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New early biomarkers for acute venous thromboembolism

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TITLE

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TÍTULO

Novos biomarcadores precoces para o tromboembolismo venoso agudo

CANDIDATE TO PhD

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Thesis for Doctoral Degree (PhD)

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“A Faculdade não responde pelas doutrinas expendidas na dissertação.”

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Abbreviations

CFHR5- complement factor H related 5 protein

CRP- C-Reactive Protein

CTEPH- chronic thromboembolic pulmonary hypertension

DVT- deep vein thrombosis

ED- emergency department

Glut-1- glucose transporter 1

GWAS- genome-wide association studies

HBA- Hospital Beatriz Ângelo

HMDB- Human Metabolome Database

IL- interleukins

INSA- Instituto Nacional de Saúde Dr Ricardo Jorge

LC-MS- mass spectrometry coupled with liquid-chromatography

miRNA- MicroRNA

MP- microparticles

MPO- myeloperoxidase

MPV- mean platelet volume

MS- mass spectrometry

MVC- mean corpuscular volume

NET- neutrophil extracellular traps

PAI-1- Plasminogen Activator Inhibitor-1

PE- pulmonary embolism

PASP- pulmonary artery systolic pressure

PTS- post thrombotic syndrome

RBC- red blood cell

RDW- red cell distribution width

TCA- tricarboxylic acid

VTE- venous thromboembolism

VWF- von Willebrand factor

Abstract

Venous thromboembolism (VTE) is one of the most prevalent cardiovascular diseases with a wide spectrum of clinical heterogeneous presentations that depends on complex imaging exams for a definite diagnosis, which are limited in the emergency setting. Biologic biomarkers could provide a fundamental aid in the diagnosis of the acute expressions of VTE, but good performance biomarkers are lacking in the daily medical practice. As so, the discovery and validation of new biomarkers for VTE are needed.

This thesis resulted from the investigation question of whether there could be new blood biomarkers for the early diagnosis of acute VTE.

To answer this question, a comprehensive review of the existing literature was completed, focusing on the existing 'omics' derived data, as we thought this would be the technological approach to be used in the discovery of new biomarkers. First, we focused on reviewing and meta-analysing two distinct therapeutic strategies in acute pulmonary embolism diuretics and fluid expansion, among normotensive patients with right ventricle dysfunction, and the results revealed significant evidence in favour of the use of furosemide. Secondly, we meta-evaluated studies measuring haematological indices in patients with and without definite diagnosis of acute PE and found a significant discriminatory value of mean platelet volume (MPV) for the diagnosis of acute PE at the emergency department (ED). From the literature review, we also observed that there are still a limited number of studies with the aim of identifying blood biomarkers for VTE through the application of omics platforms (metabolomics and proteomics) and the majority have been using plasma as a study sample.

Thus, we decided to design a clinical longitudinal prospective study to investigate the 'omics' characteristics of acute VTE patients, with the objective of discovering new blood derived diagnostic biomarkers by using plasma and red blood cells (RBCs) as study sample. The study was implemented in one ED, in which a cohort of patients diagnosed with acute deep vein thrombosis (DVT) or acute PE and patients with a non-confirmed VTE as controls, totalizing 64 patients, were recruited. A biobank of more than 500 blood derived samples was created. When analysing the clinical and demographic features of these patient cohorts, we found significant differences between VTE patients and controls. Specifically, we were able to demonstrate that acute VTE patients had significantly higher red cell distribution width (RDW) than controls.

As an initial omics approach, we performed a metabolomic untargeted analysis to identify and quantify metabolites in both plasma and RBCs. This study is one of its kind, by analysing the metabolome of acute VTE patients during the initial hours of symptomatic disease, and by including metabolomics of RBCs. Our overall results demonstrated, for the first time, that RBCs and plasma effectively have distinctive metabolomic signature profiling associated with acute VTE. However, the plasmatic signature was not completely discriminatory of acute VTE, showing some metabolic

superposition between VTE and controls. In contrast, RBCs of acute VTE expressed significant discriminatory metabolites from non-VTE, representing the first published results of RBCs metabolome profiling in this disease. Even more important, three of these RBCs discriminatory metabolites revealed a very good performance on the ROC curve testing. These results may represent the first blood biomarker diagnostic panel to early acute VTE ever published.

To further validate this unique blood diagnostic biomarker panel for acute VTE, more studies in larger multicentred populations will be needed. The critical interpretation of these results along with a proposal for future investigations, combining metabolomics and proteomics followed by clinical validation, constitutes the final chapter of this thesis.

In conclusion, the changing of the paradigm of the early diagnosis of acute VTE may be fostered by 'omics' in general. Our results on metabolomic profile of acute VTE are very promising for the discovery of the 'troponin for acute VTE' in the future.

Resumo

O tromboembolismo venoso é uma das doenças cardiovasculares mais prevalentes com um amplo espectro de apresentações clínicas que depende da realização de exames imagiológicos complexos para o seu diagnóstico, limitados no contexto de urgência. Os biomarcadores biológicos poderiam constituir um apoio fundamental para o diagnóstico das manifestações agudas de tromboembolismo venoso, mas faltam biomarcadores com bom desempenho na prática clínica. Como tal, são necessárias a descoberta e validação de biomarcadores novos ou previamente existentes para o tromboembolismo venoso.

A presente tese resultou da pergunta de investigação sobre a existência de novos biomarcadores sanguíneos para o diagnóstico precoce do tromboembolismo agudo.

A fim de responder a esta questão, foi realizada uma revisão exaustiva da literatura focada nos achados derivados das abordagens ‘ómicas’, uma vez que pensámos que esta poderia ser a abordagem metodológica a seguir na descoberta de novos biomarcadores. Durante esta revisão explorámos duas questões em particular. Em primeiro lugar, comparámos duas estratégias terapêuticas distintas- diuréticos e expansão de volume, na embolia pulmonar aguda em doentes normotensos com disfunção ventricular direita aguda, que revelou evidência significativa no sentido da utilização de furosemida. Em segundo lugar, analisámos índices hematológicos e encontramos um valor discriminatório significativo para o volume plaquetário médio (MPV) no diagnóstico de embolia pulmonar aguda em contexto de serviço de urgência.

Decidimos desenhar um estudo clínico prospetivo longitudinal para investigar as características ‘ómicas’ dos doentes com tromboembolismo agudo, com o objetivo de descobrir novos biomarcadores sanguíneos de diagnóstico. O estudo foi implementado num serviço de urgência e comparou doentes com o diagnóstico de trombose venosa profunda dos membros inferiores ou embolia pulmonar aguda, com doentes com uma suspeita de tromboembolismo venoso agudo não confirmado, num total de 64 doentes recrutados. Foi criado um biobanco com mais de 500 amostras biológicas derivadas do sangue. Como principal abordagem aos nossos objetivos, realizámos uma análise metabolómica ‘untargeted’ para identificar e quantificar metabolitos no plasma e nos glóbulos vermelhos. Este estudo é o primeiro a analisar a metabolómica de doentes com tromboembolismo venoso agudo nas primeiras horas de apresentação, e a considerar o metaboloma dos glóbulos vermelhos. Os nossos resultados revelaram, pela primeira vez, que os glóbulos vermelhos e o plasma têm uma assinatura metabolómica distinta no tromboembolismo venoso agudo. O perfil do plasma não foi completamente discriminatório, apresentando algumas sobreposições entre doentes e controlos. Pelo contrário, os glóbulos vermelhos de doentes com tromboembolismo venoso agudo expressaram metabolitos distintos dos controlos, representando os primeiros resultados publicados do metaboloma dos glóbulos vermelhos nesta doença. Ainda mais, encontramos três metabolitos dos

glóbulos vermelhos com uma 'performance' muito boa nos testes de curvas ROC. Estes resultados poderão representar o primeiro painel de biomarcadores diagnósticos precoces do tromboembolismo venoso agudo

Concomitantemente, na análise das características clínicas e demográficas da nossa coorte, encontrámos diferenças significativas entre doentes com tromboembolismo venoso agudo e controlos. Em particular, demonstrámos que os doentes com tromboembolismo venoso agudo tinham 'red cell distribution width (RDW)' significativamente superior aos controlos.

Para explorar esta possibilidade de um painel diagnóstico único para o tromboembolismo venoso agudo serão necessários mais estudos, incluindo estudos de validação com métodos laboratoriais facilmente acessíveis em populações multicêntricas maiores. A interpretação crítica destes resultados, em paralelo com uma proposta de próximos passos do nosso projeto de investigação, constitui o capítulo final desta tese.

Em conclusão, a mudança do paradigma do diagnóstico precoce do tromboembolismo venoso agudo pode ser suportada pela metabolómica e ómicas em geral. Os nossos resultados sobre o perfil metabolómico do tromboembolismo venoso agudo são muito promissores da descoberta da nova 'troponina do tromboembolismo venoso agudo'.

Outline of the Thesis

The present thesis focuses on identification of new biomarkers for the early diagnosis of acute venous thromboembolism (VTE), particularly in its clinical expressions of pulmonary embolism and/or lower limb deep vein thrombosis.

- In Chapter 1, the objectives of the thesis are presented.
- In Chapter 2, the introduction section, presents a complete overview of the state of the art of diagnostic and therapeutic principles applied to the various clinical presentations of acute VTE and the current investigation approaches in the discovery of new biomarkers in venous thrombosis, with a focus on the role of omics research. The methodological aspects associated with patients' recruitment and biobank creation, and the study design used for the investigation of acute VTE biomarkers are also presented.
- In Chapter 3, the main results of the thesis are presented, specifically clinical data results and metabolomic profiling of acute venous thromboembolism.
- In Chapter 4, the results are integrated, and the most resulting significant data are discussed, leading to the main conclusions of the present thesis. Further steps in the continuing research project are, finally, pinpointed.

Chapter 1: Objectives

Acute VTE represents a daily challenge to clinicians worldwide, particularly to emergency services. The lack of a typical clinical presentation and the need of time consuming and somewhat inaccessible imaging exams impose diagnostic inaccuracies and inefficiencies that shall be overtaken by new methods.

This thesis results from a starting question of whether there are new biomarkers for the early diagnosis of acute VTE, yet to be discovered, identifiable in blood derived samples by 'omics' methods.

The first step of the work reflected in this thesis was to review and present as a critical appraisal approach the existing evidence on diagnosis and management of acute VTE during the early symptomatic phase of the diseases, expressly pulmonary embolism (PE) and deep vein thrombosis (DVT), with a special focus on existing diagnostic biomarkers and on actual and possible 'omics' contributions to the knowledge on the VTE. During the comprehensive analysis of the state-of-the-art evidence, we decided to analyse some aspects in particular. Firstly, we explored the therapeutic approach to normotensive PE patients with acute right ventricle dysfunction. Secondly, we investigated the role of haematologic indexes as a diagnostic marker of acute PE. Finally, a comprehensive review of the literature on the 'omics' contributions to the diagnosis of acute VTE was completed.

After a thorough consideration of all these aspects, we decided to design and implement one clinical study with the aim of exploring the 'omics' characteristics of acute VTE. The study was submitted to ethical approval at Hospital Beatriz Ângelo-Loures- Portugal and authorized. It recruited adult patients presenting to the emergency department (ED) with clinical symptoms and signs suggestive of acute PE or acute TVP and compared their features according to the confirmation- acute VTE group- or exclusion- control group- of the initial diagnostic suspicion. Our objectives for the study were to compare clinical and demographic variables between the two groups; to compare 'omics' profiling of plasma and red blood cells (RBCs) between the groups; to create a biobank for blood derived samples from all recruited patients to be used in future other 'omics' investigation and validation studies.

We chose 'untargeted' metabolomics as the first omics approach to evaluate our sample cohort. The absence of coherent evidence on possible potential diagnostic metabolites for this disease led us to the exclude a 'targeted' metabolomic analysis. Due to the laboratory's technological limitations at that time, the proteomic approach had to be postponed. Meanwhile, during the writing of this thesis, proteomic data were obtained and are currently under biostatistical analysis, which justifies the non-inclusion of these data in the present thesis.

To achieve our objectives, another option to mention was the choice of the blood components to study. Plasma is the most common blood derivate used in clinical practice for diagnostic purposes, and therefore, its use was considered in our

metabolomics study. On the contrary, RBCs have been scarcely explored for diagnostic biomarkers' discovery. However, since recent evidence has been associated quantitative and qualitative RBC abnormalities with venous thrombosis, we decided also to include this sample to accomplish our objectives.

Our findings were published in the international medical literature and were overwhelming considering our initial investigation question of the present thesis. We have discovered a new panel of blood metabolites that have a very good performance to discriminate between patients with acute VTE and non-acute VTE with similar clinical presentation, in a very initial phase of the episodes.

To discuss the relevant results to our objectives, in the perspective of possible pathways to propose for the continuing next steps of our research, we present our points of view in a final chapter of the thesis.

Chapter 2: Introduction

2.1 Spectrum of venous thrombosis

Thrombosis may affect any branch of either the arterial or venous circulation. Venous thrombosis is commonly referred to as venous thromboembolism (VTE) as a consequence of the clinical expressions of the disease that commonly consist of a local thrombus on the affected territory and one or more emboli originated from that primary clot and taken to distant locations by the venous circulation. Thrombosis may affect superficial veins, a minor and self-limited disease with an usually benign clinical course and without severe complications, and beyond the scope of this thesis. Deep venous circulation thrombosis, generally referred as VTE, is the most severe manifestation of thrombotic venous disease, both for its local thrombotic damage and for embolism to distant venous territories. VTE occurs in every segment of the deep venous circulation, including deep vein thrombosis of the leg or pelvis, pulmonary embolism (PE), cerebral sinus, superior cava, mesenteric, renal, hepatic and portal veins. Except for lower limb deep vein thrombosis, generally abbreviated to DVT, and PE, all the others venous thrombotic occurrences are rare and will not be explored in the thesis.

VTE is the result of an acute cascade of events that may be symptomatic or asymptomatic and may resolve completely or evolve to a chronic form of the disease. The chronic complications of venous thrombosis are beyond the scope of the project subjacent to this thesis and, therefore, will not be discussed.

Venous thromboembolism, as an aggregate of PE and DVT, is amongst the most common causes of cardiovascular disease worldwide¹, with a major impact on health indicators. VTE is estimated to be responsible for 300.000 deaths in Europe every year and a similar number of fatalities in the United States². Even in face of these expressive numbers, there is an important lack of public awareness for the disease and much less data on its epidemiology when compared to arterial thrombotic entities³. A 25-year observational study in an American population⁴ revealed an overall incidence of 142/100,000 for VTE, and a sub-group analysis demonstrated that PE has a similar incidence (78/100,000) to stroke (88/100,000). In terms of mortality, after 5 years from the first PE episode, only one in every two patients has survived the disease. The mortality for DVT is significantly lower, although important still, reaching a 5- years 27% fatality rate⁵. Moreover, the total burden of the disease is not restricted to the related mortality.

PE may be complicated by chronic thromboembolic pulmonary hypertension (CTEPH) in up to 6% of patients^{6,7}, whom suffer from pulmonary hypertension and, ultimately, right ventricle failure. Despite efforts to lower these sequelae, including aggressive treatment with systemic thrombolysis in the hyperacute stage of PE for eligible patients, they have shown no efficacy to prevent CTEPH⁸. On the other hand, post

thrombotic syndrome (PTS) is the most frequent chronic sequelae of DVT and has shown to affect half of the patients in the first year after the first DVT episode, despite early anti-coagulation treatment⁹. PTS is usually manifested by chronic lower limb pain, fatigue and oedema, and it is associated with clinical severity in about 10% of patients¹⁰, who suffer from recurrent leg ulcers. This health problem is particularly aggravated by ageing, as the risk of VTE increases 100 times from individuals younger than 20 years to older than 80 years¹¹.

The VTE spectrum ranges from high risk related to unprovoked episodes, occurring without any identifiable risk or triggering factor, to provoked VTE. The risk of VTE is multifactorial and provoking factors have been thoroughly studied. Provoked VTE is associated with triggering factors that may be permanent, dependent on demographic or clinical characteristics of the patient, or transient, usually present for the three months before the event at least, and mostly as a consequence to acute clinical conditions¹². Provoked events may occur after immobilization, major trauma, some orthopaedic surgery, or prolonged hospitalization, considered strong risk factors. Other low to moderate predisposing conditions, include cancer, anticonception, thrombophilia, pregnancy or advanced age^{13–15}.

When there is none predisposing factor or condition to VTE, we are dealing with an unprovoked VTE, which accounts for half of all VTE^{16,17}. As a whole, hospitalization is the most important known risk factor for VTE, not only during the admission but also extending its role as high-risk until 19 days after-discharge period¹⁸, along with institutionalization¹⁹. Nevertheless, the high proportion of spontaneous, unprovoked VTE episodes, not related to medical care nor any other triggering factors, is still very high. Unprovoked VTE represents a difficult management before the initial diagnosis as there are no initial epidemiologic clues to its suspicion, and as we will discuss in the next section, the clinical picture is heterogenous and nonspecific. Finally, unprovoked acute PE may be associated with a 50% higher death risk than provoked PE, non-cancer related²⁰, which evidences its potential severity. In this context, diagnosis of acute VTE is a daily challenge to hospital and out-of-hospital physicians and other healthcare professionals worldwide.

2.2 Diagnosis and clinical management of acute venous thromboembolism

Symptoms associated with VTE are nonspecific and not rarely very subtle, which is particularly true for PE. For a definite diagnosis of DVT and PE it is mandatory to obtain imaging exams, for this cannot be achieved by clinical examination alone. As a rule, international guidelines all agree and suggest a sequential approach for the diagnosis of VTE that begins with clinical suspicion.

PE is usually suspected before a history of PE dyspnoea, chest pain, presyncope or syncope, or haemoptysis¹⁵. Chest pain presentation to the emergency department (ED) leads to investigation of PE in approximately one third of patients, although it is only confirmed in less than 3% of them²¹. Similarly, syncope has an even lower rate of confirmed PE rates although it is a common symptom leading to the diagnostic hypothesis of PE^{22,23}. Even dyspnoea, considered a classical feature of PE, is present in only one out of every three PE cases at presentation²⁴. The so-called 'classic PE-triad' of chest pain, dyspnoea, and haemoptysis occurs in less than 10% of PE patients, which underlines the actual difficulties faced by physicians at the assisting healthcare facilities²⁵.

Regarding lower extremity DVT, the presence of unilateral swelling or cramping in the thigh or calf, along with other concurrent symptoms and signs including warmth, redness, or a palpable cord, are common triggers to a diagnostic work up for DVT. No individual sign has both high sensitivity and specificity, and taken together, clinical features accurately diagnose only about 20% to 40%²⁶ of DVT in symptomatic patients, with the highest positive predictive accuracies for DVT of 0.66²⁷. The differential diagnosis includes skin and soft tissue infections and superficial venous thrombosis, that may present with undistinguishable clinical features.

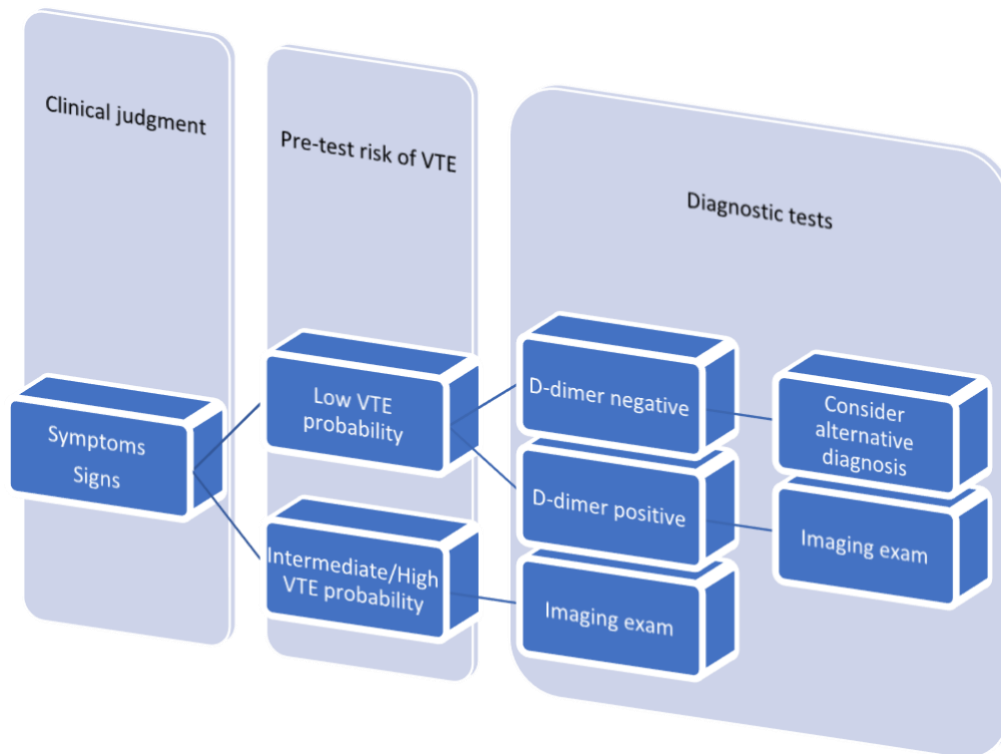
To solve these unsatisfactory performances of clinical history-based diagnosis for both PE and DVT, there has been an effort in developing clinical prediction rules to support daily practice. Combined predisposing factors and clinical characteristics are integrated into prediction rules to classify suspected PE or DVT patients into distinct categories of pre-test probability. These rules, before any laboratory or imaging test be performed, indicate classes that correspond to a low, intermediate, or high probability of acute VTE.

Revised Geneva rule and Wells rule are the most frequently used in PE suspicions. The proportion of patients who are initially considered for PE diagnosis and end with confirmed PE can be expected to be around 6-9% in the low-probability category, 23-26% in the moderate-probability category, and 49-76% in the high probability category²⁸.

The most well-known and used clinical prediction rule for lower limb DVT is the Wells score, that when combined with D-dimer level, gender, anticoagulation use, age, and family history of VTE, reveals a sensitivity and specificity of 82%²⁹. The existence of clinical prediction rules for both PE and DVT is widespread but is not absolute standard practice. Two thirds of physicians still prefer an unstructured pre-test assessment, usually referred to as clinical gestalt, than a clinical validated tool³⁰. This resistance results in an overestimation of VTE risk and, consequently, high rates of unnecessary referrals for ultrasonography and/or computed tomography pulmonary angiography (CTPA)³¹. Thus, clinical appreciation and pre-test assessment of DVT risk are fundamental to save low risk probability of DVT patients from performing unnecessary diagnostic tests.

The diagnosis of VTE relies on a multistep approach, beyond clinical reasoning (Figure 1).

Figure 1 - *Diagnostic algorithm for acute VTE in the ED*



D-dimer test measures a fibrin degradation product produced by the fibrinolytic response to thrombus formation, resulting from when plasmin cleaves fibrin to break down clots. D- dimer levels are increased in almost all cases of acute VTE. However, any reaction that increases fibrin also increases D-dimer levels. The diseases and health conditions that are may be associated with high D-dimer levels include pregnancy, any inflammatory condition, cancer, liver disease, trauma and surgery³².

Therefore, an elevated D- dimer is far from being unique to thrombosis, thus limiting its utility as a diagnostic biomarker of acute VTE. Initial management of unprovoked VTE usually takes place at the ED and we must consider that this setting is the one where most of those diseases are more common. The same limitations are true for in hospital VTE, because it occurs among diseased patients admitted for other situations, and frequently suffering from one or more conditions already associated with high D-dimer measurements.

The performance of D-dimer as a diagnostic biomarker is expressed by its high sensitivity and low specificity for acute PE alone, 0.97 and 0.41, respectively³³, and confirmed with age-adjusted D-dimer showing an higher sensitivity of 0.99³⁴. The

importance of these characteristics is central to its role as a rule out exam for acute PE. Even more, in patients with confirmed PE, the highest D-dimer levels are observed with the largest emboli³⁵, revealing its higher accuracy in patients with larger clot burden. Simultaneously, this heterogeneous behaviour underlines the high variability of D-dimer levels according to different clinical characteristics of PE episodes. It is currently recommended to use D-dimer assays with high sensitivity as a ruling-out strategy, to maximize the negative predictive value^{36–38} so that, combined with clinical decision rules, it avoids unnecessary additional imaging. The role of D-dimer assays in the diagnosis of suspected acute DVT has been extensively studied³⁹ and proven to have similar characteristics to those found in acute PE. In summary, the D-dimer assay is a highly sensitive but non-specific test with a high negative predictive value for DVT in different patient populations⁴⁰. Among patients with DVT, higher D-dimer values are present for proximal thrombosis, as opposed to distal calf thrombosis³⁹, and less than 7 days of symptomatic disease⁴¹.

Given its high sensitivity, the D-dimer assay can help to rule out VTE in low-risk patients, particularly when combined with the Wells scoring criteria or imaging, namely compression ultrasound (CUS) for VTE and CTPA for PE. Nonetheless, a D-dimer assay is not appropriate as a stand-alone test to diagnose VTE, because of its low specificity that is unhelpful to decide on any further investigation among patients suspected to have VTE with a positive D-dimer. False-positive D-dimer results lead to unnecessary ultrasounds and CT scans. On the contrary, negative D-dimer results can safely exclude the diagnosis of VTE without further testing⁴², with the advantages of reduced costs, time and iatrogenic complications. The present recommendation is to exclusively use D-dimer to rule out PE and DVT in patients with a low clinical probability.

Even with a very low specificity and, consequently, severe limitations for the early diagnosis of acute VTE, D-dimer is the only biomarker in common clinical use for diagnosis of VTE in the ED⁴³. Therefore, D-dimer testing is of limited value in VTE patients with a high pre-test probability because the negative predictive value of the test is reduced by the high prevalence of VTE in these sub-group that represents half the admissions of VTE episodes from ED⁴⁴. Therefore, in non-low risk pre-test probability of VTE there is the formal indication for an imaging diagnostic test, either CUS for DVT or CTPA for PE.

The ever-increasing availability of non-invasive CTPA largely contributed to the increasing ordering of an imaging diagnostic workup for PE, independent from the clinical presentation. Since its introduction in the late 1990s, CTPA resulted in an increase in PE diagnoses. Nevertheless, mortality from PE has not decreased^{4,45}, suggesting that there is overdiagnosis, due to detection of subsegmental clinically insignificant emboli and false-positives. CTPA is associated with risks of radiation exposure and contrast medium induced nephropathy and allergic reactions, apart from being relatively expensive, which strongly advises against its use in unselected populations.

CTPA is the imaging study of choice for the diagnosis of PE due to its high diagnostic performance for both this condition and alternative diagnosis, like pneumonia, tumours, or atelectasis. It is considered the gold standard diagnostic modality for PE with a sensitivity and a specificity of 0.94 and 0.98, respectively³³. Overall, evidence of an intraluminal filling defect in the pulmonary arterial tree on this exam had a sensitivity of 94% for PE. There are differences in the performance of CTPA for PE diagnosis according to pre-test probability. When patients have low or intermediate clinical pre-test probability of PE, a negative CTPA has a high negative predictive value for PE (96 and 89%, respectively) and a low positive predictive value (58%). Conversely, for high clinical pre-test PE suspicions the positive predictive value of a positive CTPA for PE is high (96%) whereas its negative predictive value is only 60%⁴⁶. In this sense, CTPA has similar negative predictive value to D-dimer for low probability pre-test patients, which favours the use of the laboratory assay instead, for its much lower costs, faster results, and higher accessibility, not to mention the unproportionally higher clinical safety. In the United States, only 2% of CTPA ordered for PE suspicions are positive for the diagnosis^{24,47}. In Europe, positive CTPA rate is 2–3 times higher⁴⁸, because ordering the exam is more accessible to physicians in the United States and because European physicians tend to adhere to guidelines more often⁴⁹. Finally, defensive medicine behaviour is subjacent to three out of five CTPA orders for PE diagnosis in the ED, in the United States⁵⁰, which strongly influences diagnostic workouts in the real world practice.

The alternative use of V/Q scan, a lower-radiation test without contrast medium use, is reserved for outpatients with low clinical probability and normal chest X-ray, and for special populations like pregnant women, iodine allergic, and severe renal failure patients. It is a relatively inexpensive test, without contraindications, but that is not readily available in most hospitals⁵¹, which is determinant for its widespread use, particularly among acutely ill patients.

The diagnosis of DVT is supported by ultrasound techniques. Two distinct lower limb ultrasound protocols, two/three-point compression ultrasound scanning (CUS) and whole leg ultrasound scanning (WLUS), are recommended to diagnose DVT in symptomatic patients. CUS is the first-line imaging test for proximal DVT suspicion cases, because it is safe, easily accessible, cost-effective, and reliable⁵². CUS sensitivity and specificity are 0.49 and 0.96, respectively³³, assuring a widespread use for DVT definite diagnosis in the presence of suggestive symptoms. CUS is the preferred modality because it is faster, easily trained by emergency physicians and has lower interobserver variability⁵³. However, this technique is not adequate to detect distal (calf) DVT, and an initial negative CUS examination in those patients imposes rescanning five to seven days later for confirmation, at the expense of discomfort for patients and high resource utilization⁵⁴. WLUS can mitigate this insufficiency in sensitivity of CUS, although it is a demanding technique both in terms of operator skills and high-end machines, thus highly conditioned in many healthcare settings.

In the chapter of treatment, although we are not going into details, the most transversal decision to make in the first hours after diagnosis, or before the possibility of a definite diagnosis is established, is whether to begin anticoagulation. Anticoagulants are considered to prevent clot extension and distant embolization, more commonly manifested as a PE after DVT, and should be included in the immediate measures to revert hemodynamic collapse, along with thrombolysis. On the other side of the scale there are higher bleeding risks, with potentially fatal complications, that must be weighed in the moment of the decision making⁵⁵. Initial low-molecular-weight heparin (LMWH) and direct oral anticoagulants (DOACs) are the preferred anticoagulants, with similar efficacy and safety profiles^{56,57}. Contrary to isolated DVT patients, for whom anticoagulant therapy alone is the standard care, the diagnosis of PE may imply the addition of systemic thrombolytic therapy.

Despite the lack of definite evidence, present guidelines advise for the concomitant administration of thrombolytic therapy in acute PE cases with hypotension or clinically deteriorating⁵⁸. Massive PE, defined as PE associated with hemodynamic instability, due to acute right heart failure, may evolve to a catastrophic hemodynamic collapse, and, ultimately death, with reported in-hospital mortality over 25%⁵⁹. More recently, mechanical thrombectomy has been the core of several studies as an adjunctive interventional treatment for severe acute PE with a tendency of the results to a lower mortality rates⁶⁰, raising new horizons for the reversal of this epidemiologic data. Hemodynamic collapse is the extreme clinical expression of acute right ventricle dysfunction in acute PE, but it may be represented by right ventricle dilation without hypotension, that is also associated by itself with a worse outcome than patients with normal cardiac cavities^{61,62}.

