Master Thesis

VALIDATION OF D-DIMER TESTING FROM HEPARINIZED PLASMA

Submitted by

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Statutory Declaration

I declare on my honor that I have written this dissertation independently and without assistance, that no sources other than those cited were used and that the sources used verbatim or in substance have been marked as such.

Graz,19.07.2020 *Signature (handwritten)*

Foreword

To whom it may concern……

It is almost 10 years ago as back in 2010 I joined the Medical University of Graz as a PhD student. Shortly afterwards we had a meeting with the Biobank because we wanted to establish a collection for our own research purposes. During the following years we established several internal protocols, we collected several blood samples and we had several fruitful discussions with the biobank, which led to a valuable collection of serum, plasma and whole blood coupled with clinical data. As a result of our common efforts high impact papers have been published under the umbrella of the Ludwig Boltzmann Institute for Lung Vascular Research and the Medical University of Graz. Once I finished the PhD I joined the Division of Pulmonology as a clinician, however I continued to serve as a contact person between our institute and the Biobank. Meanwhile I finished speciality training for pulmonology. Additionally I have got the opportunity to further manage our internal inquiries and to serve as a bridge between patients, laboratory and biobank staff. This was not an easy job at all. One one hand the collected samples make sense if they are collected and stored on a standardized way and are coupled with valuable clinical data and they are used. On the other hand the responsible usage of the samples requires valid scientific questions. It is the merit of the biobank and the researchers whom I am working with that these conditions are given. The Master of Science Biobanking is another merit of the Biobank Stuff who recognized the need for teaching and telling the experience they gained throughout the years. I am very pleased that I could join the courses, which provided strong bases for the future work. I would recommend this Master Study to everybody who is interested in Biobanking independently of the area of expertise. I learned that Biobanking is one of the most difficult jobs, however if it is done with perseverance and heart, success is warranted.

Vasile Foris, MD, PhD

Acknowledgement

My first acknowledgement goes to my beautiful wife Anna who encouraged me to take the opportunity and pursue for this master course. I received a lot of support from her and she is a source of joy and happiness at every moment. Special thanks go to my little son, Ádám who was missing me while I was busy with this work.

I will never forget that summer evening as I was having a beer with two of my best friends, Dr. Babicsák Botond and Dr. Bálint Zoltán while we were on vacation and our wives and children were sleeping. I told them about the Master for Biobanking and expressed my enthusiasm but also my scepticism and concerns. Both of them rather pushed me to apply and I was very happy that they shared my excitement. Thank you guys for that.

Many thanks to Gabriele Hartl who was continuously supporting us, taking care of the organisation of the modules and who always had a smile for everyone. I am very thankful for Dr. Karyne Sargsyan and Prof. Dr. Berthold Huppertz for the professional guidance through the complex topics. Both of them provided us with warm academic atmosphere, a huge amount of knowledge and they never stopped to sharing with us their valuable and unique experience. I would like to express my gratitude for Prof. Dr. Berthold Huppertz for supervising my thesis, for his great comments from the very beginning and for his patience with me.

I would like to thank to my current chief Prof. Dr. Horst Olschewski for the refreshing discussions and for showing me the publication that led to the idea of this thesis. Without the help of Dr. Florian Prüller and the staff of the Clinical Institute of Medical and Chemical Laboratory Diagnostics the measurements would not be possible. Dr. Alexander Avian provided statistical support and I am very glad that I could have him on board.

The blood samples used in this study were stored and kindly provided by the Biobank of the Medical University of Graz.

Who I should acknowledge the most, are my parents who made possible that I am here right know.

Abstract

Einleitung

D-Dimer ist ein Marker für Koagulation und Fibrinolyse, der eine hohe Sensitivität, aber eine moderate Spezifität für thromboembolische Ereignisse hat. Wir haben uns die Frage gestellt, ob D-Dimer aus heparinisiertem Plasma mit einer neuen hoch sensitiven Methode gemessen werden kann und ob die Messwerte mit pulmonaler Hypertonie (PH) assoziiert sind sowie ob D-Dimer eine prognostische Rolle bei PatientInnen mit einem Risiko für PH hat.

PatientInnen und Methoden

D-Dimer wurde aus heparinisiertem Plasma parallel mit dem Standard INNOVANCE assay und mit dem neuen high-sensitive immunoturbidimetric assay (LOCI) gemessen. N=295 PatientInnen (n=54 pulmonale arterielle Hypertonie; n=39 PH assoziert mit Linksherzerkrankungen; n=56 PH assoziert mit Lungenerkrankungen; n= 31 chronisch thromboembolische pulmonale Hypertonie; n=16 pulmonale Hypertonie infolge von unklaren oder multifaktoriellen Mechanismen; n=99 pulmonale Hypertonie Ausschluss durch Rechtsherzkatheter), die zwischen 2011 und 2017 mittels Rechtsherzkatheter (RHK) untersucht worden sind, wurden in diese Studie nach schriftlicher Einverständniserklärung prospektiv eingeschlossen. Die Plasmaproben wurden während dem RHK gesammelt, aliquotiert und bei -80°C in der Biobank Graz gelagert.

Ergebnisse

D-Dimer konnte zweifach in n=266 PatientInnen (mittleres Alter: 63±13, mPAP: 33±14 mmHg, PAWP: 11±5 mmHg, PVR: 5.2±4.1 WU, CI=2.6±0.8 l/min/m^2) mit beiden Methoden zuverlässig gemessen werden. Eine Bland Altman Analyse zeigte, dass die zwei Methoden vergleichbar sind. Während der medianen Beobachtungszeit von 48 Monaten sind n=63 PatientInnen verstorben. Nach Korrektur für Alter und Geschlecht konnte ein D-Dimer cut-off Wert von 402µg/l (LOCI) die Mortalität im Gesamtkollektiv vorhersagen (HR: 1.7 95%CI 1.0 – 2.9; p<0.033). PatientInnen über dem cut-off Wert waren älter (68 (IQR=59-75) vs. 63 (IQR=54-71)), hatten häufiger PH (56% vs. 44%) und hatten schlechtere hämodynische Werte (mPAP: 34 (IQR=24-45) vs. 27 (IQR=20-41) mmHg, PVR: 4.3 (IQR=2.4-8.6) vs. 3.2 (IQR=2.3-6.1) WU). Die Subgruppenanalyse zeigte, dass PatientInnen mit PH höhere D-Dimer Werte im Vergleich zu PatientInnen ohne PH

(324ug/l (IQR: 208 – 584) vs. 422ug/l (IQR: 237 – 719); p=0.047) hatten. D-Dimer war mit Mortalität nur in der PH Gruppe (HR 2.2, 95%CI 1.1 – 4.5; p=0.022) assoziiert.

Schlussfolgerung

D-Dimer kann aus heparinisiertem Plasma mittels hoch sensitivem Verfahren verlässlich gemessen werden. D-Dimer scheint ein neuer Prädiktor für Mortalität in einer gemischten Population mit Risiko für PH zu sein, der unabhängig von Alter und Geschlecht ist.

Abstract

Introduction

D-Dimer is a marker of coagulation and fibrinolysis activity, which has a high sensitivity but a moderate specificity for thromboembolic events. We asked the question if D-Dimer can be reliably measured from heparinized plasma and if D-Dimer levels are associated with pulmonary hypertension (PH) and if it has a prognostic role in patients with PH risk.

Patients and Methods

D-Dimer levels were assessed from heparinized plasma in parallel using the standard INNOVANCE assay and a new high-sensitive immunoturbidimetric assay LOCI. N=295 patients (n=54 pulmonary arterial hypertension; n=39 PH due to left heart disease; n=56 PH due to lung diseases; n= 31 chronic thromboembolic PH; n=16 PH due to unclear or multifactorial mechanisms; n=99 PH excluded by RHC) undergoing RHC between 2011 and 2017 were prospectively enrolled in this study after written informed consent. Plasma samples were collected during RHC and stored at Biobank Graz at -80°C.

Results

D-Dimer was reproducibly measured with both methods in duplicate in n=266 patients (mean age: 63±13 yr, mean pulmonary arterial pressure (mPAP): 33±14 mmHg, pulmonary arterial wedge pressure: 11±5 mmHg, pulmonary vascular resistance (PVR): 5.2±4.1 WU, cardiac index=2.6±0.8 l/min/m^2). A Bland Altman analysis showed that the two methods are comparable. During the median follow-up time of 48 months, n=63 patients died. After adjustment for sex and age, a D-Dimer cut-off value of 402µg/l measured with LOCI predicted all-cause mortality (HR: 1.7 95%CI 1.0 – 2.9; p<0.033) in the cohort. Patients above the cut-off level were older (68 (IQR=59-75) vs. 63 (IQR=54-71)), more likely to have PH (56% vs. 44%) and had more severe hemodynamic changes (mPAP: 34 (IQR=24-45) vs. 27 (IQR=20-41) mmHg, PVR: 4.3 (IQR=2.4-8.6) vs. 3.2 (IQR=2.3-6.1) WU). Subgroup analysis revealed that patients with PH had higher D-Dimer values than those without PH (324ug/l (IQR: 208 – 584) vs. 422ug/l (IQR: 237 – 719); p=0.047) and D-Dimer was significantly associated with mortality in the PH subgroup only (HR 2.2, 95%CI 1.1 – 4.5; p=0.022).

