without PAH patients (SSc) and 41 healthy controls from Johns Hopkins PH Program and Johns Hopkins Scleroderma Center, as previously published[25] and described in online supplementals. The expression data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE 33463. Analytical methods were performed as previously published[25]. Details are available in the online supplements.

Animal experiments

All experiments were performed in accordance with Queen's University biosafety and ethical guidelines (ROMEO/TRAQ#6016826). The procedures followed were performed in accordance with institutional guidelines. Conditional, haematopoietic heterozygous (*Tet2*$^{+/-}$) and homozygous (*Tet2*$^{-/-}$) knockouts were generated by crossing parental floxed (Tet2$^{f/f}$, B6;129S-Tet2tm1.1Iaai/J) and Vav1-Cre (B6.Cg-Tg(Vav1-Cre)A2Kio/J) mice (Jackson Laboratory, Bar Harbor, ME) and validated, as previously described[12,26]. IL-1β PAH regression experiments were performed on 5 *Tet2*$^{f/f}$ and 10 *Tet2*$^{-/-}$. 10 *Tet2*$^{-/-}$ mice (7 months old) were randomly distributed

in 2 groups of 5 age/sex-matched mice and treated with anti-IL-1β antibody (a generous gift from Novartis Pharma AG) at a dose of 10 mg/kg/week IP for 6 weeks) or IgG2a (a generous gift from Novartis Germany) as a placebo. Treatments and data collection were performed by scientists blinded to treatment groups. PAH development was assessed by echocardiography, right heart catheterization, 2-photon, confocal microscopy lung perfusion and histology, as described in online supplements. Inflammation was assessed by fluorescence-activated cell sorting (FACS) and NanoString nCounter PanCancer Immune Panel Profiling (Seattle; WA), as previously published[12] (see online supplements).

Results

Exome sequencing identifies rare deleterious variants in TET2.

Detailed characterization of the PAH Biobank and gnomAD cohort are described in a separate report[18] and in Table-S1. The cohort includes 2572 total cases: 43% IPAH, 48% APAH 4% FPAH and 5% other (Table S1). Among APAH patients, 58% (722/1239) had some form of connective tissue disease. The majority of cases are adult-onset, with a 3.7:1 ratio of females to males, typical of adult PAH. The genetically determined ancestries were 72% European, 12% Hispanic, 11% African, and smaller percentages of other ancestries.

We first limited the association analysis to 1832 unrelated European cases and 7509 non-Finnish, European controls. Using a REVEL score >0.5 to define D-MIS variants, we tested for association in three categories of variants: D-MIS, LGD or D-MIS+LGD. Among all PAH cases, we observed significant enrichment of LGD (8/1832 cases vs 4/7509 controls; RR=8.18, p=0.0005) as well as D-MIS+LGD variants for *TET2* (9/1832 cases vs 6/7509 controls; RR=6.15, p=0.00068) (Table-1A). The association was largely due to patients with IPAH (RR=10.79, p=8.483e-05) (Table-1B).

We then identified 1 additional mutation in the 740 non-European patients of the PAH Biobank (Table-2 and S3A). In the total cohort of 2572 cases, we identified 9 unique likely germline variants in 8 patients (Table-2 and S3A) and 3 somatic variants in 3 patients (Table-2 and S3B and Figure 1A). All mutations were unique, meaning the affected patients were not shown to carry variants in known PAH risk genes[7,28,29]. In addition, none of the *TET2* mutant

patient carriers had hematologic malignancies at the time of enrollment in the PAH Biobank. Two-dimensional structure of the encoded protein showed that 6 deleterious variants (both germline and somatic) localized to the conserved catalytic (TET/J-binding protein methylcytosine dioxygenase activity) domain (Figure 1B).

TET2 variant carriers exhibited increased overall inflammation compared to age/sex-matched PAH non-mutated patients, and age/sex-match controls, as assessed by measurement of 42 circulating inflammatory markers (area under the curve AUC 266.9 ±81.74 for *TET2* carriers; 117.1 ±58.9 *TET2* non-carriers; 41±5 for healthy control $p<0.05$) (Figure S2A-B). We focused on the expression of pro-inflammatory cytokines/chemokines and reported that *TET2* variant carriers had increased expression of 30 pro-inflammatory cytokines (AUC 204.3 ±72.14 versus 75.26 ±17.92 $p<0.001$) (Figure 2A-B and S2A-B and Table-S4). IL-1β expression was increased in 70% of *TET2*-mutated patients compared to their matched non-mutated PAH patients (Figure S2C).