The first objective of this thesis is the reviewing of the literature on the spectrum of evidence on acute VTE. Apart from the synthesis hereby presented, many questions were explored along this journey. One of those questions relates to the best treatment to administer to patients who present at the highest risk of mortality, but have no criteria to get systemic thrombolytics administered.

To explore new possible decisions for the treatment of this group, one article was published on the systematic review and meta-analysis of the available data comparing diuretics versus fluid expansion in the initial treatment of normotensive acute PE (Section 2.3). The publication resulted from a systematic review of trials that compared the administration of furosemide versus fluid therapy, through various protocols, in the acute and early treatment of patients with a definite diagnosis of PE that presented right ventricle dysfunction established based on echocardiographic findings and plasmatic biomarkers. The analysis revealed a significant 24-hour reduction in right ventricle dysfunction parameters with the use of diuretics compared to fluids, at the expense of a discrete elevation of creatinine levels. These data constitute new supporting evidence to the immediate treatment of patients with severe acute PE presenting to the ED in the real practice, which might be considered in the future for the international guidelines.

2.3 Comparison of diuretics and fluid expansion in the initial treatment of patients with normotensive acute pulmonary embolism: a systematic review and meta-analysis

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Comparison of diuretics and fluid expansion in the initial treatment of patients with normotensive acute pulmonary embolism: a systematic review and meta-analysis

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ABSTRACT

Background Right ventricular (RV) dysfunction is the main cause of death in patients with normotensive acute pulmonary embolism (PE). The optimal management for this subset of patients remains uncertain. This systematic review and meta-analysis focused on the comparison of diuretics and fluid expansion in patients with acute PE presenting with RV dysfunction and haemodynamic stability.

Methods A systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines considering only RTCs. The authors searched the traditional and grey literature through 1 November 2022. Meta-analysis used open source packages in R. Inverse variance fixed-effects models with OR as the effect measure were used for primary analyses. The main outcomes defined in this review protocol included pulmonary arterial systolic pressure (PASP), creatinine value changes and N-terminal pro-B-type natriuretic peptide during the first 24 hours.

Results Four studies with a total of 452 patients met the inclusion criteria. The baseline characteristics of patients were similar across all studies. Overall, patients receiving diuretics had a significant 24 hours reduction in pro-B-type natriuretic peptide (standard mean difference of −41.97; 95% CI −65.79 to −18.15), and PASP (standard mean difference of −5.96; 95% CI −8.06 to −3.86). This group had significantly higher creatinine levels (standard mean difference of 7.74; 95% CI 5.04 to 10.45). The quality of the studies was heterogeneous; two had a low risk of bias, and the other two had a high risk of bias.

Conclusions Very few studies have compared the efficacy and safety of diuretics and fluid expansion in normotensive patients with acute PE with RV failure. Overall, furosemide appears to reduce RV dysfunction in this subset of patients compared with fluid expansion. Further research is required to confirm these findings.

INTRODUCTION

Acute pulmonary embolism (PE) is the third most common cardiovascular disease and represents a significant burden to health worldwide.^{1 2} Right ventricular (RV) failure is the leading cause of mortality in PE, even in haemodynamically stable patients classified as having submassive PE. RV

WHAT IS ALREADY KNOWN ON THIS TOPIC

- ⇒ Patients with normotensive acute pulmonary embolism (PE) commonly present with right ventricular (RV) dysfunction, which is associated with increased mortality.
- ⇒ There is debate regarding the optimal treatment for acute RV failure in haemodynamically stable PE, including whether diuretics or volume loading represent a better approach.

WHAT THIS STUDY ADDS

- ⇒ In this meta-analysis of randomised controlled trials and observational comparative studies, early furosemide administration was associated with a significant reduction in acute RV failure at 24 hours, compared with volume expansion.
- ⇒ Higher creatinine levels were observed in patients treated with diuretics. However, during the decongestion phase of the RV failure treatment, increases in creatinine may reflect transient changes in glomerular haemodynamics and may even be associated with a better prognosis in the medium term to long term.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ Administering diuretics in the acute management of PE should be further investigated for the treatment of normotensive patients with acute PE with RV dysfunction.

dysfunction is a common echocardiographic finding with a prevalence of 40%–60% among unselected patients with PE.^{3 4} In haemodynamically unstable patients, the mainstay of acute management of PE is to relieve the RV afterload by means of thrombolysis, thrombectomy and anticoagulation.⁵ However, in haemodynamically stable patients, less is known about the optimal preload for the RV and the consequences of different RV filling pressures on the outcomes. There are recommendations for cautious fluid challenges to achieve euvolaemia in this subset of patients with PE under strict volume-loading monitoring.^{6–8} Excessive volume administration results in hypoperfusion of the RV. It remains

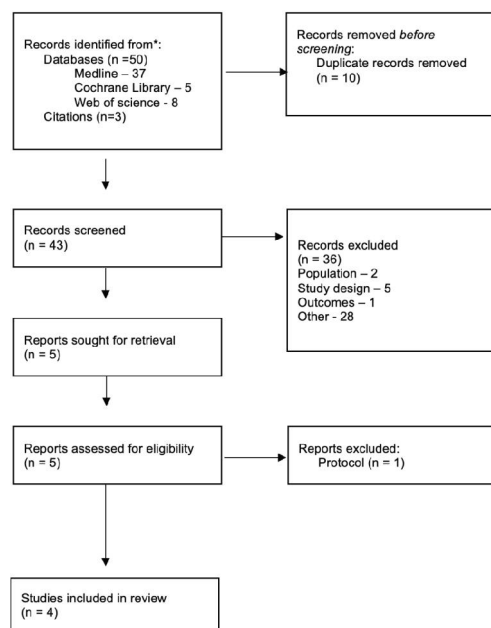


Figure 1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram.

unknown whether fluid loading-based treatments or diuretic therapy have greater benefits overall. This systematic review of randomised controlled trials (RCTs) aimed to compare fluid resuscitation and diuretic strategies in normotensive patients with acute PE presenting with RV failure.

METHODS

The systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.⁹

Search strategy

Databases MEDLINE, Cochrane Library (Central), EMBASE and Web of Science were searched for RCTs published before 1 November 2022, using a combined text and MeSH search strategy. The search terms used were acute pulmonary embolism AND heart failure AND diuretics AND (volume OR placebo OR saline OR fluid) AND (survival OR mortality OR complications). References from the relevant papers were also included.

Data collection

Two authors independently reviewed the abstracts of selected studies to determine their eligibility. The primary outcomes were pulmonary arterial systolic pressure (PASP) at 24 hours (mm Hg), N-terminal pro-B-type natriuretic peptide (NT-proBNP) at 24 hours (pg/mL), and creatinine at 24 hours (μmol/L). The selection process is described in detail in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram (figure 1).

Risk of bias evaluation

After the final selection of articles to be included, two reviewers independently assessed the risk of bias using the *ReviewManager V.5.4.1 (RevMan)* software and *Cochrane's* software for writing reviews. This platform allowed the evaluation of the following criteria: random sequence generation; allocation concealment; blinding of participants, personnel and outcomes; incomplete outcome data; selective outcome reporting; and other sources of bias. Low risk, high risk or unclear risk (either because of lack of

information or uncertainty over the potential for bias) were used to characterise the articles using different criteria. The fulfilment of the criteria was determined by consensus between the two reviewers, with no need to consult a third author to resolve any disagreements.

Data analysis and reporting

Descriptive statistics were expressed as mean and SD. For articles that used medians, the quartiles and median were converted to mean and SD to evaluate and compare the values on the same scale. Mean differences in time to proBNP normalisation at 24 hours, delta PASP at 24 hours, and delta creatinine at 24 hours between preintervention and postintervention or control treatment were compared using RevMan software. Data were expressed as values (95% CI). A value of $p < 0.05$ was considered statistically significant. To accommodate the variation in designs among the studies included in the analysis, a random-effects model was employed to combine the data. Clinical heterogeneities were assessed by two authors, and a meta-analysis was conducted with sufficient similarity between the study participants, interventions and outcomes. Statistical heterogeneity was assessed using the I^2 statistic, with values $\geq 60\%$ considered to indicate severe heterogeneity.

RESULTS

A total of 45 studies were identified, 41 were excluded due to their characteristics or methodology, and 4 studies were included in this analysis.^{10–13}

Study characteristics

The characteristics of the studies included in the meta-analysis are listed in table 1. A total of 452 patients were included in this study. All patients had RV dysfunction established based on echocardiographic findings in association with troponin and/or BNP elevation. Across all studies, the exclusion criteria were patients with a history of administration of thrombolytic agents or need for thrombolysis, diuretic and/or fluid infusion < 24 hours, catecholamine infusion, cardiogenic shock, or cardiopulmonary resuscitation. Patients with previously documented LV dysfunction or significant left-sided valvular diseases were excluded in two studies. Two studies excluded individuals with severe chronic renal disease, defined by a glomerular filtration rate of < 30 mL/min.

Patient characteristics

The number of patients included in the studies ranged from 46 to 275, without substantial disparities in sex distribution or age groups. In three studies, women predominated, with 61% being the highest percentage. Biomarker measurements at baseline for those assigned to the diuretic group consisted of creatinine between 78 μmol/L and 95 μmol/L, BNP between 342 pg/mL and 2 093 pg/mL, and PASP ranging from a minimum of 50 mm Hg to a maximum of 58 mm Hg. In the fluid group, creatinine ranged from 76 μmol/L to 92 μmol/L, BNP between 384 pg/mL and 2798 pg/mL, and PASP between 47 mm Hg and 60 mm Hg.

Study protocols and treatment regimens

All the studies used furosemide as a diuretic. The protocols consisted of a single intravenous bolus at admission, with dosages ranging from 40 mg to 80 mg, or an initial bolus of 40 mg, followed by subsequent administrations dependent on urine output. In the fluid groups, all treatment protocols used normal saline infusions with variable regimens. Across all

Table 1 Reviewed articles based on the PICO methodology

Author (year)	Study design	Population	Intervention (n)	Comparison (n)	Variables	Results
Ternacle <i>et al</i> ¹¹	Retrospective study	Submassive PE (acute PE without hypotension, but with RV dysfunction or troponin elevation)	Furosemide 40 mg intravenous and repeated every 4 hours if urine output remained <0.5 mL/kg/hour (40)	Isotonic saline solution 0.5–1 L during 30 min and repeated if necessary (30)	TTE at admission and before first intervention; only 10 patients repeated at 24 hours. Shock Index and sPESI at 24 hours, final outcome.	At 24 hours, only patients from the diuretic group had lowering of RV dilation (p=0006). Improvement in Shock Index (p<0001) was significant in the diuretic group. Patients in the diuretic group were less likely to have a severe sPESI (p=0.03) than in the fluid expansion group (p<0.0001). No significant differences were found in final outcomes.
Schouwer <i>et al</i> ¹²	Open single-centre prospective trial	Acute PE plus RV dilatation and dysfunction, and myocardial injury	Single furosemide 40 mg bolus intravenous at admission (24)	500 mL saline infusion/4 hours plus 1000 mL/day (22)	TTE at admission, at 4 hours and daily. BNP and troponin Ic at admission and every 12 hours. Clinical variables daily and at 30 days in consultation.	PASP values and reduction in RV/LV ratio were lower in the diuretic group (p=0001 and p=0,01, respectively). Normalisation of BNP was faster in the diuretic group (p=0003) and similar for troponin (p=0,59). At 4 hours, higher HR reduction was observed in the diuretic group (p=0001). Creatinine on days 1 and 2 were higher in the diuretic group (respectively, p=0.027 and p=0.019). After day 2, creatinine showed no differences. No significant differences were found in final outcomes.
Ferrari <i>et al</i> ¹³	Randomised open-label multicentre study	Acute PE with RV dysfunction	Furosemide 40 mg bolus intravenous at admission, followed by 40 mg at 4 hours if urinary output <500 mL (30)	500 mL saline infusion/4 hours plus 1000 mL/day ²⁸	TTE performed at admission, 4 hours post-treatment and then daily. Troponin, BNP and creatinine measured on admission and every 12 hours.	The decrease in PASP at 4 hours was higher in the diuretic group than in the volume expansion group (p=0006). Time to BNP normalisation was shorter in the diuretic group (p=0,05) and the number of patients with decreased BNP concentration at 12 hours was higher (p<0001). Troponin and creatinine measurements did not differ between the two groups.
Lim <i>et al</i> ¹⁰	Multicentre, double-blind trial	Acute PE, sPESI ≥1 and RV dysfunction (TTE only for randomisation)	Fluid therapy to clinician discretion plus single furosemide 80 mg bolus intravenous (135)	Fluid therapy to clinician discretion plus placebo (141)	Creatinine, BP, urinary output, sPESI at admission and at 24 hours. Duration of hospitalisation, major CV events.	At 24 hours there was a reduction of oliguria in the diuretic group (p<0001), but creatinine was higher (p<0001). sPESI improvement was similar (p=0021), as were major CV events.

BNP, B-type natriuretic peptide ; PASP, pulmonary arterial systolic pressure; PE, pulmonary embolism; RV, right ventricle; sPESI, Simplified Pulmonary Embolism Severity Index.

studies, there were no delays in the initiation of anticoagulant therapy, according to the respective medical guidelines. Other supportive measures such as oxygen therapy were also used at the physician's discretion.

Endpoints and primary outcomes

All studies had similar primary and secondary endpoints, including haemodynamic status, biomarker alterations and echocardiographic findings, with two studies adding the Simplified

Systematic review

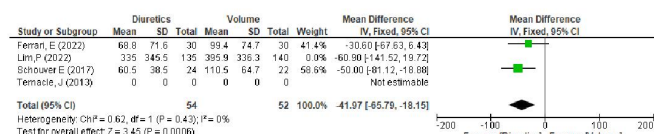


Figure 2 -Meta-analysis time to pro-B-type natriuretic peptide (proBNP) normalisation at 24 hours.

Pulmonary Embolism Severity Index. At the end of the meta-analysis, it was possible to select three comparable variables across the majority of the studies: time to proBNP (hours) normalisation at 24 hours, variation in PASP (mm Hg) values at 24 hours and creatinine ($\mu\text{mol/L}$) variation at 24 hours.

The meta-analysis demonstrated a significant reduction in proBNP levels during the first 24 hours in the furosemide group, with a standard mean difference revealing an effect estimate of -41.97 (95% CI -65.79 to -18.15) and a Z-score of 3.45 ($p < 0.0001$) (figure 2). χ^2 was 0.62 and I² was 0%, showing no heterogeneity in this data set. The funnel plot is available as online supplemental material.

At 24 hours, PASP was lower in the diuretics group, standard mean difference found an effect estimate of -5.96 (95% CI -8.06 to -3.86) and a Z-score of 5.56 ($p < 0.0001$) (figure 3). χ^2 was 2.18 and I² was 8%, showing low heterogeneity in this data set. The funnel plot is available as supplemental material.

Creatinine ($\mu\text{mol/L}$) levels at 24 hours in patients receiving furosemide were significantly higher than those receiving fluid treatments, with a standard mean difference of 7.74 (95% CI 5.04 to 10.45) and a Z-score of 5.61 ($p < 0.0001$) (figure 4). Furthermore, this data set showed no heterogeneity, with χ^2 and I² values of 1.85 and 0%, respectively. The funnel plot is available as supplemental material.

Quality evaluation

A summary of the risk assessment is shown in figure 5. The two most recent studies^{10 13} had an overall low risk of bias, as they were blind and double-blind studies, respectively. Two studies had a high risk of selection bias because of the consecutive selection of the population that was not adequately concealed. These same two studies had no blinding to the study team. In Schouwer *et al*'s study,¹² the decision between diuretics or fluid was at the discretion of the clinical treatment of the patient. Similarly, in the study by Ternacle *et al*,¹¹ one senior clinician always applied diuretic treatment, while others only applied fluid treatment. Therefore, both studies had a high risk of performance bias. The lack of randomisation also meant that both studies had a high risk of detection bias. Regarding attrition bias, only one study mentioned less than 5% of the missing data, resulting in a low risk of attrition. No evidence of selective outcome reporting that would suggest a high risk of reporting bias was found.

DISCUSSION

Our systematic review on the use of diuretics compared with fluid therapy in normotensive acute PE with RV failure included

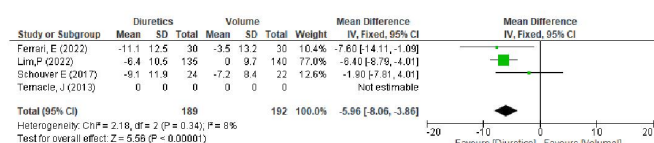


Figure 3 Meta-analysis of the delta PASP at 24 hours. PASP, pulmonary arterial systolic pressure.

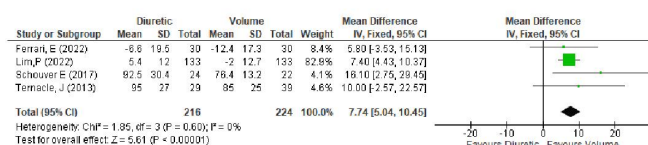


Figure 4 -Meta-analysis of delta creatinine at 24 hours.

four studies that met the predefined criteria. We found that intravenous furosemide administration was more likely to improve RV function at 24 hours than volume expansion in this subset of patients.

These findings of a significant early reduction in proBNP and PASP in haemodynamically stable patients with acute PE treated with diuretics suggest an immediate improvement in acute RV failure after initial management measures. In these early stages of right heart failure, even if there is no clinical evidence of haemodynamic deterioration, it is known that the surging RV dilation is associated with a decreased RV perfusion.¹⁴ This finding provides a rationale to support therapeutic volume-unloading strategies in the face of echocardiographic evidence of RV dilation, similar to the results we report in this study. Nevertheless, a gold standard measure for RV dysfunction secondary to PE is missing. Echocardiography assumes a relevant role in the management of acute PE for diagnosis, risk stratification and intervention guidance;^{15–17} however, this seems to be less consistent in haemodynamically stable patients.¹⁸ In normotensive patients, echocardiographic evidence of RV dysfunction is technically challenging¹⁹ and has shown low positive predictive value for PE-related in-hospital mortality.²⁰ In addition to these limitations, the current use of echocardiography for acute PE in emergency settings remains limited mainly because of the availability of skilled users and appropriate machines.²¹ As an alternative to echocardiography, biomarkers may be useful for the indirect inference of RV failure during acute PE. Cardiac biomarkers suggestive of myocardial necrosis (cTnT) and strain (NT-proBNP) are reliable as a surrogate to RV dysfunction in acute PE, in a simple and sufficient way.²² The use of NT-proBNP to guide diuretic interventions deserves further investigation to confirm its potential as a therapeutic management variable.

The benefits of diuretics do not seem to be restricted to RV-overloaded patients, as the population included in the studies was normotensive patients, independent of RV filling conditions. Numerous studies have shown that normotensive patients with PE and RV dysfunction on admission have an increased risk of short-term mortality, without evidence of any

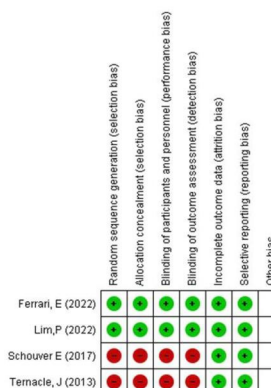


Figure 5 Risk assessment chart (ReviewManager V.5.4.1 (RevMan) software).

relationship to different preloading conditions.^{15 22–24} Persistent signs of RV dysfunction during the initial phases of PE are associated with higher in-hospital severe events and recurrent venous thromboembolism.^{4 20 25} It is our opinion that more studies should be performed with the inclusion of intravenous furosemide in the initial management of normotensive patients with PE with evidence of acute RV dysfunction, determined by imaging modalities and cardiac biomarkers, as well as by evaluating hard outcomes such as mortality.

Importantly, fluid infusion resulted in lower creatinine levels during the first 24 hours. Creatinine increases have been linked to poor short-term and long-term outcomes in patients with acute PE, serving as a predictor of mortality,²⁶ in addition to other risk factors including RV dysfunction.²⁷ However, during the decongestion phase of the RV failure treatment, increases in creatinine may reflect transient changes in glomerular haemodynamics and may even be associated with a better prognosis in the medium term to long term.²⁸ Therefore, higher creatinine values observed in the furosemide groups within the first 24 hours may not be of negative significance. To confirm this hypothesis, additional studies are needed to assess creatinine levels and renal dysfunction over extended periods of time.

This meta-analysis presents several limitations. First, the systematic review and meta-analysis are restricted by the small number of studies. Second, there is ongoing debate regarding the appropriate method for quantifying RV dysfunction and a definite answer has yet to be determined. One commonly used parameter (PASP) is operator dependent and has produced controversial results, leading to current recommendations for RV dysfunction diagnosis through the use of tricuspid annular plane systolic excursion instead. Third, mortality could not be included as an outcome due to insufficient information. Finally, the variability in quality scores among the four studies included in this analysis imposes cautious interpretation of the results.

CONCLUSION

There have been limited studies conducted to compare the benefits of diuretics versus fluid expansion in the early treatment of normotensive acute PE. Nevertheless, it appears that early intravenous furosemide may reduce RV dysfunction within the first 24 hours. Further research is needed to compare both therapeutic strategies over longer periods of follow-up in order to determine the potential roles of diuretics in the acute management of haemodynamically stable patients with acute PE.

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Patient consent for publication Not applicable.

Ethics approval Not applicable.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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2.4 Gaps in the diagnosis of acute venous thromboembolism

The main implication of an acute VTE suspicion is the need to decide on the emergent starting of anti-coagulation and, in selected patients, fibrinolytics amongst the most severe patients. PE is a fatal disease in 30% of untreated patients⁶³ and anticoagulation treatment dramatically reduces mortality to 2% of patients assisted in the hospital⁶⁴. The main challenge of acute VTE diagnosis is to immediately identify all patients that may benefit from treatment while minimizing the burden of diagnostic tests performed on patients without confirmed VTE and the risks of therapeutic adverse events.

Initial clinical evaluation is determinant for the pathway of VTE. As none of the available diagnostic tests for VTE are highly accurate, basal clinical assessment categorizes patients according to a predicted probability of the disease and increases the diagnostic performance of subsequent diagnostic tests. According to Bayes' theorem, the probability of one disease in patients with a negative or positive test depends both on the test accuracy and the disease prevalence in the tested population⁶⁵. The superiority of clinical decision rules versus clinical gestalt supports the use of one of the available VTE prediction rules as a first step to the final diagnosis.

Independent from the chosen rule, all with similar efficacy⁶⁶, there is a low adherence to their implementation in everyday clinical practice. Around half the patients with a confirmed PE are inappropriately managed, and the lack of clinical probability scoring is independently associated with this inadequateness⁶⁷. One of the main reasons for this fact is the difficulty of physicians to memorize the scores, even when using simplified versions and smartphone based calculators⁶⁸. Moreover, these scores are only useful when the physician has the clinical suspicion of PE to proceed to specific imaging investigation, which is not equally accessible to all healthcare facilities and contexts.

The problem of assuring the correct treatment to all VTE patients without prejudice of non-VTE treated patients, is not adequately solved by the available diagnostic tools. D-dimer is the only laboratory test for patients with suspected VTE. This laboratory test is far from being a good diagnostic biomarker, intrinsic to its very low specificity, and has been proposed as a 'filter' to the use of complex imaging tests, in combination with pre-test probability. More importantly, D-dimer is essential to manage VTE low risk patients as it supports the immediate clinical decision of no anti-coagulation treatment and of discharge patients to outpatient follow-up, or to investigate an alternative diagnosis.

For the half part of patients admitted to ED within the high-risk pre-test probability category, there are no diagnostic biomarkers and the only option is the completion of laborious imaging exams. When considering low-risk pre-test with negative D-dimer and high risk patients together, an imaging exam is required in 70% of VTE all suspected patients⁶⁹.

Apart from themes of costing and resource management, CUS and CTPA pose problems of exposure to adverse events, delays in time-to-treatment and contraindications in certain subgroups of patients. The mean diagnostic delay for PE ranges from 2.5 to 11.9 days and is of 7.7 days in the ED⁷⁰. Similarly, rates for lower limb DVT patients diagnosed less than 72 hours from presenting symptoms is less than 25%⁷¹, being arrival to disposition time independently associated with delay of the diagnosis⁷². In conclusion, there is an imperative need of new tools for the early diagnosis of acute VTE that will assure immediate directed treatment and, therefore, decrease the still high mortality rates of the first hours of the disease

2.5 Research of previously existing biomarkers in venous thrombosis

Research on VTE has been directed to evaluate the potential utility of various previously existing biomarkers as new biomarkers for VTE diagnosis.

As we move into the era of precision medicine, diagnostic biomarkers to detect specific diseases or diseases' subtypes are expectedly the future common rule in the clinical practice of healthcare professionals. The National Institutes of Health (NIH) defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"⁷³. As for European Medicines Agency and World Health Organization (WHO), the definition of a biomarker covers a measure of a physiological process, pathological process or response to a treatment⁷⁴. An ideal biomarker must be reliable, safely and easily measured, highly sensitive and specific, and cost efficient⁷⁵. At the stage of clinical utility, correct biomarkers must target the improvement of patient outcomes, reinforcing its biologic plausibility and impacting clinical decision making. Considering diagnostic biomarkers, that are critical to distinguish a patient with a particular disease within a population of healthy or diseased individuals, the assessment of clinical performance is determinant⁷⁶. There is no perfect performance of any biomarker test, with both 100% sensitivity and specificity.

Biomarkers may derive from existing laboratory tests newly applied to diagnostic purposes and assessed in terms of clinical performance to conclude on its accuracy to diagnose a disease. Contemporary technologic evolution led to the worldwide investigation of new potential biomarkers. Thus far, there is no single biomarker nor panel of biomarkers to exclusively use in the diagnosis of acute VTE. This is true for pre-existing molecules validated in VTE populations and for new candidates to diagnostic biomarkers. Various well known blood existing cells or substances have been targeted as novel biomarkers for VTE including hematologic indices, selectins, microparticles, interleukin-10 and other inflammatory markers.

Recent research towards a biomarker for acute VTE has explored the potential role of hematologic indices for diagnostic and prognostic intentions. Red blood cells (RBCs), neutrophils and platelets are measured in various dimensions with common laboratory machinery available at most hospitals and clinical settings. This easy access has raised interest on the study of the relations between hematologic indices and venous thrombotic diseases.

RBCs have been long standers as part of thrombosis and haemostasis, although generally presumed to have a more passive role in thrombosis than other blood components. Erythrocytes induce a prothrombotic state in blood by increasing blood viscosity and forcing platelets towards the vessel wall, and participate in thrombin generation⁷⁷. Therefore, RBCs related variables are of interest to analyse in the context of VTE. DVT has been investigated for RBCs count, haemoglobin, haematocrit, red cell distribution width (RDW), mean corpuscular volume (MCV), mean

haemoglobin concentration and mean corpuscular haemoglobin concentration, total leucocyte counts with differential leucocyte count, absolute neutrophil, lymphocyte count and monocyte counts, and platelet count and mean platelet volume (MPV). Data on these indices revealed that RDW was the only variable consistently found to be significantly associated with VTE, shown to be higher in patients than in controls^{78–80}.

RDW is a measure of the size variation and an index of the heterogeneity of the RBCs (i.e. anisocytosis), used in the clinical practice to classify anaemia. Recent studies have shown that RDW is associated with increased mortality in a number of cardiovascular disorders such as coronary artery disease, stroke, peripheral artery disease, heart failure, pulmonary embolism, and pulmonary arterial hypertension⁸¹. Moreover, RDW levels were associated with higher all-cause mortality in unselected critically ill patients⁸². The rationale beyond those results is not completely established, as it is unclear whether anisocytosis might be the cause, or an epiphenomenon to processes as inflammation or oxidative damage, instead. Other studies found an association of RDW>14.6%-15% at admission and early PE-related mortality in patients with acute PE, establishing a prognostic role for this index^{83–85}. In the perspective of diagnostic usefulness, a RDW >18.9% was associated with the presence of confirmed PE in the first hours of symptomatic patients admitted to the ED⁸⁶, although with a unsatisfactory receiver operating curve (ROC), characterized by very high specificity but very low sensitivity. Measurement of RDW can be done with readily available, easy, inexpensive tests at hospital admission, which could constitute important values for the diagnosis of acute VTE. Repeated evidence on higher RDW in the initial assessment of patients with DVT or PE deserve further research, particularly in larger populations and in combination with other clinical and laboratory variables.

Mean platelet volume, a measure of platelet size that is available in usual blood counting, is another hematologic index investigated as a predictive of thrombosis. The relevance of platelets to the haemostatic process, particularly in primary haemostasis for generating stable blood clots and stopping blood leakage from injured vessels, is unequivocal. Therefore, platelets derived variables are natural potential candidates to play a role in the diagnosis of venous thrombosis. The values of MPV have been noted to be increased in cardiovascular disease, peripheral artery disease and cerebrovascular disease⁸⁷. Reliable evidence has been provided that larger platelets are more functionally active and that increased MPV values may be an index of platelet hyperactivity⁸⁸. Consequently, high MPV may represent a risk factor for hypercoagulability and arterial and venous thrombosis. Higher MPVs were found in patients with acute myocardial infarction and highest values predicted mortality in this group^{89,90}, constituting an innovative insight into the diagnostic and prognostic role of MPV in arterial thrombosis. Similarly, there is supporting evidence of an increased MPV in patients with acute stroke, that reinforce the association between MPV and arterial acute thrombotic events⁹¹.

The possible role of MPV in VTE has been the aim of recent investigations. Patients with VTE were found to have higher MPV, but the ROC analysis revealed poor sensitivity and specificity^{92,93}. An increased MPV value is independently associated with a 2.4-fold higher risk of death in VTE, more specifically 2.3-fold in patients with DVT and 2.9-fold in PE⁹⁴. Increased MPV was demonstrated to be significantly correlated with Wells score for PE pre-test probability, and was suggested as a substitute to clinical prediction rules in the determination for the need of imaging exams⁹⁵. Taken together, several lines of evidence warrant further investigation on the establishment of the importance of enlarged platelets in thrombosis. Considering that MPV is an easy, rapid, and inexpensive laboratory parameter, the characterization of its performance as a diagnostic biomarker for VTE or venous thrombosis sub-groups will expectedly result in wide implementation in the hospital settings.