Conclusion

D-Dimer can be reliably measured using heparinised plasma using high sensitivity assays. D-Dimer appears to be a new age- and sex- independent predictor of mortality in a mixed population with PH risk.

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Glossary and Abbreviations

art. pCO_2	arterial partial pressure of carbon dioxide
art. pO_2	arterial partial pressure of oxygen
$AVDO_2$	arteriovenous oxygen difference
BMI	Body Mass Index
BMPR2	Bone Morphogenetic Protein Receptor type II
BNP	Brain Natriuretic Peptide
CI	cardiac index
CO_2	carbon dioxide
COPD	Chronic obstructive lung disease
CRP	C-reactive protein
CT	Computer tomography
CTEPH	Chronic Thromboembolic pulmonary hypertension
EK	Ethics Committee
ECG	Electrocardiogram
ELISA	Enzyme-Linked Immunosorbent Assay
ERS	European Respiratory Society
ESC	European Society of Cardiology
GFR	Glomerular Filtration rate
Hb	Hemoglobin
HR	Heart rate
INR	International Normalized Ratio
IPAH	idiopathic pulmonary arterial hypertension
mPAP	mean pulmonary arterial hypertension
NT-proBNP	N-terminal pro brain natriuretic peptide
NYHA	New York Heart Association
PAH	pulmonary arterial hypertension
PAWP	pulmonary arterial wedge pressure
PVR	pulmonary vascular resistance
py	Pack-Years
PZ	Prothrombin time
RAP	right atrial pressure
RDW	red cell distribution width

RHK	right heart catheterization
WU	Wood Units

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1. Introduction

1.1 Pulmonary hypertension

Pulmonary hypertension (PH) is a hemodynamic condition characterized by pathologic increase of the pressure in the pulmonary arteries. Patients with PH present with unspecific symptoms like shortness of breath by exercise, chest pain, weakness, fatigue, syncope, etc. which is the reason why the diseases is often diagnosed late. It takes usually more than one year from the beginning of the complaints until patients get a definite diagnosis. If left untreated, the prognosis is poor.

Pathophysiologically there are two mechanisms that play a role: vasoconstriction and pulmonary arterial remodeling which affects all the vascular layers, intima, media and adventitia (**Figure 1**).

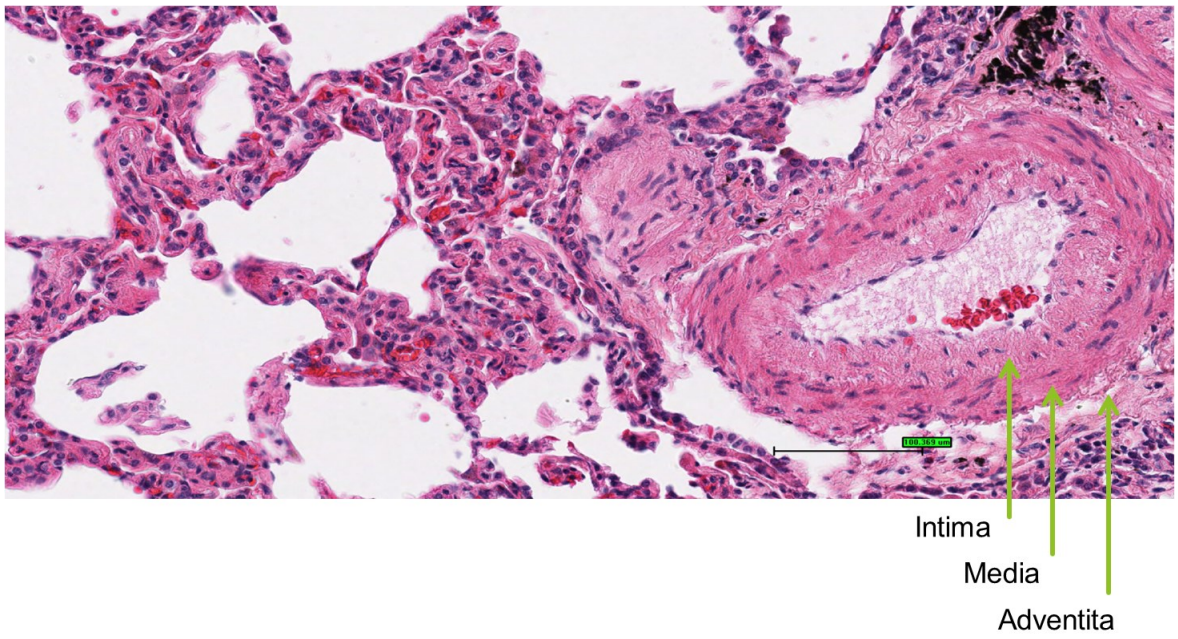

Figure 1.

Hematoxylin & Eosin staining of a lung from a patient with idiopathic pulmonary arterial hypertension who underwent lung transplantation. Arrows point to the remodeled vessel components. The staining and the picture were made by the

author and is part of his own collection. The figure has not been published elsewhere.

Physiologically the mean pulmonary arterial pressure (mPAP) is 14 ± 3.3 mmHg (1). Once the mPAP increases above 20 mmHg it is already considered as pathologic (2). Patients presenting with mPAP values between 20 and 25 mmHg have a poor prognosis as compared to those with normal hemodynamics or patients with PH (3-5). Although current guidelines define pulmonary hypertension as a mPAP ≥ 25mmHg, recently it has been proposed that the threshold should be reduced to 20 mmHg (2,6). There is evidence that patients presenting with mPAP values between 20 and 25 mmHg have already a pulmonary vasculopathy (7). According to the recent report of the World Meeting of Pulmonary Hypertension in Nice 2018 the hemodynamic definition of the precapillary PH is an elevation of the mPAP > 20mmHg and a pulmonary vascular resistance (PVR) of ≥ 3 Wood Units (WU) assessed by right heart catheterization (RHC) (2).

The gold standard for measuring the pulmonary arterial pressure is the RHC. During RHC with the use of Swan Ganz Catheter other hemodynamic values are assessed as well, which are important for the assessment the pulmonary hemodynamics.

In the clinical practice for initiation of a targeted therapy the threshold of mPAP ≥ 25mmHg is still being used according to the 2015 ESC/ERS Guidelines for the diagnosis and treatment of pulmonary hypertension, which is a result of the Joint Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS) endorsed by the Association for European Paediatric and Congenital Cardiology (AEPC) and the International Society for Heart and Lung Transplantation (ISHLT) (6).

The clinical classification is complex and relies mostly on the underlying pathologies. Currently five major groups are distinguished: Group 1: pulmonary arterial hypertension (PAH), Group 2: PH due to heft heart disease, Group 3: PH due to lung diseases and/or hypoxia, Group 4: chronic thromboembolic pulmonary hypertension (CTEPH) and Group 5: PH due to unclear and/or multifactorial mechanisms (**Table 1**).

Table 1.

Updated clinical classification of pulmonary hypertension

1 PAH

1.1 Idiopathic PAH (IPAH)

1.2 Heritable PAH

1.3 Drug- and toxin-induced PAH (aminorex, fenfluramine, metamfetamines, etc.)

1.4 PAH associated with:

1.4.1 Connective tissue disease

1.4.2 HIV infection

1.4.3 Portal hypertension

1.4.4 Congenital heart disease

1.4.5 Schistosomiasis

1.5 PAH long-term responders to calcium channel blockers

1.6 PAH with overt features of venous/capillaries (pulmonary venoocclusive disease, pulmonary capillary haemangiomatosis) involvement

1.7 Persistent PH of the newborn syndrome

2 PH due to left heart disease

2.1 PH due to heart failure with preserved left ventricular ejection fraction

2.2 PH due to heart failure with reduced left ventricular ejection fraction

2.3 Valvular heart disease

2.4 Congenital/acquired cardiovascular conditions leading to post-capillary PH

3 PH due to lung diseases and/or hypoxia

3.1 Obstructive lung disease

3.2 Restrictive lung disease

3.3 Other lung disease with mixed restrictive/obstructive pattern

3.4 Hypoxia without lung disease

3.5 Developmental lung disorders

4 PH due to pulmonary artery obstructions

4.1 Chronic thromboembolic PH

4.2 Other pulmonary artery obstructions (sarcoma, leiomyoma, hydatidosis, etc.)

5 PH with unclear and/or multifactorial mechanisms

5.1 Haematological disorders (Chronic haemolytic anaemia, Myeloproliferative disorders)

5.2 Systemic and metabolic disorders (pulmonary Langerhans cell histiocytosis, Gaucher disease, glycogen storage disease, neurofibromatosis, sarcoidosis)

5.3 Others (chronic renal failure, fibrosing mediastinitis)

5.4 Complex congenital heart disease

For the screening of PH the most adequate non-invasive screening tool is a transthoracic echocardiography which can reliably detect signs of right heart failure. It uses a combination of parameters like tricuspid regurgitant velocity, tricuspid annular plane systolic excursion (TAPSE), right ventricular size, interventricular septal function, inferior vena cava diameter, etc. Additional tools for screening are also used like cardiopulmonary exercise stress tests, which can quantify the degree of relative hypoperfusion of the lung and the systemic circulation. One of the most important parameters is the peak oxygen uptake (peak VO_2) which is also used in the practice for follow-up. Imaging with high-resolution computed tomography (HR-CT) is routinely performed to exclude other structural abnormalities of the lung and has an essential role in the initial work-up. In the presence of a significant lung disease CT might differentiate between Group 1 and Group 3 PH. Additionally, there a signs suggestive of PH like right ventricular dilation, right atrial dilation, enlarged main pulmonary artery or an elevated main pulmonary artery/ascending aorta diameter ratio (8).

