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Novel diagnostic and prognostic methods for cancer and cancer-associated thrombosis

DOCTORAL THESIS

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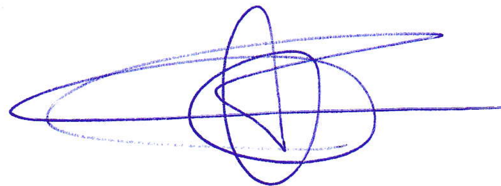
CERTIFIES:

That this Doctoral Thesis entitled:

"NOVEL DIAGNOSTIC AND PROGNOSTIC METHODS FOR CANCER AND CANCER-ASSOCIATED THROMBOSIS"

Has been developed under her supervision by the graduated with a bachelor's degree in Biochemistry and Biomedical Sciences, Julia Oto Martínez.

In witness whereof and for all relevant purposes, this certificate is issued at Valencia on December 13, 2021.



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A mis padres.

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ABSTRACT

Cancer is the second leading cause of death in Spain. Collaterally, venous thromboembolism (VTE), as a complication of cancer, consumes a great part of its healthcare budget and, more importantly, it is the second cause of death in these patients. However, limited tools are available to identify high risk patients. Additionally, a simple, minimally invasive and economical diagnostic methods for bladder cancer are also lacking. For that aim, harmful techniques are used like CT scan with high radiation exposure and invasive procedures like cystoscopy. Moreover, a hypercoagulable state seems directly related to a large tumor burden and poor prognosis. The overall aim of this Doctoral Thesis is to explore the clinical utility of novel diagnostic and prognostic methods for cancer and its thrombotic complications. In the first part of this Doctoral Thesis, we focused on the role of urine miRNAs as bladder cancer biomarkers. We identified miR-29c-3p as the most stable miRNA and was therefore used as normalizer. We adjusted an ordinal logistic regression model for the diagnosis and stratification of BC using the urine miRNA expression levels of patients and controls. This model included 7 miRNAs: miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p and miR-21-5p. In the second part of this Doctoral Thesis, we focused on the study of novel biomarkers for cancer-associated thrombosis. We analyzed the predictive potential of miRNAs and neutrophil activation markers of thrombotic events in patients with pancreatic cancer and patients with glioma and meningioma. In pancreatic cancer, we obtained a profile of 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) able to estimate the risk of potential VTE at diagnosis with targets involved in the *pancreatic cancer* and *complement and coagulation cascades* pathways. In the study of the neutrophil activation makers, we obtained a new predictive model of VTE with calprotectin as predictor. Regarding the study of cancer-associated thrombosis in intracranial tumors, in glioma patients, we adjusted and validated a predictive model for post-surgical pulmonary embolism (PE) with 6 miRNAs: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p, and another with cfDNA and myeloperoxidase as predictors. Furthermore, we combined both types of biomarkers and obtained an improved model using myeloperoxidase and miR-140-3p as predictors. In meningioma patients we fitted and validated a predictive model with 6 miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p. In conclusion, we

propose several profiles of biomarkers for the diagnosis of bladder cancer and for the identification of oncologic patients at high risk of suffering a thrombotic event.

RESUMEN

El cáncer constituye la segunda causa de muerte en España. El tromboembolismo venoso (TEV), una complicación del cáncer, conlleva gran gasto del presupuesto sanitario y representa la segunda causa de muerte en estos pacientes. Sin embargo, las herramientas actuales disponibles para la identificación de pacientes oncológicos con elevado riesgo trombótico son limitadas. Adicionalmente, no existen métodos simples, mínimamente invasivos y económicos de diagnóstico de cáncer vesical. Por este motivo, se utilizan técnicas dañinas como la tomografía computarizada la cual implica una elevada dosis de exposición a radiación y procedimientos invasivos como la cistoscopia. Además, un estado hipercoagulable parece tener una relación directa con una mayor carga tumoral y un peor pronóstico. El objetivo principal de la presente Tesis Doctoral es explorar la utilidad clínica de nuevos métodos diagnósticos y pronósticos para el cáncer y sus complicaciones trombóticas. En la primera parte de la Tesis, nos hemos centrado en el papel de miRNAs en orina como biomarcadores de cáncer vesical. Hemos identificado al miR-29c-3p como el miRNA más estable por lo que fue utilizado como normalizador. Hemos ajustado un modelo de regresión logística ordinal para el diagnóstico y estratificación de cáncer vesical utilizando la expresión de miRNAs en orina de pacientes y controles. Este modelo incluyó la expresión de 7 miRNAs: miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p y miR-21-5p. En la segunda parte de la Tesis, nos centramos en el estudio de nuevos biomarcadores para la trombosis asociada a cáncer. Analizamos el potencial predictivo de los miRNAs y de marcadores de activación de neutrófilos en pacientes con cáncer pancreático y pacientes con glioma y meningioma. En cáncer pancreático, obtuvimos un perfil de 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p y miR-103a-3p) capaz de estimar el riesgo de TEV al diagnóstico con dianas incluidas en las rutas *pancreatic cancer* y *complement and coagulation cascades.* En el estudio de los marcadores de activación de neutrófilos, obtuvimos un nuevo modelo predictivo de TEV con la calprotectina como variable predictora. Respecto al estudio de trombosis asociada a cáncer en tumores intracraneales, en pacientes con glioma, ajustamos y validamos un modelo predictivo de embolismo pulmonar (EP) postquirúrgico con 6 miRNAs: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p y miR-140-3p y otro con cfDNA y mieloperoxidasa como predictores. Además, hemos combinado los dos tipos de marcadores y hemos obtenido un modelo con mayor capacidad predictiva

que incluye a miR-140-3p y a la mieloperoxidasa como predictores. En pacientes con meningioma, ajustamos y validamos un modelo predictivo de EP postquirúrgico con 6 miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p y miR-23b-3p. En conclusión, proponemos diferentes perfiles de biomarcadores para el diagnóstico de cáncer de vejiga y para la identificación de pacientes oncológicos con elevado riesgo de trombosis.

RESUM

El càncer constitueix la segona causa de mort a Espanya. El tromboembolisme venós (TEV), una complicació del càncer, representa la segona causa de mort en aquests pacients i comporta una gran despesa sanitària. No obstant això, les eines disponibles actualment per a la identificació de pacients oncològics amb elevat risc trombòtic són limitades. Actualment, no existeixen mètodes diagnòstics per al càncer de bufeta senzills, mínimament invasius i econòmics. Per aquest motiu, s'utilitzen tècniques nocives com la tomografia computada la qual implica una elevada dosi d'exposició a radiació i procediments invasius com la cistoscòpia. A més, un estat hipercoagulable sembla tindre una relació directa amb una major càrrega tumoral i un pitjor pronòstic. L'objectiu principal de la present Tesi Doctoral fou explorar la utilitat clínica de nous mètodes diagnòstics i pronòstics per al càncer i les seues complicacions trombòtiques. En la primera part de la Tesi, ens hem centrat en el paper dels microRNAs (miRNAs) en orina com biomarcadors de càncer de bufeta. Hem identificat al miR-29c-3p com el miRNA més estable per la qual cosa va ser utilitzat com a normalitzador. Hem ajustat un model de regressió logística ordinal per al diagnòstic i estratificació de càncer de bufeta utilitzant l'expressió de miRNAs en orina de pacients i controls. Aquest model va incloure l'expressió de 7 miRNAs: miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p i miR-21-5p. En la segona part de la Tesi, ens centràrem en l'estudi de nous biomarcadors per a la trombosi associada a càncer. Analitzàrem el potencial predictiu dels miRNAs i de marcadors d'activació de neutròfils en pacients amb càncer pancreàtic i pacients amb glioma i meningioma. En càncer pancreàtic, vàrem obtindre un perfil de 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p i miR-103a-3p) capaç d'estimar el risc de TEV al diagnostic dels pacients els quals tenen dianes incloses en les rutes biològiques *pancreatic cancer* y *complement and coagulation cascades*. En el estudi dels marcadors d´activació de neutròfils, vàrem obtenir un altre model predictiu de TEV amb la calprotectina com a variable predictora. Respecte a l'estudi de trombosi associada a càncer en tumors intracranials, en pacients amb glioma, ajustàrem i validàrem un model predictiu d'embolisme pulmonar (EP) incidental postquirúrgic amb 6 miRNAs (miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p i miR-140-3p) i un altre amb cfDNA i mieloperoxidasa com a predictors. A més, vàrem combinar els dos tipus de marcadors i vàrem obtenir un model amb major capacitat predictiva que inclou al miR-140-3p i la