Having in mind this hypothesis, we decided to explore the existing evidence on the performance of MPV as an early diagnostic biomarker for acute PE. To do so, we completed a systematic review of the published data on measurements of MPV in patients presenting to ED with symptomatic PE versus comparing control groups (Section 2.6). Our results were published, showing that there is a significant increase in MPV of patients with acute PE compared to controls, with an effect estimate of 0.84 (95% CI: 0.76–0.92) and a Z-score of 20.25 ($P < .00001$). However, data retrieved from studies had significant heterogeneity. The exploratory ROC curve analysis performed sub-optimally for predicting acute PE, with an optimal cut-off of 8.5 fl revealing a sensitivity of 80% and a specificity 60%. In summary, MPV may be considered for inclusion in diagnostic algorithms in the future.

2.6 Diagnostic Role of Mean-Platelet Volume in Acute Pulmonary Embolism: A Meta-analysis and Systematic Review

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Diagnostic Role of Mean-Platelet Volume in Acute Pulmonary Embolism: A Meta-analysis and Systematic Review

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ABSTRACT

BACKGROUND: Acute pulmonary embolism (PE) is the third most fatal cardiovascular disease. PE is frequently misdiagnosed due to its clinical presentation's heterogeneity and the inexistence of biomarkers for its immediate diagnosis. Mean platelet volume (MPV) has shown a potential role as a biomarker in acute PE. In this analysis, we aimed to systematically compare the MPV in patients with and without definite diagnosis of PE, in emergency departments.

METHODS: Embase, PubMed and Medline were searched for relevant publications, in English. The main inclusion criteria were studies which compared MPV in patients with acute PEA versus a control group.

RESULTS: Thirteen studies consisting of a total number of 2428 participants were included. Of the participants included, 1316 were patients with confirmed acute PE, and 1112 were assigned to the control group. MPV was significantly higher in patients with acute PE than in controls (RR: 0.84, 95% CI: 0.76 – 0.92; $P < .00001$). There was a significant heterogeneity in the data.

CONCLUSIONS: This analysis showed higher MPV to be associated with acute PE immediate diagnosis. These data show promise for the use of MPV as a readily available biomarker for the diagnosis of acute PE at the emergency department.

KEYWORDS: MPV, mean platelet volume, thrombosis/embolism, biomarker, coagulation, pulmonary embolism

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Introduction

Pulmonary embolism (PE) is the third most fatal cardiovascular disorder. The main contributing factor is a persistently high and increasing incidence of PE cases in the general population, with around 0.4 symptomatic cases per 1.000 person-years.¹

Fatality rates of PE are still unacceptably high and are responsible for a short-term case fatality rate of 3.9% to 12%¹⁻⁴ among clinically recognizable cases, not to mention the sudden death clinical presentation cases, that may represent 20% to 25% of all PE cases.^{5,6} As these events can be silent and misdiagnosed, an early and accurate diagnosis of acute PE is vital for initiating emergent directed therapy and preventing deaths. The delay in the diagnosis of acute PE is a common feature of the disease as almost one third of patients are diagnosed only after 5 days from the onset of symptoms,⁷ equally divided between patient delays in getting medical care and long time from medical assistance to diagnosis. The heterogeneity and lack of specific symptoms and signs of PE clinical presentation may contribute to the physicians' attributable delay to diagnosis alongside limited access to the definite diagnostic test, only assured by the pulmonary CT scanning.⁸ The current diagnostic approach to acute PE, entailing a laborious and non-specific integration of clinical judgment, laboratory D-dimers results

and diagnostic imaging, could be revolutionized by the discovery of new sensitive and readily available biomarkers.

In recent years, platelet indices have been studied on cardiovascular diseases, and an elevated mean platelet volume (MPV), in particular, has been associated with coronary artery disease and with cardiovascular risk factors, of which diabetes and metabolic syndromes gathered the most evidence.⁹⁻¹¹ Although MPV value is automatically available to the prescribing physician from any ordinary electronic blood cell count, this parameter is only exceptionally used in the clinical practice. Nevertheless, recent attentions have focused on MPV as a potential diagnostic and prognostic biomarker in several conditions.¹² A recent meta-analysis reported a higher value of MPV in deep vein thrombosis patients, compared to control groups, but it included studies with mixed deep vein thrombosis and pulmonary embolism populations, and heterogenous clinical presentations such as acute and non-acute phases of the diseases.¹³ Contradictory results and shortness of studies justify why the role of MPV in diagnosing acute PE is still uncertain and, therefore, undervalued in clinical practice.

This study aims to systematically review publications on MPV determinations in acute PE patients and to conduct a meta-analysis on differences of MPV between acute PE



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patients and controls. We hypothesized that MPV is increased in patients with acute PE.

Methodology

Protocol

This systematic review and meta-analysis were conducted by Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendation¹⁴ (Supplementary Material).

Clinical question

The PICO model was used to define our clinical question: **Population** – Clinical studies of patients with acute PE; **Intervention** – MPV detected by any laboratory method; **Comparison** – Control patients who are (1) Emergency department controls (ruled out for acute PE diagnosis after imaging tests), or (2) Non-emergency department controls (inpatient or outpatient clinic patients without acute PE suspicion, or healthy); **Outcome measure** – Primary outcome: effect size (MPV in acute PE patients compared to controls). Secondary outcomes: assessment of quality and heterogeneity between studies.

Study design

All study types in which MPV was measured and compared to a control.

Search strategy and data extraction

A highly sensitive search strategy was conducted in EMBASE, PubMed and Medline without date restrictions. The keywords used for systematic searches were deep vein thrombosis, pulmonary embolism (PE), and MPV. Search terms were (Pulmonary Embolism [MeSH] OR Venous Thrombosis [MeSH] OR Venous Thromboembolism [MeSH]) AND (Mean Platelet Volume [MeSH] OR Blood Platelets [MeSH]). The last search was performed on 02/03/2020 and we re-ran the searches at final analyses' procedures.

Study selection and inclusion criteria

We looked for studies assessing the measurement of MPV for acute PE in adult patients. Two independent reviewers (the authors) conducted title and abstract screening procedures of the initial search results and independently evaluated eligibility of the retrieved articles. Only those in which MPV was measured at the exact moment of the acute PE provisional diagnosis and whose PE diagnosis was confirmed by contrast thoracic angiographic tomography were finally included for analysis. This review was limited to studies comparing acute PE patients with control individuals, and only included studies where the association of MPV and acute PE was explicitly investigated.

Study selection and data collection process

The titles of publications were independently reviewed by the authors and eligible abstracts were evaluated, in a standardized manner. The studies fulfilling the inclusion criteria were full-text read and finally selected for inclusion in the analysis. Further studies identified through references were equally evaluated. Lack of consensus between authors were solved through discussion and there was no need of a third reviewer's moderation.

Data items

Data extracted from each study included: authors, year of publication, type of population (emergency department patients, or other clinical settings), type of study, number of patients and controls, characterization of controls (recruitment and inclusion criteria), age of patients and controls, MPV measurement method, and values of MPV for patients and controls.

Quality assessment

Quality assessment of the observational retrospective and prospective studies was carried out by the Newcastle-Ottawa scale (NOS)¹⁵ where scores were attributed in number of stars quantitatively related to positive parameters.

Risk of bias and applicability were evaluated in Review Manager (RevMan 5.3, The Cochrane Collaboration, Oxford, UK). Domains were graded as low risk, high risk, or uncertain risk respecting biases. The studies were stratified in terms of risk of bias: low risk, when a maximum of 1 domain was "uncertain" or "high"; moderate risk when 2 or 3 domains were "uncertain" or "high"; and high risk when at least 4 domains were "uncertain" or "high."¹⁶

Statistical analysis

Standardized mean differences (SMDs) were considered for MPV within each study. $SMD > 0$ demonstrates an increased level of MPV.

Heterogeneity across studies was quantified using the I^2 statistics and an $I^2 \geq 50\%$ pointed to significant heterogeneity.¹⁷ Publication bias was analyzed through Egger's Regression test and bias visualization was assessed using funnel plot.

Statistical analysis was carried out by the RevMan (software version 5.3) and R (software version 3.6.1, package "metaphor" and "PRROC"). The data are expressed as a value (95% confidence interval [CI]). $P < .05$ was considered statistically significant.

Results

Study selection

From a total of 42 studies, we isolated 13 studies which fulfilled all the inclusion criteria and had complete data on MPV values

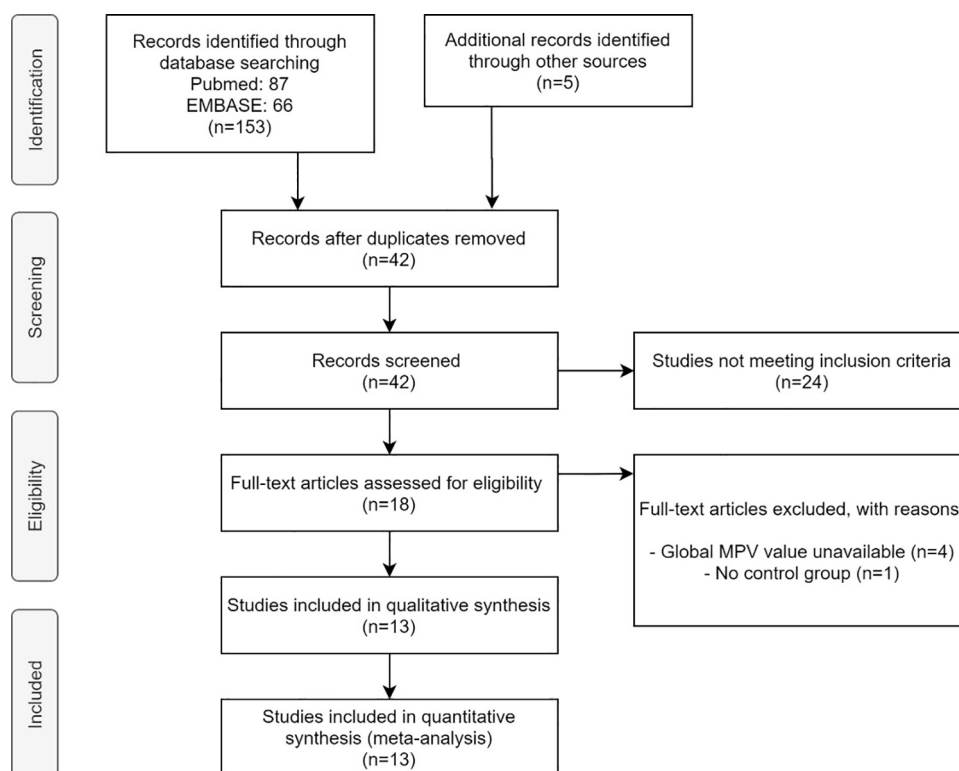


Figure 1. Search process.

at the time of acute PE diagnostic hypothesis for a patients' group. The complete searching process is shown in Figure 1.

Study characteristics

A total of 13 studies were included in the meta-analysis^{18,19,20-30}. There were 4 prospective cohort studies and 9 retrospective case-control studies for a total of 2428 participants, including 1316 patients and 1112 controls. In 7 studies, controls were specified as patients presenting suspected pulmonary embolism, although not confirmed after imaging tests results, which represented 576 individuals of the control group. The other control sub-groups were represented by healthy adults (229 individuals) or other emergency department patients (307 patients). 11 studies were performed in an emergency department and 1 study was conducted in the hospital ward. Only 1 study did not specify the clinical department.

Risk of bias within studies

No publication bias was identified through Egger's test ($P = .696$), and low evidence of publication bias was observed through a funnel plot, as shown in Figure 2.

Quality assessment

The overall assessment of the 13 studies revealed a classification of 5 or more stars in 11 studies (Table 1), but only 2 studies included healthy and community controls (the same studies) and none was blinded to cases.

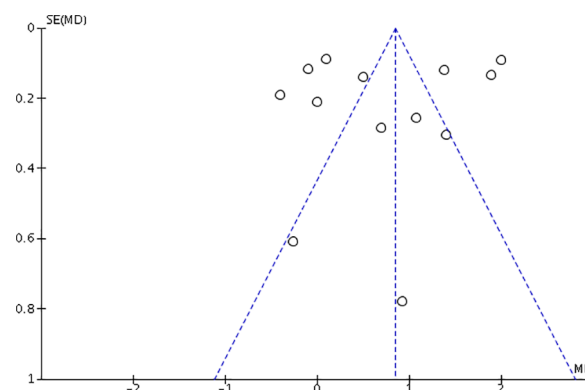


Figure 2. Begg's funnel plot – Publication bias.

Results for MPV

Meta-analysis of acute PE versus controls using standard mean difference found an effect estimate of 0.84 (95% CI: 0.76–0.92) and a Z-score of 20.25 ($P < .00001$) demonstrating a significant increase in MPV of patients with acute PE compared to controls (Figure 3). However, χ^2 was 439.06 and I^2 was 97%, showing significant heterogeneity in this dataset.

ROC curve analysis

The ROC curve analysis of MPV when predicting acute PE were constructed on an exploratory basis (Supplementary Material). The optimal cut-off values for MPV when predicting acute PE was 8.5 fl (sensitivity 80%; specificity 60%).

Table 1. General properties and MPV features of the studies.

STUDY	DEPARTMENT	CONTROLS	TOTAL (N)	ACUTE PE (N)	CONTROLS (N)	MPV ACUTE PE (MEAN)	MPV CONTROLS (MEAN)	NOS
Gunay	ER and outpatient clinic	Healthy	113	63	50	10.92 ± 1.37	10.23 ± 1.61	*****
Hilal	ER	Other ER	371	209	162	8.0 ± 1.1	7.9 ± 0.59	*****
Huang	Non specified	Non specified	145	70	75	9.91 ± 1.4	8.84 ± 1.68	***
Icli	ER and outpatient clinic	Suspected DVT, ruled out	293	98	98	9.9 ± 0.6	7.9 ± 0.7	*****
Kostrubiec	Wards	Ophtalmologic and HTN	292	192	100	10 ± 1.2	10.1 ± 0.8	*****
Moharamzadeh	ER	Suspected DVT, ruled out	173	125	48	10.38 ± 8.59	9.46 ± 1.11	*****
Talay	ER	Suspected DVT not confirmed	315	150	165	9.42 ± 1.22	8.04 ± 0.89	*****
Varol	ER and outpatient clinic	Suspected cardiac disease	177	107	70	9.9 ± 1.0	8.1 ± 0.8	*****
Abd El-Hady	ER and outpatient clinic	Suspected DVT, ruled out	70	50	20	8.9 ± 1	7.5 ± 1.2	*****
Çevik	ER	Suspected DVT not confirmed	128	61	67	9.73±1.19	10.13 ± 0.94	***
Erdal In	ER	Healthy	187	108	79	8.9 ± 1.1	8.4 ± 0.8	*****
Farokhi	ER	Suspected DVT, ruled out	141	16	125	8.6 ± 2.39	8.86 ± 1.19	*****
Sunnetcioglu	ER	Suspected DVT, ruled out	120	67	53	8.4 ± 1.2	8.4 ± 1.1	*****

Abbreviations: DVT, deep vein thrombosis; ER, emergency room; HTN, hypertension; MPV, mean platelet value; NOS, Newcastle-Ottawa scale; PE, pulmonary embolism.

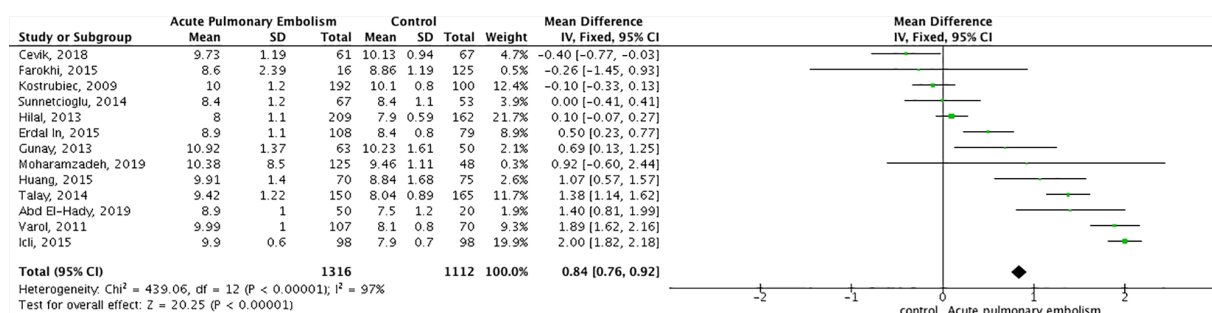


Figure 3. Forest plot for mean platelet volume: acute PE patients versus controls.

Discussion

To our knowledge, this is the first meta-analysis on the MPV values of acute PE at emergency department presentation. Our analysis showed significantly higher MPV in acute PE patients. However, likely due to diverse laboratory methods in MPV measurement, clinical manifestations' heterogeneity in acute PE, and diverse controls, we found substantial heterogeneity and risk of bias across the studies. Furthermore, different time-frames from sampling to storage – which were not documented – may have influenced MPV values. Nonetheless, diagnostic criteria for patient selection did not vary between studies.

Data on the role of MPV in acute PE is misleading. We know that variation of platelet size occurs in different arterial and venous thrombotic conditions, but it is not clear whether the sizing changes are a cause of a pro-thrombotic status or the consequence of the presence of an intra-vascular thrombus, which may lead to an increase of younger and bigger platelets on blood samples. We found the best cut-off value for the predictive value of MPV for acute PE to be 8.5 fl and this can be interpreted as a consequence of increased platelet aggregation.

Along with evidence on the influence of genetic polymorphisms³¹ and lifestyle factors³² on MPV (although these being assumed to contribute to pathogenic mechanisms of cardiovascular diseases known to develop under their influence), studies on acute phases of venous thrombosis rise additional associative hypotheses. MPV has been shown to increase in acute thrombotic conditions after platelet consumption and accelerated platelet turnover,³³ making it a potentially good hyper-acute diagnostic marker for PE. Additionally, inflammatory mediators seem to give feedback to the bone marrow that results in changes towards a higher MPV and prothrombotic phenotype. This reinforces that higher MPV is a consequence of acute thrombosis and may be detected in the beginning of acute PE pathologic processes.^{34,35}

The pragmatic question raised with this meta-analysis results is: how can we gather MPV to other variables in the early diagnosis of acute PE?

Some particular aspects deserve special considerations when it comes to biomarkers, and to MPV as an acute PE biomarker specifically. First, technical conditions for MPV determination represent a great amount of results variability. MPV values may be affected by venipuncture and sample storage conditions, indicated by significant different results in

samples stored at room temperature for more than a 3-hour period,³⁶ which corresponds to an easily accepted time-frame of delayed refrigeration for real clinical conditions. Similarly, platelet exposure to EDTA, the uniformly used anticoagulant in clinical laboratories, results in an increase of up to 50% on MPV determinations.³⁷ In the analytical phase, several other factors contribute to differences in the MPV measurements by different automated analyzers, either impedance or optical counters,³⁸ and, in our opinion, the daily use of MPV as a diagnostic marker for acute PE shall not precede the optimization of guidelines for its quantification.

Second, we share Norris et al¹² concerns about the small difference on MPV absolute results between both arterial and venous thrombosis patients, and controls. The narrow diagnostic range of MPV for acute PE complicates the definition of unquestionable cut-off values for its use in clinical practice, although this being already the case for d-dimers, the only blood biomarker used to determine PE diagnostic probability before imaging exams. In our opinion, this points out that investigation on the diagnostic efficacy of well-defined MPV measurements for acute PE in an early stage of the disease is justifiable by the present evidence. In summary, we believe that MPV values should not be used as a single diagnostic tool of acute PE. However, this parameter deserves further investigation as a potential biomarker of this potential suddenly fatal event, in the light of clinical probability scores' validation efforts.

Our meta-analysis has certain limitations. First, the majority of studies included represent small and somewhat heterogeneous populations. Although we were very strict to select only absolutely clear acute PE events in the patient's group, we had to accept some heterogeneity of controls. In fact, while some controls were healthy individuals, others were acute patients who presented to an emergency department and were suspected of having acute PE at admission. Even though these varied controls may have had some impact on the studies' results, their exclusion would have implied a significant loss of patients included in the meta-analysis. Second, most of such studies were retrospective, which may have limited their conclusions. Moreover, missing information on counters and technical protocols, on some demographic variables, on risk factors to venous thrombosis, or on time from symptoms to MPV determination, limited our evaluation on clinical heterogeneity. Third, there

was no sufficient available information to conduct robust analyses of cut-off points of MPV for acute PE diagnosis.

The existence of a new biomarker like MPV for the immediate diagnosis of acute PE, available at all emergency departments and other acute and chronic care facilities, even in the prehospital setting, could help to support the clinical decision of immediate administration of anti-coagulants before imaging results become available, thus potentially saving thousands of lives every year, worldwide.

Conclusion

Our systematic review and meta-analysis reveal that MPV may be a promising biomarker for the immediate diagnosis of acute PE. Given the current gap of diagnostic biomarkers of acute PE, further research regarding MPV's utility in this context shall be pursued.

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Supplemental material

Supplemental material for this article is available online.

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2.7- Research of new lines of biomarkers in venous thrombosis

P-selectin, an adhesion molecule found in platelets and endothelial cells, is emerging as one of the novel biomarkers for VTE. Elevated soluble P-selectin levels, that refers to the plasma released form of P-selectin, have been demonstrated in patients with DVT both at the presenting symptoms and at one week after heparin treatment^{96,97}. The association of P-selectin and Wells score present a positive predictive value of 100% and a negative predictive value of 96%⁹⁸. In the absence of the gold-standard imaging tests, soluble P-selectin added to D-dimer and Wells score rules out 29% of suspected DVT cases⁹⁹. If we consider the specific population of cancer associated VTE, P-selectin is an independent factor to the occurrence of an acute VTE event¹⁰⁰, promising a potential benefit from a future diagnostic approach for this high risk population that includes this molecule.

P-selectin for PE diagnosis is less well established. The levels of soluble P-selectin and glycoprotein IIb/IIIa complex (PAC-1) were detected to be high in acute PE patients during the hyperacute phase¹⁰¹. These are well-established markers of platelet activation, meaning that the presence of high levels in the first hours of symptomatic acute PE may indicate early platelet activation. Moreover, P-selectin was correlated with right ventricle dysfunction, a severity indicator in acute PE, which may represent an eventual advantage of this biomarker as a diagnostic tool in the ED. In this context, P-selectin is a serious candidate to diagnosis of acute VTE in the early hours of the symptomatic phase of the diseases in the near future, as long as widely available laboratory techniques are implemented¹⁰².

Fibrin monomers, produced from fibrinogen by thrombin-mediated cleavage, are another participant in the coagulation cascade that were implicated in the process of intravascular hyperactivity of coagulation in disseminated intravascular coagulation or DVT¹⁰³. Considering the hypothesis of fibrin monomers as biomarkers of acute VTE, several studies evaluated the levels of this molecules for the early diagnosis of postoperative DVT^{104,105} and determined a positive predictive value of 63%–73%, although with low specificity (73.2%). As fibrin monomer derives from fibrinogen in hypercoagulable conditions, it could be considered as a potential biomarker of the pre-D-dimer phase of VTE and studied in conjunction with D-dimer for a better specificity in the diagnosis of acute VTE¹⁰³.

The possible relation between thrombosis and inflammation has long been explored, mostly among arterial thrombotic diseases, but also amidst venous thrombotic conditions. Immune-thrombosis, or thrombo-inflammation, refers to the microvascular thrombotic response to pathogenic triggers and contributes to the innate immune reaction to foreign pathogenic invaders^{106,107}, suggesting that thrombosis under certain circumstances has a major physiological role in immune defence. It is hypothesized that VTE may derive from dysregulation of this immune response. Furthermore, platelets interact with leukocytes for the initiation of venous thrombus

formation, unlike arterial thrombus genesis. The formation of neutrophil extracellular traps (NETs) results from platelet-neutrophil interaction and are the main responsible for the stabilization of venous clots during the first hours of the thrombotic processes, corresponding to the histopathological findings of organizing thrombus (before organized thrombus that appears some days after)¹⁰⁸. NETs release forms web-like structures with a complex composition of extracellular DNA, histones, and granular proteins¹⁰⁹, and they may contribute to thrombosis by forming a “scaffold”, which induces platelets adhesion, activation, and aggregation, recruits RBCs, and maintains the stability of thrombus together with fibronectin, fibrinogen, and von Willebrand factor (VWF)¹¹⁰. Thrombus composition is similar between patients with PE and DVT, contributing to the conviction that NETs are part of the inflammatory response in thrombus development. The clinical significance of NETs biomarkers in VTE has been investigated for plasma DNA¹¹¹, myeloperoxidase (MPO), citrullinated histone H3 (H3Cit-DNA) and neutrophil elastase (NE)¹¹². The level of plasma MPO had a diagnostic performance of 0.78 for VTE¹¹³, but the remaining products were inferior to D-dimer in terms of diagnostic accuracy. On the contrary, normotensive acute PE patients presented 370% higher H3Cit-DNA than healthy controls¹¹⁴, underlining the impact of NETs formation on acute PE. Thromboembolism is one of the deadliest comorbidities associated with cancer. The higher risk for VTE in this sub-group was associated with H3Cit, which is an independent predictor of VTE in patients with pancreatic and lung cancer¹¹⁵. MPO was also positively correlated with colorectal cancer¹¹⁶ and levels of plasma DNA-histone and DNA were significantly higher in hepatocellular carcinoma patients with VTE¹¹⁷. This evidence suggests that NETs may contribute to coagulation activation and increased risk of cancer-associated thromboembolism and is a target of investigation on new biomarkers of diagnosis and prognosis.

Microparticles (MPs) are bioactive sub-micron (0.1–1 μm) membranous vesicles released from blood and endothelial cells, with the highest proportion constituted by platelet microparticles¹¹⁸, and it was previously associated with VTE¹¹⁹. MPs activate coagulation by means of monocyte-derived MPs and platelet-derived MPs, that contain surface proteins responsible for the thrombus propagation. MPs high levels in plasma have been associated with the risk of a first VTE¹²⁰, although it is not known if MPs are a true risk factor for VTE or simply a marker of hypercoagulability. A cut-off point of 16.5 nmol/l for MPs can significantly differentiate DVT patients with 100% sensitivity and 100% specificity¹²¹. On the other hand, there are contradictory results which could be explained by analytical issues, as the methods used in measuring MPs levels or the type of specimen used for MPs assay⁹⁸. Over time, there were discovered different MPs as a potential target for biomarker investigation, but it is difficult to consider them as a diagnostic option as there is no consensus on their measurement and results are contradictory.

C-Reactive Protein (CRP) is the mainstay of acute inflammatory response measurement used in clinical practice worldwide. The diagnostic usefulness of CRP

in VTE diagnostic workup is contradictory. CPR levels with D-dimer did not improve the rate of negative imaging studies in PE¹²². CRP>5mg/dL on admission was associated with an higher mortality and higher bleeding rates¹²³ among patients with acute VTE, and the junction of CRP to clinical scores increased their prognostic capacity, suggesting that CRP level on-admission may identify high-risk VTE patients. It is consensual that CRP is inaccurate to VTE diagnosis due to its low specificity, that is even more accentuated in the acute presentations, because of many other diseases associated with high CRP levels, particularly inflammatory states and diseases¹²⁴. Clinical studies revealed conflicting on the prognostic value of CRP in acute PE patients, also. Subsequently, there is still no established utility for CRP in the course of VTE management.

Few studies have evaluated the diagnostic and prognostic role of interleukins (IL) for VTE patients. A few studies focused on the role of IL, important influencers of inflammatory proteins like tissue factor, in VTE¹⁰³. IL-6, IL-8 and prothrombin fragment F1 + 2 levels have been reported to be increased at the time of DVT diagnosis¹²⁵. Nevertheless, other studies found heterogeneous results, and none of them assessed their diagnostic role^{126,127}. Further research is needed before defining an appropriate positioning of IL in the clinical approach to VTE.

As with other biomarkers, the diagnostic role of fibrinogen in VTE patients has not been sufficiently evaluated. Fibrinogen was found higher during the acute phase of DVT than healthy controls¹²⁸ and significantly higher in patients with unprovoked VTE than in those with risk-associated VTE and controls¹²⁹. These were small sized isolated studies, with results published more than a decade ago, and that were not repeated in another circumstances, limiting in a critical way the interpretation of a potential place for fibrinogen as VTE biomarker.

Many of the referred biomarkers are very promising, but, currently, they cannot be used to make decisions in the management of patients with VTE. The lack of established cut-offs impedes the implementation of clinical studies to validate them, and analytical issues are far from being solved through uniformization of procedures and accuracy of the results. Nowadays, in the absence of an objective method other from D-dimer, there are no further biomarkers available that can confirm or exclude the diagnosis of DVT or PE in one patient. Biomarkers are very important to clinical decision making, justifying the expectations on the discovery of novel biomarkers to identify patients with acute VTE.

2.8 Multi-omics and venous thrombosis

Current biomedical research has been critically influenced by two essential pillars: big data analysis to discover new patterns of biologic behaviour, and ‘omics’ results that jointly refers to biologic disciplines focusing on the high-throughput identification and quantification of huge numbers of molecular measurements within a tissue or cell¹³⁰. Technologies like metabolomics, genomics, transcriptomics, or proteomics, have been extensively applied to biologic systems and reveal biologic processes with high resolution. The growing knowledge on the capabilities of each emerging ‘omics’ technology, together with the machinery evolution of the laboratory devices and development of new friendly software tools for bioinformatic analysis, leveraged their utilization in the most various clinical and translational fields of investigation. The integration of different ‘omics’ derived data has created an even more recent field of biologic research known as multi-omics.

Genomics, the most mature of the ‘omics family’, developed a framework for mapping genome and identify gene expressions profiling on a global basis, thus carving the way for a better knowledge on normal physiology and of human disease¹³¹. These developments occurred during the first decade of the new millennium and was made possible by the technologic evolution of high-throughput genotyping and complex biostatistics tools, along with focused efforts in financing this biological discipline. At the same time, hopes on genomic explanation for most known human diseases were confronted with real limitations of genomics. In fact, phenotypes naturally occurring depend on several interacting alleles that express themselves differently according to their context and environment, which seems to be true both simple and complex diseases¹³². As a natural consequence, systems biology, by that meaning the biomedical research approach centered in methods to gather information and put the pieces together to understanding the larger picture¹³³, has recurred to other complementary omics data to integrate with genomic studies, to identify molecular patterns associated with disease.

As the core machinery of human biology, proteins have been an important target of new studies with high-throughput methods, in the origin of proteomics discipline. Mass spectrometry-based methodologies revolutionized the identification and quantification of proteins in cells and body fluids, strongly contributing to the understanding of protein expressions along the continuum of healthy-to-diseased processes.