The therapy consists on one hand of targeted therapy and on the other hand on supportive therapy (9). Calcium channel blockers are used for only a small group of IPAH patients who fulfill certain criteria. Targeted therapies are currently approved for Group 1 (PAH) and Group 4 (CTEPH). There are currently more than 10 drugs approved for the therapy of PAH which target the endothelin pathway (ambrisentan, bosentan, macitentan), inhibit phosphodiesterase 4 (sildenafil, tadalafil) or support prostacyclin release (iloprost, beraprost, epoprostenol, treprostinil, selexipag). The soluble guanylate cyclase stimulator riociguat is approved for both PAH and CTEPH.

1.2 Biomarkers in pulmonary hypertension

Due to the unspecific symptoms and the heterogeneity of PH, there is currently no reliable specific blood derived biomarker that is used in routine. Currently, brain natriuretic peptide (BNP) and the N-Terminal fragment NT-proBNP are widely used for risk stratification; however, they lack specificity and in the absence of a manifest right heart failure they might show normal values. Some screening methods are available (10); however, blood derived biomarkers would be ideal noninvasive tools for risk stratification and prognosis. Circulating progenitor cells are one of the several examples that are emerging as novel markers (11) (**Figure 2**).

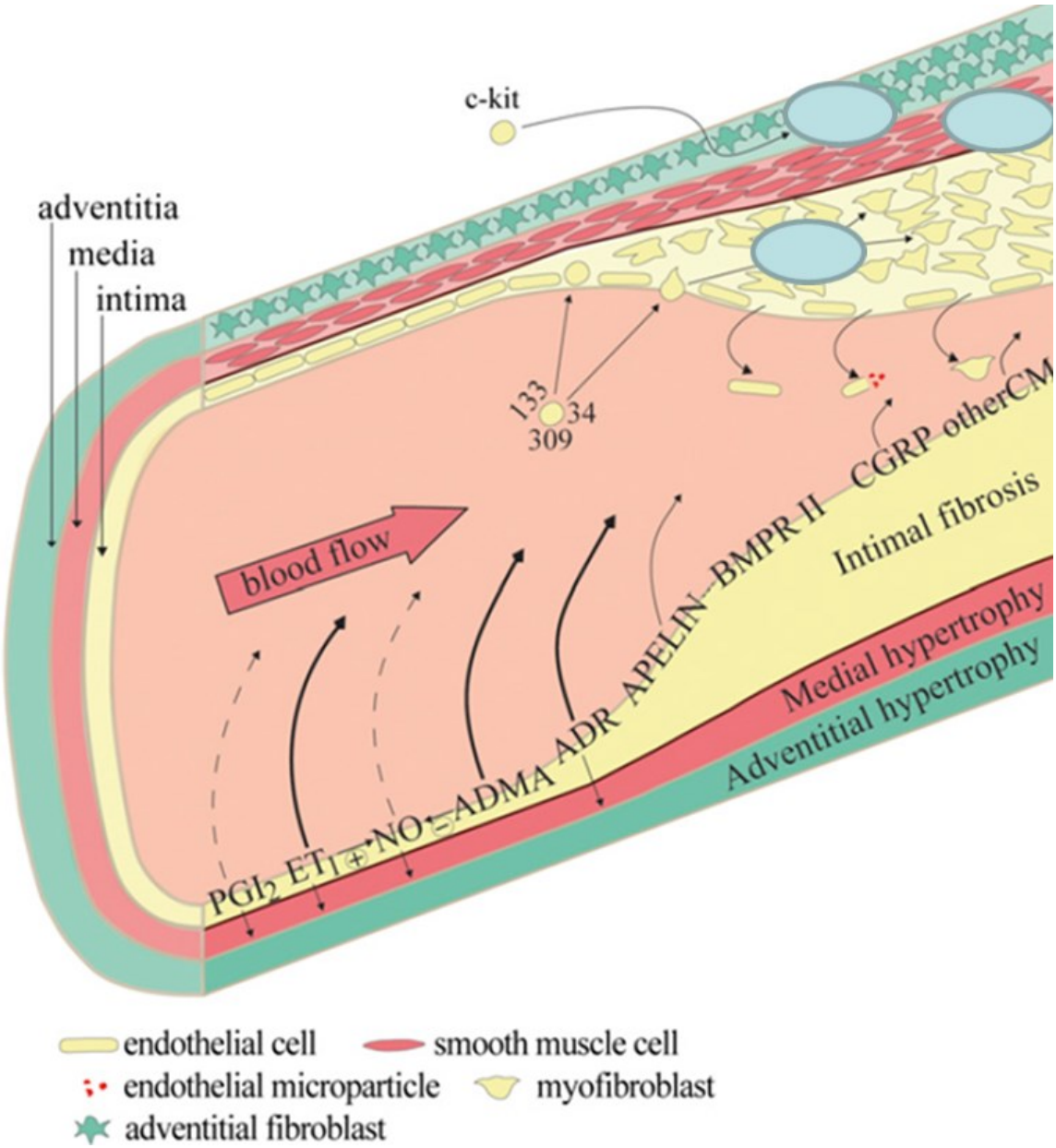

Figure 2. *(Modified from Foris V et al. Chest 2013 (12))*

A cross section of a remodeled vessel is depicted. Arrows show release of mediators as potential biomarkers. Circulating progenitor cells expressing CD133, CD34, CD309 or c-kit can be also found in the different vessel layers. Abbreviations: PGI_2=prostaglandin, ET1=endothelin 1, NO=nitric oxide, ADMA=assymetric dimethylarginine, ADR= adrenomedullin, BMPRII=bone morphogenic receptor II, CGRP=calcitonin gene related peptide, CM=circulating microparticles.

Diagnostic markers.

There are currently no blood-derived biomarkers that detect the presence of pulmonary hypertension. Studies addressing this challenging issue were performed on small case series and the markers still miss validation (13-16). Basic laboratory tests are routinely performed by the initial diagnostic work-up including blood count, international normalized ratio (INR), creatinine, sodium, potassium, ALAT/ASAT, bilirubine, BNP and NT-proBNP. These support differential diagnosis and guide clinicians; however, none of them has a diagnostic value for PH. Extended laboratory investigations like thyreotropin (TSH), troponin, uric acid, iron status and other variables according to individual patient needs are suggested by the guidelines (6).

Therapy response markers

The most reliable predictor of therapy response is a positive vasoreactivity during right heart catheterization. Patients are exposed to inhaled nitric oxide (NO) and a positive vasoreactivity is defined by a drop of mPAP ≥ 10mmHg to reach an absolute value of mPAP ≤ 40 mmHg and increase or no change of the cardiac output (CO) (Sitbon). These patients, the so-called “responders” can be treated with high doses of calcium channel blockers and there is strong evidence that they have a very good prognosis with sustained haemodynamic improvement (17,18).

Prognostic markers

Markers of prognosis are very important for risk stratification. Mixed venous oxygen saturation (SvO_2), right atrial pressure (RA) and cardiac index (CI) are good predictors of prognosis; however, these values are obtained by RHC which is invasive. Although right heart catheterization is the gold standard for diagnosis of PH it is invasive and requires hospitalization. There are currently several non-invasive methods that can be used in the follow-up of patients with PH; however, there is still an unmet need for further non-invasive markers. Blood derived biomarkers would be useful tools due to their broad availability. For example, markers of iron homeostasis or red cell distribution width have been shown to be prognostically relevant in IPAH (19, 20). There are several good quality reviews

that summarized recent data about biomarkers in PH (12, 21, 22). Novel pathways and their players are currently under investigation (23) (**Figure 3**).