mieloperoxidasa com a predictors. En pacients amb meningioma, ajustàrem i validàrem un model predictiu d´EP incidental postquirúrgic amb 6 miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p i miR-23b-3p. En conclusió, proposem diferents perfils de biomarcadors per al diagnòstic de càncer de bufeta i per a la identificació de pacients oncològics amb elevat risc de trombosi.

LIST OF ABBREVIATIONS

3'UTR	3' untranslated region
Ago2	Argonaut protein
AT	Antithrombin
AUC	Area under the receiver operating characteristic curve
BC	Bladder cancer
CCDC25	Coiled-Coil Domain Containing 25
cfDNA	Cell-free DNA
CI	Confidence interval
CIS	Carcinoma *in situ*
DECC	Distal extrahepatic cholangiocarcinoma
DNA	Deoxyribonucleic acid
DVT	Deep vein thrombosis
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
ESA	Erythropoiesis-stimulating agents
F2	Gene that encodes for prothrombin
FDR	False discovery rate
FVL	Factor V Leiden mutation
GSTM1	Gene that encodes for glutathione S-transferase mu 1
H3Cit	Citrullinated histone 3
HMWK	High-molecular-weight kininogen
MIBC	Muscle-invasive bladder cancer
miRNA	microRNA
MPO	Myeloperoxidase
mRNA	Messenger RNA

MTHFR	Gene that encodes for methylenetetrahydrofolate reductase
NAT2	Gene that encodes for acetylator N-acetyltransferase 2
NETs	Neutrophil extracellular traps
NMIBC	Non muscle-invasive bladder cancer
nt	Nucleotide
OR	Odds ratio
PAD4	Peptidyl-arginine deaminase 4
PAI-1	Plasminogen activator inhibitor-1
PC	Protein C
PDAC	Pancreatic ductal adenocarcinoma
PE	Pulmonary embolism
PMA	Phorbol 12-myristate 13-acetate
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
PS	Protein S
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
RR	Relative risk
rRNA	Ribosomal RNA
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TNM	Tumor, Node, Metastases Classification of Malignant Tumors

tPA	Tissue plasminogen activator
uPA	Urokinase plasminogen activator
VTE	Venous thromboembolism
VWF	von Willebrand factor
ZPI	Protein Z-dependent protease inhibitor

1. INTRODUCTION

1.1. ASSOCIATION BETWEEN CANCER AND THROMBOSIS

Cancer is a devastating disease with high rates of prevalence and mortality. According to the latest data available from the GLOBOCAN study corresponding to the year 2018, 18.1 million people were diagnosed for the first time with cancer and 9.6 million people died from this disease [1].

Hemostasis is the compendium of processes that control blood fluidity and the integrity of the vascular system. It involves a large number of complex reactions and interactions between blood components and the vessel wall. All these mechanisms are essential for normal hemostasis, are balanced and interrelated. When this balance tips in favor of clotting, thrombosis occurs, while if it is unbalanced in favor of slowing down clot formation, hemorrhage can occur. In this Doctoral Thesis we will focus on thrombosis. Thrombosis is the formation of a blood clot within blood vessels that can occur in both the arterial and venous systems. Venous thrombosis can be of two types: on the one hand, deep vein thrombosis (DVT) occurs when the thrombus is located in a deep vein, generally in the lower limbs, although it can occur in other atypical locations such as upper limbs, portal system, retina, etc. On the other hand, pulmonary embolism (PE) occurs when this thrombus detaches and reaches the pulmonary circulation where it obstructs blood flow. Both events are included within venous thromboembolism (VTE), being PE the most serious pathological situation.

The incidence of VTE is 104 to 183 cases per 100,000 in habitants and has a high recurrence rate, since approximately 30% of patients suffer a recurrent thrombotic event in the first 10 years [2]. In addition, it is associated with a reduction in survival and with a high healthcare cost. VTE is a multifactorial disease that is caused by a combination of genetic risk factors (mutations in genes that encode proteins involved in the hemostatic system, mainly factor V Leiden *F5* c.1691G> A p.Arg506Gln, and the prothrombin mutation *F2* g.20210G> A) and environmental (such as cancer, surgery, pregnancy or puerperium, trauma, immobilization, etc.) [3].

Considering the risk of the different factors, Anderson FA and Spencer F classified the risk factors for VTE into high-risk factors (with an Odds Ratio [OR] ≥10), moderate-risk factors (OR between 2 and 9) and low-risk factors (OR<2). High-risk factors include leg or hip fracture, hip or knee replacement, major general surgery,

major trauma, and spinal cord injury. Moderate-risk factors are arthroscopic knee surgery, central venous catheter, chemotherapy, congestive heart failure or respiratory failure, hormone replacement therapy, cancer, oral contraceptives, stroke, puerperium, previous history of thromboembolism and thrombophilia. Finally, low-risk factors include bed rest for a period greater than 3 days, immobilization (long trips by car or plane, etc.), age, laparoscopic surgery, obesity, pregnancy and varicose veins [4].

Among the causes of death related to the cardiovascular system, VTE is the third leading cause preceded by acute coronary syndrome and cerebrovascular accidents, and is responsible for more than 3 million deaths per year worldwide [5]. Data from the Spanish National Institute of Statistics for 2018 indicate that cancer represents the second cause of death in Spain (26.35%) behind diseases of the circulatory system (28.26%). This fact highlights the severity of cancer-associated thrombosis, since both constitute the two main causes of death in our country.

Armand Trousseau first described the relationship between cancer and thrombophlebitis in 1865. He observed, very astutely and advanced of his time, a "particular condition" in the blood, which he considered could correspond to an "excess of fibrin, and an increase of white blood cells". He also speculated that this effect could be the cause of thrombosis and that this hypercoagulable state was evident in many other disorders [6]. Today, over 150 years after Trousseau´s studies, the association between cancer and thrombosis is a hot topic within the field of oncology and hemostasis.