Correlations between genome and proteome are promising in the explanation of common diseases mechanisms. There is proven influence of genetic variation on human protein levels¹³⁴ and clues to a causal relation between genetic traits and protein levels, although it is not clarified if protein level modifications may be related to the etiology of diseases or is just a consequence of diseased processes. Be either one of the explanations the reason for the variation of proteins measurements,

proteomics and genomics data integration may well be a successful approach to the discovery of new protein biomarkers of diseases¹³⁵.

The most recent member of this omics family is metabolomics, dedicating to the study of small molecules. Metabolites reflect both environmental and biologic derived modifications and is anticipated as an important method to bridge the gap between genotype and phenotype¹³⁶. Metabolomics inherited most proteomics mass spectrometry know-how and adapted it to the analysis of small metabolite abundance. The hypothesis generating untargeted approach of metabolomic studies is the most used, with the aim of quantifying both known and unknown molecules, that can lead to new insights of diseases and is becoming the paradigm for metabolic phenotypes. Compared to other omics, metabolomics results in abundant data results with low material necessary, thus an excellent candidate to the discovery of new biomarkers^{137,138}.

Different types of omics data are available in the development of new potential biomarkers for the diagnosis of acute VTE. Nevertheless, several studies have proposed biomarker candidates for acute VTE, but none have yet translated into clinical implementation.

In the last decade of the 20th century, technologic development of genetic tests was in the basis of the firstly described genetic associations with venous thrombosis. Factor V Leiden variant was described as a single mutation to coagulation factor V and found to be responsible for decreased activated protein C clot limiting properties. This resistance to activated protein C, an endogenous procoagulant, determines a 7 to 10-fold increase in the risk for DVP¹³⁹. Moreso, this same mutated Factor V revealed a variable association with cerebral vein thrombosis¹⁴⁰ and was associated with an higher risk for retinal vein thrombosis¹⁴¹. This mutation is inherited as autosomal dominant transmission, and is present in 20–25% of venous thromboembolic diseases¹⁴², although there is a different impact depending on the condition of heterozygous carriers, with a 3-7 times higher acute VTE risk, or homozygous carriers, reported to have a 50-100 times higher risk¹⁴³.

Caucasian studies verify a prevalence of 3 to 7% of factor V Leiden heterozygosis among general population and a 20% prevalence among VTE patients, representing a challenge in terms of correct management and prevention, especially before a first VTE episode. In fact, while it is consensual not to screen healthy individuals for factor V Leiden mutation, it is the source of continued discussion whether it is necessary to screen the mutation among patients during every first episode VTE events. The correct attitude towards long-term anticoagulation in these patients is controversial. Although the incidence of recurrent VTE for heterozygous factor V Leiden mutation is not significantly different from non-carriers, and guidelines establish only three months of anticoagulant treatment in cases of provoked VTE, physicians are influenced by the positivity of the mutation and tend to prolong anti-coagulation beyond the recommended¹⁴⁴.

The second most common inherited mutation associated with VTE, present in 2% of the general population¹⁴⁵, is prothrombin G20210A, known from 1996¹⁴⁶. The presence of this gene mutation results in increased levels of prothrombin and thrombin, implying an increased risk of VTE. The carriers of prothrombin G20210A present an increased risk of splanchnic vein thrombosis¹⁴⁷, cerebral vein thrombosis¹⁴⁸, in addition to DVT and PE. The corresponding hypercoagulable state translates into an increased the risk of VTE 3 to 4-fold for heterozygotic carriers and up to a 50-fold increase in the rare homozygotics¹⁴⁹. Prothrombin G20210A carriers do not have an higher risk of VTE recurrence (OR 1.45, 95%CI, 0.96–2.21), challenging the need for investigation of this mutation among VTE patients and for determining the need of long-term anticoagulation in these patients¹⁵⁰. Like factor V Leiden, prothrombin G20210A gene mutation investigation has no role to play in the diagnosis of acute VTE in the emergency settings.

Finally, another classic risk factor genetically associated with a higher risk for acute VTE is Plasminogen Activator Inhibitor-1 (PAI-1), which inhibits fibrinolytic activators and compromises the dissolution of fibrin clots. The PAI-1 4G/5G polymorphism has been described in a recent meta-analysis as a potential biomarker of VTE risk¹⁵¹, although the results of studies investigating a possible relation between PAI-1 hyperexpression and the risk of VTE have been controversial. PAI-1 was described in 3.4% of healthy individuals and its detection in unselected population was not associated with risk of VTE¹⁵². In the same direction of the previous genetic mutations analysed, the presence of PAI-1 has no impact in the decision making of the clinical management of acute VTE, within the diagnostic workup.

The development and availability of genotyping arrays in the early 21st century lead to a wide movement of simultaneous multiple variant research at reasonable costs. Genome-wide association studies (GWAS) investigated 172 variants in 24 clotting-related genes for association with an incident VTE in peri-menopausal and post-menopausal patients, and identified tissue factor pathway inhibitor (TFPI) associated with higher VTE risk, plus five variants associated with VTE in F5, F11, and protein C (PROC)¹⁵³. GWAS allowed the identification of three single nucleotide polymorphism (SNPs) strongly associated with DVT in CYP4V2, antithrombin (SERPINC1), and GP6¹⁵⁴, associations of risk variants in chromosomes 1q24.2 and 9q at the F5 and ABO loci¹⁵⁵.

Genome sequencing and interpretation led to the evidence that one single gene mutation may interfere in different metabolic pathways and have heterogenous phenotypic expressions, thereby contradicting the initial conviction that a single gene controls a single function. Hereby the mapping of human genetics, contrary to initial expectations, did not derive into identification of the pathophysiologic pathways for most diseases and unhealthy states. The completion of human genome unveiled that the diversity of biologic systems is much beyond the list of gene products¹⁵⁶ and that exploitation of systems biology.

Transcriptomics, consisting of the various methods used to study messenger RNA and noncoding RNA resulting from genome transcription¹⁵⁷, has been rarely applied to venous thrombosis. A transcriptomic profiling of a porcine model of DVT revealed the predominance of tumour necrosis factor (TNF), NF- κ B and apoptosis pathways¹⁵⁸ that enhances the possible critical relation between thrombo-inflammatory biologic processes and thrombus formation and progression. MicroRNAs (miRNAs) are a class of noncoding endogenous single-stranded small RNA molecules with a length of approximately 21–25 ribonucleotides, that have a tissue specific expression and are different in some diseases¹⁵⁹. These molecules may be quantified by quantitative reverse transcription polymerase chain reaction, which could be a considerable optimization of diagnostic possibilities in the real practice. Recent experiments show that the level of microRNA-134¹⁶⁰, miR-28-3p¹⁶¹ and miR-1233¹⁶² in the plasma of patients with acute PE is significantly higher than non-PE patients. Therefore, miRNAs have been gaining relevance for predicting PE with a high diagnostic potential¹⁶³.

Serum miRNAs have high diagnostic performances which are encouraging for their potential as future diagnostic biomarkers for PE diagnosis. MicroRNAs measurements are reproducible and they revealed high specificity and sensitivity for some diseases such as liver diseases, tumours, coronary diseases or heart failure¹⁶⁴. Furthermore, circulating miRNAs are resistant to endogenous RNase activity, which confers them high stability in the peripheral blood collected from patients. These characteristics contribute to an high expectancy on the potential role of miRNAs as acute VTE, or at least acute PE, diagnosis. Microchips of miRNA, either alone or combined with other biomarkers of PE, might have a real importance in the daily clinical diagnosis of PE in the near future¹⁶⁵. Some encouraging results from miRNA are even applicable to distinguish acute DVT from post-thrombotic stages of the disease, specifically for circulating miRNA-320a/b¹⁶⁶. A microRNA untargeted analysis of patients with a previous VTE episode identified a specific signature that included messenger RNA targets related to thrombomodulin and platelet functions¹⁶⁷, accentuating the probable implication of these RNA products in the pathophysiology of venous thrombosis, although not useful as acute disease diagnostic biomarkers.

The comparison of arterial with venous thrombosis transcriptomics exposed both shared pathways and differently expressed ones. The functional analysis of common gene expression revealed common haemostasis, complement and coagulation cascades, blood clotting cascade and prothrombin activation¹⁶⁸ related identifiers. On the contrary, several sets of gene expression compared between venous and arterial thrombosis, including the comparison of several time-collected VTE samples to acute myocardial infarction samples, resulted in a classifier consisting of 107 genes with 100% of accuracy. The differences were referred to products predominantly involved in biological processes related to cell maintenance, cell proliferation and immune regulation. Transcriptomic evaluation of Krüppel-like family (KLF) members, regulators of proinflammatory activation in the vasculature, in mice with vena cava thrombosis

identified matrix metalloproteinases differently expressed in VTE animals that could be a target of diagnostic biomarker development¹⁶⁹.

Metabolomics has raised scientific interest by its integration with other -omics profiles (transcriptomics, genomics, proteomics, lipidomics) to generate more comprehensive knowledge of venous thrombosis and general cardiovascular biology. Metabolomics has emerged as a biology discipline, among other high-throughput biology areas, to connect genotype and phenotype by metabolite screening. Metabolomic profiling consists on identification and quantification of molecules under 1.5KDa either at cellular levels or peripherally, at plasma. Making use of complex analytic techniques, such as nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled with different chromatographic methods [liquid-chromatography (LC-MS) or Gas-chromatography (GC-MS)] and bioinformatics, metabolomic processes quantify the very diverse metabolites¹⁷⁰. The chemical structure and variation in abundance of human metabolome is unique to an individual in specific circumstances and, contrary to the genome and transcriptome, is not completely traceable. On the other side, the footprints of diseases and health conditions in terms of metabolites is a promising new window into physiologic and pathologic processes. At present, there are about 40.000 metabolites listed at Human Metabolome Database (HMDB), but only 1.7% of them were mapped to a metabolic pathway¹⁷¹. This is a major pitfall for the interpretation of metabolomic analysis results and its integration into clinical practice, as most compounds quantified at biologic samples have an unknown biologic significance.

MS-based methods are the mainstay of metabolomic quantification and LC-MS is the most versatile among them¹⁷². Untargeted metabolomics, by opposition to the targeted approach, is an approach that does not follow a pre-identified list of metabolites, but identifies and quantifies as many metabolite peaks as possible, instead¹⁷³. The resulting highly complex data from this exploratory methodology, with thousands of simultaneous results not compatible with manual interpretation, are analysed by metabolomic software packages that include statistical tools applicable to high-throughput techniques. Although most metabolites remain unidentified, the crucial potential of untargeted metabolomics relates to the clarification of unanswered biologic processes and the revelation of new insights into unknown metabolic pathways¹⁷⁴.

Animal studies on venous thrombosis, in rabbits, concluded that lactic acid, glycine, glutamate, cysteine glutathione disulphide, glutamine, and lysine, were the most abundant metabolites inside the venous thrombus, while citric acid, glucose 6-phosphate, nicotinamide adenine dinucleotide phosphate+ (NADP+), tryptophan, and fructose 6-phosphate were decreased¹⁷⁵. These differently metabolites, quantified with an exploratory untargeted approach, are generically involved in glycolytic pathways, redox-related reactions, purine nucleotides and tryptophan metabolic processes. The increase in lactate and decrease in glycolytic metabolites levels suggest an increased glycolysis inside the thrombus secondary to low oxygen supply. More importantly, lactate is a modifier of blood coagulation, independent from acidemia¹⁷⁶, establishing a coherent physiopathology process that may be explored in

the future, either for biomarker development or for the discovery of new treatment targets.

The glucose transporter 1 (Glut-1) expression on erythrocytes of human venous thrombus, aspirated from patients with DVT, was different across different stages of the thrombus progression¹⁷⁵. More specifically, the erythrocyte Glut-1 expression was negatively correlated with the time after the onset of DVT, guarantying the rationale for further studies on its potential as early acute DVT biomarker. Considering purine metabolism, the higher levels found on venous thrombus may be the consequence of pH and oxygen altered in that setting, according to authors. Metabolomic studies in a mouse model identified higher levels of TMAO (or trimethylamine N-oxide) in the serum of venous thrombotic animals¹⁷⁷. TMAO is a metabolite produced by gut bacteria, and is a propeller of platelet reactivity, that increases with aging, and may be a precursor of platelet thrombi, both in arterial and venous circulation¹⁷⁸.

Having these results into consideration, there might be new perspectives on the influence of diet and gut microbiome on the pathogenesis of VTE. In fact, the influence of gut microbiome on arterial and venous thrombosis has been recently accentuated by convergent results from animal and human studies¹⁷⁹. Nevertheless, TMAO is not suitable as a diagnostic biomarker due to its extremely biologic high variability, with all dietary interferences¹⁸⁰.

Another metabolomic profiling of acute venous thrombosis in mice¹⁸¹, showed discriminatory metabolites that were involved in energy, lipid and purine metabolic pathways. The energetic changes suggested by altered levels of components of the tricarboxylic acid (TCA) cycle, carnitine and triglycerides, are attributed to a disturbed carnitine metabolic pathway, which is implicated in several inflammatory phenomena¹⁸² and oxidative stress.

Pulmonary embolism induced in pigs revealed a similarly altered plasmatic metabolomic matrix, with differently expressed metabolites from pathways involved in glycolysis, intermediates of TCA cycle, lipid metabolism, and ketone bodies¹⁸³. Again, hypoxic stress alters the energetic metabolism with a glycolytic shift and through alternative ways of ATP as oxidative phosphorylation rate is reduced, like TCA cycle, while it protects cells from accumulation of reactive oxygen species (ROS). Ketone bodies may also result from hypoxic responses and work as an alternative energy source, besides having a possible role in brain protection from hypoxic insults¹⁸⁴. The increase of lipolysis intermediates relates to the augmentation of lipidic breakdown from adipose tissue induced by hypoxia. Apart from some insights into the pathophysiologic steps of the thrombotic events, these metabolites could be targeted by some complementary studies using different analytical methods to validate the potential of either one of them, or a panel of more than one, as diagnostic biomarkers.

A metabolomic analysis on samples from a registry on venous thrombosis¹⁸⁵ concluded that adult male patients with at least one episode of DVT had lower plasma levels of acylcarnitine, sampled after the clinical episode, and that acylcarnitine have

anticoagulant activity related to an ability to bind and inhibit factor Xa. In this sense, acylcarnitine seems to play a role in the risk of VTE, but data on the kinetics of these metabolites during the early stages of the clinically symptomatic disease have not been studied, in the acute phase, to explore its potential as a diagnostic biomarker. The serum analysis of metabolomic data from patients with an episode of DVT several weeks before the collecting moment, found differences in amino acids, specifically glutamate and valine, with high efficacy for discriminating between the thrombotic and control groups¹⁸⁶. The transportation of these findings to the clinical practice must consider the resolution of questions unanswered such as the validity of these metabolites to distinguish between groups during the acute disease.

Plasma from patients with a previous DVT occurring three months before sample collection was analysed by a targeted metabolomic strategy and concluded that triglycerides were differently quantified in patients when comparing with controls and that microbiota metabolites were associated with DVT recurrence¹⁸⁰. The authors discussed the possible involvement of lipids as potential regulators of oxidative and inflammatory responses of the post-VTE biologic and the emergent association of gut microbiome to DVT relapses.

Human studies on metabolomic profile of DVT are scarce, and in the acute phase even lesser. One study at an ED collected samples from PE patients during the first 24 hours from the imaging diagnosis and divided them in groups of low or medium/high risk PE. There were 50 metabolites that differed these two groups, including intermediates of TCA cycle, fatty acid metabolism, purine metabolism, glycolysis, nucleotide, and amino acid pathways, not dissimilar to the results from animal models¹⁸⁷. These findings may be further investigated in future research projects, because risk stratification after PE diagnosis is central to the choice of treatment modalities and to the decision making about the discharge destiny¹⁵. The absence of a control group without VTE to compare with those individuals is a limitation for the use of these data to develop a biomarker panel for the early diagnosis of PE, when we must distinguish patients with acute DVT from patients without this disease in the presence of very heterogenous clinical presentations.

Another study performed in patients with cerebral venous thrombosis, an unusual presentation of DVT, during the acute phase, revealed more than 300 different metabolites when comparing to healthy controls, of whom branched chain amino acids (BCAAs) were validated and revealed a very good performance¹⁸⁸. The untargeted metabolic matrix of patients with hospital-acquired VTE, including DVT and PE in similar proportions, after initial admission for a blast trauma episode, identified the tryptophan pathway as differently expressed¹⁸⁹. Tryptophane is bioavailable to the kynurenine pathway, involved in inflammation, oxidative stress and immune changes observed in patients with multiple arterial diseases and is believed to play a major role in the early inflammatory processes of arteriosclerosis¹⁹⁰, but it was not studied in venous thrombosis.

The metabolome of acute stroke compared to chronic post-stroke patients, also revealed a different expression on amino acid metabolism, unveiling a possible role for these metabolites in diagnosing acute arterial or venous thrombotic diseases. Even more, it suggests that arterial and venous thrombotic diseases may share common features in the acute symptomatic phases, which is an interesting point to the drawing of the pathophysiologic processes of human thrombosis.

Indeed, metabolomics in patients with arterial cardiovascular diseases has brought some new data into possible metabolic pathways that can lead to some new conclusions on the common processes to arterial and venous thrombosis. Coronary artery disease plasmatic metabolic profiling, including different phenotypes of ischemic heart disease, revealed several modified metabolic pathways, including reduced phospholipid catabolism and TCA cycle, and increased amino acid metabolism and short-chain acylcarnitine¹⁹¹. These footprints found in patients with arterial cardiovascular disease have also shown differences when considering the various forms of coronary artery disease severity, pointing to a possible role in separating patients according to their clinical severity. In terms of prognostic biomarkers, elevated plasma branched-chain amino acids (BCAA) levels on admission were associated with worse early prognosis. Similarities between arterial and venous thrombotic profiles may express neighbouring oxidative and inflammatory responses during cellular hypoxia and must be taken into consideration when developing new biomarker panels, to avoid a low specificity for DVT diagnosis. Concomitantly, we should pursue new data on the hypothesis of a common pathologic base for both clinical distinct entities, that could modify the paradigm of cardiovascular risk management and, even, the treatment management of clinical events of thrombosis.

Proteomic analysis of venous thrombosis patients aims to simultaneously identify hundreds of proteins in a biologic sample. Blood plasma proteome, the circulating proteome, is a challenge derived from high dispersion of the concentration of different proteins, in such a way that 1% of proteins represents more than 90% of the proteome¹⁹². A proteomic targeted profiling of plasma from patients presented to the ED with an acute DVT and or PE diagnosis was compared to outpatients, and resulted in four target candidates higher in VTE patients when using EDTA¹⁹³. The antibodies represented SULF1, leukocyte surface antigen CD47, adenosine receptor A2a and P-Selectin and the the main captured protein in their presence was complement factor H related 5 protein (CFHR5). The characteristics of CFHR5 may constitute important advantages for increasing the diagnostic accuracy of D-dimer for acute VTE diagnosis in the VTE-likely group, thus avoiding negative imaging results.

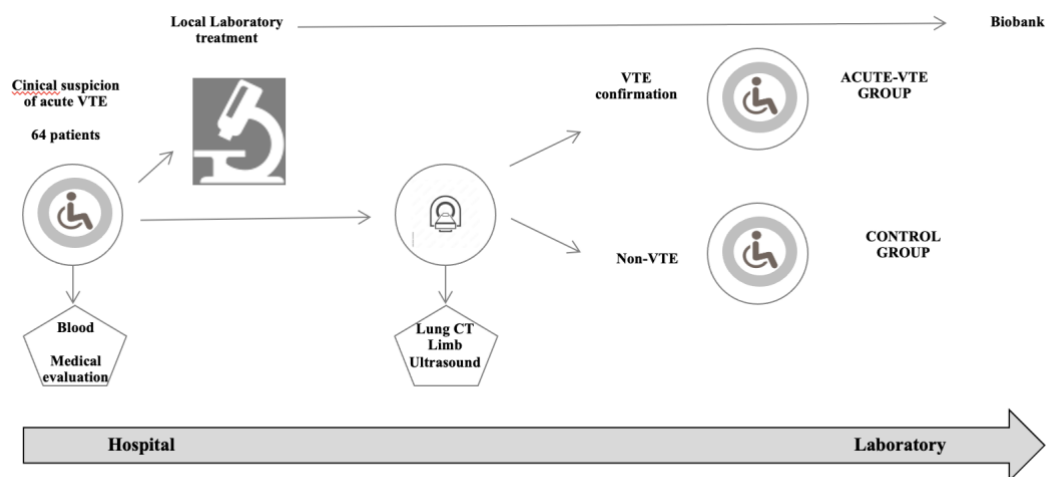
Another unanswered question on VTE is whether PE and DVT are similar diseases with common biologic processes. In fact, proteomic analysis of acute PE compared to other forms of DVT showed different proteins according to the clinical manifestations supporting the hypothesis that distinct acute VTE presentations constitute pathophysiological specific entities¹⁹⁴. Acute PE proteomic profiling evidenced

pathways usually related to respiratory and chronic inflammation, especially Interferon- γ (IFN- γ), polypeptide *N*-acetylgalactosaminyltransferase 3 (GALNT3), and β -nerve growth factor, unlike DVT¹⁹⁵. This proteome underlines a possible relation between host defence processes and venous thrombosis. The identification of different proteomics to the expressions of clinical heterogeneous VTE diseases, including isolated PE and DVT-PE association, related to different platelet activation patterns¹⁹⁶, supports the existence of specific post-transcription biologic phenomena that impacts the manifestations of an eventually common basal propensity condition. Plasma clot from patients with acute PE had distinctive proteins, mostly involved in the density of the clot, like apolipoprotein B-100, platelet glycoprotein Ib, lipopolysaccharide-binding protein, and histones H3 + 4¹⁹⁷. At present, there is still a gap of knowledge to ascertain whether DVT and PE are different expressions of the same disease or they are distinct diseases.

2.9 Study design and original research on acute venous thromboembolism

The thesis hereby presented has the main intention to report and discuss the findings resulting from a clinical trial, which we briefly introduce.

Figure 2- Overview of the study design.



Blood samples were taken for consecutive patients during a regular blood withdrawal. A medical evaluation took place according with usual clinical practice and, posteriorly, data recorded at a clinical research platform, exclusive to the study. Those samples were treated at the local laboratory with a pre-defined protocol and stored at -80°C. Ultimately, samples were transported to the biobank and stored for a maximum of 20 years. The samples were prepared and analyzed, and bioinformatics and biostatistical tools and techniques were used for data analysis.

The objectives of the study were as follows:

- **Primary Objective-** To discover diagnostic plasma biomarkers in patients admitted at the ED with acute VTE episodes.
- **Secondary Objectives**
 - Apply metabolomics techniques based on MS complemented by orthogonal techniques (LC) to identify and quantify potential metabolites candidates to plasma biomarkers of acute VTE at hyper-acute phase (at the time of clinical suspicion).
 - To characterize demographic and clinical features of acute VTE patients admitted at ED.
 - To correlate the plasma metabolomic profile with the clinical characterization of acute VTE patients.
 - To apply statistical-learning modelling and bioinformatics analysis for exploration of putative associations between the plasma metabolomic profile of acute VTE and possible pathophysiological processes.
 - To create one biobank.

The outcomes for the investigation objectives included:

- **Primary outcome** - identification and quantification of potential metabolite biomarker expression in blood samples from acute VTE patients.
- **Secondary outcomes**
 - For the secondary objective of applying metabolomics techniques based on MS complemented by orthogonal techniques (LC) to identify and quantify potential metabolites candidates to plasma biomarkers of acute VTE at hyper-acute phase (at the time of clinical suspicion), the outcome measure was determination of an expected 10-20 potential biomarkers.
 - For the secondary objective of characterization of demographic and clinical features of acute VTE patients admitted at ED, the outcome measures comprised clinical and demographic characteristics in relation to acute VTE episodes.
 - For the secondary objective of applying statistical-learning modelling and bioinformatics analysis for exploration of putative associations

between the plasma metabolomic profile of acute VTE and possible pathophysiological processes, the outcome measure was a biomarker signature for acute VTE disease.

- For the secondary objective of creating one biobank, the outcome measure was standard biobanking processes with quality control by INSA.

For the enrolment of participants, the following criteria were used:

Inclusion Criteria

1. Subject was 18 years of age or older at time of enrolment.
2. Subject was diagnosed with suspected acute VTE (DVT or PE) in the ED or transferred to the ED (e.g from a clinic or outside hospital) with a diagnosis of suspected or confirmed acute VTE.
3. Subject was indicated for a diagnostic imaging exam (thoracic angiotomography and/or lower limb venous Doppler ultrasound) during the same emergency episode
4. Subject has signed the informed consent.
5. Subject was able to understand study procedures and comply with them for the entire length of the study

Exclusion Criteria

1. All candidates meeting any exclusion criteria at baseline were excluded from study participation.
2. VTE suspicion raised more than 24 hours after admission to the ED.
3. Subject with life expectancy of less than three months.
4. Inability or unwillingness to comply with the requirements of the protocol as determined by the investigator.
5. Pregnancy or breastfeeding.
6. Other medical condition that, in the investigator's judgment, was associated with increased risk to the subject or might interfere with study assessments or outcomes.
7. Participation in another clinical trial and/or receipt of investigational drugs within four weeks prior to ED visit.
8. Inability or unwillingness of individual or legal guardian/representative to give written informed consent.

Subjects were free to withdraw from the study at any time for any reason or determined to be withdrawn by the investigator, but there were no withdrawals.

Sample sizing has unique issues in the context biomarker discovery in human samples. Due to the complexity and inevitable environmental variability of human metabolomics (time of day, season, external stressors, etc.) and preanalytical bias (mode of collection, storage, etc.), researchers working with human samples need a large sample set to overcome patient-to-patient variability. The only viable way to increase the statistical power of clinical studies is to increase the number of patient samples in the discovery phase. For MALDI-based, discovery-phase human studies it is recommended a minimum sample size of ~30 per classification group during the discovery phase, being this rational for minimum sampling of 30 acute VTE and 30 controls, to accommodate the high biological variability of the human population^{198–201}. This number of samples generally offers >90% statistical confidence in single markers (with P values of <0.01) and the ability to use some forms of multivariate analysis.

Multiple time points samples were intended to be collected at startup, including immediately after clinical suspicion of VTE (hyper-acute phase), immediately after confirmation of VTE by imaging modalities (acute phase), and 2 weeks after VTE diagnosis (sub-acute phase). During the implementation, COVID 19 pandemics modified the circuits of every patient, especially with respiratory-related symptoms or signs, and we rearranged the collecting procedure accordingly. In this scenario, we gave up pf the sub-acute phase procedures, as patients were transferred to admission, where general investigation projects were suspended by national authorities. Additionally, we implemented the 24-hour after diagnosis collection of samples, limited to patients still admitted to the same ED by that time.

Controls included every patient with an initial clinical suspicion of acute VTE, after the standard medical evaluation, and that completed a CTPA and/or lower limb Doppler ultrasound. As using more than one type of controls adds to the confidence in the resulting data²⁰⁰, control samples collected from patients with various other diseases mimicking acute VTE, under study, was expected to augment the robustness of the analysis.

All patients were recruited at the ED of a general hospital (HBA). This was determined by the fact that samples and controls for metabolomic discovery-based research must be collected and handled as similarly as possible.

Enrolment period was meant to occur between 1 October 2019 and 30 September 2021, in the initial version of the protocol. Again, Covid 19 pandemics imposed several timing changes, with a structural impact in the study protocol, and recruitment occurred

from October 2020 to January 2022, after attaining the necessary adaptations of the initial protocol to the new situation.

This study was a single arm study, without applicable randomization.

Sample collection occurred only during weekdays, between 8a.m. and 1p.m., to assure the homogenous treatment of the samples by the specifically trained local laboratory staff.

All eligible patients were to be offered enrolment for sample and data collection within the study. The participating physicians assessed patients to determine their eligibility for sample and data collection within the study based on the selection criteria. The moment of clinical suspicion of acute VTE determined the collection of one blood sample, and data registering. The same was applicable to 24-hour after diagnosis of acute VTE (for patients only).

After collection, the blood samples were directed to the local laboratory where there is a specific technical protocol for their treatment (Appendices). Briefly, peripheral blood collected by venepuncture into anticoagulant-free tube was allowed to clot for a maximum of 30 min at ambient temperature and serum was isolated by centrifugation at $2000 \times g$ for 10 min and immediately aliquoted and stored at -80°C .

Clinical data were be collected on a dedicated database only accessible to investigators. A medical evaluation was performed as usual practice to each patient, and, posteriorly there was the registration of extracted data from clinical files on a dedicated platform. Data collected intended to include anthropometric measurements, blood pressure, heart rate and pulsed oximetry (with very common missing data), electrocardiographic data, clinical pathology values, and CTPA and ultrasound reported findings. Furthermore, data on comorbidities and pre-admission medication, were limited to spontaneously registered facts by the attending physician.

Biobank

The study subjacent to this thesis included the creation of a biobank for biological samples in accordance with Helsinki Declaration. Informed consent was be obtained from every patient. The protocol was approved by the Ethics Committee and Administration of participating centres, of Hospital Beatriz Ângelo (HBA) and Instituto Nacional de Saúde Dr Ricardo Jorge (INSA) (Appendices).

Collected blood samples were treated with the same protocol for separation of plasma and RBCs within the first four hours after blood taking, as mentioned. Properly separated samples were immediately stored at -80°C , at local laboratory (HBA), and then regularly transported directly to INSA, for a maximum 30 minute trip, in a frozen

container, for the reduction of biologic variability unrelated to the medical condition proteomics.

Every sample is identified and catalogued for location and selection purposes. Identification is anonymized, with identification with a number that is matched to the patient in a list located at the Ethics Committee of HBA. Similarly, the number that identifies the samples is coincident with clinical data identification labelling (without patient personal data identification).

Biobank is located at INSA and follows necessary certifications.

According to the informed consent procedures, the biobank is available for the project investigations for a 20-year long period of time, since sample entrance.

Chapter 3: New biomarkers for acute venous thromboembolism

The main results obtained from the study were published as two different articles and answer the main objective of the present work.

The first original article compares and discusses the clinical and demographic variables between acute VTE patients and non-acute VTE patients. Data from recruited acute VTE patients and controls (non-acute VTE patients) were retrieved from clinical electronic medical records by the investigators and registered in an electronic platform with restricted access.

We included 58 patients in the analysis which corresponded to the initial cohort with the exclusion of actively infected patients, due to the high interference of infectious diseases on the values of hematologic indices. The 48 patients with a diagnosis of acute VTE had similar demographic and clinical characteristics than 10 patients with other acute illnesses, including vital signs like heart rate and pulsed oxygen saturation, accentuating the fragility of anamnesis to the diagnosis. Additionally, blood analytic parameters classically associated with acute VTE, including leukocyte counting, CRP and even D-dimer, did not differ significantly between the two groups.