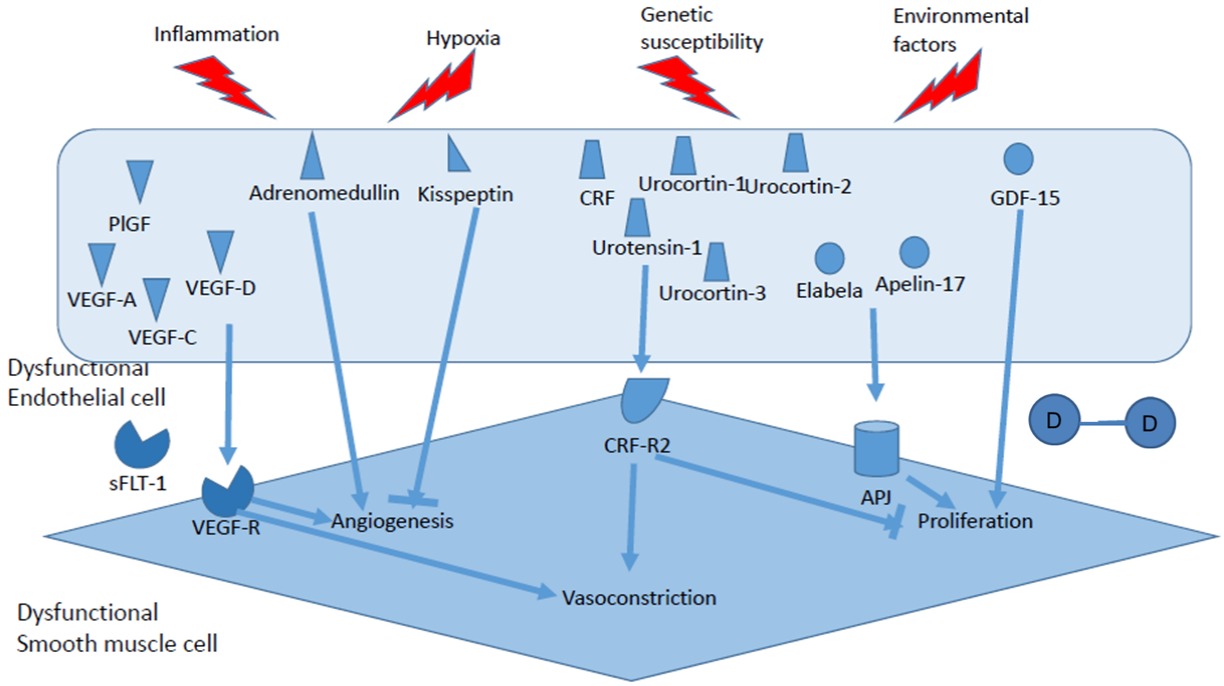

Figure 3.

External stimuli like inflammation, hypoxia, genetic susceptibility or environmental factors may trigger endothelial dysfunction. This may lead to release of mediators that serve as triggers for smooth muscle cell dysfunction, vasoconstriction, proliferation and dysfunctional angiogenesis. Abbreviations: PlGF=placental growth factor, VEGF=vascular endothelial growth factor, sFLT-1=soluble Fms like tyrosine kinase, VEGFR=vascular endothelial growth factor receptor, CRF=corticotropin releasing factor, CRF-R2= corticotropin releasing factor receptor 2, APJ= apelin receptor, GDF-15= growth differentiation factor-15.

It has been postulated and there is growing evidence that there is a possible involvement of endogenous fibrinolysis in patients with PH (24). As an example, in a small study including 12 patients with IPAH D-dimer levels assessed by ELISA were significantly higher as compared to matched healthy controls (25, 26). However, it is not known if other PH Groups also exert high D-Dimer levels

and if there is a difference between subgroups of PH patients regarding their D-Dimer level.

There is convincing data from a recent Australian study about patients with stable coronary heart disease where D-Dimer predicted long-term cause-specific mortality including cardiovascular events and cancer (27). The study included 7863 patients who were initially participating in a randomized clinical trial taking either pravastatin or placebo ((Long-Term Intervention with Pravastatin in Ischaemic Disease). The authors showed that after adjustment for about additional 30 risk factors high D-Dimer was associated with increased risk for a cardiovascular event. During the long follow-up of 16 years, higher D-Dimer was an independent predictor of all-cause mortality.

The question if D-Dimer represents a prognostic marker in PH has not been answered yet.

1.3 Importance of D-Dimer testing

D-Dimer is a degradation product of fibrin and is produced as a result of fibrinolysis once a blood clot is present (**Figure 4**). Elevated D-Dimer values in plasma are found because of a simultaneous activation of coagulation and fibrinolysis. D-Dimer testing is routinely performed in the clinical practice once a thrombosis or an embolism is suspected. The negative predictive value of the test is high, meaning that in case of normal D-Dimer values a deep venous thrombosis or an acute pulmonary embolism is unlikely (28). Conversely, elevated D-Dimer has a low positive predictive value and cannot be used for confirmation of a thromboembolic event (28). Basically, there are no differences between incidences of the diseases where D-Dimer testing is relevant. However, pregnant women represent a very special population where D-Dimer has a high negative predictive value for pulmonary embolism (28). During pregnancy or the post-partum period D-Dimer measurement and clinical prediction rules are to be considered to rule out pulmonary embolism. D-Dimer is often elevated in patients who are hospitalized, in those who have severe infections or cancer (28).

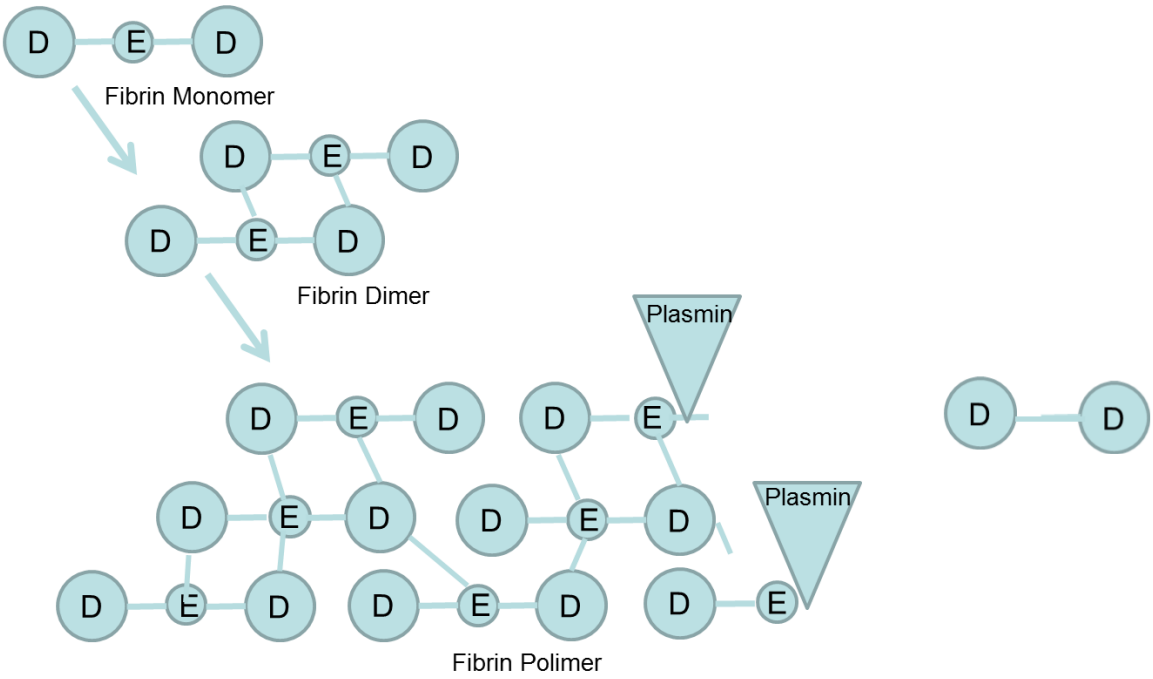

Figure 4.

The activation of the coagulation cascade leads to polymerization of fibrin that in turn is cleaved by plasmin due to concomitant activation of fibrinolysis. D fragments as cleavage products dimerize and form the so-called D-Dimer.

The 2019 European Society for Cardiology (ESC) Guidelines for the diagnosis and management of acute pulmonary embolism developed in collaboration with the European Respiratory Society (ERS) suggest that D-Dimer should be used in patients with a low or intermediate clinical probability (29). In these cases an elevated D-Dimer should trigger the further assessment for thromboembolism.

D-Dimer levels below 500 µg/l (0.5 mg/l) are considered as normal (29). D-Dimer cut-off values adjusted for age can be used instead of fixed cut-off values in the decision trees for ruling out pulmonary embolism or deep venous thrombosis. In patients aged > 50 years the age adjusted cut off with the formula “age x 10 µg/l” should be used (29).

1.4 Challenges of D-Dimer testing

In primary care medicine point-of-care D-Dimer tests are routinely used which have the advantage of not referring the patients to a central laboratory. The limitation of these assays is that they have a lower sensitivity and lower negative predictive value compared to laboratory based tests. Current guidelines suggest the use of point of care tests only in patients with a low pre-test probability (29).

The measurement of D-Dimer levels is routinely performed from plasma tubes that are prefilled with a specified amount of natrium citrate (Na-Cit). However, there are several preanalytical variables that can have a major impact on the results of the measurements. For example, the tubes have to be filled completely, the tubes should not be filled by phlebotomy as the first ones and by mixing the tubes haemolysis may often occur. If Li-Hep plasma would be suitable for D-Dimer testing as well one could save material, time and costs resulting from the avoidance of the Na-Cit blood collection tubes.

Point of care tests are also available; however, these tests have a lower sensitivity. In order to overcome some of these limitations new high sensitive assays are needed and currently are being developed.

Recently, new methods became available for D-Dimer measurement, which use heparinised plasma instead of Na-Cit plasma. Some of these methods have already been tested in bigger patient populations (30); however, clinical validation is still missing.

Currently there are several tests available on the market for D-Dimer determination. INNOVANCE has a fast turnaround time, validated diagnostic sensitivity of ≥98% the required sample volume relies between 8–15 µl and the measuring range is 0.17–4.40 mg/l FEU (31).

One of the most promising ones, the high sensitive method using a quantitative latex-enhanced immunoturbidimetric immunoassay called LOCI has not been validated yet for clinical use; however, preliminary data are very promising.