Thus, thrombosis is the second most frequent cause of death in cancer patients due to their hypercoagulable state and a cancer-associated VTE is related to a lower survival in these patients [7]. The strong association between cancer and VTE means that approximately 20% of the new cases of VTE diagnosed are associated with cancer, often hidden, being VTE the first clinical manifestation [8]. In the largest study conducted to date, which includes 1,824,316 patients recruited from a total of 133 health centers, Khorana AA et al. [9] observed that 4.1% of cancer patients develop VTE, although the frequency of thrombosis differs according to the type of tumor, which will be discussed later. Therefore, there is a growing concern among oncologists regarding the early diagnosis of VTE in cancer patients, the management of primary thromboprophylaxis, and anticoagulant therapy once the thrombotic event has occurred to avoid both thrombotic recurrences and bleedings [10-12]. Despite the complexity of

cancer-associated thrombosis, there is a consensus regarding the main risk factors. Thus, Khorana AA and Connolly GC [13] described the main risk factors for cancer-associated thrombosis.

1.1.1. Cancer-related factors

1.1.1.1. Primary site of cancer

The primary tumor location is considered a risk of thrombosis in a wide variety of studies. Although the incidence of thrombotic events may vary between the different populations studied, the tumors types with a higher frequency of thrombosis are: pancreas (5.3-26%), stomach (6.8-13.6%), gynecological (2.8-25%), lung (1.8-13.6%) and brain tumors (1.6-26%) [13]. It should be noted that those tumors with the highest prevalence, even with a low frequency of VTE, can contribute significantly to the total percentage of cancer-associated thrombosis. For example, Paneesha S et al. [14] described prostate cancer as one of the four most common types of cancer (along with breast, colorectal and lung cancer) among a cohort of outpatients who had suffered VTE. Although this type of tumor does not present a high VTE risk compared to pancreas or head and neck tumors [OR pancreatic cancer=9.65, OR head and neck tumors=8.24 and OR prostate cancer =1.94] it must be taken into account in the global count of cancer-associated thrombosis due to its high prevalence.

1.1.1.2. Stage of cancer

Sallah S et al. [15] evidenced through a multivariate analysis that an advanced tumor stage (stages III and IV) is associated with a greater probability of suffering a thrombotic event [Relative Risk (RR): 3.2; 95% Confidence Interval (CI): 1.9-5.2]. However, an advanced stage may be related to a worse functional status of the patient. In fact, in outpatients with good performance status receiving chemotherapy, tumor stage is not considered a predictor of VTE [16].

1.1.1.3. Time span from cancer diagnosis to thrombotic event

In cancer patients, the risk of VTE is higher in the immediate period after tumor diagnosis. In the first 3 months after tumor diagnosis the OR for VTE is 53.5 (95% CI: 8.6-334.5), between the first 3 months and one year the OR is 14.3 (95% CI: 5.8-35.2), between the first year and the third it is 3.6 (95% CI: 2-6.5) and between the third and fifth year it is 3 (95% CI: 1.5-5.7). Finally, after 15 years, the risk of VTE decreases to an OR of 1.1 (95% CI: 0.6-2.2) [17].

1.1.2. Treatment-related factors

1.1.2.1. Cancer therapy

The use of chemotherapeutic agents is associated with an increased risk of VTE. Compared with the general population, patients undergoing chemotherapy treatment have a 6 -fold higher risk of VTE , while cancer patients without this type of treatment have a 4-fold higher risk [18].

1.1.2.2. Erythropoiesis-stimulating agents and transfusions

Cancer patients are frequently given erythropoiesis-stimulating agents for the treatment of anemia. In a systematic review where information was collected from 57 randomized trials, it was concluded that treatment with darbepoetin or epoetin, two erythropoietin-stimulating drugs, significantly increased the risk of VTE (RR=1.67, 95% CI: 1.35-2.06) [19]. Blood transfusions are commonly used as an alternative to erythropoiesis-stimulating agents in cancer patients with anemia. However, red blood cell and platelet transfusions are associated with an increased risk of venous and arterial thrombotic events and mortality in hospitalized cancer patients (OR=1.60, 95% CI: 1.53–1.67and OR=1.20, 95% CI: 1.11–1.29, respectively) [20].

1.1.2.3. Surgery and hospitalization

As previously described, surgery and its corresponding postoperative period are well known risk factors for VTE. Particularly, in cancer patients a high incidence of VTE has been observed as a consequence of surgery [21]. The risk of DVT and PE in cancer patients is 2- and 3-fold higher, respectively, when compared with non-cancer patients undergoing the same procedures [22]. Furthermore, hospitalization also increases the risk of VTE in cancer patients who have not undergone a surgical procedure (OR=2.34, 95% CI: 1.63-3.36) [23]. Epidemiological studies show that, before the use of thromboprophylaxis during hospital stays, 55 to 60% of VTE occurred in the hospital or in the first 90 days after hospital discharge [24].

1.1.2.4. Central venous catheters

The use of central venous catheters is vital in many aspects of cancer treatment, including intravenous drug administration and sampling. Its use, however, can trigger a thrombosis that can lead to the interruption of chemotherapy treatment, other intravenous medication, as well as cause other comorbidities such as PE and post-

phlebitic syndrome [21]. It is estimated that the incidence of DVT related to the use of catheters in cancer patients varies from 0.3% to 28%, while when analyzed by venography the incidence of DVT increases from 27% to 66% [25]. Regarding PE, an incidence of 15% to 25% has been evidenced in patients with cancer and upper limb DVT associated to the use of catheters, although the rate of PE increases to 50% when an autopsy is performed and PE was subclinical [25].

1.1.3. Patient-related factors

1.1.3.1. Comorbidities

The presence of comorbidities influences the risk of VTE in cancer patients. The comorbidities most related to the risk of VTE in hospitalized patients are kidney disease (OR=1.53, 95% CI: 1.49-1.58), infection (OR=1.77, 95% CI: 73-1.81), arterial thrombosis (OR=1.45, 95% CI: 1.39-1.52) and anemia (OR=1.35, 95% CI: 1.32-1.39) [9].

1.1.3.2. Prior history of venous thromboembolism

A previous history of thrombosis is a risk factor for developing a cancer-associated thrombosis. In a study of cancer patients undergoing surgery, those who had previously suffered a thrombosis had a higher risk of developing another VTE (OR=5.98, 95% CI: 2.13-16.80) [22]. Likewise, previous arterial thrombotic events are also related to new thrombotic events (OR=1.45, 95% CI: 1.39-1.52) [9].

1.1.3.3. Prothrombotic mutations

The presence of mutations in the genes that encode for the proteins involved in coagulation may represent a risk factor for VTE in cancer patients. Pihusch R et al. [26] analyzed the presence of thrombophilic mutations:the factor V Leiden mutation (FVL) in the factor V gene (*F5*c.1691G> A p.Arg534Gln), g.20210G>A in the prothrombin gene (*F2*) and c.665C> T in the methylenetetrahydrofolate reductase (*MTHFR*) gene, in patients with gastrointestinal cancer and evaluated their association with the risk of VTE. Those patients with the FVL mutation had a greater predisposition to develop VTE (RR=4.4, 95% CI: 1.3-14.9).