The significant differences were limited to the total platelet counting, higher in acute VTE, and RDW, with the same tendency. Taking into consideration previous descriptions of an higher $RDW \geq 14\%$ at admission in patients with acute VTE, the ROC curves for this cut off were completed and revealed a poor overall performance with an area under the curve of 0.685 (95% CI, 0.535-0.834).

However, when analysing patients with isolated acute DVT, comparing to controls, $RDW \geq 14\%$ had a sensitivity of 76.9%, a specificity 100%, and accuracy 85.7%. Considering the low sample size for the effects of analysing the performance of RDW, which was not the objective of the study protocol, the results have to be cautiously interpreted. The tendency of RDW to discriminate acute DVT patients from other ED patients may suggest a potential role as a new parameter of predictive clinical scores, added to the already existing ones. The specificity revealed for this particular subgroup of patients, if confirmed, would mean that $RDW < 14\%$ would rule out the possibility of an acute DVT, thus directing physician's diagnostic pathway to considering other diagnosis and sparing patients with unavailable immediate ultrasound the side effects from empiric anti-coagulation.

It is my belief that the repetition of the association of a higher RDW to VTE diagnosis in several studies should guarantee the investigation of a clinical predictive score that includes this RBC parameter. The relevance of an eventual result on this direction would be that it is readily adoptable worldwide, as we already have this parameter available every time we ask for a blood counting in our daily routine.

The second original article is the main report on the results obtained so far in the course of our research project.

Our discovery approach was based on an untargeted metabolomic analysis of plasma and RBCs obtained from a total of 62 patients admitted to the ED with acute VTE suspicion, of whom 50 had this diagnosis confirmed.

After metabolite extraction and sample preparation, we used ultrahigh-performance liquid chromatography system (UHPLC) in conjunction with quadrupole time-of-flight mass spectrometer for the LC–MS/MS analysis (TIMS-QTOF). Data extracted were processed and mapping of the identified metabolites proceeded to statistical analysis.

We identified a total of 91 metabolites in plasma and 55 metabolites in RBCs, after decontamination (for a complete listing of the metabolites consult Appendices). Overall, metabolome of the plasma from acute VTE patients expressed a tendency to a particular signature, but with a limited performance as discriminatory biomarkers between these patients and controls. Among the 21 different metabolic pathways potentially associated with significant differences, the metabolic pathway of d-glutamine and d-glutamate was found to be the most relevant pathway and possessed the strongest impact on the acute VTE phenotype ($p=0.001$, false discovery rate = 0.06).

The most surprising results came from RBCs metabolome. Long-time assumed as a near passive spectator of the thrombotic cascade of events, RBCs revealed a distinct metabolomic profiling in acute VTE patients, that can differentiate these episodes in the ED from other acutely diseased patients. Specifically, adenosine 3',5'-diphosphate (AUC: 0.983), glutathione (AUC: 0.923), and adenine (AUC: 0.91), tested with high performant ROC curves, raising justified hopes of a potential utility as clinical biomarkers in the near future.

Multiple questions arise from the different metabolites quantified in the study, including the mechanistic meanings for a better knowledge of acute VTE. Despite these speculative scientific explanations for the findings, detailed in the discussion of the published article, I think that the implications of the results for the clinical practice is the most appealing point of reflexion. If we try to measure one of these metabolites, or a panel with more than one, by the use of easily available machines that may be widely diffused across multiple healthcare facilities and settings, we could aspire to a new biomarker development for the actual medical practice. Without minimising the limitations of this study, there are reasons to create new study protocols for validation of these metabolites.

3.1 Predictive Value for Increased Red Blood Cell Distribution Width in Unprovoked Acute Venous Thromboembolism at the Emergency Department

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Abstract

Acute venous thromboembolism (VTE) is a common worldwide disease admitted to emergency departments (ED), usually presenting as pulmonary embolism or lower limb deep vein thrombosis (DVT). Due to the lack of typical clinical and biomarker diagnostic features of unprovoked VTE, early identification is challenging and has direct consequences on correct treatment delay. Longitudinal, prospective, observational study. Patients admitted to ED with a suspicion of unprovoked acute VTE between October 2020 and January 2021 were included. Clinical and laboratorial variables were compared between VTE positive and negative diagnoses. Red cell distribution width (RDW) cut point was determinate through a receiver operating characteristic analysis. RDW accuracy, sensitivity, and specificity were calculated. Fifty-eight patients were analyzed. And 82.8% of suspected patients with VTE were diagnosed with an acute thrombotic event confirmed by imaging examination. In patients with VTE, RDW at admission in ED was higher than with other diagnosis, respectively, 14.3% (13.2-15.1) and 13.5% (13.0-13.8). Platelet count was the only additional characteristic that revealed difference between the 2 groups ($264 \times 10^9/L$ for VTE and $209 \times 10^9/L$ for non-VTE). Logistic regression models showed good discriminatory values for $RDW \geq 14\%$, with an area under the curve (AUC) = 0.685 (95% confidence interval, 0.535-0.834). These findings were more pronounced in isolated DVT, with a sensitivity of 76.9%, specificity 100%, and accuracy 85.7%. Our study demonstrated a significant association between an early high RDW and the diagnosis of acute unprovoked DVT. $RDW \geq 14\%$ has an independent predictor of unprovoked VTE in adult patients.

Keywords

red cell distribution width, erythrocytes indices, venous thromboembolism

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Introduction

Pulmonary embolism (PE) and lower limb deep vein thrombosis (DVT) are the most frequent clinical expressions of acute venous thromboembolism (VTE), resulting in an annual incidence equal to that of stroke.¹ Acute VTE episodes have a significant and constant impact on health resources, accounting for 1 million emergency visits in the United States, being half of them admitted to hospital.² VTE episodes occurring out-of-hospital, compared to those acquired in hospitals, are about 40 times less frequent but have a wide range of related risk factors and morbidities.³ Due to the heterogeneity of these demographic and clinical characteristics, particularly in PE cases, and the absence of specific and sensitive biomarkers, VTE diagnosis in the emergency departments (ED) is a high challenge to physicians. No single clinical symptoms

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are predictive or exclude PE, although the lack of dyspnea slightly reduces the probability of PE.⁴ The clinical presentation of VTE is unspecific,⁵ particularly for unprovoked cases, characterized by the absence of any transient or persistent environmental risk factor.⁶ When patients are admitted to the ED, the risk of VTE is assessed by clinical gestalt or clinical scores according to symptoms and existing risk factors.⁷ This risk assessment is the first diagnostic step after suspicion of VTE and it is usually followed by D-dimer measurements to determine whether further investigations are required. Small or intermediate clinical probabilities and negative D-dimers can exclude PE⁸⁻¹⁰ and DVT,¹¹ but the results of positive D-dimers have a low-positive predictive value and are not useful for VTE diagnosis.¹²⁻¹⁴ The key to diagnosis of PE and DVT is still to perform imaging examinations, computed tomography (CT) angiogram, and Doppler ultrasound, respectively. Accessibility, radiation exposure, time-to-exam and high costs are some of the critical aspects that are due to the need of such examinations. The absence of alternative diagnostic biomarkers for VTE has a direct impact on the percentage of 60% undiagnosed cases among all VTE-attributable deaths.¹⁵

Efforts have been made to increase the knowledge on the diagnostic and prognostic importance of red cell indices of cardiovascular diseases. Red blood cell distribution width (RDW) is a measurement of size heterogeneity of circulating erythrocytes,¹⁶ which has been studied as a prediction tool for cardiovascular and thrombotic diseases, including PE,¹⁷⁻¹⁹ although some conflicting results have prevented it from being used in everyday practice. The diagnostic role of RDW for acute VTE is even less well understood. A previous retrospective study on VTE found an association between high RDW at admission to ED and acute VTE, and determined a cutoff of 14.6%, with an Negative Predictive Value (NPV) of 0.85 for DVT and 0.91 for PE, suggesting that RDW could supplement clinical preliminary examinations to exclude acute VTE attacks.²⁰ This study, even considering some limitations, has set a possible threshold of RDW > 14% at admission for distinguishing VTE from non-VTE patients in acute care setting.

In response to the same questions, we decided to investigate the early RDW values in patients admitted to the ED for suspected acute unprovoked VTE episodes and compare clinical characteristics and biomarkers of patients with confirmed and excluded VTE diagnosis.

Methods

Study Design and Settings

We conducted a prospective longitudinal, observational study aiming to describe and compare clinical and laboratory parameters of patients admitted in an ED with suspected VTE, including PE and DVT, from October 2020 to January 2021.

Participants

The study included patients admitted in the ED who were clinically suspected of an acute VTE and referred, according to usual clinical practice, for imaging examinations for diagnosis confirmation.

Inclusion criteria were individuals aged ≥ 18 years with unprovoked suspected acute VTE cases (DVT or PE) admitted in the ED, or transferred to the ED (eg, from an outpatient clinic or other hospital) with a diagnosis of suspected acute VTE <24 h. Patients were excluded if there was VTE suspicion more than 24 h before admission to the ED, life expectancy of <3 months, active neoplasia, pregnancy or breastfeeding, COVID-19 diagnosis, and fever at admission.

Ethical Considerations

The study was conducted in accordance with current International Council for Harmonisation Good Clinical Practice guidelines, and according to Declaration of Helsinki principles. The study protocol was approved by Hospital Beatriz Angelo Ethical Committee and Board. Written informed consent was obtained from all enrolled subjects.

Procedures and Data Collection

Data were collected from patients' medical records. All patients were screened for the presence of VTE and depending on the clinical suspicion thoracic angiotomography for PE and/or lower limb venous Doppler ultrasound for DVT were performed according to usual clinical practice.

The VTE diagnosis was ruled out, if the imaging examinations were negative for the initial suspicion, and another diagnosis was established.

Clinical, Biochemical Parameters, and Imaging Examinations

Collected data included: demographic data (sex and age), present and past medical history, previous medication, physical examination, vital signs, peripheral oxygen saturation, and laboratory parameters such as red blood cells, hemoglobin, hematocrit, RDW, platelets, total leukocyte count, creatinine, D-dimer, and C-reactive protein. All patients performed an imaging examination in the first 24 h after admission to the ED, according to physician's dispositions. The imaging considered to include patients in the analysis consisted of thoracic CT angiography for PE-suspected cases and lower limb Doppler ultrasound for DVT suspicions. Patients were allowed to perform both examinations in the same period.

Statistical Analysis

Continuous data were presented as median and interquartile ranges, mean and standard deviation (SD), maximum and minimum. Discrete variables were presented as absolute and relative frequencies. The normality of the data was assessed using the Shapiro-Wilk test.

Between-group (VTE vs non-VTE) comparisons of continuous variables were performed using Mann-Whitney U tests. Categorical variables were compared using Fisher's exact test.

A receiver operating characteristic curve analysis was used to evaluate the diagnostic value and to define the diagnostic cutoff value of RDW concentrations. Using the cutoff point defined, the RDW were compared between VTE versus non-VTE by the Fisher's exact test.

A logistic regression model was implemented to interrogate the significance of the association of RDW in patients with VTE, considering demographic and other clinical variables.

A significant level of 0.05 was considered for all the analysis.

SPSS 26.0 statistical software was used to analyze the data.

Results

Patients' Characteristics and VTE Diagnosis

A total of 59 participants were initially enrolled. One patient abandoned ED without any imaging examination and was excluded. Of the 58 patients included in the analysis, 44 completed thoracic CT angiography and 33 lower limb Doppler ultrasound with diagnostic results of adequate quality, with 19 patients carrying out both examinations. The characteristics of 58 patients are summarized in Table 1. There was a total of 48 patients (82.8%) with an acute VTE diagnosis and the remaining 10 patients received an alternative diagnosis. There was no significant difference between patients with

VTE and without VTE diagnosis in terms of clinical presentation. Considering 44 patients who completed a thoracic CT angiography, 35 (80%) presented features of acute PE, as shown in Table 2. Of those patients who performed a lower limb Doppler ultrasound, 70% (25 cases) presented were diagnosed with acute DVT. According to the imaging examinations, 21% (10 cases) of patients with VTE were diagnosed with both acute PE and acute DVT.

In terms of laboratory investigation, there were significant differences on platelet count and RDW between VTE and non-VTE patients. There were no differences in hemoglobin, hematocrit, leukocytes, and D-dimer. Since RDW was the variable showing the strongest association with acute VTE in the univariate analysis, we focused on RDW for further analyses.

All patients were discharged from the hospital.

Diagnostic Performance of $RDW \geq 14\%$

The diagnostic accuracy of $RDW \geq 14\%$ for determining an acute VTE, as quantified by the area under the curve, was 0.685 (95% CI, 0.535-0.834; Figure 1). At a threshold of $\geq 14\%$, RDW ruled in 47% of individuals, with a sensitivity of 55.6%, specificity 100%, and accuracy 79.0% (95% CI, 65.6%-89.0%). For isolated PE $RDW \geq 14\%$ had a sensitivity

Table 1. Study Participants' Baseline Characteristics.

Characteristic	All patients	Patients with VTE	Patients without VTE	P value*
Age, y, median (IQR), maximum-minimum (N)	65.0 (44.5-84.5) 19-90 (N = 58)	64.5 (42.8-83.8) 19-89 (N = 48)	72.5 (55.8-88.0) 27-90 (N = 10)	.415
Female, N (%)	38 (65.5) (N = 58)	31 (64.6) (N = 48)	7 (70.0) (N = 10)	1.0#
Standing heart rate at rest, bpm, mean $\pm$ SD	88.0 (76-102) 50-130 (N = 48)	88.0 (76-103) 50-130 (N = 41)	91.0 (82-101) 53-127 (N = 7)	.597
Oxygen saturation (SpO ₂), %, mean $\pm$ SD	97.0 (95-98) 85-100 (N = 46)	97.0 (95-98) 85-100 (N = 39)	96.0 (96-98) 96-98 (N = 7)	.618
Hemoglobin, g/dL	13.4 (12.3-14.5) 7.3-15.8 (N = 48)	13.4 (12.5-14.5) 7.3-15.8 (N = 41)	13.9 (11.6-14.1) 11.2-15.3 (N = 7)	.597
Hematocrit, %	42.2 (36.9-44.3) 24.6-47.4 (N = 48)	42.4 (37.2-44.3) 24.6-47.4 (N = 41)	39.8 (36.2-42.7) 33.9-45.8 (N = 7)	.683
RDW, %	14 (13.1-14.7) 11.4-25.4 (N = 48)	14.3 (13.2-15.1) 11.8-25.4 (N = 41)	13.5 (13.0-13.8) 11.4-13.9 (N = 7)	.004
Total leukocyte count, 10 ¹² /L	10.2 (7.6-11.6) 4.2-18.8 (N = 48)	10.08 (7.5-11.4) 4.2-18.8 (N = 41)	10.32 (7.8-13.5) 7.3-15.2 (N = 7)	.683
Neutrophil %	69.6 (63.4-76.7) 37.6-88.3 (N = 48)	69.8 (63.5-76.5) 37.6-88.3 (N = 41)	69.0 (57.4-81.6) 44.4-82.2 (N = 7)	.683
Lymphocyte %	19.9 (13.7-25.4) 2.9-50.7 (N = 48)	19.6 (14.2-25.0) 2.9-50.7 (N = 41)	20.2 (10.4-32.6) 10.3-43.4 (N = 7)	.683
Platelet count, 10 ⁹ /L	256.5 (210-354) 167-791 (N = 48)	264.0 (212.5-355) 174-791 (N = 41)	209.0 (190-230) 167-441 (N = 7)	.041
C-reactive protein, mg/dL	2.6 (0.8-5.7) 0.2-40.2 (N = 43)	2.3 (0.8-4.7) 0.2-40.2 (N = 37)	9.5 (1.4-19.5) 0.4-22.6 (N = 6)	.346
D-dimer, μ g/mL	6.9 (3.3-10.8) 0.9-32.5 (N = 41)	7.2 (3.8-11.3) 1.3-32.5 (N = 35)	2.2 (0.9-10.8) 0.9-16.2 (N = 6)	.413
Creatinine	0.9 (0.8-1.8) 0.7-2.3 (N = 47)	0.9 (0.8-1.1) 0.6-1.8 (N = 41)	1.0 (0.8-1.8) 0.7-2.3 (N = 7)	.867

Abbreviations: IQR, interquartile range; RDW, red cell distribution width; VTE, venous thromboembolism.

* Mann-Whitney U test.

Fisher's exact test.

Table 2. Results of Imaging Examinations for Location of VTE (N = 48) and Diagnosis of Non-VTE (N = 10).

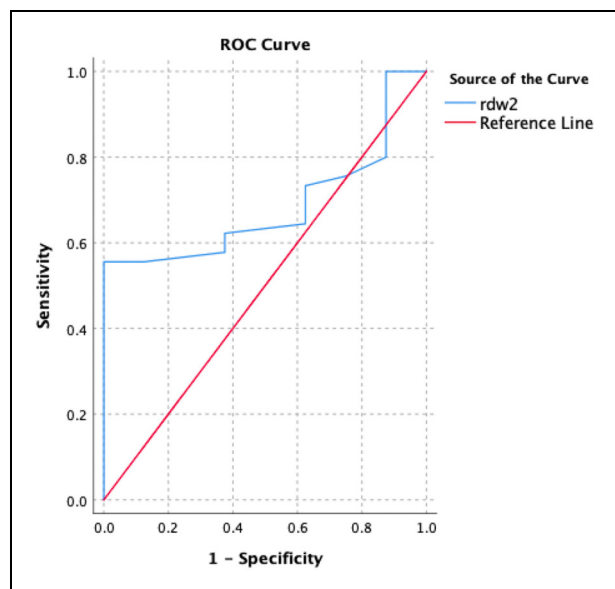
Location of disease or diagnosis	Number of patients
PE	35
Main pulmonary artery trunk	4
Bilateral multiple segmental	18
Unilateral multiple segmental	6
Segmental single	3
Subsegmental	5
DVT	25
Proximal	18
Distal	7
No VTE	10
Erysipelas	4
Superficial phlebitis	3
Anxiety	1
Pericardial effusion	1
Tachycardia	1

Abbreviations: DVT, deep vein thrombosis; PE, pulmonary embolism; VTE, venous thromboembolism.

Table 3. RDW $\geq 14\%$ Sensitivity, Positive Predictive Value, Specificity, Negative Predictive Value, and Accuracy.

	VTE	PE	DVT
Sensitivity (%; 95% CI)	55.6 40.0-70.4	40.9 20.7-63.7	76.9 46.2-94.9
Positive predictive value (%; 95% CI)	100 86.3-100	100 66.3-100	100 69.2-100
Specificity (%; 95% CI)	100 63.1-100	100 63.1-100	100 63.1-100
Negative predictive value (%; 95% CI)	71.6 51.5-86.9	38.1 18.1-61.6	72.7 39.0-94.0
Accuracy (%; 95% CI)	79.0 65.689.0	56.7 37.5-74.6	85.7 63.7-97.0

Abbreviations: CI, confidence interval; DVT, deep vein thrombosis; PE, pulmonary embolism; RDW, red cell distribution width; VTE, venous thromboembolism.

**Figure 1.** ROC analysis for RDW.

Abbreviations: RDW, red cell distribution width; ROC, receiver operating characteristic.

of 40.9% and the specificity of 100%, with an accuracy of 56.7%. And, for isolated DVT RDW $\geq 14\%$ had a sensitivity of 76.9%, specificity 100%, and accuracy 85.7% (Table 3).

Logistic regression analysis confirmed RDW as a predictor of VTE, being RDW $> 14\%$ and age the only independent predictors of acute VTE.

Discussion

Our study aims to improve the early, rapid, and accurate diagnosis of patients who present to the ED with a suspicion of acute

VTE. We evaluated the diagnostic value of clinical and laboratory findings at the time of ED presentation, keeping in mind the need for urgent recognition of acute VTE in this setting, for the emergent initiation of anticoagulation and, in the catastrophic situations, fibrinolytics therapy.²¹⁻²³ Our findings showed that RDW value and platelet count were the only variables with a significant association with acute DVT. A high RDW ($\geq 14.0\%$) at admission to ED was significantly associated with the presence of acute DVT, independent of other clinical and laboratory variables, including D-dimer. These findings are consistent with those of previous general population studies or retrospective series,²⁴⁻³⁰ that utilized healthy controls for comparison. RDW was demonstrated to be higher than normal cutoffs in such diverse conditions as cardiac stent thrombosis,³¹ heart failure,³² inflammatory diseases,³³ or acute exacerbation of chronic obstructive pulmonary disease.³⁴ One of the studies²⁴ described the association of an RDW $> 14\%$ to acute deep and superficial vein thrombosis at an ED at the admission, but it mixed unprovoked and provoked VTE—by cancer, trauma, infection, surgery, and other causes—which can be very different populations in terms of RDW. More so, it used outpatients on routine follow ups as controls, expected to present normal RDW, thus not completely reliable as a comparator if we consider that RDW is higher in many of the diseases presenting to the ED. To the best of our knowledge, this is the first study to document that high RDW is associated with the presence of VTE among acutely ill patients who present clinical features suspicious of acute unprovoked VTE, thus distinguishing confirmed from excluded VTE cases. The detailed analysis of RDW $> 14\%$ confirmed its high specificity and positive predictive value for distinguishing between acute VTE and other alternative acute diseases, in line with the aforementioned results. We could also observe that all patients admitted to the ED who were diagnosed non-VTE conditions had higher than normal RDW measurements, but RDW value was always inferior to 14%. In the light of these data, we propose that RDW $> 14\%$ could be tested as an early biomarker to predict acute VTE among acutely ill patients.

For unprovoked VTE attacks, which make up to a half of all acute VTE episodes³⁵ and present a much higher risk of recurrence compared to patients with VTE provoked by a major transient risk factor,^{36,37} baseline risk assessment of patients with suspected VTE on ED admission may be particularly difficult. The lack of typical clinical characteristics, which our study also found, increases the risk of unprovoked VTE being missed. The use of a high D-dimer should be discouraged to differentiate acute VTE in situations of acute medical or surgical illnesses, even with cutoff adjustments.³⁸⁻⁴⁰ Considering the high prevalence of high D-dimer values in these circumstances, ordering costly, often unavailable, and potentially harmful, contrasted imaging tests for exclusion of VTE is necessary. Our study found that an RDW > 14% alone, for both PE and DVT, had a significant positive predictive value, which is comparable to the association of clinical assessment and D-dimer for low-risk patients. We have arguments to suggest that this diagnostic parameter should be further studied, along with clinical characteristics and risk factors, to augment both the specificity and sensitivity of scores in the early diagnosis of acute VTE. The low specificity demonstrated by D-dimer for both PE and DVT,^{12,41} and that contrasts with its significant negative predictive value, limits its usefulness among acute ED patients. By contrary, further evidence on a high positive predictive value of RDW > 14% in the first hours of acute VTE, particularly in DVT that showed more consistent results when we analyzed separate subgroups of venous thrombosis, may add some value to the existing algorithms. In fact, the combination of both D-dimer and RDW measurements may, eventually, contribute to a higher predictive value of clinical and easily ready blood tests to the diagnosis before imaging examinations. Being this confirmed, we could expect a strong impact in clinical settings where imaging examinations are not immediately accessible and the decision making for beginning anticoagulation is needed.

Our study had several limitations. The first one is directly related to COVID-19 pandemics. During COVID-19 pandemics, worldwide ED were re-arranged to handle patients with potential or confirmed SARS-CoV-2. There was a modification of accessibility to EDs during COVID-19 pandemics, overloaded with COVID-19 cases that consumed most healthcare resources, preventing patients from receiving clinical care. ED visits declined 44%,⁴² and clinical assessment of all incoming patients were likely altered from the “usual” procedures. Furthermore, disorders like PE and DVT, which require extensive workups, may have been significantly affected by the reduction in one-third of the diagnostic examinations. Although in Portugal 48% reduction in ED visits did not impact the mix case of visiting patient,⁴³ little is known about the use of diagnostic imaging tests in the ED. In the light of these details, we could advise against extrapolating the findings to a population not affected by the COVID-19 pandemic. The same caution should be applied due to our small-sized and single-center population. Although we collected data on concomitant diagnosis (ICD-10), we were not exhaustive and lack data on nutritional status, shown to modify RDW among unhealthy populations.²⁰ Finally, we would like

to address the dearth of information regarding patient outcomes. Aside from the mortality outcome, we lacked information on the clinical evolution during the in-hospital stay and after hospital discharge. The analysis of those variables could provide some insight into the potential prognostic significance of RDW over the course of the illness.

In summary, we have demonstrated that high RDW and platelet count, 2 parameters that are inexpensive and easily obtainable, were independently associated with an increased risk of venous thrombosis. Further research is necessary to determine whether this association is causative. Future studies should target the evaluation of these variables as predictors of a recurrent event, which could assist in future decisions regarding anticoagulation and prophylaxis.

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Authorship

Conception, study design, and analyses were conducted by CF and AM. Data collection was conducted by CF, VS, and FF. The first draft of the article was written by CF. All authors critically reviewed and revised multiple drafts of the article and approved the final article before submission.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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3.2 Acute venous thromboembolism plasma and red blood cell metabolomic profiling reveals potential new early diagnostic biomarkers: observational clinical study

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RESEARCH

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Acute venous thromboembolism plasma and red blood cell metabolomic profiling reveals potential new early diagnostic biomarkers: observational clinical study

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Abstract

Background Venous thromboembolism (VTE) is a leading cause of cardiovascular mortality. The diagnosis of acute VTE is based on complex imaging exams due to the lack of biomarkers. Recent multi-omics based research has contributed to the development of novel biomarkers in cardiovascular diseases. Our aim was to determine whether patients with acute VTE have differences in the metabolomic profile compared to non-acute VTE.

Methods This observational trial included 62 patients with clinical suspicion of acute deep vein thrombosis or pulmonary embolism, admitted to the emergency room. There were 50 patients diagnosed with acute VTE and 12 with non-acute VTE conditions and no significant differences were found between the two groups for clinical and demographic characteristics. Metabolomics assays identified and quantified a final number of 91 metabolites in plasma and 55 metabolites in red blood cells (RBCs). Plasma from acute VTE patients expressed tendency to a specific metabolomic signature, with univariate analyses revealing 23 significantly different molecules between acute VTE patients and controls ($p < 0.05$). The most relevant metabolic pathway with the strongest impact on the acute VTE phenotype was D-glutamine and D-glutamate ($p = 0.001$, false discovery rate = 0.06). RBCs revealed a specific metabolomic signature in patients with a confirmed diagnosis of DVT or PE that distinguished them from other acutely diseased patients, represented by 20 significantly higher metabolites and four lower metabolites. Three of those metabolites revealed high performant ROC curves, including adenosine 3',5'-diphosphate (AUC 0.983), glutathione (AUC 0.923), and adenine (AUC 0.91). Overall, the metabolic pathway most impacting to the differences observed in the RBCs was the purine metabolism ($p = 0.000354$, false discovery rate = 0.68).

Conclusions Our findings show that metabolite differences exist between acute VTE and nonacute VTE patients admitted to the ER in the early phases. Three potential biomarkers obtained from RBCs showed high performance for acute VTE diagnosis. Further studies should investigate accessible laboratory methods for the future daily practice usefulness of these metabolites for the early diagnosis of acute VTE in the ER.

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Keywords Pulmonary embolism, Deep vein thrombosis, Venous thromboembolism, Metabolomics

Background

Venous thromboembolism (VTE) is a leading cardiovascular disease in terms of both incidence and mortality, and ranks among the top three [1]. Much less investigated than arterial thrombosis, VTE is a complex multifactorial entity manifested by a spectrum of disease that ranges from pulmonary embolism (PE), as its most severe manifestation, to deep vein thrombosis (DVT), as the most common presentation [2]. Survival after VTE is heavily reduced, both short and long term, with 6% of patients not surviving the first 30 days after the acute event [3]. Acute VTE events may be further complicated by chronic thromboembolic pulmonary hypertension in 3% of PE cases, whereas 20–50% of DVT patients develop chronic post-thrombotic syndrome [4]. The diagnosis of VTE is only possible after a considerable workup, due to the high heterogeneity of symptoms [5, 6]. The absence of available diagnostic biomarkers necessitates an approach in the emergency setting based on clinical risk factors to determine the indication for gold-standard imaging exams, such as thoracic CT angiography and lower limb Doppler ultrasound. These imaging modalities are essential for a definitive diagnosis, but they present several challenges, including accessibility issues worldwide, high resource expenditure, long wait times for results, and unsafe radiation exposure [7]. The use of a single biomarker, or a panel, to distinguish patients with acute VTE is critical to modify the negative outcomes still associated.

Multomics analysis of venous thrombosis, propelled by the use of contemporary high-throughput approaches, contributed with recent data on possible biological processes involved in VTE [8]. Acute PE

plasma metabolomics revealed significant differences in metabolites associated with energy imbalance and signal transduction mediators [9]. Concomitantly, DVT fresh thrombus metabolic profiling detected high levels of metabolites involved in glycolysis, purine and tryptophan metabolism, and redox reactions [10]. The search for new biomarkers for early and accurate VTE diagnosis is a challenge and with scarce data available, but we know from studies in other areas that easily accessible biologic samples are considered as the most interesting carriers of the new biomarkers [11]. On the other hand, biomarkers in serum or urine are usually present at low concentrations, depending on advances in highly sensitive analytical techniques to its identification and quantification.

In this context, we decided to compare the metabolic profile of plasma and RBCs obtained from patients admitted to an emergency room (ER) for acute VTE with those of non-acute VTE patients with the same presenting symptoms. Our aim was to identify metabolites differentially associated with acute VTE during the early stages of the disease that could integrate a potential future panel of diagnostic biomarkers.