2. Aims

The hypothesis of the study is that D-Dimer can be reliably measured by the new high sensitive LOCI technique and elevated D-Dimer is a marker of poor prognosis in patients with PH. Therefore, the aims are as follows:

- To prove that D-Dimer testing can be reliably performed using the new high sensitive assay LOCI
- To establish D-Dimer as a prognostic biomarker in patients who underwent right heart catheterization To assess the value of D-Dimer in follow-up of patients who underwent follow-up right heart catheterization

3 Patients and Methods

Blood was obtained from the vena cava superior from patients who underwent right heart catheterization between 2011 and 2017 at the Department of Internal Medicine, Division of Pulmonology at the Medical University of Graz, Austria. All subjects gave their written informed consent and the study was approved by the Ethical Committee of the Medical University of Graz (23-408 ex 10/11).

Patient data were collected and stored in the RDA (Research Documentation and Analysis) system. Mortality data was obtained from Statistik Austria. At the timepoint of analysis the samples were anonymised.

Right heart catheterization (RHC) was performed whenever a suspicion of pulmonary hypertension was made based on non-invasive screening tools, including transthoracic echocardiography (TTE). The same experienced team performed the procedure in a standardized manner. Patients were hospitalized usually one day prior to the RHC where the perioperative procedures including blood tests, electrocardiogram (ECG), chest X Ray, lung function, six-minute walk test, and TTE tests were performed. All subjects gave their written informed consent for the procedure. The monitoring of the patients included ECG, peripheral oxygen saturation (SpO_2), and blood pressure. After sonography of the cervical region with special focus on the internal jugular vein a local anaesthesia was performed. The venepuncture was done using the Seldinger technique. A fluid filled balloon tipped 4F Swan Ganz Catheter was then introduced and pressure curves were visualized on a Philipps monitor and followed. The catheter was inserted until the wedge position was reached. The first hemodynamic measurement was followed by blood gas analysis from ear (arterialised blood), vena cava superior (venous) and pulmonary artery (mixed venous blood).

Inclusion criteria:

- Written informed consent for participating in the study
- Written informed consent for storage of blood samples in the Biobank
- Age > 18 years
- Technically reliable and plausible hemodynamic measurements
- Available Li-Hep plasma

Exclusion criteria:

- Missing written informed consent for participating in the study
- Missing written informed consent for storage of blood samples in the Biobank
- Withdrawal of any of the above mentioned consents
- Age < 18 years
- Technically unreliable hemodynamic measurements
- Missing Li-Hep plasma

N=295 (pulmonary arterial hypertension: n=54, PH due to left heart disease: n=39, PH due to lung diseases: n=56, chronic thromboembolic PH: n=31, PH due to unclear or multifactorial mechanisms: n=16, PH excluded by RHC: n=99) patients who underwent RHC between 2011 and 2017 were prospectively included in this study (**Figure 5**).

Plasma samples were collected during RHC after the first measurement into Li-Hep Tubes (Vacutainer) and the tubes were put immediately in ice cold water. The samples were transferred within 1 hour for processing in the central laboratory where the centrifugation and aliquoting was executed. The aliquots were than stored at the Biobank Graz at -80°C.

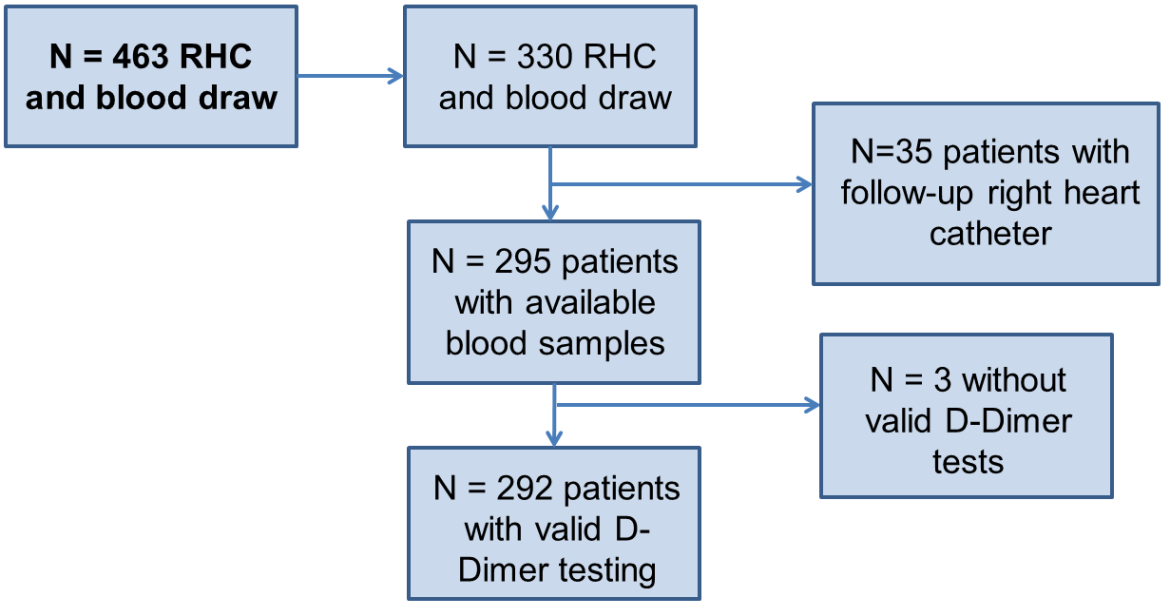

Figure 5.

During the initial interrogation of the database based on the right heart catheterizations (RHCs) n=463 examinations were identified. Out of these n=330 RHCs were included due to available complete dataset at that time point. N=35 RHCs were identified as being performed as follow-up examinations. Out of 295 samples n=3 had to be excluded due to technical failure.

Prior to D-Dimer measurements samples were identified and requested from the Biobank Graz. D-Dimer levels were assessed in duplicate at the Clinical Institute of Medical and Chemical Laboratory Diagnostics using two assays on the Atellica® COAG 360 System, which is a fully automated coagulation analyzer manufactured by Siemens. The analyzer measures D-Dimer plasma levels in parallel using the standard INNOVANCE® assay, which is a particle-enhanced immunoturbidimetric assay and the novel high sensitive quantitative latex-enhanced immunoturbidimetric immunoassay (Luminescent Oxygen Channeling Immunoassay (LOCI®)). The latest is considered currently the most sensitive for D-Dimer detection.

Briefly, the latex based agglutination assay uses latex particles that are coated with specific antibodies that agglutinate once the analyte is present. The agglutinates cause scattering of a light beam, which is measured by turbidimetry. The LOCI assays uses two latex bead reagents and a biotinylated analyte receptor (32, 33). One bead reagent (sensibead) is coated with streptavidin and has a

photosensitive dye. A second bead reagent (chemibead) is coated with an analyte-specific binding partner and has a chemiluminescent dye. Once the analyte is applied to the mixture, the three reactants form a bead-aggregated immunocomplex. If this complex is illuminated by light at 680 nm singlet oxygen from sensibeads is generated, which triggers a chemiluminescent reaction that is read at 612 nm.

Automatic assessment of hemoglobin (H), lipaemia (L) and icterus (I) (HIL indices) was generated by spectrophotometric assessment. A snapshot of a representative sample is provided (**Figure 6**).

Proben-ID:	B0007			**HIL-Index:** H(3): 1	
Probentyp:	PAP			I(3): 2	
Letzte Rackposition:	005319-B			L(3): 1	
Angefordert von:	---				
Bemerkung:					
Test	**Rohwert**	**Ergebnis**	**Flag**	**Datum**	**Zeit**
D-Dimer INN	14,41 mE/min	0,26 mg/l FEU		14.08.2019	16:50:44
D-Dimer LOCI	27,76 kcnt	231 µg/l FEU		14.08.2019	16:43:31

Figure 6.

A snapshot of a representative sample is depicted showing HIL indices and D-Dimer values measured by both INNOVANCE and LOCI.

Statistical analysis

For the statistical analysis the Graphpad and SPSS softwares were used. P values < 0.05 were considered as statistically significant. Values are presented as mean and standard deviation or median and interquartile range as appropriate. For the comparison of the two D-Dimer measurement methods Bland Altman analysis was used. Because of the limitation of the INNOVANCE method to detect values below 0.19 mg/l values below the lower detection limit were excluded from the Bland Altman analysis. Logarithmic values were used because of missing normality of data. The survival analysis was performed using Kaplan Mayer curves. Groups were compared using the Mann-Whitney-U test. Wilcoxon test was used for comparison of D-Dimer values in baseline and in follow-up.

4 Results

4.1 Comparison of the two methods

D-Dimer was reproducibly detected in n=292 individual patients using the LOCI method (**Table 2).** D-Dimer was reproducibly measured with both methods in duplicate in n=266 patients mean age: 63±13 yr, mean pulmonary arterial pressure (mPAP): 33±14 mmHg, pulmonary arterial wedge pressure: 11±5 mmHg, pulmonary vascular resistance (PVR): 5.2±4.1 WU, cardiac index=2.6±0.8 l/min/m^2). The difference of patient numbers of n=26 results of those patients where D-Dimer values were under the detection limit of the INNOVANCE method.