1.1.3.4. Age, sex and race

In the general population, the incidence of VTE increases exponentially with age [27]. Similarly, in cancer patients, an increase in age is a risk factor for VTE. Thus,

hospitalized cancer patients over 65 years are more likely to suffer a thrombotic event compared to the same type of patients under 65 years (OR=1.1, 95% CI: 1.0-1.1) [9]. The association of gender and the risk of VTE, despite having been widely studied, remains a matter of debate since no solid differences have been demonstrated between men and women [28]. In cancer patients, female gender has been associated with an increased risk of VTE (OR=1.14, 95% CI: 1.12-1.16) [9]. However, other studies have concluded that, gender is not related to an increased likelihood of thrombotic events [21]. Therefore, additional studies are required to ascertain the potential association between sex and thrombotic risk in cancer patients. Regarding the effect of ethnicity as a risk factor for thrombosis, White RH evidenced a 2.4-4 times lower risk of VTE in Hispanics and Asians/Pacific Islanders compared to Caucasians and African-Americans [28]. Thus, Black patients have a higher risk of VTE (OR = 1.18, 95% CI: 1.15-1.22) than Caucasians. In contrast, Asians have a lower risk of VTE (OR=0.74, 95% CI: 0.68-0.80) than Caucasians [9].

1.1.3.5. Performance status and mobility

Immobilization, which leads to venous stasis, is one of the traditionally known risk factors for VTE. Also, the functional status of oncologic patients and mobility are closely related. In a prospective study, 26.3% of patients with lung cancer undergoing chemotherapy with poor functional status and, therefore, less mobility, suffered VTE while only 12.4% of patients with better functional status suffered these thrombotic complications [29].

1.2. RISK SCORES FORCANCER-ASSOCIATED THROMBOSIS

As previously described, thrombosis is one of the main causes of death in cancer patients [9]. However, current VTE risk stratification scores for cancer patients are not optimal and in subsequent studies where they have been applied in independent cohorts of patients, they have not been validated with the expected success. For this reason, it is vital to find novel biomarkers to identify those cancer patients with a high thrombotic risk in order to provide them an adequate thromboproxylaxis. For this reason, in this Doctoral Thesis we focused on identifying biomarkers capable of identifying those cancer patients who may suffer a thrombotic event during the follow-up of their disease.

The best known tool used as a clinical prediction model is the Khorana score [30], which aims to identify cancer outpatients with an increased risk of VTE during

chemotherapy treatment. This score takes into account the following variables: type of cancer (very high risk: stomach, pancreas; high risk: lung, lymphoma, gynecological, genitourinary excluding prostate; low risk: breast, colorectal, head and neck), platelet count ≥ $350x10^9$ /L, leukocyte count ≥ $11x10^9$ /L, anemia (hemoglobin < 10 g/dL) or use of erythropoiesis-stimulating agents and body mass index ≥ 35. The "Vienna Cancer and Thrombosis (CATS) Study" [31], proposes an extended model of the Khorana score incorporating two additional biomarkers as variables, soluble P-selectin (≥ 53.1 ng/mL) and D-dimers (≥ 1.44 µg/mL), considerably improving the prediction of VTE. In their study subjects the cumulative risk of VTE at 6 months in patients with the highest risk was 17.7% (95% CI: 11-27.8%) according to the Khorana score, and 35% (95% CI: 19.7-57%) according to their extended model.

The PROTECH score [32] adds the chemotherapy of choice (gemcitabine and cisplatin-based chemotherapy) to the Khorana score. The TiC-Onco score [33] is the only model proposed to date that includes genetic variables in addition to other clinical variables (body mass index ≥ 25, family history of VTE, location and stage of the tumor). Particularly, they analyze the following single nucleotide polymorphisms (SNPs): rs2232698 in *SERPINA10* gene that encodes for the Protein Z-dependent protease inhibitor (ZPI), rs6025 and rs4524 in*F5,* and rs5985 in *F13 A1* gene which encodes for the A subunit with catalytic function of FXIII. The COMPASS-CAT model [34] includes new variables such as the use of anthracycline or anti-hormonal therapy, the time since cancer diagnosis, use of central venous catheter, cardiovascular risk factors and comorbidities (previous history of peripheral arterial disease, previous stroke, coronary artery disease, hypertension, dyslipidemia, diabetes and obesity), platelet count ≥ $350x10^9$ /L, recent hospitalization due to acute medical pathology and vascular or lymphatic compression by the tumor. The ONKOTEV model [35] incorporates the following variables into the Khorana score: previous history of VTE, metastasis, and vascular or lymphatic compression by the tumor. The model proposed by Pabinger I et al. [36] takes into account the type of tumor [tumors with low/intermediate risk of VTE (breast, prostate) *vs.* high/very high (lung, colorectal, esophagus, kidney, lymphoma, bladder, uterus, ovary)] and the concentration of D-dimer. Table 1 details the clinical and laboratory variables included in the aforementioned scores.

Table 1. **Main predictive models of VTE risk in cancer patients.** Table adapted from *Consenso multidisciplinar SEMI-SEOM-SETH sobre trombosis asociada a cáncer, más allá de las prácticas clínicas*.

	Khorana	**Vienna-CATS**	**PROTECH**	**TiC-Onco**	**COMPASS-CAT**	**ONKOTEV**	**Pabinger et al**
Number of variables included	5	5	6	5	8	8	2
Biomarkers	No	Yes	No	Yes	No	No	Yes
Type of tumor	Yes	Yes	Yes	Yes	No	Yes	Yes
Pre-chemotherapy hemoglobin<10 g/dL or use of ESA	Yes	Yes	Yes	No	No	Yes	No
Pre-chemotherapy white blood cell count>$11x10^9$/L	Yes	Yes	Yes	No	No	Yes	No
Pre-chemotherapy platelet count ≥ $350x10^9$ /L	Yes	Yes	Yes	No	Yes	Yes	No
Body mass index ≥35	Yes	Yes	Yes	Yes	No	Yes	No
D-Dimer> 1.44 g/L	No	Yes	No	No	No	No	Yes
Soluble P-selectin >53.1 g/L	No	Yes	No	No	No	No	No
Gemcitabine/platinum-based chemotherapy	No	No	Yes	No	No	No	No
Genetic variants	No	No	No	Yes	No	No	No
Tumor stage	No	No	No	Yes	Yes	Yes	No
Personal history of VTE	No	No	No	No	Yes	Yes	No
Family history of VTE	No	No	No	Yes	No	No	No
Anthracycline or anti-hormonal therapy	No	No	No	No	Yes	No	No
Time since cancer diagnosis	No	No	No	No	Yes	No	No
Central venous catheter	No	No	No	No	Yes	No	No
Presence of cardiovascular risk factors*	No	No	No	No	Yes	No	No
Recent hospitalization for acute medical illness	No	No	No	No	Yes	No	No
Compression of vascular/lymphatic structures by tumor	No	No	No	No	No	Yes	No

*Personal history of peripheral artery disease, ischemic stroke, coronary artery disease, hypertension, hyperlipidemia, diabetes and obesity. ESA: erythropoiesis-stimulating agents; VTE: venous thromboembolism.