Clinical phenotypes, including demographics, PAH medication and right heart catheterization (RHC) data, are provided in Tables 2 and 3. None of the *TET2* variant carriers (0/7) vs 140/1055 (13.2%) in the remainder of the cohort, were vasodilator responders, as assessed by standard criteria (Table-3)[30]. Consistent with the severity associated with lack of vasodilator responsiveness, the use of endothelin receptor inhibitors and soluble guanylate cyclase stimulators was increased in *TET2* variant carriers (Table-3). Two-thirds (6/10) of the patients with rare deleterious germline or somatic *TET2* variants had IPAH (Table 3 and S3) Female:male ratio and genetic ancestry of *TET2* germline and somatic carriers were similar to the overall cohort whilst the mean age-of-onset (66.9 ± 10.7years) was significantly older than that of all APAH+IPAH (48±19 years, $p<0.0001$) (Table 2). In addition the age of *TET2* subjects in our study was older than in previous reports for patients with PAH related to mutations in *BMPR2*[5,6,17] and *TBX4*[17]. *TET2* variant carriers had lower mPAP (43.3±6.5 mmHg) and PVR (6.8±3.1 Woods units) compared to all the remaining APAH+IPAH subjects in our cohort (50±14 mmHg, $p<0.0001$ and 10.5±7.0 Woods units, $p=0.02$) (Table 2).

Decreased TET2 gene expression in PAH

Having established the occurrence of *TET2* rare deleterious variants in PAH patients, we next investigated *TET2* expression in PBMCs. Gene expression omnibus (GEO) analysis was acquired from 50 SSc-PAH patients, 30 IPAH patients, 19 scleroderma without PAH patients (SSc) and 41 healthy controls. All the cases had adult-onset PAH, with genetically determined ancestries as follows: 82.1% Caucasian, 14.3% African, with smaller percentages of other ancestries. The sex ratios (female:male) were control 4.9:1, IPAH 5:1, and SSc-PAH 3.7:1 whilst all SSc patients were female (Table-S1). *TET2* gene expression, relative to healthy subjects, was decreased in 86% of SSc-PAH patients (relative expression; 0.75; $p<0.0001$); 86.7% of IPAH patients (relative expression; 0.74; $p<0.0001$) and 68% of scleroderma patients without PAH (SSc; relative expression; 0.79; $p<0.01$) (expression normalized to 1) (Figure-1C). Receiver operating characteristic curve (ROC) analysis was performed for 41 healthy control and 80 PAH patients (IPAH/SSc-PAH) and revealed a potential biomarker value for *TET2* expression (AUC:0.78; $p<0.0001$) (Figure S3A). ***Gene expression of other TET paralogs, TET1 and TET3, is not affected in PAH. Moerover,TET2 expression does not correlate with the age*** *(Table-S5A-B, Figure S3B,C)*

Experimental Tet2 depletion is associated with spontaneous development of PH in mice

We next assessed the biologic plausibility that hematopoietic *TET2* depletion could result in PAH. We performed hemodynamic measurement in 15 conditional *Tet2* KO mice ($Tet2^{-/-}$) and 15 sex and age-matched control mice ($Tet2^{f/f}$) (9 males; 6 females; age 7 to 10 months). Mice with *Vav-Cre*-mediated *Tet2* depletion spontaneously developed pulmonary hypertension, evident as a significant decrease in PAAT ($p<0.01$), an increase in RVSP ($p<0.001$), TPR ($p<0.01$), arterial elastance ($p<0.01$), adverse pulmonary vascular remodeling ($p<0.05$) and decreased perfusion of distal pulmonary arteries ($p<0.01$), compatible with obliteration of the microvasculature (Figure-3A-H and Figure-S4A-F; S5A-D; S6A). We observed no change in heart rate, stroke volume, cardiac output, RV-dP/dT_{max}, RV-dP/dT_{min} or LV function (LVS weight, E/E', MAPSE, arterial systolic and diastolic pressure, LV-EF, LVSP, LVEDP, Tau Mirsky, dP/dt_{max}, dP/dt_{min}) (Figure-S6B-F and S7A-K). Heterozygous animals ($Tet2^{+/-}$) also had increased RVSP ($p<0.05$) and TPR ($p<0.05$), with a trend toward increased arterial elastance but no changes in CO (Figure-S8A-D). We assessed PH development in 2-month-old $Tet2^{-/-}$ mice (5 males) and showed

that, compared to age-matched *Tet2*$^{f/f}$ mice, Tet2 depletion in young animals was not associated with PH phenotype (no significant differences in RVSP, TPR, CO) (Figure-S9A-C).