Table 2 *Patients´ characteristics*

	Group I	Group II	Group III	Group IV	Group V	Exclusion of PH
n	53	39	56	31	16	97
Age (years)	58.9 ± 15.6	66.8 ± 10.8	66.7 ± 10.9	62 ± 13.5	59.6 ± 12	63.5 ± 11.7
Sex (M/F)	17/36	17/22	34/22	20/11	4/12	26/71
WHO FC (I/II/III/IV)	2/25/24/2	5/10/24/0	1/14/35/6	2/11/17/1	0/3/10/3	19/44/33/1
mPAP (mmHg)	42 ± 15	37 ± 11	39 ± 10	43 ± 15	39 ± 11	19 ± 3
PAWP (mmHg)	9 ± 3	19 ± 4	10 ± 3	9 ± 4	9± 4	9 ± 3
RAP (mmHg)	8 ± 4	12 ± 5	8 ± 4	9 ± 7	9 ± 7	6 ± 3
PVR (WU)	8 ± 5	4 ± 2	6 ± 3	9 ± 4	7 ± 4	2 ± 1
CI (mmHg)	2.6 ± 0.9	2.5 ± 0.7	2.5 ± 0.6	2.1 ± 0.4	2.8 ± 0.8	2.8 ± 0.9
TAPSE (mm)	19 ± 6	17 ± 5	19 ± 6	17 ± 4	18 ± 5	22 ± 5
6MWD (m)	376 ± 123	295 ± 105	291 ± 104	358 ± 117	306 ± 125	393 ± 98
peak VO_2 (%)	51 ± 20	45 ± 14	64 ± 48	56 ± 38	42 ± 22	70 ± 25
NT-proBNP (pg/ml)	880 (275 – 1757)	1946 (1146 – 3255)	762 (378.8 – 2713)	1033 (237 – 2615)	2246.5 (272 – 3956)	204 (78 – 531)

D-Dimer LOCI (µg/l)	397 (212-674)	402 (210 – 869)	495 (280 – 670)	300 (199 – 642)	363 (236 – 1414)	332 (211 – 623)
OAC (Y/N)	30/23	20/19	24/32	26/5	3/13	32/67

Table legend: *Group 1: pulmonary arterial hypertension (PAH), Group 2: PH due to left heart disease, Group 3: PH due to lung diseases and/or hypoxia, Group 4: chronic thromboembolic pulmonary hypertension (CTEPH) and Group 5: PH due to unclear and/or multifactorial mechanisms*

Abbreviations: PH= pulmonary hypertension, M=male, F=female, WHO FC= World Health Organisation Functional Class, mPAP= mean pulmonary arterial pressure, PAWP= pulmonary arterial wedge pressure, RAP=right atrial pressure, PVR=pulmonary vascular resistance, WU= Wood units, CI=cardiac index, TAPSE= tricuspid annular plane systolic excursion, 6MWD= six minute walking distance, peak VO_2=peak oxygen uptake, NT-proBNP= N-Terminal pro-Brain Natriuretic Peptide, LOCI= Luminescent Oxygen Channeling Immunoassay, OAC= oral anticoagulation, Y=yes, N=no

Bland Altmann analysis of the two test methods revealed that there are no big systematic differences between LOCI and INNOVANCE (**Figure 7**); however, INNOVANCE showed systematically higher D-Dimer values (0.13mg/l).

INNOVANCE could not detect values below 0.19 mg/l (lower detection limit) and these samples were excluded from the analysis which resulted in n=266 measurement pairs. LOCI proved to be highly sensitive showing technically reliable D-Dimer values in the samples where INNOVANCE gave a result of 0.19mg/l. The lower detection limit of LOCI proved to be 0.027 mg/l. There was, however, a strong correlation between D-Dimer values measured with INN vs LOCI (**Figure 8**.).

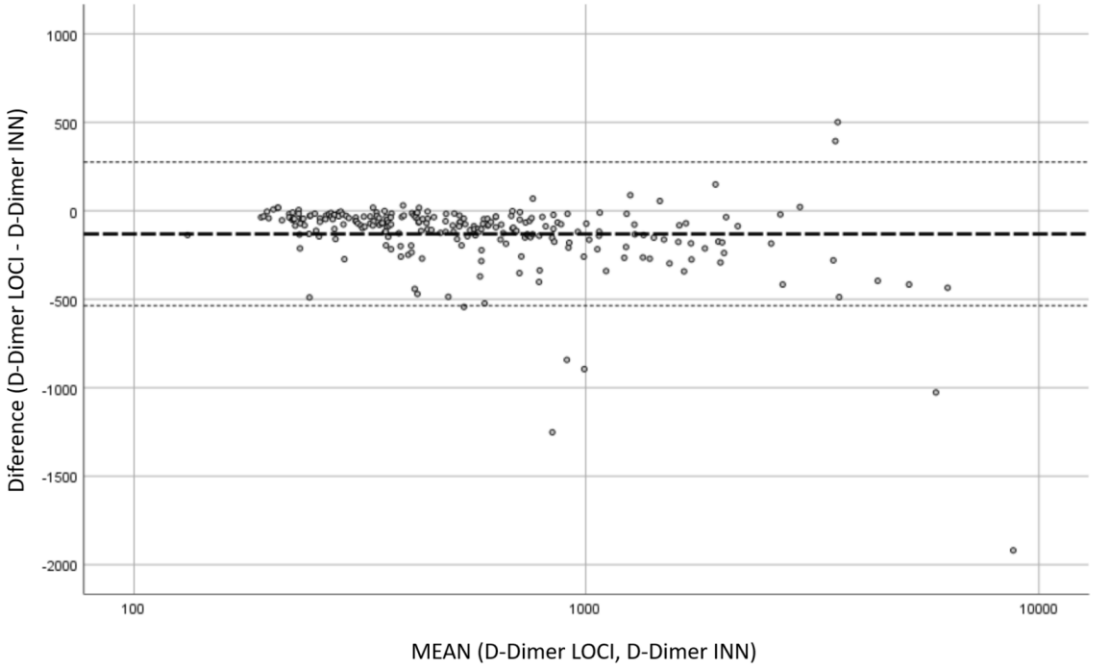

Figure 7.

Bland Altman plot of the two D-Dimer test methods. Differences (Y-axis) are plotted against the averages of the two techniques (X-axis).

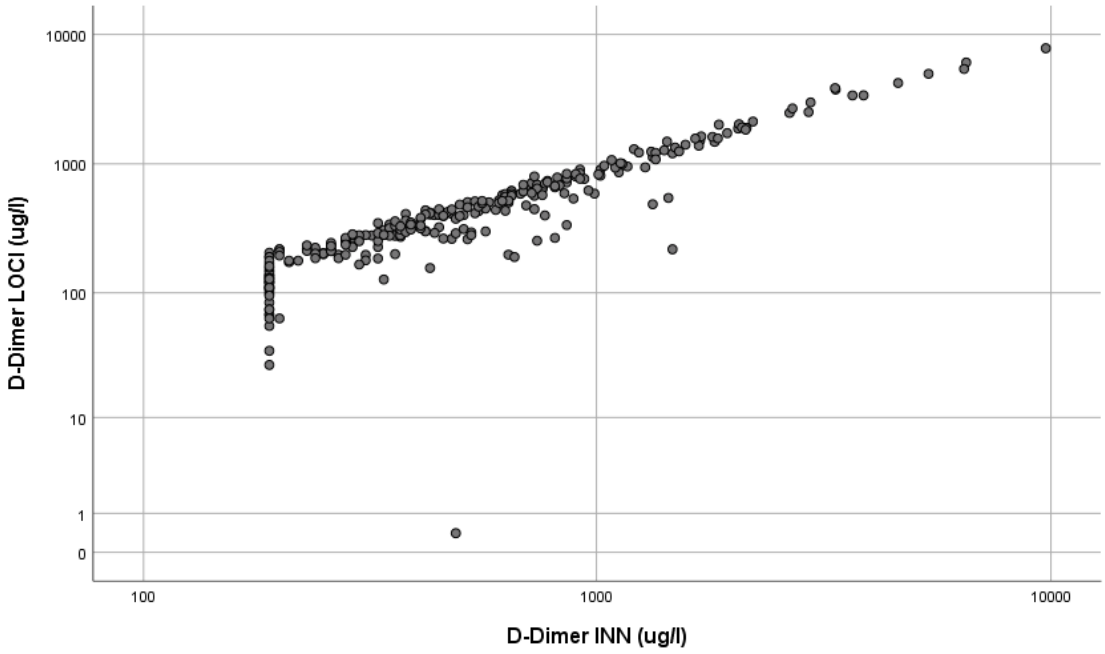

Figure 8.

Correlation analysis of logarithmic D-Dimer values by means of INNOVANCE and LOCI

4.2 D-Dimer as a prognostic marker

The median follow-up time was 48 months, within this period n=63 patients died. After adjustment for sex and age, a D-Dimer cut-off value of 402µg/l predicted all-cause mortality (p=0.033) in the whole study group (**Figure 9**). Patients above the cut-off level were older (**Table 3**) with 68 (IQR=59-75) vs. 63 (IQR=54-71) yr, and were more likely to have PH (56% vs. 44%) and had more severe hemodynamic changes (mPAP: 34 (IQR=24-45) vs. 27 (IQR=20-41) mmHg, PVR: 4.3 (IQR=2.4-8.6) vs. 3.2 (IQR=2.3-6.1) WU).

