Contribution of inflammation and impaired angiogenesis to the pathobiology of CTEPH

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DATA SUPPLEMENT

MATERIEL AND METHODS

Study population

Proximal pulmonary vascular material was obtained from 52 consecutive patients diagnosed with CTEPH, who underwent a PEA between April 1, 2004 and January 31, 2009 at the University Hospital in Leuven, Belgium. Healthy subjects were recruited out of screening programs from the departments of urology and gynaecology. The study protocol was approved by the Institutional Ethics Committee of the University Hospital Leuven and all participants gave written informed consent.

Patient characteristics

Medical records of the patients were reviewed and the following data were extracted: gender, age, weight and length, modified New York Heart Association (NYHA) functional class, and 6 min walking distance (6MWD). Any history of acute pulmonary embolism and/or deep venous thrombosis has been recorded. The presence of established thrombotic risk factors including lupus anticoagulant/antiphospholipid antibodies, protein S and C deficiency, activated protein C resistance, including Factor V Leiden mutation, prothrombin gene mutation, and antithrombin deficiency was recorded as previously described [1]. Elevated circulating factor VIII and hyperhomocysteinemia were also reported. At study entry, patients completed a questionnaire that provided information about smoking habits, any history of diabetes mellitus and hypertension. Right heart catheterization was performed at diagnosis, during and 3 days after surgery, to measure right atrium pressure (RAP), mean pulmonary arterial pressure (mPAP), pulmonary vascular resistance (PVR) and cardiac index (CI). Regarding the 41 patients with a history of acute pulmonary embolism (PE), the age of the lesions was calculated as the time lapse between the first acute PE episode and the PEA. The persistence of pulmonary hypertension after PEA was recorded and defined as previously described [2], by a mPAP ≥ 35 mmHg 2-3 days postoperatively. The time between diagnosis and start of any PAH targeted

treatment for persistent pulmonary hypertension, and between diagnosis and death was calculated. The observation period was between April 1st, 2004 and October 31st, 2013, i.e. 9.5 years. Adverse outcome events include start of a PAH-targeted therapy, persistent PH after PEA and all-cause mortality.

Blood Samples

Blood samples from the 52 patients included were collected on EDTA at the time of diagnostic right heart catheterization and plasma was prepared. Blood samples from 20 healthy controls were collected during a consultation. Plasma levels of various circulating inflammatory mediators, including CRP, cytokines such as IL-1β, IL-6, IL-10, chemokines, such as MCP-1 and MIP1-α, matrix metalloproteinase MMP-9, and growth factor VEGF were measured at diagnosis, before PEA. CRP levels (Tina-quant C-Reactive Protein, Roche Diagnostics) were determined in the University Hospital routine lab. Upper limit of normal (ULN) was 5 mg.L-1. Interleukin (IL)-1β, IL-6, IL-10, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein1-α (MIP1-α), matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor-A (VEGF-A) levels were measured in the plasma of 20 CTEPH patients and 20 healthy subjects using MSD Multi-Spot assay system, according to the supplier protocols.

Tissue collection and sampling

At the time of PEA, proximal pulmonary vascular material from 52 patients was collected and photographed; 103 specimens (left and right side for 51 patients, right side for one patient) were macroscopically classified according to the “Jamieson classification” [3]. The vascular material was then fixed in 4% paraformaldehyde for further histochemistry and immunohistochemistry analysis.

Histopathology

After being fixed, tissue was paraffin-embedded and 7-µm sections were performed. Sections were deparaffinized and rehydrated. Nuclei and cytoplasm were labeled by hematoxylin and eosin, respectively. Histochemistry of elastic and collagen fibers was performed using the Verhoeff's van Gieson and Masson’s trichrome methods, respectively. Freshly formed fibrin was labeled using phosphotungstic acid. Lipid deposits were labeled using oil red-O. Immunohistochemistry analysis was