PH development was associated with increased inflammation in the lungs of *Tet2*$^{-/-}$ mice, evident as macrophage accumulation ($p<0.01$; Figure-3I) and dysregulation of 61 inflammatory markers (59 upregulated and 2 downregulated, Table-S6). Seventeen inflammatory markers had a change >1.8-fold (up-regulation of *Il1b, Cxcr2, Csf3r, C5ar1, Fpr2, Amica1, Ccr1, Mmp9, Cd33, Itgam, H2-Q10, Arg2, Clec4n, Il1rn, Rsad2, Il1r2* and downregulation of *Ccr6*) (Figure-3J). Tet2 is a critical regulator of DNA methylation. It is not surprising that we observed an increased DNA methylation (5mC) level in bone marrow and lung tissue of *Tet2*$^{-/-}$ mice (Figure-S10A-C). *Tet2* depletion was confirmed by PCR and by decreased Tet2 protein in the lung tissue of *Tet2*$^{-/-}$ mice (Figure-S11A-B). Thus, hemopoietic *Tet2* depletion is sufficient to increase DNA methylation and exacerbate inflammation and induce PH in mice, providing biological plausibility for the importance of the mutations and pathway perturbations we found in patients.

IL1-β blockade reverses the PH phenotype in Tet2 depleted mice.

Having established that PH development in *Tet2* mutated mice is associated with increased inflammation, we assessed the potential therapeutic effect of anti-inflammatory therapy. We treated 5 *Tet2*$^{-/-}$ mice (3 males and 2 females) with anti-IL-1β antibody (IP; 10 mg/kg/week; 6weeks) and 5 age/sex-matched *Tet2*$^{-/-}$ mice with IgG2a isotype control (IP; 10 mg/kg/week; 6weeks) at the age of PH onset (7 months) assessed by decreased PAAT (Figure-S12A). After 6 weeks of treatments, we first confirmed that animals treated with placebo developed PH evident as increased Fulton index, decreased PAAT, increased RVSP, mPAP, and TPR index (Figure-4A-E and Figure-S12A). Anti-IL-1β antibody treatment prevented weight loss observed in *Tet2*$^{-/-}$ mice and improved PH hemodynamics and phenotype parameters to healthy *Tet2*$^{f/f}$, levels (PAAT, RVSP, mPAP, TPRI) (Figure-4A-E). Note that IL1-β antibody treatment induced no kidney or liver toxicity (Table S7). Our results suggest that targeting inflammation through IL1-β blockade improves PH in *Tet2*$^{-/-}$ mice and confirms the contribution of inflammation (especially IL1-β) in the etiology of PH related to *Tet2* depletion.

Discussion

We used the largest cohort of PAH patients available to identify *TET2* as a novel gene that is mutated in PAH. *TET2* mutation, observed in 0.39% of PAH cases (40% APAH, 60% IPAH), is associated with a 6.15-fold increased risk of PAH, relative to the gnomAD control database. *TET2* mutations occurred in patients who were confirmed to be free of mutations in established PAH genes. Based on their allele fraction in peripheral blood, 75% of TET2 mutations are predicted to be germline versus 25% somatic. In an independent cohort, a decrease of TET2 expression was found in >86% of APAH and IPAH patients. Finally, we reported that conditional hematopoietic Tet2 knockout is sufficient to induce PH in mice.