There was no difference between the groups regarding lung function variables, renal function or right ventricular function assessed by echocardiography (TAPSE).

Subgroup analysis revealed that patients with PH had higher D-Dimer values than those without PH (p=0.047) (**Figure 10**). Also, D-Dimer was significantly associated with mortality in the PH subgroup (p=0.022), but not in patients without PH.

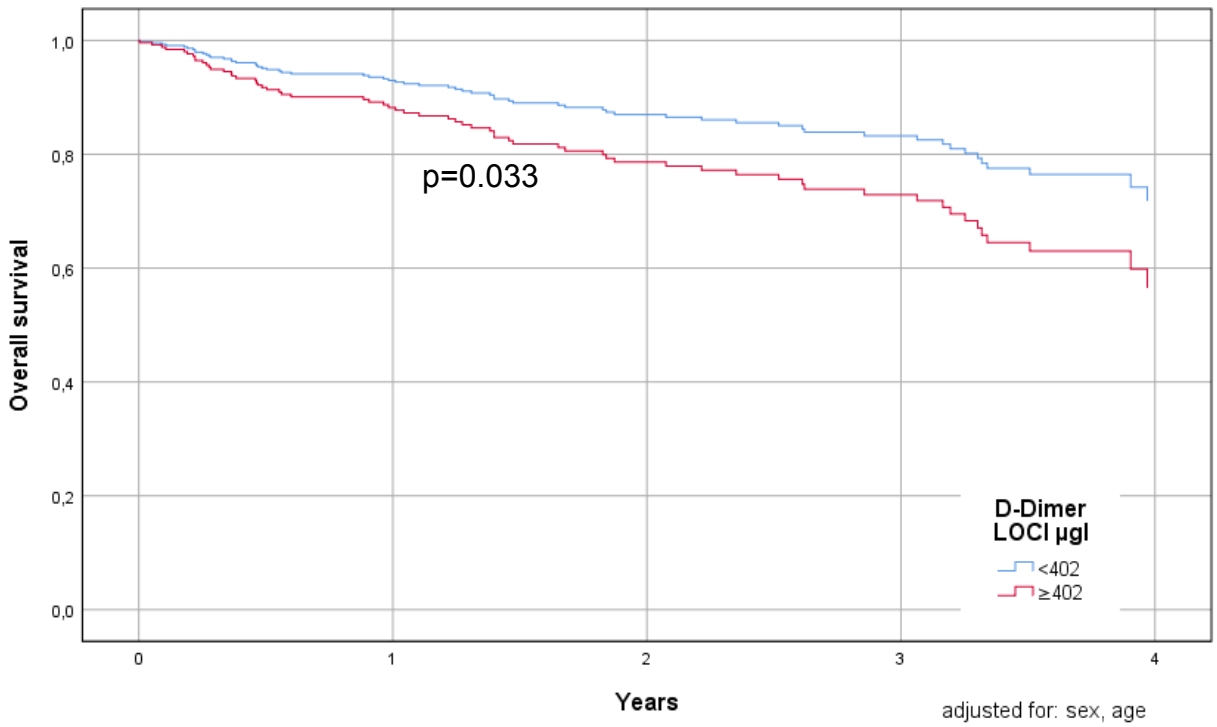

Figure 9

Kaplan-Meier analysis for D-Dimer cut-off = 402 ug/l measured by LOCI

Table 3. *Patients´ characteristics based on D-Dimer cut-off values*

D-Dimer cut-off		<402 Median (IQR), n (%)	≥ 402 Median (IQR), n (%)	p Value
Age		63 (54-71)	68 (59-75)	<.001
Sex	M	58 (49.2%)	60 (50.8%)	.532
	F	92 (52.9%)	82 (47.1%)	
PH	Y	80 (44.0%)	102 (56.0%)	0.001
	N	70 (63.6%)	40 (36.4%)	
BMI		26.0 (22.8-30.1)	28.20 (23.71-31.64)	.067
Creatinine (mg/dl)		0.95 (0.81- 1.15)	.97 (.77-1.26)	.646
Uric acid mg/dl		6.1 (4.9-7.1)	6.4 (5.0-8.0)	.044
NT-pro BNP (pg/ml)		491.5 (112.5-1372.0)	821.5 (363.0-2636.0)	<.001
CRP (mg/l)		2.6 (1.4-4.9)	4.7 (2.2-12.0)	<.001
INR		1.09 (1.02-1.27)	1.11 (1.02-1.27)	.956
Albumin (g/dl)		4.1 (3.9-4.4))	3.9 (3.6-4.2)	<.001

RDW (%)	14.10 (13.30-15.50)	14.75 (14.00-16.60)	<.001
GFR (%)	74.11 (57.30- 87.62)	65.68 (49.85-87.90)	0.129
FVC (%)	87.4 (73.2-102.7)	85.1 (68.8-100.7)	0.232
FEV1 (%)	81.3 (61.0-95.0)	74.9 (58.3-92.0)	0.304
FEV1/FVC	75.43 (66.63-80.46)	73.20 (67.49-79.00)	0.364
HR	72 (65-82)	75 (65-84)	0.211
meanPAP (mmHg)	27 (20-41)	34 (24-45)	0.004
PAWP (mmHg)	9 (7-12)	10 (7-14)	0.323
RAP (mmHg)	6 (4-8)	7 (4-11)	0.103
PVR (WU)	3.23 (2.30-6.10)	4.31 (2.44-8.58)	0.03
CI (l/min/m^2)	2.54 (2.05-3.03)	2.51 (2.03-2.89)	0.808
art pO2 (mmHg)	66.8 (59.3-73.5)	64.4 (56.7-70.8)	0.016
venSO2 (%)	68.4 (63.7-73.0)	65.9 (59.2-70.9)	0.001
TAPSE (mm)	20 (16-23)	19 (14-23)	0.588
6MWD (m)	379.5 (313.0-467.0)	327.0 (244.0-383.0)	<.001

peak VO2 (%pred)	59.33 (44.54-81.00)	49.72 (33.83-67.07)	0.011

Abbreviations: IQR=interquartile range, M=male, F=female, Y=yes, N=no, BMI=body mass index, NT-proBNP= N-Terminal pro-Brain Natriuretic Peptide, CRP= C-reactive protein, INR= international normalized ration, RDW= red cell distribution width, GFR= glomerular filtration rate, FVC= forced vital capacity, FEV1= forced expiratory volume in the first second, HR= heart rate, mPAP= mean pulmonary arterial pressure, PAWP= pulmonary arterial wedge pressure, RAP=right atrial pressure, PVR=pulmonary vascular resistance, WU= Wood units, CI=cardiac index, art pO_2= arterial partial pressure of oxygen, ven SO_2 = mixed venous saturation, TAPSE= tricuspid annular plane systolic excursion, 6MWD= six minute walking distance, peak VO_2=peak oxygen uptake

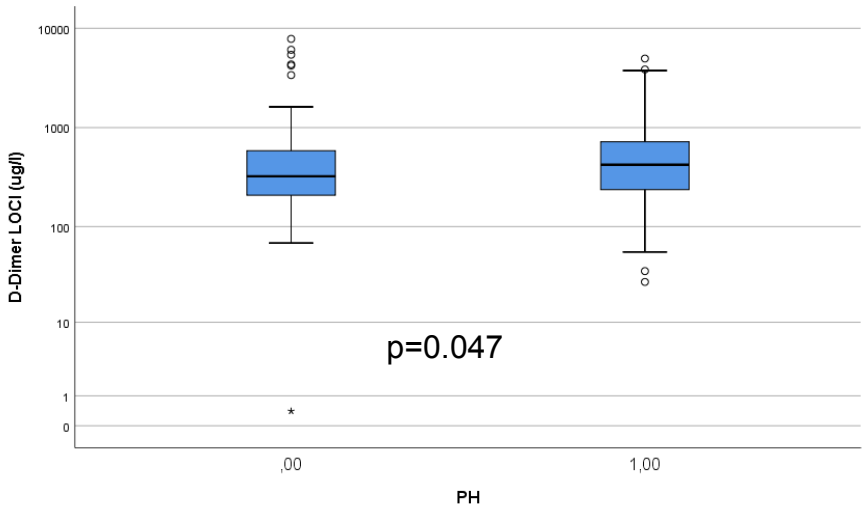

Figure 10

D-Dimer levels in patients without PH and patients with PH

4.3 D-Dimer in follow-up

In a subgroup of patients who underwent follow-up right heart catheterization (n=35) D-Dimer levels measured by INNOVANCE showed no differences (p=0.063) (**Figure 11**). D-Dimer values measured by LOCI patients with PH show slightly lower values in the follow-up (p=0.034).