Methods

Study population

Consecutive adult patients ($n=62, \geq 18$ years), attending the ER of Hospital Beatriz Angelo, Lisbon, between 8 a.m. and 1 p.m., with symptoms/signs of suspected acute PE or acute DVT and without any diagnosed active cancer, pregnancy, or recent prolonged immobilization and/or surgery, were recruited for this study (Fig. 1). The suspicion of VTE was clinically established by the attending physician. All patients were submitted to definite

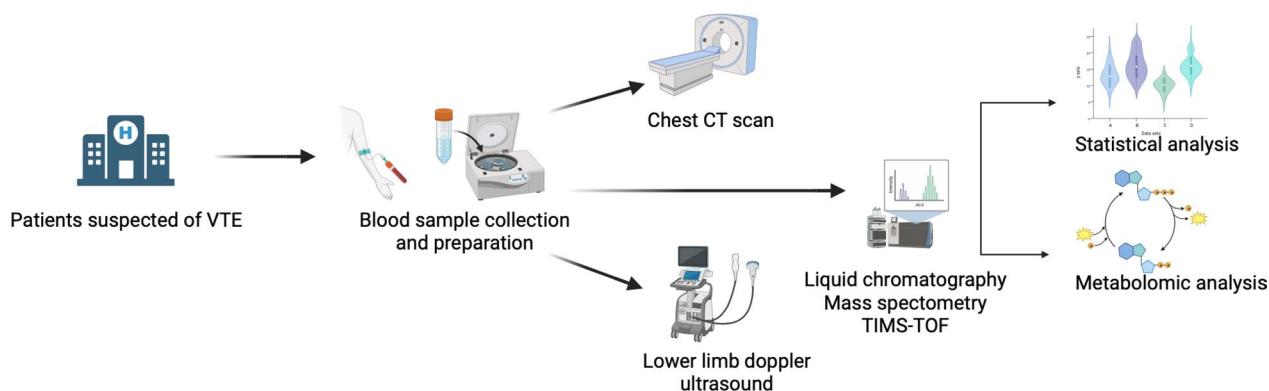


Fig. 1 Study workflow

diagnostic imaging, chest CT angiography for PE suspicion and lower limb Doppler ultrasound for DVT, according to usual practice. All patients were discharged or transferred to another department with an established final diagnosis. Demographic and clinical data referring to the first 24 h were recorded. Ethics Review Board of Hospital Beatriz Ângelo approved the study with the number 0421 (2020) and this ethical approval was recognised by the Ethical Commission of National Institute of Health Dr Ricardo Jorge (INSA). The samples will be stored until 20 years after collection. All patients included in this study gave informed consent for the purposes of research.

Samples from the Biobank

Sample preparation

Every sample obtained was processed until a maximum of four hours after collection and submitted to the same sample treatment protocol. In short, 5 mL of whole blood was collected using K2EDTA tubes, which were centrifuged at 3000 rpm for 10 min at 4 °C. The plasma was separated from whole blood and immediately stored at optimal conditions. After removal of the buffy coat (saved for later analysis), RBCs were washed with 1×PBS using centrifugation at 1800 rpm for 5 min at 4 °C. The supernatant was discarded, and the wash was repeated one more time. The resulting aliquots of plasma, RBCs and white blood cells were all stored at -80 °C. These samples were regularly transported to the biobank, taking a maximum of 30 min during transportation, and were moved inside dry ice containers.

Metabolomics assays

Preparation of RBC samples

The samples were prepared as described by Gehrle et al. [12]. Briefly, 10 µL of RBCs was added to 90 µL of cold methanol/acetonitrile/water (5:3:2 v/v/v). The mixture was shaken at 700 rpm for 30 min at 4 °C and immediately centrifuged at 18,213 g and 10 min, 4 °C. The upper phase was collected and evaporated by speed vacuum.

Preparation of plasma samples

Metabolites were extracted from plasma (10 µL) using 90 µL of 74.9:24.9:0.2 (vol/vol/vol) acetonitrile/methanol/formic acid as reported by Hernández-Alonso P et al. [13]. The samples were vortexed at 1 min, 4 °C and centrifuged at 10 min, 18,213 g, 4 °C. The upper phase was collected and evaporated by speed vacuum.

Metabolite extraction and sample preparation

Plasma or RBC samples were resuspended at room temperature, and 100 µL of the samples from each group was mixed with 300 µL methanol (≥99.9%, LC-MS

CHROMASOLV) for protein precipitation, vortexed and incubated at -20 °C for 2 h. The samples were vortexed again and centrifuged at 14,000 rpm for 15 min, and the supernatants were transferred to new glass vials. The supernatants were evaporated in a SpeedVac EZ-2 Plus (GeneVac, Ipswich, UK) at 35–40 °C. To prepare the extracts for liquid chromatography coupled to mass spectrometry (LC-MS) analysis, they were first resuspended in 200 µL of deionized water containing 0.1% formic acid (LC-MS CHROMASOLV, Honeywell, Seelze, Germany) and vortexed for 2 min for complete mixing. The extracts were then filtered using a 0.45 µm hydrophilic nylon syringe filter for LC-MS/MS analysis. Equal volumes (10 µL) of 30 samples from each study group were collected and pooled for quality control (QC) and placed in the autosampler at 4 °C to analyse the reproducibility of the analysis.

Ultrahigh-performance liquid chromatography coupled with electrospray ionization and quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS)

An ultrahigh-performance liquid chromatography system (UHPLC) (Bruker Daltonik GmbH, Bremen, Germany) was used in conjunction with a quadrupole time-of-flight mass spectrometer for the LC-MS/MS analysis (TIMS-QTOF). The system was equipped with an electrospray ionization (ESI) source, a solvent delivery systems pump (Elute UHPLC HPG 1300), an autosampler, and a thermostat column compartment. Windows 10 Enterprise 2016 LTSB was used as the computer operating system. The computer operating system was Windows 10 Enterprise 2016 LTSB, and the software used was Bruker Compass HyStar 5.0 SR1 Patch1 (5.0.37.1), Compass 4.1, Version 6.2. Two different mobile phases were utilized: one with water and 0.1% formic acid (A) and the other with acetonitrile and 0.1% formic acid (B). The gradient program was as follows: 0–2 min, 99% A: 1% B; 2–17 min, 99–1% A: 1–99% B; 17–20 min, 99% B: 1% A, and the flow rate was fixed at 0.25 mL/min. Subsequently, 20–20.1 min 99% B to 99% A; 20.1–28.5 min, 99% A: 1% B at 0.35 mL/min flow rate; 28.5–30 min, 99% A: 1% B at 0.25 mL/min. A Hamilton® Intensity Solo 2 C18 column (100 mm×2.1 mm×1.8 m) was employed for separation. The oven temperature was maintained at 35 °C. The drying gas flow rate was 10.0 L/min (220 °C), the capillary voltage was 4500 V, and the nebulizer pressure was 2.2 bar in the ESI. The collision energy ranged from 100 to 250% and was set at 20 eV with a 500 V end plate offset for MS2 acquisition. For the external calibration, sodium formate was used. For the calibrant sodium formate, the auto MS scan segment used for acquisition ranged from 0 to 0.3 min, and for auto MS/MS, it ranged from

0.3 to 30 min. The acquisition was performed in positive mode at 12 Hz in both segments, and the automatic in-run mass scan range was 50–1300 *m/z*. With a cycle length of 0.5 s and a threshold of 400 cts, the width of the precursor ion was 0.5. After three spectra, the active exclusion was removed and released after 0.2 min. Mass calibration was performed prior to analysis according to the manufacturer's recommendations using external mass calibration (10 mM sodium formate calibrant solution). TRX-2101/RT-28-calibrants for Bruker T-ReX LC-QTOF (Nova Medical Testing Inc.) were injected before sample analysis to check and test the performance of the column, reversed-phase liquid chromatography (RPLC) separation, multipoint retention time calibration, and the mass spectrometer. Additionally, TRX-3112-R/MS Certified Human serum for Bruker T-ReX LC-QTOF solution (Nova Medical Testing Inc.) was prepared from pooled human blood and injected before sample analysis to check the performance of the LC–MS instruments. The analysis was performed using a randomized sequence order with five injections of solvent A (0.1% formic acid in deionized water) sample at the beginning of the sequence for apparatus equilibration, followed by five injections of the pooled QC sample. Additionally, one QC injection was performed every (9–10 samples) to minimize the carry-over and evaluate the reproducibility of the analysis and monitor batch effects [14].

Data processing and analysis

The MS data (Bruker Daltonik GmbH, Bremen, Germany) were preprocessed through the MetaboScape® 4.0 program. The T-ReX 2D/3D approach employed the peak area to quantify the feature after bucketing the processed data with an intensity threshold of 1000 and a peak length of 7 spectra. The calibration for mass spectra was performed in 0–0.3 min, and chromatographic peak widths less than 0.03 min were not included in the analysis. The auto MS/MS scan was performed using the average method, with scan parameters ranging from 0.3 to 25 min of retention time and 20 to 1300 *m/z* of mass. LC–MS/MS–QTOF was used to examine all of the study samples in duplicate. Unreliable features were removed using the QC samples. Human Metabolome Database (HMDB) 5.0 metabolomics database was used to map the MS/MS spectra and retention time to identify the metabolites. The selected metabolites were filtered by picking a higher annotation quality score that indicates the MS/MS score, *m/z* values, best retention time, mSigma, and analyte list spectrum library, and the annotation procedure was followed to identify the compounds with MS/MS using library matching. The peak intensity of each metabolite

obtained was used for quantification of the data matrix, and only significant compounds with a *p* value of <0.05 were selected from HMDB 5.0.

Statistical analysis

Clinical and demographic variables were described using absolute and relative frequencies for categorical variables and median and interquartile ranges (IQR) for continuous variables. Comparisons between confirmed acute VTE patients and control patients were performed by means of one multifunctional metabolomics data analysis platform with univariate and multivariate tools. The preanalytical filter procedure included the exclusion of exogenous contaminants (p.e., paracetamol, caffeine, L-DOPA, metoprolol, and buffer solution) and metabolites quantified in less than one-third of the samples. We used the metabolomics analysis protocol MetaboAnalyst 5.0 [15, 16]. After checking for normality of the distribution of untargeted LCMS-derived data from the RBCs and plasma samples, they were mean-centered and UV-scaled. Only variables present in at least 60% of any group and with intensity of at least 4.0×10^3 were included for analysis. Principle component analysis (PCA) and hierarchical clustering were performed for unsupervised multivariate statistical [17]. We performed orthogonal partial-least squares discrimination analysis (OPLS-DA) as the supervised method to identify important variables with discriminative power. OPLS-DA models were validated based on multiple correlation coefficient (R^2) and cross-validated R^2 (Q^2) in cross-validation and permutation test by applying 2000 iterations ($p > 0.001$). The significance of the biomarkers was ranked using the variable importance in projection (VIP) score (> 1) from the OPLS-DA model [18]. The loading plots and variable influence on projection (VIP) plots were inspected to differentiate the phenotypic groups. Hierarchical clustering analysis was performed based on the degree of similarity of metabolite abundance profiles and similarly patterned abundant metabolites were positioned together. The heat map and dendrogram indicated this distribution of abundance profiles. Univariate analysis consisted of fold change, and we considered a fold change > 1.5 to select differentially abundant metabolites. Pathway enrichment analysis used the Mummichog algorithm [19] in the “Functional Analysis” module in Metaboanalyst 5.0. A *p* value cut-off of 0.05 was used to consider metabolite matching. Receiver operating characteristic (ROC) curves were used to assess the performance of different metabolites in diagnosing acute VTE, and the Least Absolute Shrinkage and Selection Operator (LASSO) algorithm [20] was used to select biomarker candidates, while logistic regression was used

to assess model performance based on ROC curves. In this exploratory metabolomics study, p values < 0.05 were considered statistically significant. All quantified metabolites that were matched by m/z were searched in HMDB library [21]. Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Characteristics of study subjects

In total, 62 patients admitted to the ER were included in this study when initial suspicion of acute VTE was clinically determined. The clinical and demographic characteristics of the patients are detailed in Table 1.

No significant difference between patients with confirmed acute VTE diagnosis and patients with other nonacute VTE diagnoses was observed. Red cell distribution width (RDW) and platelet hematologic parameters showed a tendency for higher values among acute VTE cases. A diagnosis of acute VTE was confirmed in 50 patients, and the remaining patients were attributed to another final diagnosis (Table 2). Acute PE, with or

Table 1 Demographic and clinical characteristics of acute VTE and control patients

	Acute VTE	Control	p
Age (years) n	50	12	0.108
Median [IQR]	64.5 [44.25–81.75]	72.5 [66.25–88.0]	
Female sex n (%)	32 (64.0)	7 (58.3)	0.748
Smoking n (%)	4 (8.0)	1 (8.3)	0.970
Diabetes n (%)	4 (8.0)	1 (8.3)	0.970
Hormone treatment n (%)	4 (12.5)	0	0.323
Anti-thrombotic drugs n (%)	2 (4.0)	2 (16.7)	0.109
Erythrocytes (*10 ¹² /L)	42	10	0.291
Median [IQR]	4.6 [3.98–4.81]	4.25 [3.76–4.71]	
Hemoglobin (g/dl) n	42	10	0.935
Median [IQR]	13.4 [12.47–14.50]	12.65 [10.92–13.95]	
RDW ^a (%)	42	10	0.079
Median [IQR]	14.30 [13.08–15.00]	13.50 [13.08–14.12]	
Leucocytes (*10 ¹² /L)	42	10	0.725
Median [IQR]	10.01 [7.37–11.37]	11.09 [7.85–13.92]	
Platelets (*10 ⁹ /L) n	42	10	0.079
Median [IQR]	263 [213–355]	202 [184–245]	
D-dimers (mg/L) n	36	8	0.803
Median [IQR]	7.06 [3.84–11.04]	2.24 [1.06–14.38]	
Creatinine (mg/L) n	42	9	0.846
Median [IQR]	0.90 [0.76–1.07]	1.05 [0.85–1.99]	
CRP ^b (mg/L) n	38	9	0.416
Median [IQR]	2.26 [0.77–4.52]	13.87 [1.39–19.50]	

^a Red cell distribution width

^b C-reactive protein

Table 2 Diagnosis of acute VTE and control patients

	Acute VTE (n = 50)	Control (n = 12)
Pulmonary embolism	34	na
Deep vein thrombosis	24	na
Pulmonary embolism and deep vein thrombosis	12	Na
Erysipela	0	4
Superficial phlebitis	0	3
Sepsis	1	1
Pneumonia	0	1
Atrial fibrillation	0	1
Pericardial effusion	0	1
Anxiety	0	1
Neoplasia	1	0

na not applicable

without concomitant DVT, was the most prevalent diagnosis and represented 68% of all acute VTEs.

Metabolomics assays identified and quantified a total of 112 metabolites in plasma and 68 metabolites in RBCs. After eliminating the contaminants in the preanalytical steps, a total of 91 were identified and quantified in the plasma assays and 55 metabolites in the RBCs (Additional file 1: Tables S1 and S2).

Plasma metabolome

As shown in Fig. 2, plasma from acute VTE patients expressed a tendency toward a specific metabolomic signature by both PLS-DA and hierarchical clustering analyses (Fig. 2a). The most important metabolites defining this signature (Fig. 2c, d) were allantoinic acid, pipecolic acid, citramalic acid, uric acid, etiocholanolone, succinylacetone and biotin. The discrimination between acute VTE and non-acute VTE by using a hierarchical clustering algorithm (Fig. 2b) with the 25 molecules with the lowest p value revealed a limited performance, due to some acute VTE patients clustering together with nonacute VTE patients.

When applying univariate analyses, there were 23 significantly different molecules ($p < 0.05$, 11 upregulated in acute VTE patients, 12 downregulated) appeared (Fig. 3 and Table 3).

To define the capacity of these specific metabolites as potential biomarkers, ROC curves were generated, and we selected those with higher areas under the curve (AUCs) (Fig. 4). The best AUC were obtained by pipecolic acid (AUC: 0.758), citramalic acid (AUC: 0.738), uric acid (AUC: 0.728) and ethiocolanolone (AUC: 0.72). These ROC's revealed that none of the metabolites had high (AUC > 0.9) por very good (AUC: 0.8–0.9) performance.

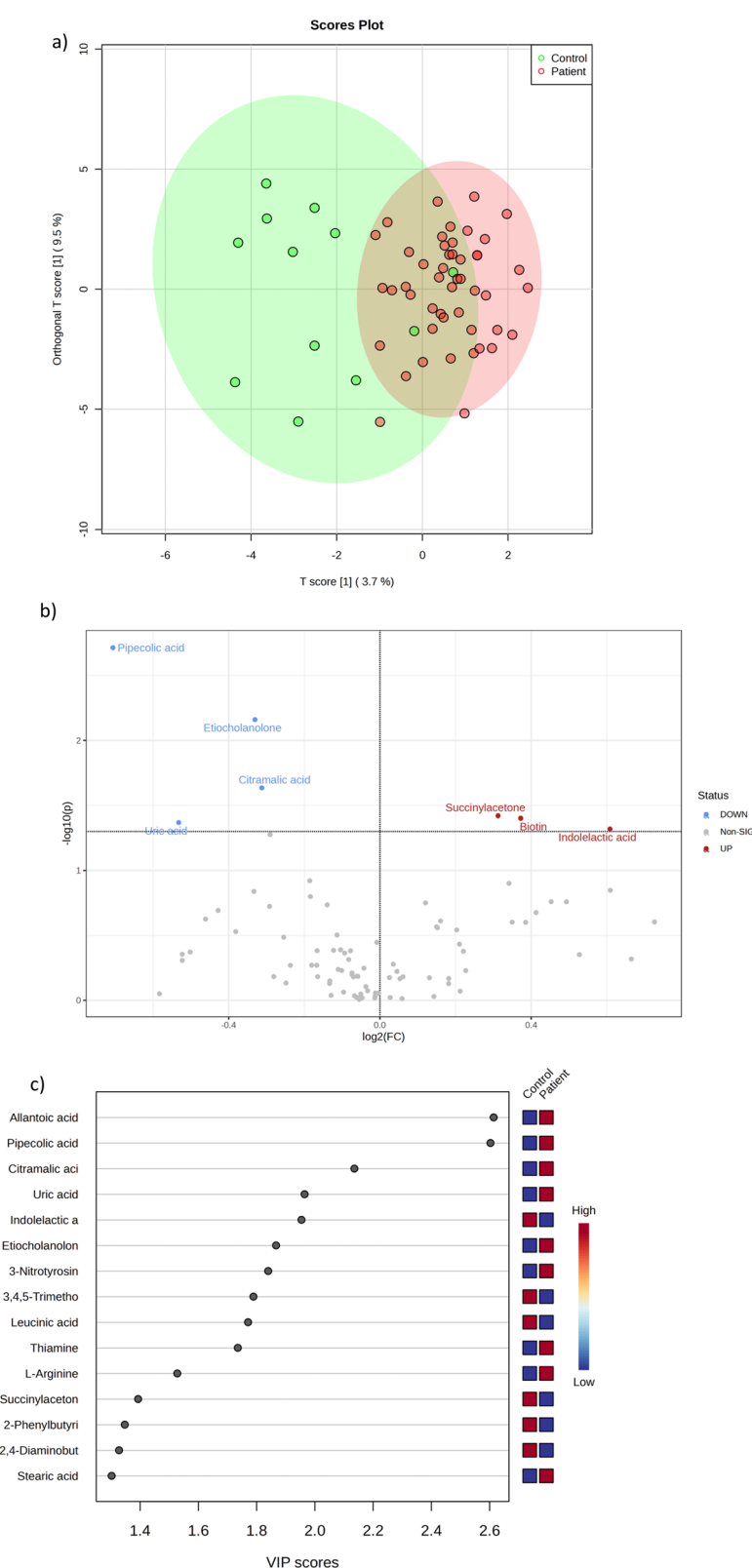


Fig. 2 Patients with acute VTE exhibit a plasma metabolomic signature. **a** PLS-DA analyses show that the metabolome is able to discriminate between acute VTE patients and control patients, with those metabolites contributing to separation shown in **b** the volcano plot and **c** VIP score graph. **d** Heatmap hierarchical clustering analyses using the 25 metabolites with the lowest *p* value indicate that there is not perfect clustering between groups

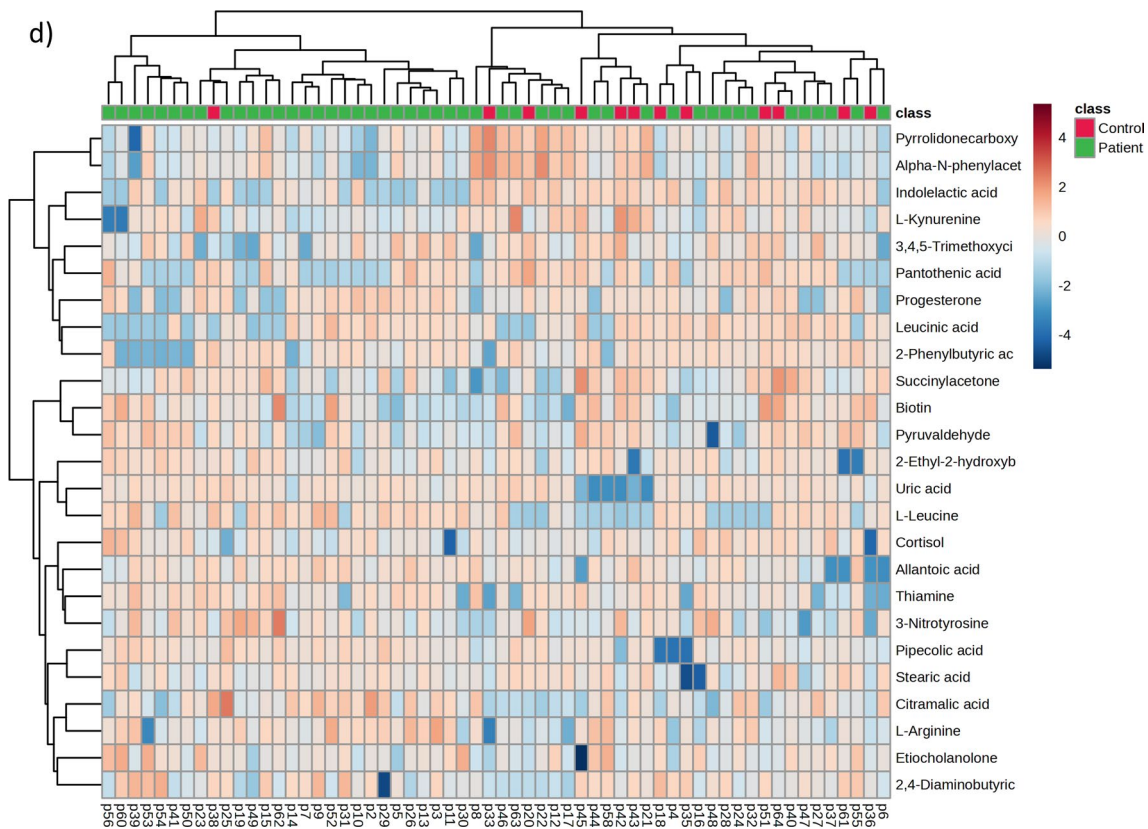


Fig. 2 continued

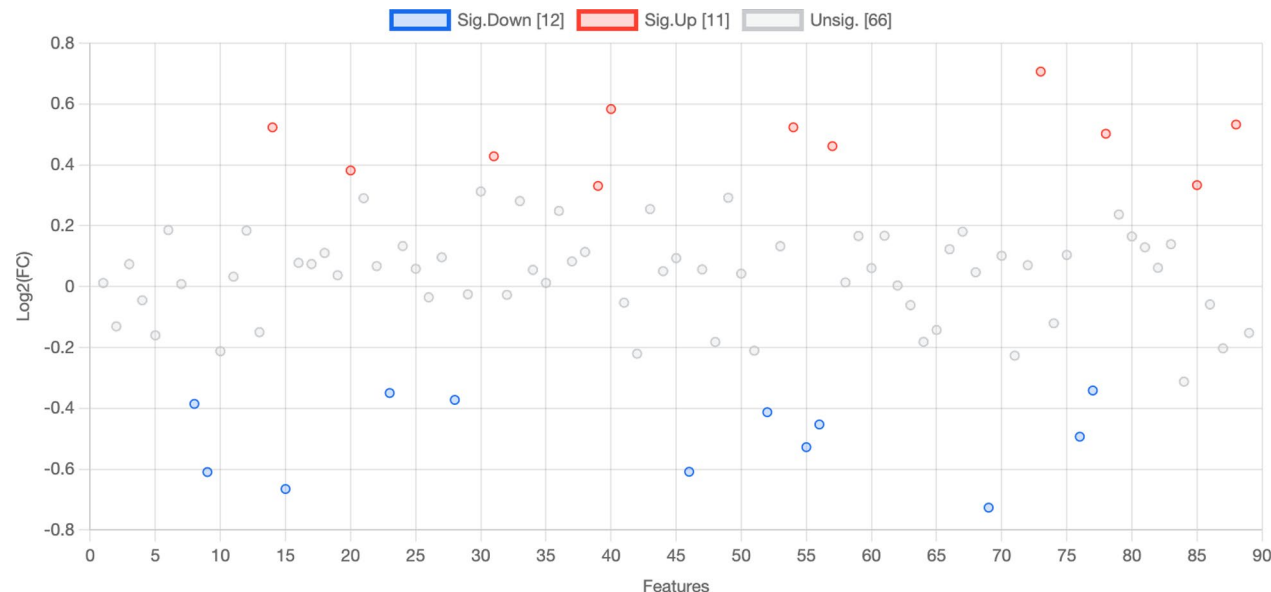


Fig. 3 Plasma of acute VTE and control patients exhibited 23 significantly different metabolites

Table 3 Plasma metabolites significantly different between acute VTE and control patients

	log2(FC ^a)
Pantothenic acid	−0.72629
Pipelicolic acid	0.70643
4-Hydroxyproline	−0.66
3,4,5-Trimethoxycinnamic acid	−0.61
Indolelactic acid	−0.61
Hippuric acid	0.58
Uric acid	0.53
L-Glutamine	−0.53
L-Fucose	0.52
4-Aminophenol	0.52
Quinaldic acid	0.50
Pyrrolidonecarboxylic acid	−0.49
L-Leucine	0.46
L-Kynurenine	−0.45
Cortisol	0.43
Leucinic acid	−0.41
2-Phenylbutyric acid	−0.39
Adenosine monophosphate	0.38
Biotin	−0.37
Alpha-N-phenylacetyl-L-glutamine	−0.35
Pyrvaldehyde	−0.34
Thiamine	0.33
Etiocolanolone	0.33

^a Fold change

Overrepresentation analysis (ORA) was performed, and one-tailed *p* values were provided after adjusting for multiple testing for enrichment analysis (Fig. 5). There were 21 different metabolic pathways evident from the analysis, with the top 5 represented by D-glutamine and D-glutamate metabolism, purine metabolism, nitrogen metabolism, thiamine metabolism, and valine, leucine and isoleucine biosynthesis. Importantly, the metabolic pathway of D-glutamine and D-glutamate was found to be the most relevant pathway and possessed the strongest impact on the acute VTE phenotype (*p*=0.001, false discovery rate=0.06).

RBC metabolome

Orthogonal partial least squares-discriminant analysis (PLS-DA) of RBC metabolites, validated by principal component analysis, identified a consistent metabolomic signature of acute VTE (Fig. 6a). The values of the variable impact importance factor (VIP) of the first component in PLS-DA, considered only for metabolites with

VIP>1 and *p*<0.05, are shown in Fig. 6c. The analysis of the raw data generated a volcano plot (Fig. 6d) and a visual heatmap of the top 25 differentially abundant metabolites (Fig. 6b).

According to the volcano plot, it can be concluded that the acute VTE group had 20 high metabolites and 4 low metabolites compared to the nonacute VTE group (Fig. 7 and Table 4).

To determine the diagnostic efficiency of the above 23 potentially differentially abundant metabolites, we generated subject ROC curves and obtained 5 differentially abundant metabolites with a high performance for differentiating acute VTE from nonacute VTE patients. After exclusion of exogenous bioactive metabolites (pyridine and 3,4,5-trimethoxycinnamic acid), there were 3 metabolites with high-performance ROC curves, including adenosine 3',5'-diphosphate (AUC: 0.983), glutathione (AUC: 0.923), and adenine (AUC: 0.91), whose ROC curves are shown in Fig. 8. The multivariate ROC analysis (Fig. 8d) presents a quasihigh performance for only 3 simultaneous metabolites (AUC: 0.894) and coherently high performance for 5 metabolites (AUC: 0.918 with CI 0.667–1).

The enrichment analysis of RBC metabolites differentially expressed between acute VTE and non-acute VTE patients resulted in the identification of a total of 20 different pathways (Fig. 9). The metabolic sets most impacting the differences observed were purine metabolism (*p*=0.000354, false discovery rate=0.68), glutathione metabolism, aminoacyl-tRNA biosynthesis, and beta-alanine metabolism.

Discussion

Acute VTE still represents a major diagnostic challenge to physicians worldwide. In this study, 23 plasma metabolites and 24 RBC metabolites were significantly different between acute VTE and nonacute VTE patients. The results of this analysis support the usefulness of high-throughput metabolomic methods to new biomarker findings in cardiovascular diseases [22–24]. The findings of RBCs analysis, in particular, represent novel inaugural data obtained from untargeted LC–MS of patients in the acute clinical phases of VTE. Chromatography is a widely used approach to the discovery of new biomarkers due to its ease of operation, inexpensiveness, selectiveness, reproducibility and low limits of detections [25]. The use of LC–MS in this study reinforces its role in the discovery of new potential biomarkers in VTE. The use of LC–MS in this study reinforces its role in the discovery of new potential biomarkers in VTE.