Epigenetic mechanisms link genes and environment. They include changes in DNA methylation, histone acetylation and production of micro-RNAs. TET enzymes are key regulators of DNA demethylation, catalyzing the conversion of the methylated nucleotide 5-methylcytosine into 5-hydroxymethylcytosine. By subtraction of methyl groups on DNA and association with histone deacetylases[32], TET enzymes contribute to the epigenetic regulation of gene expression. Our group and others showed that hyper-methylation of specific target genes contributes to the development of PAH[8,9,33]. Supporting our observation, *TET2* loss of function leads to increased DNA methylation whilst *TET2* hematopoietic depletion results in inflammation and cardiac dysfunction[34,35]. We observed increased adverse pulmonary vascular remodeling in the lungs of *Tet2*$^{-/-}$ mice (Figure 3D), consistent with previous observations implicating *Tet2* depletion in development of atherosclerotic lesions[36]. We also confirmed that hematopoietic *Tet2* depletion increases DNA methylation (Figure-S10) and inflammation[12] evident as macrophage accumulation, and increased expression of inflammatory mediators (*Il1b, Cxcr2, Csf3r, Ccr1, Mmp9, Cd33, Itgam, Il1rn, Il1r2)* in *Tet2*$^{-/-}$ lungs (Figure-3I, J). These factors are known to be associated with the development of PAH[37]. Validating our observations, we reported a global increase of pro-inflammatory markers in the blood of human PAH patients with *TET2* deleterious variants compared to age/sex-matched non-carriers (Figure 2). The fact that this mouse spontaneously develops PAH experimentally links hematopoietic *Tet2* inactivation to vascular remodeling and inflammation, two common features of the PAH phenotype[37].

We generated conditional hematopoietic *Tet2* mutated mice to mimic *TET2* deleterious variants in human patients and the ubiquitous decrease in TET2 expression observed in the peripheral blood cells of PAH patients. We showed that homozygous *Tet2-/-* mice spontaneously developed a PH phenotype and notably manifested a profound loss of the pulmonary microvasculature. Heterozygous *Tet2+/-* mice have a genotype closer to the human condition described in this article and have a less pronounced phenotype ($p<0.05$), suggesting gene dose-effect response. This observation experimentally shows that, in an animal model, a total or partial loss of Tet2 function can induce vascular remodeling secondary to increased inflammation.

Age of onset of PAH is significantly higher in patients harboring somatic and germline *TET2* variants compared to the remainder of the cohort (66.9 ±10.7 VS 48 ±19 years) (Table 2). Consistent with this observation, older (7 month) *Tet2*$^{-/-}$mice spontaneously developed PH, whilst we did not detect significant PH in younger (2 month) *Tet2*$^{-/-}$mice (Figure 3 and Figure S9). This observation suggests that aging contributes to PH related to *TET2* mutations in both humans and mice. We believe a second hit may be required to elicit clinically evident PAH, such as occurs in CHIP and myeloid cancer risk. Indeed, *Tet2* normally restrains inflammation, but an initial trigger may be required to induce inflammation. Thus, *TET2* depletion exacerbates inflammation when it occurs in a pro-inflammatory environment. Chronic inflammation associated with aging might potentially be such a second hit[38] and thereby elicit clinically evident PAH in *TET2* deleterious variant carriers.

Mutations in 12 established risk genes and 5 recently-validated risk genes predispose to PAH, with *BMPR2* mutations as the most common genetic cause of HPAH and IPAH[7,18]. Here we identify an additional novel PAH gene and report that predicted deleterious germline and/or somatic variants of *TET2* underlie 0.39% of PAH cases. Our limited read depth, using whole exome data, likely underestimates the prevalence of somatic mutations (i.e. clonal hematopoiesis of indeterminate potential, CHIP). These *TET2* mutations are observed in patients who were shown to be free of the other known PAH risk genes variants. Interestingly, *TET2* mutations also occur in APAH patients, who are known to have more severe inflammatory states and greater mortality rates than other PAH groups[39]. Similarly, we reported that *TET2* variant carriers exhibit a pro-inflammatory phenotype compared to age/sex-matched, non-carrier, PAH patients. It is possible

that patients with APAH might also be inflamed as a result of their connective disease. However, the restricted sample size of this sub-study (n=10 patients with or without *TET2* mutations) and the variability in blood cytokine levels amongst patients, required us to perform our analysis on all mutation carriers, not just those with IPAH. Thus, we cannot exclude some confounding effects related to the inclusion of 4 patients with APAH and *TET2* mutations in this cohort.

Unlike our study, the NIHR BioResource-Rare Diseases PAH study, which investigated deleterious variation in 1048 IPAH/FPAH patients, did not enroll non-European subjects or APAH patients and did not report deleterious variants in *TET2*[40]. Our study has a ~2.5-fold larger sample size, used exome rather than genome sequencing, specifically assessed somatic mutations, and included APAH patients (49% of the 2572 patients). This is the first gene associated with APAH aside from those associated with congenital heart disease.