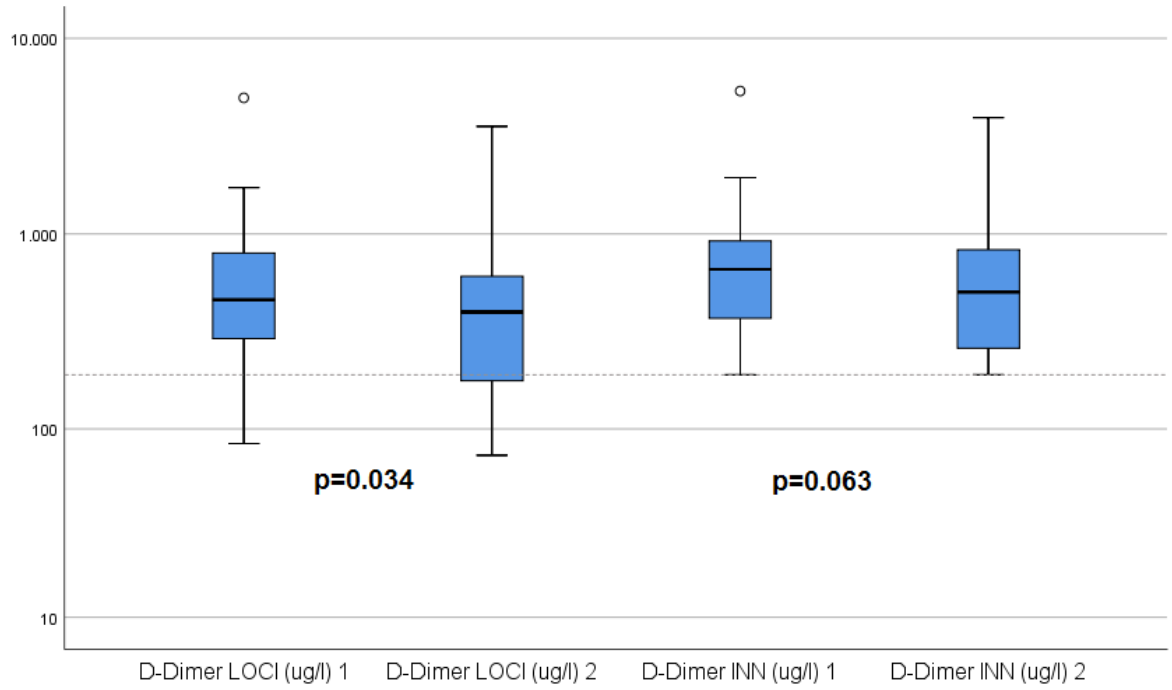

Figure 11.

D-Dimer levels in follow-up 1= baseline 2=follow-up

5 Discussion

This study shows for the first time that elevated D-Dimer values are associated with mortality in a mixed collective of patients with pulmonary hypertension and patients at risk for pulmonary hypertension. Looking into detail and performing subgroup analysis it was evident that the effect was driven mainly by patients with manifest PH. This suggests that D-Dimer may be a novel blood derived biomarker for prognosis in this patient population. D-Dimer measurements are routinely performed in the clinical practice, hence it is widely available and there is a good potential for translation (34). Nonetheless, it is not surprizing that not only the previously published cohort suffering of coronary arterial disease showed prognostic relevance but also patients with pulmonary hypertension (27).

Prognostic markers play an important role in the management of PH. During the last years, it has been more in focus of attention to develop prognostic scores that combine different aspects of the disease (35). The current PH guidelines propose a risk stratification model including BNP/NT-proBNP, however the model has not been extensively evaluated (9). A modified risk stratification model has been retrospectively evaluated in newly diagnosed PAH patients in three independent databases, namely of the French registry, Swedish registry and the German registry COMPERA (36-38). The French score which included invasive parameters classifies patients according to the number of low-risk criteria consisting of World Health Organization (WHO)/New York Heart Association (NYHA) functional class (FC), 6MWD, right atrial pressure and cardiac index. Their non-invasive method ranks PAH patients according to the number of low-risk criteria at diagnosis present for WHO/NYHA FC, brain BNP/NT-proBNP and 6MWD. The Swedish and German scores mix invasive and non-invasive data for 6MWD, WHO FC, NT-proBNP, RAP, cardiac index and mixed venous oxygen saturation using the ESC/ERS risk thresholds (low, intermediate and high risk). All these models showed a better prognostic outcome for patients with a higher number of low-risk criteria or patients in the “low-risk” strata versus other strata, at both baseline and follow-up. It is again showed that BNP nad NT-proBNP are the only blood derived biomarkers that could be included. BNP and NT-proBNP are released as result of myocardial stretching and if elevated a myocardial stress is present. D-Dimer as a prognostic biomarker would mirror another aspect of the

disease, the endothelial dysfunction. Pathophysiologically, endothelial dysfunction may be a driving factor for D-Dimer elevation (24). D-Dimer production and the elevation of D-Dimer occurs because microthrombi occlude small pulmonary vessels. This idea has been already postulated and there is histologic evidence for it (39).

These scores have been developed and validated only for Group 1 PH. In this study D-Dimmer was not tested for prognosis in the individual groups due to the relatively low sample size and the low number of mortality events.

It would be definitely interesting to see if there is a difference regarding D-Dimer levels between the subgroups of patients. The same limitation applies here as for the previous question, relatively low number in the individual groups and no statistical power. Although sample size is an obvious limitation, the collective represents real world data without any selection bias. It is, however, tempting to speculate that patients with CTEPH have higher values because CTEPH often occurs after acute pulmonary embolism. Interestingly, D-Dimer levels were in a similar range as those patients where PH was excluded by means of RHC. A possible explanation is that once the thrombi get organise within the vessel walls there is no active fibrinolysis.

Oral anticoagulation is often present in this collective mainly due to other indications as PH. It is unclear how concomitant medication changes D-Dimer values.The question remains open if patients with PH develop higher D-Dimer values due to worsening of the disease. Another question is that if targeted therapy, which has a disease modifying effect, would be able to change the disease course and in parallel D-Dimer values as well. In this study a slight decrease in the follow-up was found, however this result is limited to one measurement method. Due to the obvious limitation of small sample size and the small difference, the clinical relevance is questionable.

Another issue is the significance of D-Dimer values in patients with a borderline elevation of mPAP (20-25mmHg). Such patients have a worse prognosis than patients with normal haemodynamics and there is currently a growing interest to identify those patients with borderline mPAP who may develop a manifest disease (40).

There are reports showing that D-Dimer values may be genetically determined; however, these studies have also limitations (41, 42). A genome wide association

study conducted among 21,052 European-ancestry adults showed that three genes were associated with D-Dimer levels. One of these (F3) was newly found, and it codes for coagulation factor III (41). It has been shown that D-Dimer is a predictor of cardiovascular disease in African-Americans and there might be a sex-specific and African ancestral genetic background found in the *F3* and *HBB* loci which contribute to higher levels of D-Dimer among women and African-Americans. (42).

D-Dimer is not routinely tested in the clinical practice because of its low positive predictive value, which is why studies assessing its role in other diseases are missing. However, once a clinical suspicion of pulmonary embolism is present according to the prediction rules D-Dimer is assessed. This is the reason why in the current pandemic due to the novel coronavirus SARS CoV-2 many patients referred to the hospital due to shortness of breath underwent D-Dimer testing. Retrospective analysis of the data showed that D-Dimer is a marker of poor prognosis for the coronavirus disease (COVID-19) (43,44). Reports where D-Dimer was associated with poor prognosis for pulmonary embolism and deep venous thrombosis are widely available (45,46)

The LOCI assay for D-Dimer is not approved yet for clinical use; however, the presented data demonstrated that there is a very good correlation with values measured with the INNOVANCE. Deeper analytical comparisons are obviously to be done; however, this is beyond the scope of the present study. Clinically validated samples for approved indications like pulmonary embolism are the key for future validation studies.

Latex agglutination assays have the advantage over classical ELISAs that they do no involve washing steps, which reduce assay duration in a significant way. Moreover, it enables automatization more conveniently, which is useful in the clinical practice. The LOCI adds further advantages to the agglutination assay. Besides short time to result and the possibility of automation, it is highly precise and sensitive and requires small sample volumes. Although not reported here as an extra result, the study supports existing experience about the short time to the first result, meaning that the results can be achieved within minutes if the LOCI method is used (47).

The question remains open what do low D-Dimer values mean; however, if we use these already existing and known values in the follow-up it may find its

suitable role. Subclinical events may be detected by high sensitive assays, which provide additional tools for early recognition. There is hence a good chance that D-Dimer may become an early diagnostic marker.

One challenge remains the recruitment of patients because of the rarity of the disease. Multicenter studies should aim to collect these rare but valuable samples where biobanking is playing a crucial role. In order to validate a biomarker independent cohorts are needed, which is challenging since the disease is rare and good characterized cohorts are key for clear data. A good example for that is the validation of endostatin as a prognostic marker for pulmonary hypertension which has been previously shown in small cohorts and is currently under investigation in a large cohort in the United States of America using samples from a PH biobank (48, 49). Similar collaborative efforts in the United Kingdom led to discovery of a combination of nine circulating proteins that could identify patients with PAH who are at high risk of mortality (50).

6 Conclusion

- LOCI reliably detects D-Dimer in Li-Hep plasma in a real world collective

- D-Dimer is a predictor of mortality.

- D-Dimer appears as a new age- and sex- independent predictor of mortality in a mixed population undergoing RHC and in patients with PH and may provide a novel insight in mechanisms leading to an impaired prognosis in pulmonary vascular disease.

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