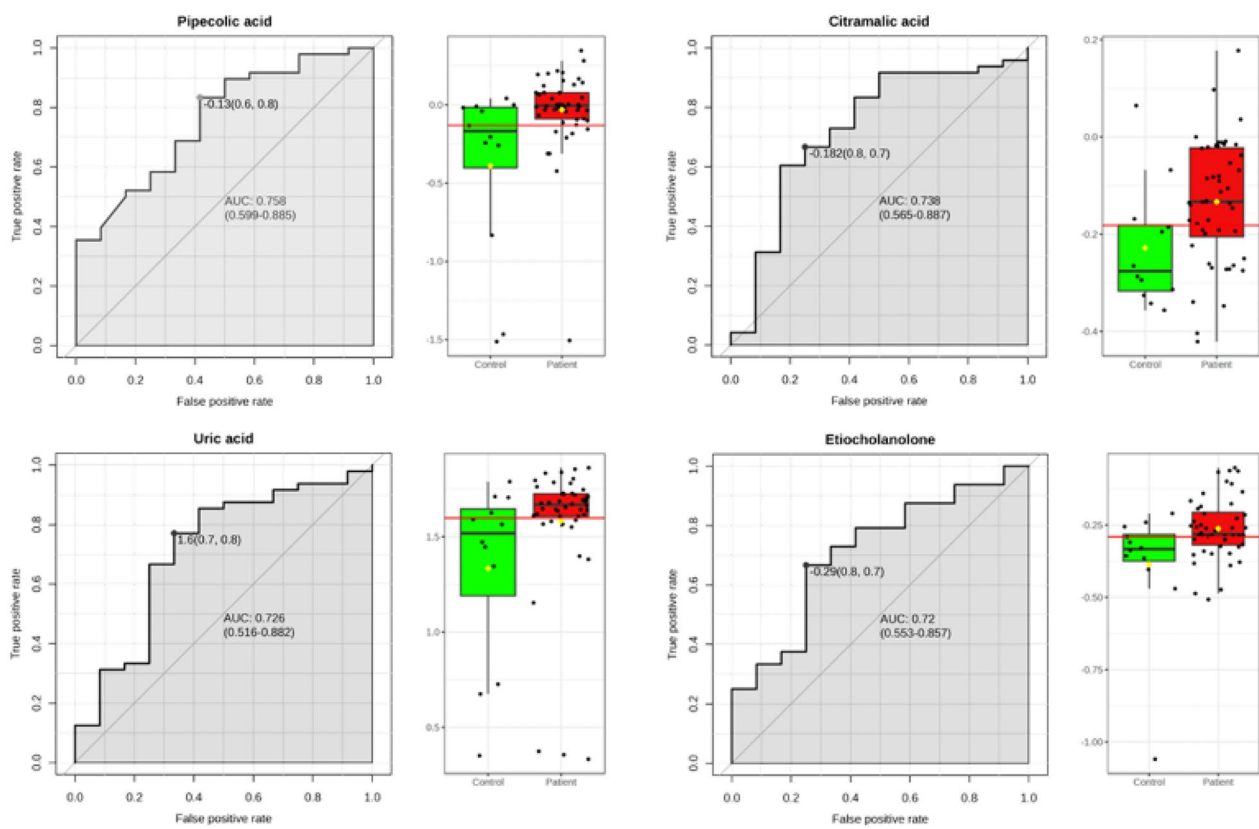


Fig. 4 ROC curves of those plasma metabolites with higher AUC values

Plasma metabolome revealed a limited diagnostic performance for acute VTE in this population. Previous untargeted plasma metabolomic assays identified changes in acylcarnitines in post-VTE patients [26, 27], which are involved in the regulation of sugar and lipid metabolism [28]. In our study, the plasma metabolome did not show significant changes in acylcarnitine metabolites, which could be explained by the fact that we analyzed patients in the early acute phases of VTE and the available comparable data were obtained from patients several weeks to months from discharge.

In contrast, the RBC metabolomic signature was clearly discriminatory of acute VTE patients. The role of RBCs in thrombosis extends from interactions with platelets, fibrinogen and coagulation proteins, as they incorporate the initial clot structure [29], modulation of the clot mechanic properties [30], to redox equilibrium and response to extreme conditions such as hypoxia [31]. Nevertheless, new approaches to its metabolism are expected to unshadow some other potential functions [32]. Adenosine 3',5'-diphosphate (PAP) acts as a sulfuryl group transferring cofactor between compounds with

PAP binding sites [33], and its targets in human cells are unknown [34]. PAP is converted to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) by sulfotransferase and then back to PAP, impacting different bioprocesses, such as cell communication, growth and development, and defense [35]. PAP inhibits sulfotransferase even at micromolar concentrations and, consequently, sulfation events on substrate molecules, including heparan sulfate [35]. Heparan sulfate, a ubiquitous protein ligand involved in the regulation of blood coagulation [36], was identified on normal mature human RBCs and is probably involved in heparan sulfate-sensitive adhesion of pathogens such as malarial parasites [37]. In this study, PAP was found to be higher in the RBCs of patients with acute VTE than in those of patients without VTE. The impact of RBC PAP accumulation on sulfation dysregulation and the risk for acute VTE deserves further investigation. The absence of previous data on metabolic profiling of RBCs from acute VTE patients prevents us from comparisons with other studies and increases the cautiousness that we shall use to interpret and generalize them.

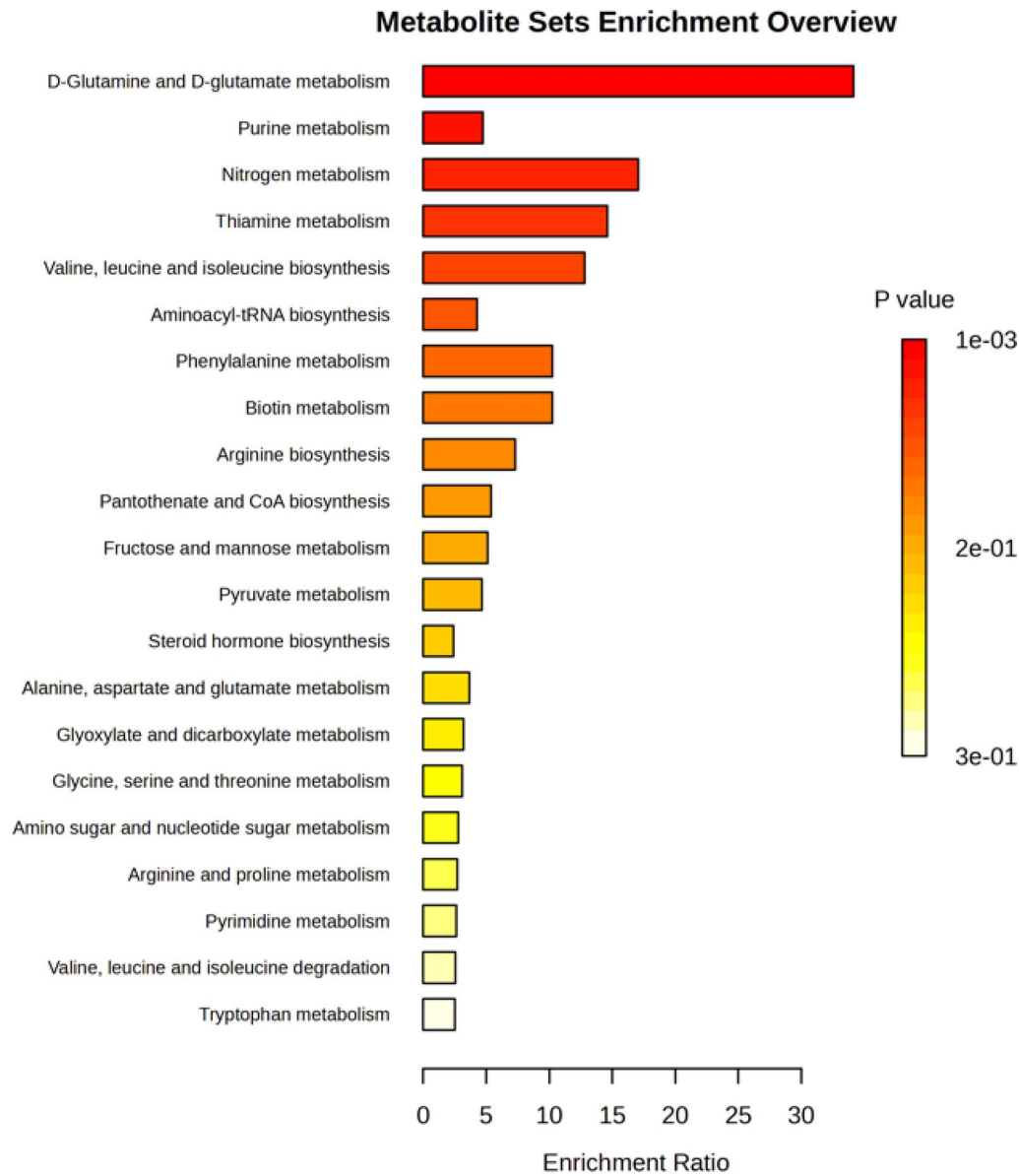


Fig. 5 Metabolic pathways identified considering the metabolites differentially expressed in the plasma of acute VTE and controls

All adenine nucleotide metabolites were upregulated in RBCs. ADP is a key intracellular component of the adenosine triphosphate (ATP) cycle that is abundant in RBCs, platelets and other cells. ADP plays an important role in energy metabolism, nucleic acid synthesis, cell signaling and gene expression [38], while its exit from cells results in platelet activation and aggregation [39–41]. Adenosine, derived from adenine, is an inhibitor of platelet activation [39] that is usually low in extracellular environments and increases in response to hypoxia, inflammation, and tissue injury [42]. Adenosine was found to be

increased in the vein wall of DVT models and decreased in the serum of severe PE patients [43, 44]. Although we did not evaluate plasma, our findings of increased adenosine in RBCs of acute VTE patients lead us to the hypothesis that during a venous thrombosis event, complex cross-talk between plasma and cells, such as RBCs and endothelial cells, may take place in the regulation of adenosine signaling. The acute VTE patients in our study exhibited significantly higher levels of RBC-derived glutathione, and the oxidative/antioxidative balance shifted towards the oxidative status in venous thromboembolism

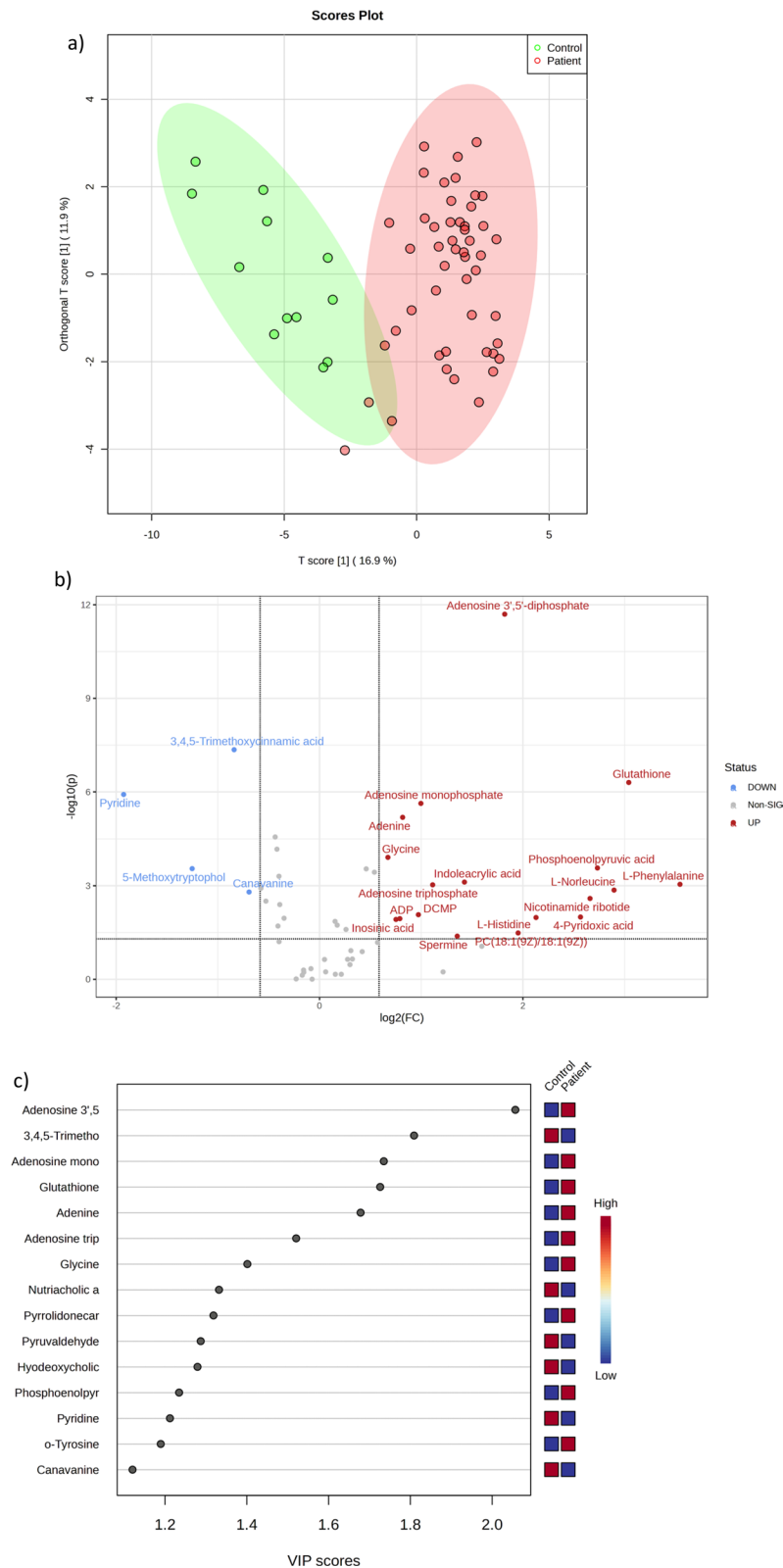


Fig. 6 RBCs of the patients with acute VTE exhibit a distinct metabolomic signature. **a** PLS-DA analyses show that the metabolome is able to discriminate between acute VTE patients and control patients, with those metabolites contributing to separation shown in the volcano plot (**b**) and VIP score graph (**c**). **d** Heatmap hierarchical clustering analyses using the 25 metabolites with the lowest *p* value (Wilcoxon test) indicate that there is good clustering between groups

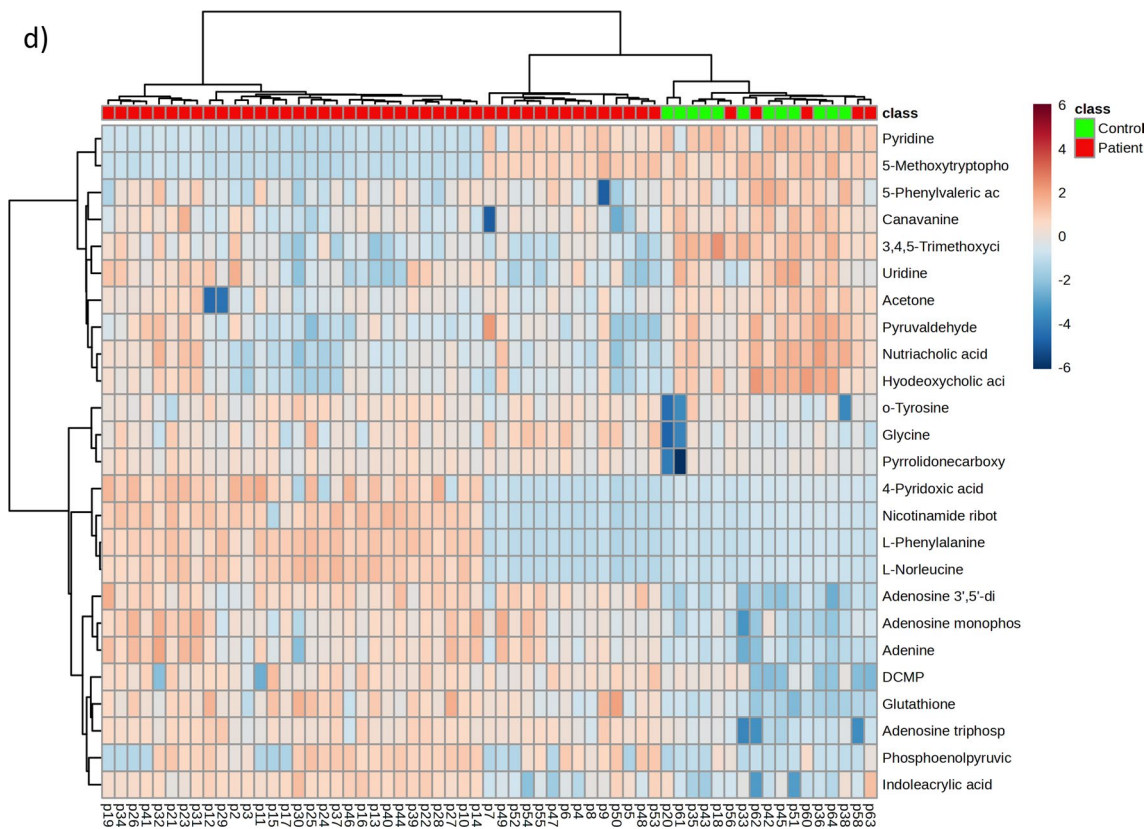


Fig. 6 continued

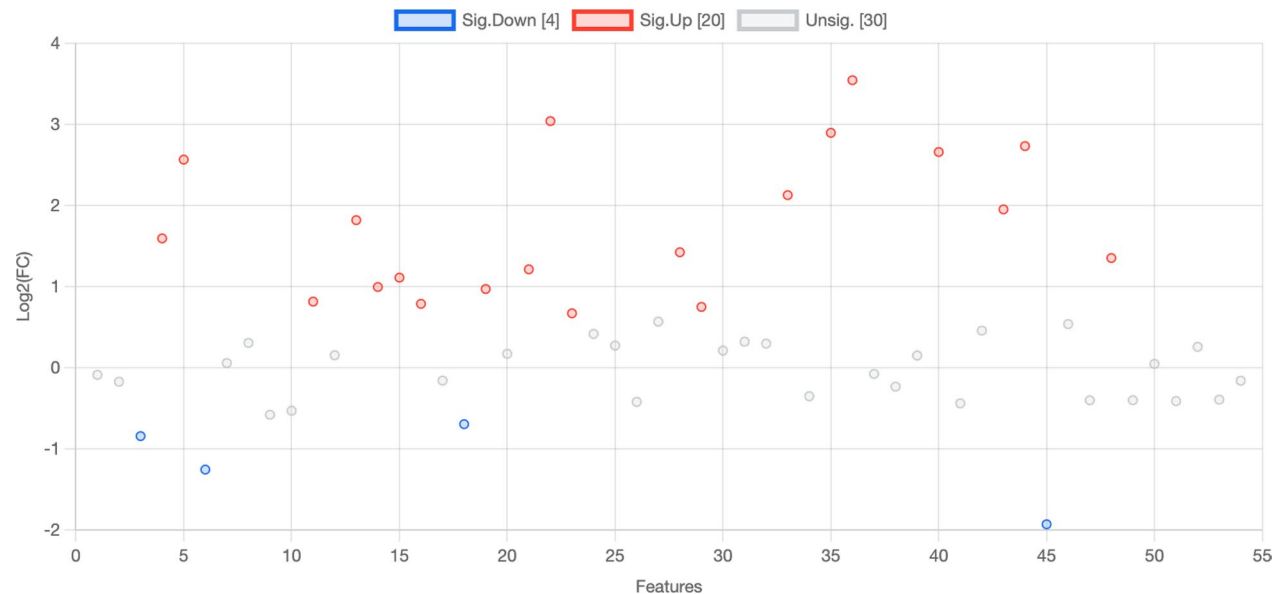


Fig. 7 RBCs from acute VTE patients exhibited 24 significantly different metabolites

Table 4 RBC metabolites significantly different between acute VTE and control patients

	log2(FC ^a)
Adenosine 3',5'-diphosphate	1.82
3,4,5-Trimethoxycinnamic acid	−0.84
Glutathione	3.04
Adenosine monophosphate	0.99
Adenine	0.82
Glycine	0.67
Phosphoenolpyruvic acid	2.73
5-Methoxytryptophol	−1.25
Indoleacrylic acid	1.42
L-Phenylalanine	3.54
Adenosine triphosphate	1.11
L-Norleucine	2.90
Canavanine	−0.69
Nicotinamide ribotide	2.66
DCMP	0.97
4-Pyridoxic acid	2.57
L-Histidine	2.13
ADP	0.79
Inosinic acid	0.75
PC(18:1(9Z)/18:1(9Z))	1.95
Spermine	1.35

^a Fold change

[45, 46]. Notably, glutathione metabolism can be the potential origin of free radicals interfering with platelet redox status and acting as modulators of platelet function [47]. In the preclinical stages of VTE, inflammation of the endothelium is one of the first phenomena and is responsible for high oxidative stress, with increased glutathione production [48]. Glutathione may thus have a potential role as part of a diagnostic biomarker panel for acute VTE in future studies.

The question of whether arterial and venous thrombosis are just two different clinical manifestations on the continuum of the same disease [49] remains to be answered. Plasma metabolomic studies from patients diagnosed with coronary syndrome identified upregulation of 2-OH-butyric acid, a glutathione presynthesis byproduct under hypoxia [50, 51], in line with our findings of higher glutathione levels in RBCs from acute VTE patients. Purine metabolism has also been associated with unstable angina [52, 53], metabolic syndrome and all-cause mortality [54–56], and venous thrombosis [57, 58]. A similar trend was observed in both plasma

and RBCs of our acute VTE, reinforcing possible pathophysiological common pathways. Finally, glutamate and glutamine metabolites, which were higher in the plasma of our acute VTE patients, were previously found to be independently associated with type 2 diabetes [59], stroke and composite cardiovascular disease [60]. There may be reasons to believe in common pathways for arterial and venous acute thrombotic conditions that may be explored by multi-omics.

Despite their promise as early biomarkers for acute VTE, the measurements of new biomarkers are usually costly and dependent on complex laboratory techniques and methods [61]. Clinical metabolomics for biomarker screening of acute VTE should aim for a widely available, fast and inexpensive laboratory analysis that can be measured in real practice. Nucleotides such as adenine can be quantified by LC/MSMS from blood and various tissues and cells [62, 63], with the use of simple extraction and quantification protocols that can be a future solution to daily practice. Glutathione determination is already possible with current clinical laboratory methods and even with simple methods that could allow future minimally invasive monitoring [64]. The daily use of LC–MS-based metabolomics is not possible in most hospitals. Nevertheless, we believe that this technology, similar to other omics-based technologies, will lead to the discovery of pathophysiological pathways occurring during acute VTE that should enhance hyperacute treatment and the prognosis of these patients. Further translation of these biomarkers to the clinic will certainly require the development of more cost-effective technologies.

Our study has limitations. The groups presented large differences in the size and variance. However, by using controls that are acutely ill patients presenting the same symptoms as acute VTE, we increased our possibilities to identify reliable diagnostic/predictive biomarkers for this pathology. The ROC curves showed high diagnostic accuracy for RBC metabolite data, which confirmed that assumption. The PLS-DA model statistics and heatmap for acute VTE did not result in a perfect distinction between them and nonacute VTE, thus partially limiting the interpretability of the enrichment analysis to the most significant findings, especially in plasma. Similarly, differences in metabolomics profiles due to body mass index could entail changes in the metabolome. Unfortunately, this study does not use a confirmatory cohort, which might be useful in the future for enhancing the robustness of the findings.

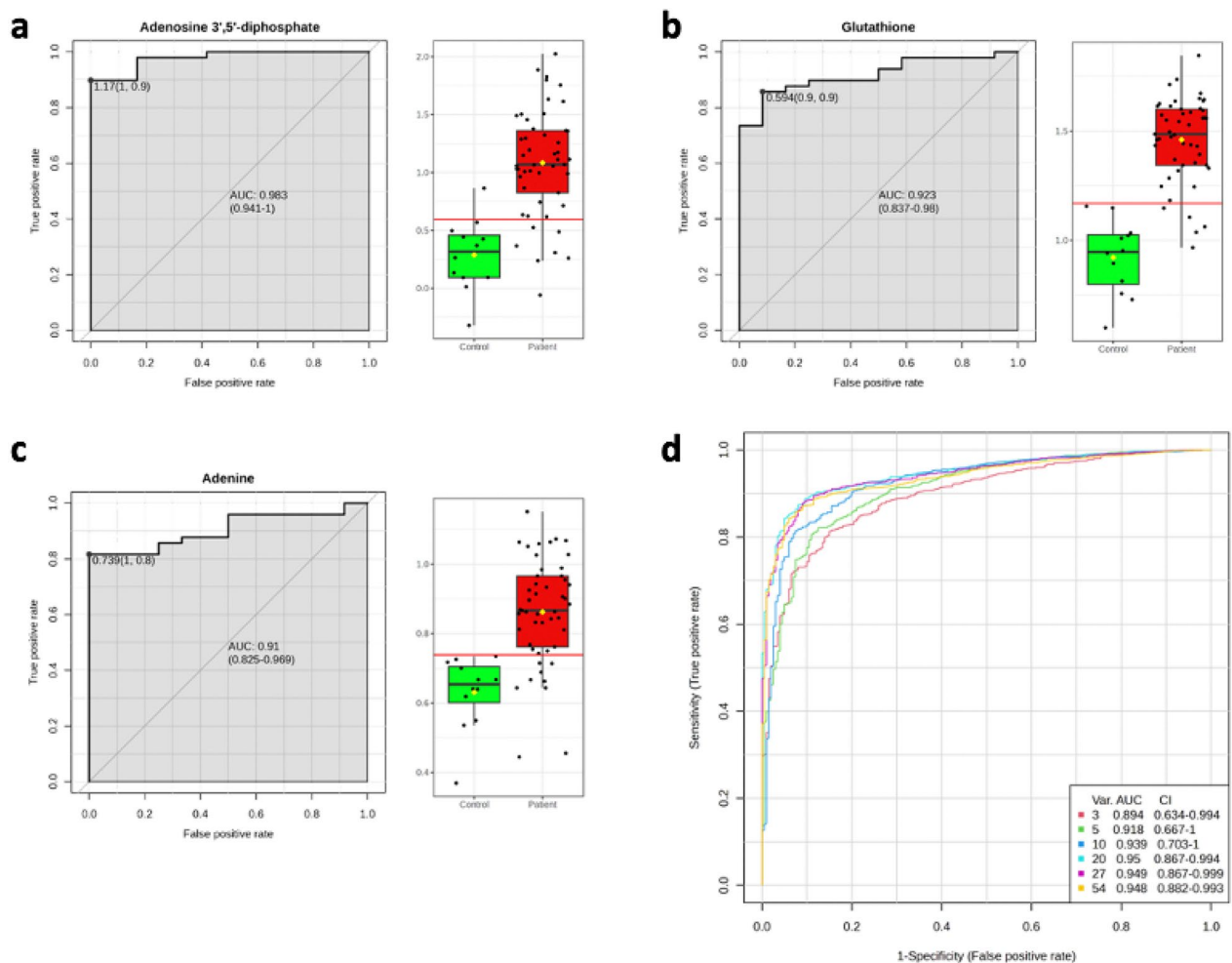


Fig. 8 ROC curves of biologically relevant RBC metabolites with AUC > 0.9 values (a–c) and multivariate ROC analysis (d) with confidence intervals of the six models

Conclusions

This discovery based metabolomic study using patient plasma and RBCs, clearly suggests that acute VTE presents a specific metabolic signature. The differences in early phases of the disease have potential diagnostic predictive value. Our findings identified a panel of RBCs metabolites that potentially represents a set of diagnostic

biomarkers to differentiate patients presenting with an acute VTE episode from other patients. These results should be validated by larger targeted metabolomic studies. Acute venous thrombosis revealed a plasmatic and RBCs metabolomic profile suggestive of disturbances in nucleotide metabolism and of excessive oxidative stress which are common to arterial thrombosis, and point to the existence of some shared pathophysiological processes.

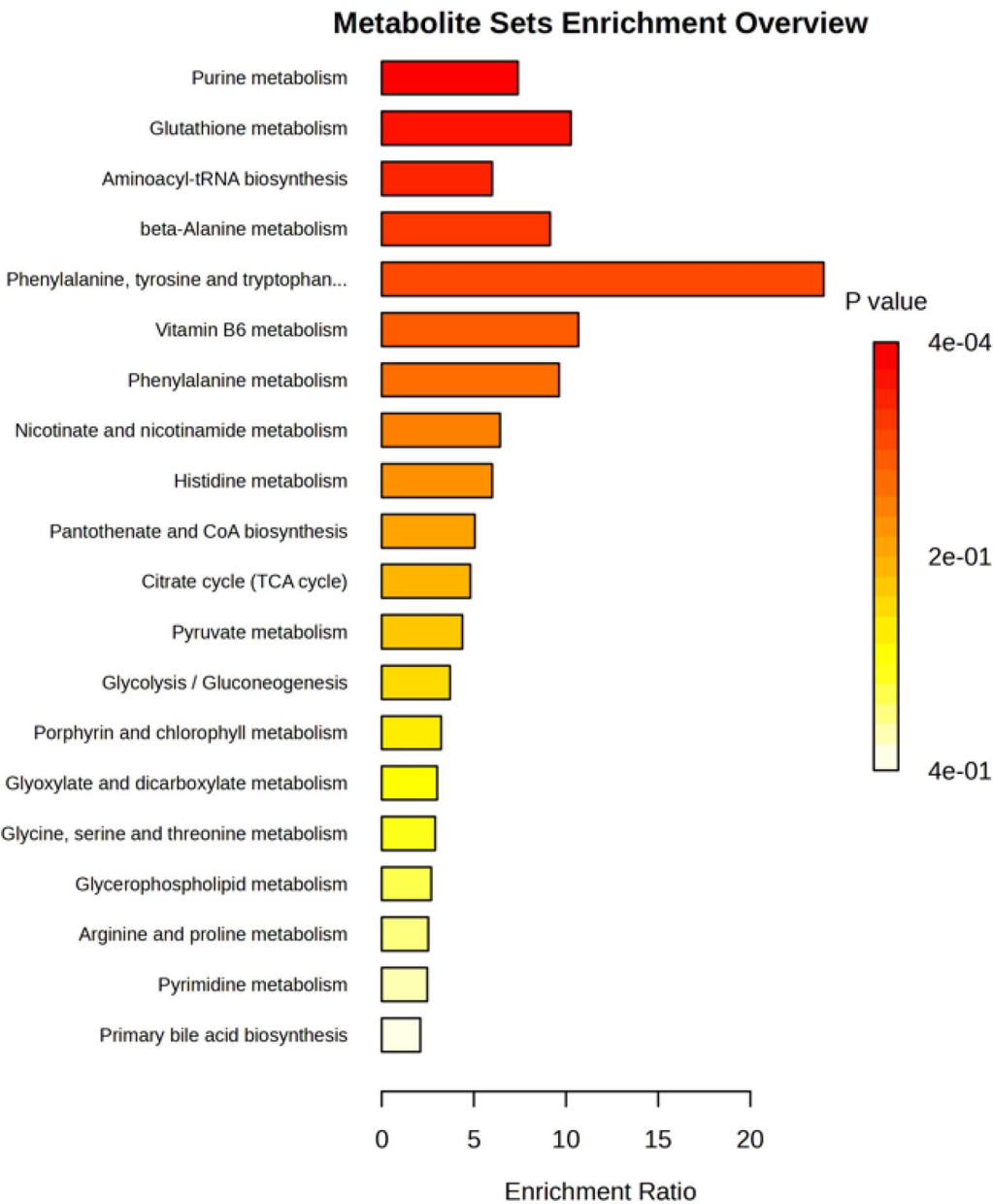


Fig. 9 Enrichment analysis of the metabolic pathways identified considering the RBC metabolites differentially expressed between acute VTE and controls

Abbreviations		ORA	Over representation analysis
AUC	Area under the curve	PCA	Principle component analysis
DVT	Deep vein thrombosis	PE	Pulmonary embolism
ER	Emergency room	RBCs	Red blood cells
ESI	Electrospray ionization	RDW	Red cell distribution width
HMBD	Human metabolome database	ROC	Receiver operating characteristics
INSA	National Institute of Health Dr. Ricardo Jorge	RPLC	Reversed-phase liquid chromatography
LC–MS	Liquid chromatography coupled to mass spectrometry	UHPLC	Ultra-high-performance liquid chromatography
MS	Mass spectrometry	UHPLC-ESI-QTOF-MS	Ultra-high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry
OPLS-DA	Orthogonal partial-least squares discrimination analysis	VTE	Venous thromboembolism

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-024-04883-8>.