Over 86% of PAH patients have decreased *TET2* expression, suggesting an acquired mechanism of *TET2* gene dysregulation in PAH. Consistent with this speculation, hypoxia, as well as metabolic disorders observed in PAH, are also associated with pathologic regulation of *TET2* expression[41]. The difference between the rarity of *TET2* mutations versus the ubiquitous dysregulation of *TET2* expression mirrors what is seen with *BMPR2* in PAH. *BMPR2* mutations in non-hereditary PAH occur in the minority of subjects whereas *BMPR2* mRNA and protein expression is downregulated in most forms of human and experimental PAH[5,6].

TET2 is one of the two most commonly mutated genes in CHIP, which is associated with an increased risk of atherosclerosis and elevated inflammation[42]. CHIP mutations lead to elevation of interleukin 1β[43]. The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS)[31], which investigated the effect of a monoclonal antibody targeting interleukin-1β (Canakinumab) in cardiovascular diseases, identified the presence of CHIP (and *TET2* mutation) in 8.8% of the cohort. Patients with a somatic mutation in *TET2* showed a greater magnitude of risk for major adverse cardiovascular events (MACE); however, they also displayed an improved therapeutic response to canakinumab. We showed that conditional hematopoietic *Tet2* depletion is associated with accumulation of IL-1β in the lung of mice (Figure 3J) and reported that IL-1β blockade reverses PH in *Tet2*$^{-/-}$ mice (Figure 4). In PAH patients, elevated serum levels of IL-1β correlate with a worse outcome and targeting this cytokine, in a preclinical model of PAH,

improved the disease[44]. An IL-1β receptor antagonist is currently in phase 1 clinical trial in PAH (NCT03057028). As observed for CANTOS and in *Tet2*$^{-/-}$ mice, our results suggest that genetic investigation of *TET2* variants and expression might be relevant to predict the likelihood of benefit from IL-1β based therapy. Thus, discovery of this new gene mutation in PAH may have therapeutic consequences.

CHIP is also a precursor to myeloid neoplasms such as MDS and AML[13,15]. Intriguingly, previous studies have shown that PAH is observed in 15-48% of patients with MDS, where *TET2* is one of the most common mutated genes[45]. We demonstrated that hematopoietic *Tet2* depletion is associated with PAH and pulmonary vascular remodeling in mice. The same mutant mice ultimately develop an MDS-like disease[26], which experimentally confirms the link between *Tet2* depletion, PAH and MDS; also suggesting a relationship between CHIP and PAH. The moderate severity of pulmonary hypertension reported in our animals (RVSP 30.2 ± 1.3mmHg; Figure-3A) and the milder severity of the hemodynamic derangement in *TET2* mutant patients (Table-2) suggests this gene mutation might result in a less symptomatic state in patients, which would be hard to detect without active surveillance. Borderline elevation of systolic PAP (29mmHg at rest) was recently reported in a cohort of 34 MDS patients, lending support to our experimental observations[46].

Our observations suggest that CHIP and PAH are two manifestations of similar mutations. Whether the manifestation of the mutations will be vascular or hematopoietic (or both), likely reflects a complex interplay of factors including: mutation burden, mutation-related inflammatory consequences, and cellular/tissue distribution of the mutations. The *Tet2*-knockout mouse is an accepted model of human myeloid neoplasia and our discovery that PAH develops spontaneously as these mice age, indicates that both age and TET2 deficiency may also interact to yield different disease manifestations.

Limitations

Using the largest PAH cohort to date, we reported enrichment of rare deleterious variants for *TET2* among 2572 PAH patients. However, we did not investigate the occurrence of *TET2* mutations in other forms of pulmonary hypertension. This limitation precludes any conclusion

regarding the specificity of the mutation to group 1 PH versus patients with group 2-5 PH. Similarly, as observed for *BMPR2* mutation, which is mainly reported in PAH but also observed in cancer[47] (e.g. gastric, colorectal, breast cancer), we acknowledge that *TET2* mutation might not be specific for PAH. Indeed, it is known that somatic mutations in *TET2* are common in MDS and AML and can portend poor prognosis[48]. However, none of our patients had known active myeloid neoplasms, and we have provided strong evidence of the contributions of deleterious mutations in *TET2* to PAH's etiology.