Despite representing a great advance in the stratification of the thrombotic risk in cancer patients, all the previously mentioned clinical scores have limitations [37-41] and current tools available for VTE risk prediction and monitoring are not adequate. Van Es N et al. [38] compared 3 scales (Khorana, Vienna, PROTECH) in 876 cancer patients. The ROC curves of the 3 scales analyzed had an area under the ROC curve (AUC) that ranged between 0.50 and 0.57, so none of them showed high predictive power. Tafur AJ et al. [37] analyzed 7,948 patients with cancer-associated thrombosis and determined that the Khorana scale does not predict VTE recurrence neither major bleeding nor mortality among this type of patients. Metcalf RL et al. [39] studied a cohort of 910 patients with gastroesophageal cancer and 1,299 patients with colorectal cancer in whom they observed an incidence of VTE of 9.7% and 8.9%, respectively. The Khorana score had a low sensitivity for thrombotic events in patients with colorectal cancer and did not discriminate between patients with low and high thrombotic risk, although it should be noted that these scales should only be used in the type of patients for which they were designed.

In addition to the aforementioned scales, Jara-Palomares L et al. [42] designed a diagnostic score for occult cancer in patients with a previous history of VTE. They followed-up a total of 5,863 VTE patients for two years, 444 of whom were eventually diagnosed with occult cancer. Thus, they proposed a hidden cancer risk model in these patients that includes the following clinical and laboratory variables: male sex, age over 70 years, suffering from chronic lung disease, anemia, platelet count $\geq 350x10^9/L$, recent surgery and previous history of TEV.

Scales have also been proposed to ascertain the risk of VTE recurrence in cancer patients, such as the Ottawa score [43]. This scale, proposed by Louzada ML et al., includes various clinical parameters with the aim of identifying cancer patients with a high risk of VTE recurrence during the first six months of anticoagulant treatment. This scale is dichotomous, classifying patients as high- and low-risk. The prothrombotic variables were female sex, lung cancer and previous thrombotic event, and the variables that lowered the risk were breast cancer and TNM=1 according to TMN classification of malignant tumors. Subsequently, these same authors proposed a modification of the Ottawa score in which they grouped patients with stages TNM=1+2 and TMN=3+4, classifying the patients as low-, intermediate- and moderate-risk. However, Alatri A et al. [40] studied a total of 11,123 patients with cancer and VTE to whom they applied the

modified Ottawa score and obtained a low specificity, sensitivity and positive predictive value, with an AUC = 0.58.

All of the aforementioned indicates that the available scores for predicting cancer-associated thrombosis must be refined or new predictive models for VTE in cancer patients must be developed, since the existing ones present serious limitations as evidenced by studies in which attempts have been made to validate these models in independent cohorts of patients. It is necessary to optimally stratify those patients who have a high risk of VTE to provide them an adequate thromboprophylaxis, as well as to identify those patients with a low risk of thrombosis in order to avoid unnecessary treatment and adverse bleedings.

1.3. BLADDER CANCER

Bladder cancer (BC) is one of the most common types of tumors in the world. In fact, BC represents 3% of all tumors in adults and is the most lethal urological tumor. According to the GLOBOCAN 2018 study, there are more than 540,000 BC new cases in the world and 200,000 deaths annually [1]. Regarding the pathological anatomy of the tumor, more than 90% of BC patients present a transitional cell carcinoma, 5% present squamous cell carcinoma and less than 2% present a bladder adenocarcinoma [44]. Furthermore, the incidence of BC is 3 to 4-fold higher in men than in women [45]. 70% of new BC diagnoses present non-muscle-invasive bladder cancer (NMIBC) [46], characterized by being a superficial type of tumor. The pathological stages that include are Ta (papillary, 70%), T1 (infiltration of the tumor in the lamina propria, 20%) and carcinoma *in situ* (CIS, 10%). Muscle-invasive bladder cancer (MIBC) include T2, T3, and T4 stages [44]. Figure 1 shows the different grades and stages of BC and their degree of invasion. Moreover, up to 80% of NMIBC patients relapse at 5 years and 30% of Ta patients progress to MIBC [44].

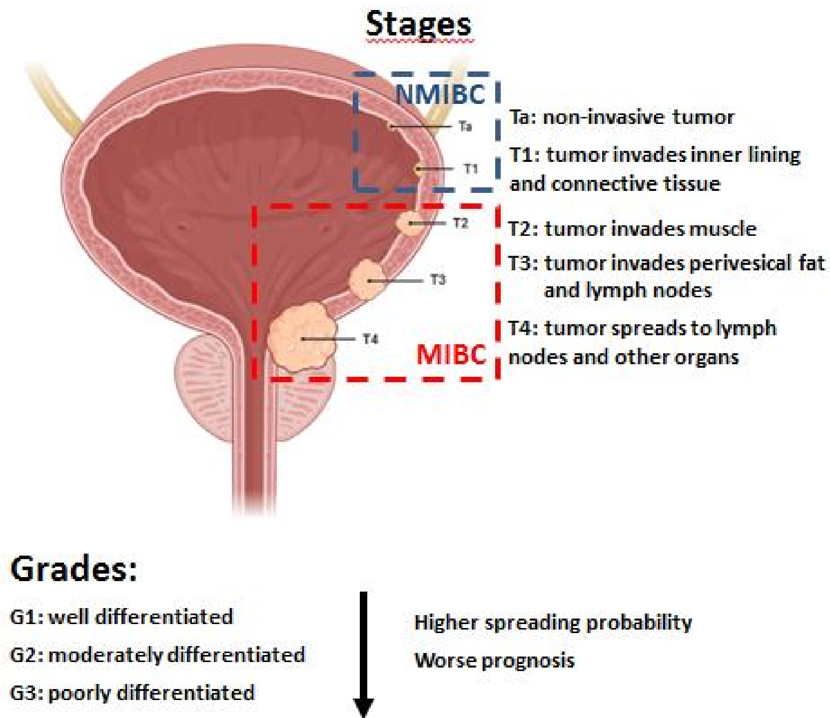

Figure 1. BC stages according to tumor location (non-muscle-invasive bladder cancer [NMIBC, stages Ta and T1] and muscle-invasive bladder cancer [MIBC, stages T2, T3 and T4]) and tumor grades (G1: well differentiated cells, G2: moderately differentiated cells and G3: poorly differentiated cells).

At present, cytology and cystoscopy are the most widely used methods for BC diagnosis and follow-up. However, cystoscopy is an invasive procedure that can lead to side effects such as urinary tract infections, bleeding, and pain. Cytology is a non-invasive procedure that tests the urine for tumor cells. It is a simple and cheap method, although it has a low sensitivity, especially in low-grade BC cases [44].

Risk factors for developing BC are classified into genetic and environmental. There is a great genetic predisposition to develop BC since the risk of first-degree relatives of patients is two-fold higher than those without relatives with BC. Inherited genetic factors for BC include the genetic slow acetylator N-acetyltransferase 2 (*NAT2*) variants and glutathione S-transferase mu 1 (*GSTM1*)–null genotypes, as well as mutations in oncogenes and widely related tumor suppressor genes such as *FGFR3*, *PIK3CA*, *RAS* and *TP53* [47, 48]. Regarding the environmental risk factors for BC, tobacco smoking is the one that confers the highest risk [49]. There is a direct pathophysiological relation between BC and smoking, since it contains aromatic amines such as b-naphthylamine

and polycyclic aromatic hydrocarbons, which are known to cause BC [47]. The second most important risk factor for BC is the occupational risk. In certain professions, workers are exposed to carcinogens such as aromatic amines, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons. These compounds are found in paints, dyes, metals and petroleum products, so the professions in which these substances are managed are exposed to this risk [47]. As in other types of cancer, BC is also influenced by dietary factors, such as the volume of fluid ingested, coffee, and the type of diet [47]. Environmental contamination is also a known risk factor for BC, like the presence of arsenic in drinking water [50]. There are also differences in the risk of BC according to sex, being more frequent in men than in women as indicated above, and socioeconomic level since a higher level of poverty has been related to a lower survival [47]. Although there are few studies on the impact of different ethnic groups on BC, a lower 5-year survival has been found in Black ethnic groups compared to Caucasians, Hispanics, and Asians/Pacific Islanders (70.2%, 82.8%, 80.7% and 81.9%, respectively) [51]. Finally, the presence of other disorders and exposure to ionizing radiation or pharmacological agents can predispose to BC tumorigenesis, such as chronic urinary retention, chronic inflammation, schistosomiasis or the use of oral hypoglycemic agents for the treatment of diabetes [47].