Additional file 1: Table S1. List of the 91 metabolites identified in plasma (without contaminants). **Table S2.** List of the 55 metabolites identified in RBC's (without contaminants).

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Author contributions

CF and DP wrote the original draft. CF, KW, YL, JZ, and HZ performed the research. FV, JS, JM, NS, HH, MS and CF analyzed the data. CF, DP, RM, and VG designed the experiment and revised the manuscript.

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Data availability

Not applicable.

Declarations

Ethics approval and consent to participate

The clinical trial part was approved by the Research Committee of the Hospital Beatriz Angelo and the Ethical Commission of INSA.

Consent for publication

This study has not been published before, and this publication has been approved by all authors.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplemental materials (for article ‘Acute venous thromboembolism plasma and red blood cell metabolomic profiling reveals potential new early diagnostic biomarkers: observational clinical study’)

Table S1 List of the 91 metabolites identified in plasma (without contaminants)

32233223232311111111111111111111111111231(R)-

Lipoic acid

17a-Estradiol

1-Methyladenosine

2,3-Diaminopropionic acid

2,4-Diaminobutyric acid

2-Ethyl-2-hydroxybutyric acid

2-Hydroxy-3-methylbutyric acid

2-Phenylbutyric acid

3,4,5-Trimethoxycinnamic acid

3-Hexenedioic acid

3-Methylindole

3-Nitrotyrosine

4-Aminohippuric acid

4-Aminophenol

4-Hydroxyproline

4-Pyridoxic acid

5-Hydroxy-L-tryptophan

5-Methoxytryptophol

Acetone

Adenosine monophosphate

Allantoic acid

All-trans-retinoic acid

Alpha-N-phenylacetyl-L-glutamine

Androstenedione

Aspartyl-lysine

Asymmetric dimethylarginine

Benzoic acid

Biotin

Canavanine

Citramalic acid

Cortisol

Creatinine

Cyclic AMP

Dihydrothymine

DUMP

Elaidic acid

Estradiol

Ethanolamine

Etiocholanolone
Hippuric acid
Homocysteine
Hydroxyindoleacetic acid
Hyodeoxycholic acid
Hypoxanthine
Indoleacrylic acid
Indolelactic acid
Isovalerylcarnitine
L-Acetylcarnitine
L-Arginine
L-Carnitine
L-Cystathionine
Leucinic acid
Levulinic acid
L-Fucose
L-Glutamine
L-Kynurenine
L-Leucine
L-Methionine
L-Norleucine
L-Tryptophan
L-Valine
m-Coumaric acid
Myristic acid
N-Acetyl-L-phenylalanine
Naringenin
Nutriacholic acid
Oleic acid
o-Tyrosine
Pantothenic acid
Paraxanthine
PC(18:1(9Z)/18:1(9Z))
p-Cresol
Pipelicolic acid
Progesterone
Pyridoxal
Pyrrolidonecarboxylic acid
Pyrvaldehyde
Quinaldic acid
Ribothymidine
Saccharopine
Sphinganine
Sphingosine
Stearic acid
Succinylacetone

Thiamine
Tyramine
Urea
Uric acid
Uridine
Xanthosine

Table S2 List of the 55 metabolites identified in RBC's (without contaminants)

(R)-Lipoic acid
2-Phenylbutyric acid
3,4,5-Trimethoxycinnamic acid
3-Phenylbutyric acid
4-Pyridoxic acid
5-Methoxytryptophol
5-Methylcytidine
5'-Methylthioadenosine
5-Phenylvaleric acid
Acetone
Adenine
Adenosine
Adenosine 3',5'-diphosphate
Adenosine monophosphate
Adenosine triphosphate
ADP
Androstenedione
Canavanine
DCMP
Deoxyguanosine
Diacetyl
Ethanolamine
Glutathione
Glycine
Glycylproline
Guanosine 5'-diphosphate
Guanosine monophosphate
Hyodeoxycholic acid
Hypoxanthine
Indoleacrylic acid
Inosinic acid
Isovalerylcarnitine
L-Acetylcarnitine
L-Carnitine
L-Histidine
Linoleic acid
L-Norleucine
L-Phenylalanine
L-Tryptophan
m-Coumaric acid
Nicotinamide ribotide
Nutriacholic acid
o-Tyrosine
PC(18:1(9Z)/18:1(9Z))

Phosphoenolpyruvic acid
Pyridine
Pyrrolidonecarboxylic acid
Pyruvaldehyde
Spermine
Sphinganine
Succinylacetone
Tyramine
Uric acid
Uridine
Uridine 5'-monophosphate

Chapter 4- Discussion and future perspectives

The final goal of the present thesis is to integrate the relevant findings of the research works, both of systematic reviewing of the scientific literature and of one clinical trial and put on perspective the impact they might have in clinical practice of early acute VTE diagnosis.

Acute VTE remains a challenging diagnosis in the early symptomatic stages, which considerably impacts on the immediate management of patients in either category of low- risk or high-risk of death and severe complications.

More than 150 years from the inaugural elaborations of Virchow on the clinical presentations of venous thrombotic diseases and their intrinsic pathologic processes, the answers to mechanistic of biologic systems and diagnostic biomarkers are far from resolute. Although recent logarithmic increases in research on cellular and molecular mechanisms subjacent to the occurrence of venous thrombus have contributed to new lines of investigation on biomarkers, there is still a considerable gap between laboratory research and daily assistance to patients in the real-world clinical practice.

The discovery of adequately performing biomarkers for acute PE and DVT will certainly diminish the rates of undiagnosed episodes, and, consequently, as we are dealing with a potentially fatal disease, shall reduce acute and chronic VTE related mortality. In the era of easily accessible and manageable anti-coagulation, diagnostic dilemma is one of the most relevant unsolved problems. In the presence of very heterogenous clinical presentation symptoms and signs, which is especially true for acute PE, the possibility of reaching a diagnosis should involve a quick, easy, widely available and unexpensive test.

An inspirational example for what could be the near future history of acute VTE diagnosis is the role of troponin in the early diagnosis of acute myocardial infarction. The surging of this laboratory test in the initial management of this fatal disease has rewritten its chapters on morbidity and mortality. The very inner motivation to begin the work reflected on this thesis was my belief that this 'acute PE troponin' is possible to surge in the next years to clinical practice, and that this could contribute to that future.

Bearing this objective in mind, a great and urgent challenge in acute VTE is the identification of very early measurements of high acute venous thrombotic burden and the accurate definition of each patient prognosis, defining whom may be the patients with the most benefits of an immediate and, eventually, more invasive treatment modality. Reliable and validated biomarkers, ideally measured with minimally invasive techniques, may bridge these gaps to routine clinical practice.

Currently, imaging gold-standard exams to the diagnosis of acute VTE episodes impose routines based on long standing hours before the diagnosis, not available on a 24/24h basis in most healthcare settings and associated with considerable costs and

adverse reactions. Not arguing that CTPA and venous ultrasound have very good diagnostic accuracy in the majority of acute VTE diseased populations, they delay the beginning of sometimes lifesaving thrombolytics and anti-coagulants. Not to mention its restriction to urban hospital facilities, where the resources for an uninterrupted service availability are more probable. As a whole, it would be 'game changing' to discover new diagnostic biomarkers that would be used as a first line procedure in the initial approach to acute VTE.

For these reasons, we decided to develop a research project to investigate new biomarkers for the diagnosis of acute PE and acute DVT at presentation to the ED.

To evaluate what biomarkers are already available to the diagnosis of acute VTE or under investigation to introduce them in the clinical practice, we established Goal 1 of this thesis – Synthesis of evidence. Apart from exposing the relevant data on D-dimer testing potentialities and limitations, we made a comprehensive critical appraisal of the articles on various aspects of acute VTE, from epidemiology to clinical management.

During this process of continuing updating, combined with personal reflexion on the patients I treated along this period and the initiatives to investigate acute PE from my medical students (of Faculty of Medicine at University of Algarve), it emerged the investigation hypothesis of comparing diuretics and fluid expansion for the treatment of the acute PE patients with right heart failure not in shock.

We performed a systematic review and identified 53 articles that resulted in four final randomised controlled trials (RCTs) that compared fluid resuscitation and diuretic strategies in normotensive patients with acute PE presenting with right ventricle failure, and they were included for the meta-analysis. The 452 patients included in the studies had right ventricle dysfunction as determined by echocardiographic measurements and either troponin or proBNP elevations, which we considered a strength of this work for it intended to homogenise the population under investigation and facilitate extrapolation of the results to the clinical practice.

Although we found important differences in the administration protocols of both furosemide and fluids, the common findings of a significant reduction of pulmonary artery systolic pressure (PASP) after diuretics, with an effect estimate of -41.97 (95% CI -65.79 to -18.15) and a Z-score of 3.45 ($p < 0.0001$), and proBNP levels, with an effect estimate of -5.96 (95% CI -8.06 to -3.86) and a Z-score of 5.56 ($p < 0.0001$), within the first 24 hours after medication are interesting. One important point was the low heterogeneity of the studies included that reinforces the potential relevance of these findings.

These results provide a rationale to support more studies on therapeutic volume-unloading strategies in the face of echocardiographic evidence of early right ventricle dilation, like the results reported in other studies, even in non-overloaded patients. If the results are reproduced in specific trials, we might expect that there will be a mention to diuretics use in the immediate management of acute PE in the future international guidelines.

In the continuum of the literature reviewing, we hypothesised that hematologic indices, subject to several clinical trials exploring their potentialities as VTE biomarkers, could be explored in a more focused perspective.

Bearing in mind that acute PE causes most deaths related to VTE, we decided to investigate if MPV might have a diagnostic role in the early hours of PE symptomatic disease. In our opinion, although there are other reviews of the behaviour of MPV in acute VTE, they distinct clinical forms of VTE, including acute and sub-acute or chronic stages.

Our systematic review of 153 studies allowed to retrieve 13 RCTs for a meta-analysis, summing a total of 2428 patients. The analysis resulted in a significant increase in MPV of patients with acute PE compared to controls, with an effect estimate of 0.84 (95% CI: 0.76–0.92) and a Z-score of 20.25 ($P < .00001$). Besides a significant heterogeneity of the studies under analysis, the results expressed a relevant tendency of MPV augmentation in acute PE patients.

The ROC curve analysis for MPV in predicting acute PE was deceptively low performant, with optimal cut-off values for MPV of 8.5 fl revealing sensitivity 80% and specificity 60%. The high heterogeneity of the dataset imposes some caution in the interpretation of the results, but the unequivocal evidence of high MPV measurements in acute PE raise a potential role in the early diagnosis of acute PE, in conjunction with other predictors that increase the diagnostic accuracy.

As we had the opportunity to discuss in the published text, there are important limitations to the interpretation of these results that prevent it from adapting the practice of MPV use to diagnose acute PE in the ED. Nevertheless, along with other studies in with results in the same direction, it is my opinion that hematologic indices may have an important role as an adjunctive to the non-invasive and immediate diagnosis of acute VTE, namely by being added to other clinical variables in a new pre-imaging score that could reinforce the ruling out of patients, thus avoiding unnecessary CTPAs and ultrasounds and resulting in an early discharge of non-acute VTE patients.

After these comprehensive evaluations, I decided to proceed with the investigation for biomarkers to detect acute VTE. Biomarkers discovery is a hard and demanding task, with multiple possible approaches. I decided to begin with an exploratory approach to identify relevant molecules specifically modified in the blood of acute VTE patients that could be the basis for a targeted study in future validation research, conducted either by us or others. This was the mind mapping that led to an “omics” approach.

The high throughput technologies used by omics medicine allow the blind questioning of biologic samples and the acquisition of abundant data ready for bioinformatic and biostatistics analytical tools. These areas of Biology knowledge made a leap forward in biomarker's discovery, not just by designing new analysis tools to work those data and retrieve robust results, but also by insisting on user friendly software that made it possible for ‘non-experts’ investigators to use them and obtain their own results.

After some reflexions on the best way of making some research work around these ideas, I hypothesised that blood from acute VTE patients could have a specific biologic signature with identifiable proteins or metabolites that could be used as early diagnostic biomarkers of this disease in the future.

As an answer to the investigation hypothesis, we implemented one observational longitudinal study to investigate new potential early diagnostic biomarkers of acute PE and DVT. This led to our second goal of the thesis, represented by methodological relevant points of the study protocol.

We started the study protocol in one general hospital nearby Lisbon and recruited a relatively large cohort of 64 patients with presenting features suspicious of an acute VTE episode in the ED. The recruitment basically aimed the inclusion of all consecutive adults admitted to that ED and in whom the assistant physician, based on clinical and demographic characteristics only, hypothesised acute DVT or PE as a probable diagnosis and decided to proceed with imaging investigation directed to this diagnosis.

The moment of the clinical suspicion of acute VTE was the moment zero for the inclusion and was followed by all procedures of enrolment. For included patients, there was a blood collection, with the sampling of one EDTA tube differently identified among the other tubes requested by usual practice procedures and was transported to the laboratory within a maximum of 30 minutes from collection to be immediately prepared for aliquots storage. These steps of the protocol were very rigid, resulting in major limitations to recruitment schedules that were limited to weekdays during a small slot of morning hours, when there were a small group of three laboratory staff adequately trained in sample preparation protocol before the beginning of the study. Nevertheless, it is our conviction that this is a major strength of the project, as we thus assured small variations in the metabolome due to extrinsic factors (time in the tube before preparation exposed to EDTA, temperature variation periods, biologic processes occurring in the samples after collection, circa diary variation of metabolome). Considering our discovery approach, it was critical to assure that the results of metabolites identified in the samples were relevant to the pathologic ongoing processes and not an artefact from collecting and preparation processes.

The Biobank that resulted from this process, located at INSA, is a library of more than 500 sample aliquots that were ready to be analysed in any time. Some of these samples were used in the studies presented in this thesis and hundreds samples are still kept stored up to 20 years to be hopefully used in future studies, according to the ethical approval. This was, in fact, a hard task that was successfully completed, and, in my opinion, it was one of the main achievements of the project.

I will not go into details of Covid 19 constraints affecting the course of the project but I would like to make notice the enormous efforts of every people involved in the study, both at the hospital and at INSA, to guarantee that our mission would be complete

independent of Covid 19 pandemics'-imposed changes of all our daily professional and organizational routines.

During our research project we found a major setback: lack of funding. Our initial idea was to analyse RBCs and plasmatic samples with an untargeted proteomic approach. Nevertheless, the lack of financial funding to support this analysis, after some unsuccessful applications to government and private grants, impeded it and implied a delay in the initial chronogram.

Finally, there came a solution of analysing metabolites, instead of proteins, and we pursued with the project in that direction. In the end, we might have been strengthened by this evolution, as we found a new collaboration for our project, which we believe will participate in future research pathways after this study.

As the main goal of this thesis, we analysed data from the included patients and reported the results in the form of two already published articles.

In the study of the clinical characteristics of the acute VTE patients (35 PE and 25 DVT episodes) compared to non-acute VTE patients (10 patients with other diagnosis), in whom there was this initial suspicion, but imaging exams ruled out this diagnosis, I found relevant differences between the two groups. The small sized population for these purposes was a major limitation to this study, especially the control non-acute VTE group, but the resulting significant difference was a reason to consider its sharing with the scientific community.

To strengthen our study, we excluded patients with known active infections because inflammatory host defence responses to infection trigger major blood detected modification, both in cellular elements and plasmatic fractions, thus compromising the interpretation of the results and the performance for diagnosis accuracy. In this way, we were able to demonstrate that acute VTE patients had higher RDW than controls.

Moreover, we showed that if we focus on a threshold of $RDW \geq 14\%$, previously explored by other investigators as a diagnostic marker for VTE, we can expect a poor accuracy for acute VTE aggregate, but it revealed a good accuracy for isolated DVT diagnosis in the ED.

In this context, I believe that RDW, which is a parameter that we already have available when asking for blood counting in our daily routine, may be integrated in future diagnostic algorithms for acute DVT without any additional cost or resources. Furthermore, the 100% specificity for both isolated PE and DVT suggests that for each patient presenting to the ED with symptoms/signs suspected to derive from acute VTE that reveals a $RDW < 14\%$ should be thoroughly investigated for other diagnosis, and spared any VTE diagnostic CTPA and ultrasound imaging.

These findings might not be that relevant in the daily practice because D-dimer occupies this step in the acute VTE diagnosis, with similar specificities and sensitivities among acute VTE populations. However, RDW does not imply additional costs as it is

available at hematologic counts routinely performed, unlike D-dimer testing, which could be an advantage to be considered in future guidelines.

In an exploratory approach, we raised the possibility that acute VTE patients and non-acute VTE patients could have distinct metabolomic signatures that could help to differentiate them.

For this aim, we analysed and compared the plasma and the RBCs of acute VTE (50 patients) and controls (12 patients) with metabolomic assays, to find significant differences that could separate them at the moment of the admission to the ED. The laboratory assays were performed in previously prepared samples, that were treated with the same protocols and by the same staff members of INSA laboratory. There was laborious testing along the process of metabolite extraction from RBCs and plasma samples that strictly respected rigorous quality criteria, and that I partially had the opportunity to watch. I think this strict process of sample preparation was a major determinant of the quality of the final data extraction, as it must have assured the correct selection of all metabolites in all the samples while avoiding undesirable contaminants and biologic processes setbacks that are fundamental to the final dataset and, consequently, to the conclusion withdrawn from their analysis. The laboratory metabolomic assays occurred in Sharjah Institute for Medical Research, at United Arab Emirates, according to international certificated practices that allowed the meticulous collection of data to analyse.

In the following step, data were submitted to multiple statistical analysis based on the open software MetaboAnalyst5.0, in which I had the opportunity to actively participate. This proximal collaboration contributed to some adjustments to the first statistical analysis and to investigate new questions generated by the ongoing process of statistical analysis, which I believe that contributed to a better understanding of the outstanding results and to an approach to the analysis that aimed to fill the gaps of the real medical practice.

We identified and quantified a total of 112 metabolites in plasma and 68 metabolites in RBCs, limited by the technical methods used, considering a potentially range of some hundreds of metabolites identified/quantified for larger spectrum methods.

We identified 24 differently abundant metabolites in the RBCs of acute VTE patients compared to non-acute VTE and after meticulous analysis for diagnostic performance we found three biologic meaningful metabolites with high performance for distinguishing acute VTE patients from non-acute VTE ones.

We believe this is a stunning discovery because it opens the door to targeted metabolomics approaches to validate the results, and to the research of easy-use technology-based measurements that can be adopted in the routine of healthcare facilities. RBCs have revealed a highly discriminatory signature in acute VTE, which is a surprising result due to the absence of any previous metabolome investigation of

RBCs from this disease and to the assertiveness of the statistical results for their diagnostic accuracy. These three metabolites, namely adenosine 3',5'-diphosphate, glutathione and adenine were determinant for the metabolic pathways that resulted in significant differences between patients and controls, particularly purine metabolism, glutathione metabolism, aminoacyl-tRNA biosynthesis, and beta- alanine metabolism.

Independent from the biologic processes that underpin these changes, I think that these metabolites merit further investigation in the context of acute VTE, including their behaviour in larger, multicentre populations, across all times of the day/night, and by using measurement technologies that may be easily accessible in the hospital setting. Should these results be reproduced and validated, as we expect they are for all the rigorous steps of our investigation that led to these findings, here might be part of the answer to a new 'troponin for acute VTE' paradigm.

Plasmatic tests are more compelling, generally, because of the easiest sampling handling and stability, and more widespread and developed laboratory machinery. However, and against our initial expectations, plasmatic signature for acute VTE showed more fragile results in our study. Effectively, although we found 23 metabolites significantly different between acute VTE patients and controls, the analysis of their diagnostic performance resulted in less than good discriminative power. That means that plasma reflect differences in acute VTE patients, but these may not be sufficient to be a sole discriminative feature from patients without acute VTE.

Nonetheless, there was a tendency to a signature of acute VTE in plasma and the metabolic pathway of d-glutamine and d-glutamate was found to have the strongest impact on the acute VTE phenotype. I can hypothesise that if one uses a metabolic assay that allows to identify and quantify a larger number of metabolites, or a targeted approach to plasmatic metabolites involved in of d-glutamine and d-glutamate metabolic ways, there may be still room for the surgency of new potential diagnostic biomarkers in plasma.

A compelling discussion of the results in the perspective of the biologic systems and processes involved in acute PE and DVT, reaches some hypothesis that may be in the basis of some other new research.

Firstly, the functional role of RBCs in human health and disease is obscure apart from the commonly accepted relevance in oxygen transportation and delivery to tissues, and parallel carbon dioxide elimination. As we all think of venous clots as platelet enriched, it is a rather different viewpoint to centre attentions at RBCs in venous thrombotic diseases. Our important findings of RBCs metabolome in acute VTE may not only support new exploratory pathologic processes occurring during the early days of acute venous thrombus formation, but also begin new approaches to biomarkers discovery and explore unprecedented laboratory tests that could reach physician's practice.

Secondly, inflammatory related metabolites differently expressed in acute VTE patients are consistent of previous studies on immune-thrombosis and reinforce the need of more knowledge on this cascade of events, probably years-long, to eventually identify healthy individuals with an high risk for VTE episodes and find effective preventive measures to change the unacceptable high acute VTE prevalence.

Finally, the paradigm of venous thrombosis and arterial thrombosis as completely different diseases may be challenged in the near future. As with our results, other investigators have found similar biologic processes in arterial and venous thrombosis, raising coherent hypothesis of a common pathogenesis. Omics, to my belief, will guarantee new data on this subject and, eventually, contribute to new etiology fundamentals and, ultimately, new diagnosis and treatment targets.

As a final remark, and after these discussions on the work done, I would like to reflect on the work to be done. To be honest, I always believed since the beginning of the project for this thesis, that I would reach any pertinent conclusions. Being an attentive spectator of the scientific literature on PE along these last 20 years and having spent some thousands of hospital working shifts dealing with critical care and emergency patients, I had the conviction that patients with acute PE are very different from one another, and sometimes not that different from other unhealthy patients suffering from unrelated diseases. This was my main concern when decided to investigate the subject of acute VTE, that I tried to gather the most homogenous population and that was an accurate reflexion of the daily practice realities.

I strongly believe that the biobank resultant from the process of patient selection is a unique resource for present and future ongoing research in acute VTE. As such, we already discussed within the present project team the possibilities for next steps into our mission of contributing to the discovery of a 'troponin for acute VTE'. Considering some relevant limitations, of which funding may well be the most impacting, we are beginning to explore the already available methods of quantification of the RBCs metabolites with the highest diagnostic performance in our study, to design a new study protocol to their validation. The most defying aspect of this further step would be the enrolment of multi-center patients, not only because of the costs involved, but also for the challenge in terms of sample collection and preparation homogeneity, crucial to assure that the metabolites isolated are a consequence of the true pathologic processes and not an artifact deriving from defective laboratory protocols.

By way of conclusion, my overall research goal would be to establish a reliable and accurate panel of biomarkers to diagnose early acute venous thromboembolic diseases, especially pulmonary embolism, and to integrate it in the widely accepted diagnosis algorithm. When achieved, this example of precision medicine tool will certainly help in clinical decision making in the face of a possible acute episode, in the timely initiation of disease-specific treatments and its non-initiation in other diseases'

patients that will be prevented from serious adverse events, and an individualized approach to the patient management, according to the clinical severity profile of each patient.

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Appendices

Approval from Ethics Committee of HBA



Exma. Senhora

**Dra. Cláudia Mafalda Pedroso Moita de
Macedo Febra**

Médica Coordenadora de Novas Áreas
Clínicas no Hospital da Luz, Lisboa

Loures, 17 de julho de 2020

N/Ref.º 3307/2020_MJHNO

Estudo HBA n.º 421

Correio eletrónico e PMP

Assunto: *"Proteomic discovery of new biomarkers for pulmonary embolism and deep vein thrombosis."*

Exma. Senhora Dra. Cláudia Febra,

No seguimento da submissão a este Hospital do estudo melhor identificado em epígrafe, no qual V. Exa. participa na qualidade de Investigador Principal, temos o prazer de informar que a Comissão de Ética para a Saúde (CES) do HBA considera asseguradas as questões éticas relacionadas com a realização do estudo, pelo que deliberou a sua aprovação em reunião ordinária do dia 17 de julho do corrente ano.

Com os nossos melhores cumprimentos,

A Presidente da Comissão de Ética para a Saúde do HBA

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Laboratory protocol for blood sample separation

 Instituto Nacional de Saúde Doutor Ricardo Jorge		
Departamento de Genética Humana	Procedimento Específico	DGH
Lab Proteómica	Isolamento de fracções de sangue total (plasma buffy coat RBC)- Proteómica PROTOCOLO I	Página 1 de 3

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1. Objectivo e Âmbito

Isolamento de fracções de sangue total (plasma buffy coat RBC)- Proteómica .

2. Referências

Sprague, R. *et al.*, *Deformation-induced ATP release from red blood cells requires CFTR activity*, The American Physiological Society, 1998, **275** (5), H1726-32.

Dodge *et al.*, (1963) *The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes*, Arch Biochem Biophys., 1963, 100, 119-130.

3. Definições / Siglas

Eritrócitos – Células sanguíneas bicôncavas anucleadas constituídas, essencialmente, por globina e hemoglobina, cuja principal função é o transporte de oxigénio.

RBC – Red Blood Cells (Eritrócitos).

Buffy Coat - Fração correspondente aos leucócitos e plaquetas originada após centrifugação do sangue periférico sem tradução para português

Responsabilidades

Execução: Técnico de Análises Clínicas, Técnico Superior de Saúde ou Investigador.

Verificação: Técnico de Análises Clínicas, Técnico Superior de Saúde ou Investigador.

Gestão: Coordenador de laboratório, Unidade ou Sector.

Elaborado por : Fátima Vaz

Revisto por:
Data:

Aprovado por:
Data:



4. Reagentes/Soluções, Material e Equipamento

4.1 Reagentes/Soluções

Lixívia diluída 1:10 em H₂O (Tipo II)

Meio mineral 1x (Phosphate Buffer Saline, PBS)

4.2 Material

- ☐ Gelo
- ☐ Caixa de esferovite para transporte de amostras
- ☐ Luvas
- ☐ Papel das mãos
- ☐ Pipetas descartáveis
- ☐ Pipetas de Pasteur graduada de plástico
- ☐ Pontas com filtro (200 µL e 100 µL)
- ☐ Contentor imperfurável para pontas
- ☐ Rack para microtubos eppendorf e tubos de centrifuga falcon
- ☐ Tubos de centrifuga falcon de 15 mL
- ☐ Microtubos de centrifuga eppendorf 1,5 mL
- ☐ Tubos de colheita (EDTA) de 4.9 mL

4.3 Equipamento

- ☐ Centrífuga de bancada com rotor basculante refrigerada
- ☐ Centrífuga de bancada refrigerada, com rotor fixo e capacidade de atingir 27300 x g
- ☐ *Hotte* para protecção contra agentes biológicos
- ☐ Microcentrífuga refrigerada
- ☐ Pipetas descartáveis
- ☐ Pipetadores de 1000 µL, 200 µL

5. Condições de Manuseamento e Segurança

- ☐ De acordo com o Manual de Ambiente e Segurança do Departamento de Genética .
- ☐ Deverá utilizar bata, luvas de vinil ou nitrilo e o cabelo apanhado..
- ☐ Todo o procedimento é efectuado na *hotte*.
- ☐ Deverá ter o cuidado de limpar toda a área de trabalho antes e depois de iniciar qualquer procedimento com álcool a 95 % (v/v)

6. Metodologia

6.1 Preparação, conservação e estabilidade dos reagentes

Meio mineral 1x (Phosphate Buffer Saline, PBS)

Retirar 20 mL do meio mineral 10x e perfazer até 200ml de H₂O (tipo II)

Nota: estas quantidades foram efectuadas para uma primeira tentativa de tratar apenas 2 amostras



6.2 Modo de Proceder

6.2.1 Notas preliminares

Todos os dadores devem assinar um consentimento informado preparado para o efeito

- ☐ As amostras de sangue periférico deverão ser processadas no dia da colheita e no menor intervalo de tempo possível, uma vez que o material biológico de interesse para os procedimentos subsequentes inicia um processo de degradação contínua após a colheita de sangue.
- ☐ Após a colheita do sangue, os tubos devem ser colocados em gelo para manter a integridade das amostras e evitar a sua degradação.
- ☐ A amostra deve ser processada tão cedo quanto possível.
- ☐ Todos os desperdícios provenientes da manipulação do sangue e/ou dos seus derivados devem ser colocados numa solução de lixívia 10% em recipiente fechado e deixar a descontaminar durante 24 horas antes de serem eliminados.
- ☐ Todo o material perfurante e pontas de micropipetas deverão ser colocadas no contentor apropriado

6.2.2 Isolamento de fracções (plasma, buffy coat e RBC) a partir de sangue periférico

- ☐ Recolher 4,9 mL de sangue periférico para um tubo de EDTA e colocá-los imediatamente em gelo.
- ☐ Centrifugar nos próprios tubos de colheita por 10 min a 2000 g, 4 °C (numa centrifuga basculante de modo a se tornar mais fácil retirar os leucócitos).
- ☐ Retirar o sobrenadante (**plasma**) com uma pipeta de Pasteur, descartável com o cuidado de não contaminar com as restantes fracções. Se necessário deixar parte do plasma que está em contacto com o buffy coat .
- ☐ Aliquotar em tubos eppendorf de 1,5mL.
- ☐ Recolher o **buffy coat** para novo tubo de congelação
- ☐ Pipetar com pipetas Pasteur, o pellet correspondente à fracção dos **RBC**, para um novo tubo de centrifuga de 15 mL.
- ☐ Adicionar PBS 1x até perfazer o tubo e agitar o tubo por inversão suave da mão
- ☐ Centrifugar (10 min a 1800rpm, 4°C e desprezar e descartar)
- ☐ Lavar mais 2x com PBS 1x repetindo o passo anterior;
- ☐ O pellet obtido (RBCs lavados com ~2 mL) foi dividido em 2 do seguinte modo:
- ☐ - ~1.5 mL foram aliquotados em tubos eppendorf de 1.5 mL (500uL cada alíquota) e guardados a -80°C