performed using the system EnVision™ FLEX+ (Dako). T lymphocytes and neutrophils were detected using rabbit polyclonal antibodies (Dako) against CD3 (dilution, 1:250), myeloperoxidase (dilution, 1:2000), respectively. B lymphocytes, macrophages, smooth muscle cells and endothelial cells were detected using mouse monoclonal antibodies (Dako) against CD20 (dilution, 1:500), CD68 (dilution, 1:50), α-smooth muscle actin (α-SMA; dilution: 1:200) and CD31 (dilution, 1:50), respectively. Regarding CD31 and myeloperoxidase antibodies, the low pH target retrieval solution was used and the high pH target retrieval solution for the other antibodies. A system for quantifying histopathological features as hallmarks of CTEPH was developed so that the severity of the different abovementioned staining and labelling could be compared between the different patients included. Labelling for α-SMA (SMC), CD31 (EC), CD3 (T lymphocytes), CD20 (B lymphocytes), CD 68 (macrophages) and myeloperoxidase (neutrophils) was scored by a specialized pathologist (E.V.) who was unaware of the outcome of each patient, as 0 (reaction absent), 0.5 (doubtful or minimal reaction), 1 (mild reaction, seen only at high power magnification), 2 (moderate reaction, seen at low power), and 3 (severe reaction) as previously described [4,5]. Each specimen was screened from central (main pulmonary artery) towards peripheral (lobar and segmental arteries) vascular territory. Between 2 to 7 sections were analysed per vascular territory and the highest score was recorded.

The topographic distribution of the different labelling was assessed as “superficial” (close to the residual lumen of the pulmonary artery), “trans-mural”, or “deep” (close to the native media of the pulmonary artery).

Statistical analysis

Database management and statistical analyses were performed using GraphPad Prism version 4.01. Values not normally distributed were log-transformed to normalize their distribution and expressed as geometric mean with 95% confidence interval (95% CI). Differences between more than 2 groups were analyzed using ANOVA test followed by Tukey post-hoc tests. The cut-off value of angiogenesis score to predict survival, start of medical treatment and persistent pulmonary hypertension after PEA was determined by performing a receiver-operator characteristic (ROC) analysis. Odds ratios to predict

survival, start of medical treatment and persistent pulmonary hypertension after PEA were determined by performing a Chi-square test. Survival curves for patients were contrasted with angiogenesis score above and below the cut-off value by Kaplan-Meier survival function estimate and log-rank test. P-values were for 2-sided tests. $p<0.05$ was considered statistically significant. Pearson correlations were performed between circulating inflammatory mediators and local accumulation of inflammatory cells.

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LEGENDS TO FIGURES

Figure S1. Angiogenesis and adverse outcome. CD31 immunostaining was scored in the different types of lesion and for the different stages of angiogenesis (early and late). Lesions were stratified according to persistent pulmonary hypertension after PEA, survival and start of a medical treatment. Scored overall angiogenesis according to persistent pulmonary hypertension after PEA **(A)**, survival, *p=0.009 **(B)** and start of a medical treatment **(C)**. Scored late angiogenesis according to persistent pulmonary hypertension after PEA, *p=0.03 **(D)**, survival, **p=0.001 **(E)** and start of a medical treatment, *p=0.01 **(F)**.

Figure S2. Inflammatory cells accumulation scores in different types of lesion according to inflammatory cell type. CD3, CD20, CD68 and myeloperoxidase immunostaining, in proximal pulmonary vascular material **(A),** in atherosclerotic **(B)**, neointima **(C)**, thrombosis **(D)** and recanalized lesions **(E).** ANOVA, p<0.0001; **p<0.001 *vs.* B lymphocytes; *p<0.01 *vs.* B lymphocytes, T lymphocytes and macrophages; $^{\#}$p<0.05 *vs.* B lymphocytes.

Figure S3. Inflammatory cells accumulation scores according to the type of lesion. (A) for all inflammatory cells, and specifically for B lymphocytes (B), T lymphocytes (C), neutrophils (D) and macrophages (E). **(A, B, D, E)** ANOVA, p<0.005; ***p<0.001 *vs.* neointima; **p<0.001 *vs.* atherosclerosis; *p<0.01 *vs.* atherosclerosis; §p<0.01 *vs.* thrombosis; $^{\#}$p<0.05 *vs.* atherosclerosis and thrombosis; **(C)** ANOVA, p<0.03.

Figure S4. Topographic distribution of inflammatory cells type in proximal pulmonary vascular material **(A)** and in atherosclerotic **(B)**, thrombotic **(C)**, neointimal **(D)** and recanalized lesions **(E).** ANOVA, p<0.0001, *p<0.001 *vs.* B lymphocytes; §p<0.001 *vs.* neutrophils.

Figure S5. Correlation between circulating CRP and MMP-9 and local inflammatory cell accumulation. Pearson correlation between CRP and inflammatory cells **(A)**, CRP and neutrophils **(B)**, MMP-9 and inflammatory cells **(C)**, MMP-9 and macrophages **(D)**.