In addition, BC is the tumor that generates the highest economic cost in its treatment and among those with the highest monitoring cost. In fact, the cost of MIBC is estimated at $150,000 per patient [52, 53] and the management of BC is expected to represent more 3% of cancer-related healthcare costs [52]. In 2012 in the European Union, BC represented a cost of 4.9 billion euros, 5% of the total health expenditure related to cancer [54].

In cancer patients, an early diagnosis of the disease is directly related to a longer survival. Although important advances in methods for cancer diagnosis have occurred, reliable and non-invasive markers for screening, diagnosis, prognosis or follow-up have not yet been discovered. Thus, diagnostic and monitoring biomarkers for BC are in urgent need.

1.4. PANCREATIC CANCER

Pancreatic cancer accounts for 2.5% of new cancer diagnoses and is the seventh most deadly, accounting for 4.5% of deaths among cancer patients. According to the GLOBOCAN 2018 study, there are more than 450,000 new cases of pancreatic cancer in the world and 430,000 deaths annually [1, 55]. Pancreatic cancer presents complex and multifactorial risk factors, such as alcohol consumption, obesity, diet, occupational exposure, sex, age, ethnicity, other comorbidities, genetic factors, blood group, tobacco and previous family history, although the last two are the most predominant [56].

Pancreatic cancer has the highest incidence of VTE in hospitalized cancer patients (4.3%) [57]. According to recent studies, the prevalence of VTE in patients with pancreatic cancer ranges from 12 to 36%, and the most common thromboembolic manifestations are DVT, thrombophlebitis migrans and PE [58]. Pancreatic cancer patients who suffer a VTE have a lower overall survival (median 5.8 months) than patients who do not develop VTE (10.3 months) (HR=1.45, 95% CI: 1.03-2, 04), as well as a lower progression-free survival (HR=1.82, 95% CI: 1.30-2.54). These two types of survival rates are even lower in those patients who suffer VTE during chemotherapy (HR=3.04; 95 %CI: 2.12-4.36 and HR=1.95; 95% CI: 1.32-2.87, respectively) [59].

1.5. INTRACRANIAL TUMORS: GLIOMA AND MENINGIOMA

Central nervous system tumors represent 1.6% of new cancer diagnoses and are responsible for 2.5% of deaths in cancer patients. According to the GLOBOCAN 2018 study, there are more than 290,000 new cases of these tumors in the world and 240,000 deaths annually [1, 55].

Meningiomas are the most common brain tumors in adults, accounting for 36.1% of all adult brain tumors according to the Central Brain Tumor Registry of the United States (CBTRUS) [60]. The incidence of this tumor is difficult to estimate, since it is usually asymptomatic and the diagnosis occurs incidentally. The incidence of meningioma in the United States is estimated at 7.61 cases per 100,000 inhabitants. The incidence of meningioma increases with age, going from an incidence in the population aged 55 to 64 years of 15 cases per 100,000 inhabitants to 35 cases per 100,000 inhabitants in the population aged 75 to 84 years. This tumor is twice more prevalent in women than in men [61] and 20% more frequent in Black ethnicity than in Caucasians

[62]. 80% of meningiomas are benign (grade I), 5 to 20% are classified as atypical (grade II) and 1 to 3% are malignant (grade III) [62]. However, the 5-year survival of meningioma patients is less than 70% and decreases with age. In cases where the benign tumor has been completely removed, the 5-year recurrence is 20% [61].

Glioma is the second most common type of brain tumor in adults, representing 24% of all intracranial tumors [62] and 81% of malignant brain and central nervous system tumors [60]. The incidence of glioma is 4.7 cases per 100,000 inhabitants, being slightly higher in women than in men (4.9 versus 4.5cases per 100,000 inhabitants) [63]. The histology in this type of tumor varies widely from benign grade I tumor (pilocytic astrocytoma) to locally aggressive grade IV tumor with a high risk of recurrence and progression (glioblastoma). Thus, the survival of glioma patients depends on the histology of the tumor. Patients with pilocytic astrocytoma have a 10-year survival greater than 90%, while the 5-year survival of patients with glioblastoma is 5% [62].

Patients with intracranial tumors are at higher risk of experiencing a thrombotic event compared to those with tumors in other locations [64]. In the cohort studied by Stein PD et al. [57] comprising more than 40,000,000 hospitalized cancer patients, brain tumors were the second most thrombogenic, only preceded by pancreatic cancer (3.5% and 4.3% VTE, respectively). In other studies, the incidence of VTE in patients with glioma and meningioma is higher (24% and 29.8%, respectively) [65, 66].

1.6. NOVEL BIOMARKERS OF CANCER-ASSOCIATED THROMBOSIS

1.6.1. microRNAs

microRNAs (miRNAs) are small single-stranded noncoding RNAs that regulate the translation of their target messenger RNAs (mRNAs) by binding to their 3' untranslated region (3'UTR) and preventing their translation [67]. miRNAs were discovered in 1993 by Lee RC et al. [68] in the nematode *Caenorhabditis elegans* (*C. elegans*). In these organisms, down-regulation of the LIN-14 protein is essential for progression from the first larval stage to the second and was found to be mediated by miRNAs. In 2000, two separate groups discovered that a small RNA, let-7, was essential for the development of a later larval stage to adult in *C. elegans* [69, 70].This novel method of regulating gene expression was initially thought to be a phenomenon unique to *C. elegans*, but homologues of let-7 have been discovered in many other organisms, including humans [71]. Since then, the study of miRNAs has been extended in numerous

pathophysiological processes. These noncoding RNAs of approximately 20 to 25 nucleotides (nt) in length, are derived from a larger hairpin-shaped precursor [67]. The miRBase database is the main online repository for potential miRNA sequences, their annotation, nomenclature and target prediction [72]. According to its latest version (v.22.1), a total of 38,589 miRNAs have been described, of which 2,693 mature miRNAs are present in humans.

Next, we will describe the canonical synthesis pathway for miRNAs. The biogenesis of miRNAs starts in the nucleus, where RNA polymerase II or III produces the transcription of its precursor, primary miRNA or pri-miRNA [73]. This pri-miRNA, normally composed of hundreds of nts processed in the cell nucleus by the microprocessor composed of the enzymes DROSHA and DCGR8 [74-76]. The microprocessor modifies its structure until obtaining a new structure of approximately 70 nt, the precursor or pre-miRNA. The pre-miRNA is exported from the nucleus by Exportin-5-Ran-GTP [77]. Once in the cytoplasm, RNase Dicer cleaves the pre-miRNA to its final mature length, about 20-25 nt. The functional chain of the mature miRNA is incorporated together with the Argonaut protein (Ago2) in the RNA-induced silencing complex (RISC), where it guides the RISC complex to silence its target mRNA [78] (Figure 2).