Kaasinen and colleagues have recently reported germline *TET2* frameshift mutation in a lymphoma family[49]. They did not mark observation of unusual predisposition to atherosclerosis nor abnormal pro-inflammatory cytokine or chemokine expression in this family. However, their study was conducted in a Finnish population which was not investigated in our work. The mutation (c.4500delA) reported by Kaasinen and al, was not observed in the PAH cohort. In their report, *TET2* mutation was not associated with significantly decreased *TET2* expression in peripheral blood cells. Based on this observation and our results, we can speculate that decrease *TET2* expression in peripheral blood cells is required to induce a pro-inflammatory phenotype and cardiovascular disorders.

The PAH Biobank used the REVEAL relaxed criteria for enrollment of patients deemed to have Group 1 disease by their PH specialist. This allowed enrollment of patients with a PCWP ≤18 mmHg, which is the case for patient 12-206[50]. While patient 29-016 had a PCWP of 17mmHg, which is above the conventional cut-off of 15mmHg, as defined by the World Symposium on Pulmonary Hypertension[1], this likely reflects the technical heterogeneity of PCWP measurements, rather than clinical misclassification. This patient's PAH physician integrated all data to make the best clinical diagnosis.

We provided the hemodynamic data obtained in closest temporal proximity to enrollment in the biobank. In most cases these were the diagnostic hemodynamics, obtained prior to treatment; however in one patient (19-036), the low PVR we reported reflected the fact they were already being treated with two PH-targeted medications. At the time of their diagnosis, prior to PH-targeted treatment, their hemodynamics were typical of PAH (mPAP of 67mmHg and a PVR of 9.96WU).

The use of gnomAD as a control cohort can be consider as a limitation of the study. GnomAD spans 15708 genomes from unrelated individuals sequenced as part of various disease-specific and population genetic studies. GnomAD excludes individuals known to be affected by any severe pediatric disease, as well as their first-degree relatives; however, some individuals with severe disease may still be included in the data set, albeit likely at a frequency equivalent to or lower than that seen in the general population. Thus, the 7509 non-Finnish European gnomAD cohort used in our study likely includes individuals with severe diseases, potentially including hematologic disorders or atherosclerosis. This limitation could explain the 6 patients with *TET2* variants observed in the gnomAD cohort. Regardless of this limitation we observed a 5.5-fold enrichment of *TET2* deleterious variants in the PAH cohort.

Conclusion.

We identify *TET2* as a new PAH-associated gene and highlight its importance as a potential mechanism for the component of PAH pathogenesis resulting from inflammation. The *TET2* pathway offers potential new biomarkers and therapeutic targets for PAH therapy, including canakinumab.

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Figure Legends:

Figure 1. Increased mutation and decreased *TET2* gene expression in blood from PAH patients. Topologic analysis and gene expression of the Human DNA tet methylcytosine dioxygenase 2 (*TET2*) in pulmonary arterial hypertension (PAH). **A)** Distribution of *TET2* somatic and germline deleterious variants. **B)** Locations of rare deleterious PAH patient-derived *TET2* variants within the two-dimensional protein structures. The numbers of variants at each amino acid position are indicated on the y-axis. Germline variants are shown above the protein schematic; mosaic variants are below. D-MIS, predicted damaging missense; LGD, likely-gene-disrupting (stopgain, frameshift). TET/JBP, TET/J-binding protein catalytic domain. Protein domain coordinates were modified according to UniProtKB). **C)** *TET2* gene expression in peripheral blood mononuclear cells (PBMCs) from 41 healthy control, 30 idiopathic PAH (IPAH), 50 scleroderma associated PAH (SSc-PAH) and 19 scleroderma without PAH (SSc). Violin plot was used to visualise data distribution; black line is the median; red lines are the upper and lower quartile. one-way ANOVA. *P<0.05; **P<0.01; ****P<0.0001; n.s. non-significant.