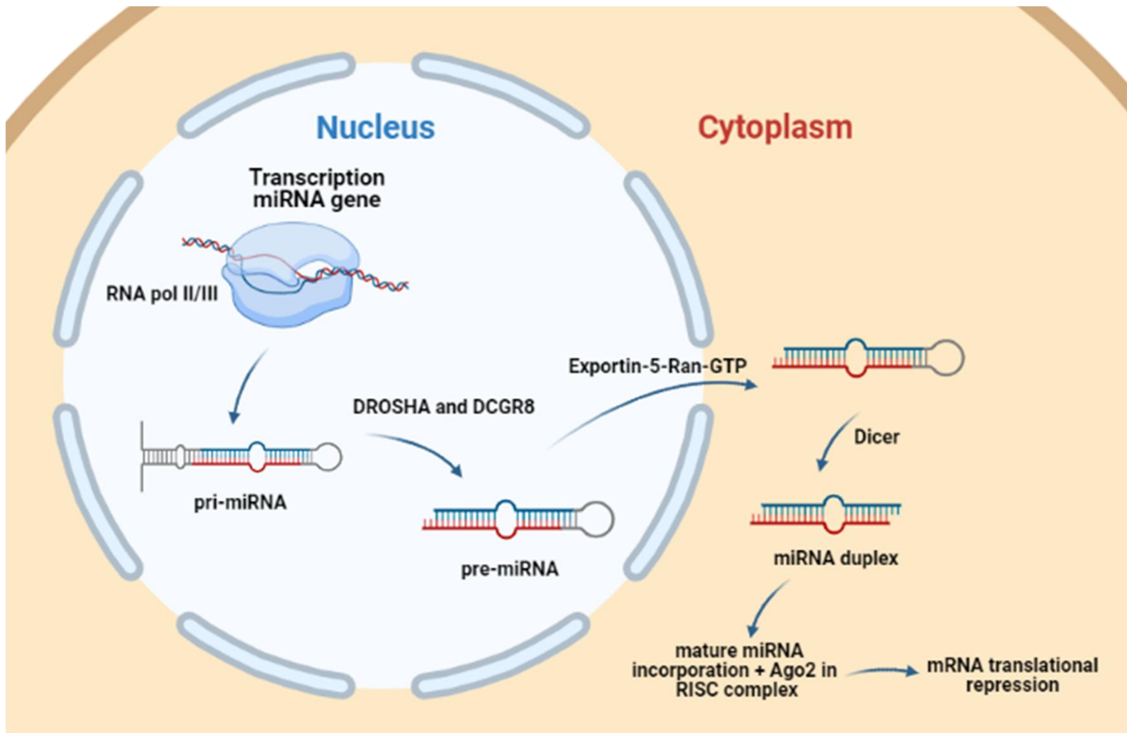

Figure 2. Biogenesis of miRNAs. miRNA biogenesis strats in the nucleus, RNA pol II/III transcribes a transcript known as primary miRNA (pri-miRNA). pri-miRNA is processed by DROSHA, and its cofactor, DGCR into smaller structures known as precursor miRNAs (pre-miRNA). Pre-miRNAs are transported out of the nucleus by Exportin 5 into the cytosol where RNase Dicer cleaves the pre-miRNA to its final mature length, about 20-25 nt, (mature miRNA). The functional chain of the mature miRNA is incorporated together with the Ago2 in the RNA-induced silencing complex (RISC), where it guides the RISC complex to silence its target mRNA. The seed-sequence of a mature miRNA binds to complementary sequences primarily located within 3'-UTRs of mRNAs resulting in post-transcriptional gene silencing.

miRNAs can control numerous biological processes, since they might be able to regulate 30 to 80% of human genes [79]. miRNAs are present in exosomes and microparticles, which are released into the extracellular space from many cell types, including tumor cells [80]. miRNAs have been postulated as excellent biomarkers due to their high stability [81] and can be found in many biological fluids, such as plasma, urine, saliva, tears, etc. [82]. Due to these characteristics, they have been proposed as biomarkers for a multitude of diseases, including cancer or thrombosis [83-86].

However, despite the numerous studies that assess the usefulness of miRNAs as biomarkers in cancer, discrepancies arise among studies for each tumor type, possibly due to the lack of standardization between the protocols used in the different laboratories. In particular, these results are not reproducible among publications due to

several factors such as the criteria in the selection of patients and controls, the use of different procedures in sample processing, the isolation of RNA, the quantification of miRNAs and the use different normalization strategies. Specifically, normalization is one of the crucial steps in miRNA studies. Various types of RNAs have been proposed as normalizers in miRNA studies: small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), miRNAs or even exogenous synthetic RNAs. However, some of these proposed normalizers differ from miRNAs in their structure and size (150 nt for snRNAs and 60–200 nt for snoRNAs, compared to 20–25 nt for miRNAs) [87]. Differences in structure and length among species of RNAs can affect the efficiency of isolation, and the processes of reverse transcription and the amplification during quantitative reverse transcription polymerase chain reaction (RT-qPCR). For this reason, the use of miRNAs stably expressed among the different study groups represents the best normalization option compared to the use of other types of RNA molecules [88]. The use of exogenous synthetic RNAs is very useful to monitor the efficiency of RNA isolation and reverse transcription, but not to normalize amplification in qPCR. To date, there is no consensus on the best normalizing miRNA for the study of the tumor types explored in this Doctoral Thesis, namely BC, pancreatic cancer, glioma and meningioma, in biofluids. The identification of miRNA normalizers is a requirement for the transfer of miRNA analysis to clinical practice.

Unlike cancer, scarce studies explore the role of miRNAs in VTE. It is known that the expression of the proteins involved in the coagulation cascade is regulated by the expression of different miRNAs. Li S et al. [89] performed an *in silico* analysis in which they determined that miR-223 had tissue factor (TF), the initiator of the extrinsic coagulation pathway, as a possible target. Through gain and loss of function assays in endothelial cells by transfecting of miRNA mimics and inhibitors, respectively, they confirmed this regulation. The review of Morelli VM et al. [86], describe how miRNAs play a complex role in the regulation of hemostasis. Figure 3 shows how miRNAs are capable of regulating different molecules throughout the coagulation cascade, both in the extrinsic and intrinsic pathways, as well as natural anticoagulants.