Figure 2. *TET2* mutation is associated with a pro-inflammatory phenotype in blood of PAH patients. Expression of 30 pro-inflammatory cytokines were assessed in blood of 9 healthy patients, 10 age/sex-matched PAH *TET2* variant carriers and 10 matched PAH TET2 non-carriers patients. **A)** *TET2* variant carriers show increased levels of 28 cytokines (IFNa2; IP-10; IL-12p40; IL12p70; IL-6; IL-1β; IL-2; Fractalkine; IFNy; IL-15; IL-1a; IL-18; IL-3; G-CSF; IL-7; TNFa; IL-17A; MIP-1a; MIP-1B; MDC; TNFB; IL-5; Flt-3L; MCP-3; IL-8; RANTES; GRO alpha) and decreased levels of 2 cytokines (MCP-1; Eotaxin) compared to matched non-carriers patients. Values are expressed as fold change compared to healthy patients. **B)** TET2 carriers display overall increased levels of pro-inflammatory markers measured by an increase of the area under the curve. One-way ANOVA. Values are expressed as mean±SEM. ***P<0.001

Figure 3. *TET2* depletion in hematopoietic cells and pulmonary hypertension in a murine model. Haemodynamic assessment of pulmonary hypertension using right heart catheterization and echocardiography of 6-15 $Tet2^{-/-}$ and age-sex matched $Tet2^{f/f}$ mice show **A)** increased right

ventricular systolic pressure (RVSP), **B)** decreased pulmonary artery acceleration time (PAAT) and, **C)** increased total pulmonary resistance (TPR) in *Tet2*$^{-/-}$ mice compared to *Tet2*$^{f/f}$ animals. **D)** Pulmonary arterial vascular remodelling was blindly quantified by the percent of wall thickness: (total diameter−internal diameter)/total diameter by immunofluorescence (smooth muscle actin); 10 arteries per mice on 5 mice per group. Pulmonary vascular wall thickness is increase in *Tet2*$^{-/-}$ mice compared to *Tet2*$^{f/f}$. Perfusion of pulmonary vessels has been assessed in *Tet2*$^{-/-}$ and *Tet2*$^{f/f}$ mice (FITC albumin perfusion, 2 photon microscopy). Perfused vessels have been clustered according to their volumes in 3 categories: small (15-225µm^3), intermediate (225-3347µm^3) and big (3347-50000µm^3). *Tet2*$^{-/-}$ mice display decreased numbers of **E)** small and **F)** intermediate (P=0.08) perfused vessels whilst **G)** number of large vessels perfused remains the same compared to *Tet2*$^{f/f}$ (number of vessels/1e^6 µm3). **H)** Representative pictures of vessels perfused by FITC-albumin in the lung of *Tet2*$^{-/-}$ and *Tet2*$^{f/f}$ (500X550µm). **I)** Compared to *Tet2*$^{f/f}$ animals, lungs from mutated mice (*Tet2*$^{-/-}$) show an elevated macrophages population measured by fluorescence activated cell sorting (FACS; F4/80; CD11b; n=6 *Tet2*$^{-/-}$ and age-sex matched *Tet2*$^{f/f}$). **J)** Quantification of inflammatory cytokines and chemokines in total lung of 3 Tet2$^{-/-}$ and age-sex matched *Tet2*$^{f/f}$ mice displays up-regulation of *Il1b, Cxcr2, Csf3r, C5ar1, Fpr2, Amica1, Ccr1, Mmp9, Cd33, Itgam, H2-Q10, Arg2, Clec4n, Il1rn, Rsad2, Il1r2* and downregulation of *Ccr6* gene expression in mutated animals. Results show change >1.8 fold Log2-transformed normalized NanoString mRNA counts. Unpaired t- test. Values are expressed as mean±SEM. n.s.: non-significant; *P<0.05; **P<0.01; ***P<0.001.

Figure 4. IL-1β blockade reverses PH in *Tet2* mutated mice. 10, 7-month*Tet2*$^{-/-}$ mice were treated with an antibody against IL-1β (*Tet2*$^{-/-}$ + IL-1β ab; IP; 10mg/kg/week; 6weeks) or IgG2a (*Tet2*$^{-/-}$ + IL-1β ab IgG2a; IP; 10mg/kg/week; 6 weeks). *Tet2*$^{-/-}$ + IL-1β ab mice showed **A)** significant increased pulmonary artery acceleration time (PAAT); **B)** decreased right ventricular systolic pressure (RVSP); **C)** decreased mean pulmonary arterial hypertension (mPAP); **D)** reduced Total pulmonary resistance (TPR) normalized by body weight associated. **E)** IL-1β antibody treatment significantly prevents weight loss observed in *Tet2*$^{-/-}$ mice treated with IgG2a. n=5 per group. One-way ANOVA; mixed effect ANOVA. Values are expressed as mean±SEM. *P<0.05, **P<0.01.