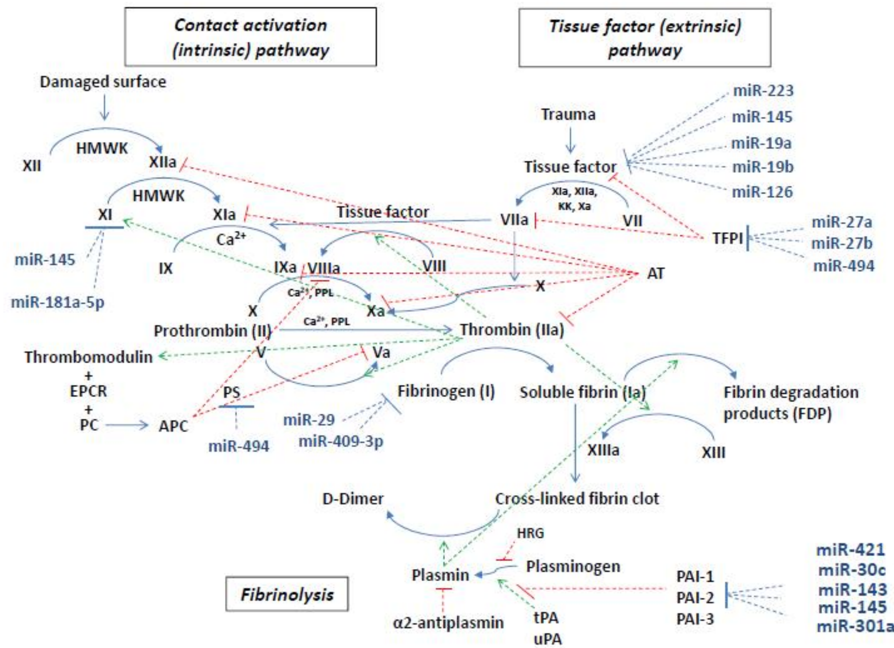

Figure 3. An overview of blood coagulation and fibrinolysis pathways and the miRNAs reported to regulate key hemostatic factors. Adapted from Morelli VM et al. [86]. HMWK: High-molecular-weight kininogen; AT: antithrombin; EPCR: endothelial protein C receptor; PC: protein C; PS: protein S; PAI-1: plasminogen activator inhibitor-1; TFPI: tissue factor pathway inhibitor; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator.

In addition to the different studies focused on the regulatory mechanisms of VTE, miRNAs have also been proposed as non-invasive biomarkers of this disease. Starikova I et al. [90] studied the expression level of 742 miRNAs in 20 patients with a previous history of VTE and 20 healthy controls matched by age and sex. They found an increase in the plasma levels of miR-10b-5p, miR-320a/b, miR-424-5p and miR-423-5p and a decrease in miR-103a-3p, miR-191-5p, miR-301a -3p and miR-199b-3p in patients compared to controls. Wang X et al. [91] analyzed the plasma levels of 179 miRNAs in 39 patients with recurrent VTE and 39 patients with a single episode of VTE. The expression of miR-15b-5p, miR-222-3p, miR-26b-5p, miR-532-5p, miR-21-5p and miR-30c-5p was increased and the expression of miR-106a-5p, miR-197-3p, miR-652-3p, miR-361-5p, miR-27b-3p and miR-103a-3p was decreased in the group of patients with recurrence. These 12 dysregulated miRNAs may arise as new non-invasive biomarkers of recurrent VTE.

However, there are hardly any studies that explore the role of miRNAs in cancer-associated thrombosis. In fact, to date, only 3 studies have been published in which miRNAs are proposed as predictors of thrombotic events in cancer patients, 2 of which are part of this Doctoral Thesis (*MicroRNAs and Neutrophil Activation Markers Predict Venous Thrombosis in Pancreatic Ductal Adenocarcinoma and Distal Extrahepatic Cholangiocarcinoma* and *microRNAs and Markers of Neutrophil Activation as Predictors of Early Incidental Post-Surgical Pulmonary Embolism in Patients with Intracranial Tumors*). The third work, a congress abstract, was conducted by Kim A et al. [92] in which they analyzed the plasma levels of 2,426 miRNAs in 21 patients with colorectal cancer. They compared 7 patients who suffered VTE in the first 6 months after cancer diagnosis with 14 patients who did not suffer any thrombotic event, as a control group. They identified a profile of 9 under-expressed miRNAs in patients compared to controls: miR-4451, miR-942-3p, miR-8063, miR-3132, miR-3118, miR-105-5p, miR-891a-5p, miR-200a-5p and miR-6832-3p. They also identified 609 target genes for the dysregulated miRNAs, which participate in important biological and signaling processes in cancer. Despite being a pilot study, these data suggest that colorectal cancer patients who will develop VTE express a specific profile of miRNAs, so they may be very useful as predictive biomarkers.

1.6.2. Neutrophil activation markers

Neutrophils are a type of polymorphonuclear leukocytes that play a main role in inflammatory processes. They are the first leukocytes to be recruited at sites where inflammatory processes are taking place and are capable of eliminating pathogens through various mechanisms: phagocytosis, degranulation of proteins with bactericidal properties contained in their granules and the formation of neutrophil extracellular traps (NETs) [93]. NETs are extracellular networks of deoxyribonucleic acid (DNA) and proteins, both cytoplasmic and contained in the granules (myeloperoxidase, MPO; calprotectin; elastase; among others), which are released by neutrophils in response to an inflammatory stimulus or in the presence of pathogens in a process called NETosis (Figure 4).

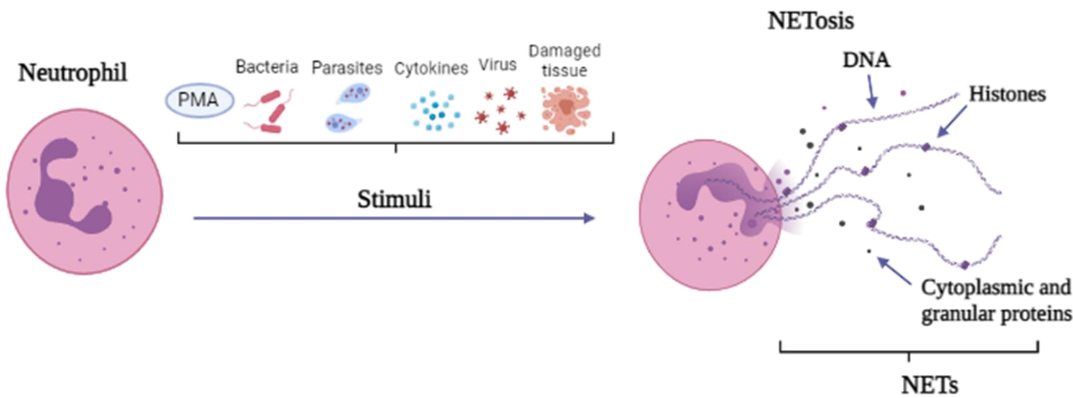

Figure 4. Upon activation, neutrophils play a prominent role in defense mechanisms by phagocytosis, degranulation and by neutrophil extracellular trap (NET) formation. NETs are extracellular networks of DNA, histones and granule and cytoplasmic proteins (calprotectin, myeloperoxidase, elastase, etc.) released by neutrophils in response to an inflammatory stimulus or to the presence of pathogens, in a process called NETosis.

NETs were discovered by Takei H et al. in 1996 [94] as a type of cell death other than apoptosis and necrosis. They studied the relation between activation and death of neutrophils using phorbol 12-myristate 13-acetate (PMA) and observed morphological changes quite different from those that take place during apoptosis or necrosis, which suggested an alternative form of cell death. First, fusion of the multilobed nucleus and decondensation of chromatin take place. The nuclear membrane ruptures while the cytoplasmic organelles remain intact. After 3 hours, the extracellular membrane ruptures due to a mechanism dependent on reactive oxygen species (ROS). In 2004, Brinkmann V et al. [95] further detailed the process and name it NETosis. The NETosis process is classified as lytic or suicidal NETosis and vital NETosis. The process first discovered by Takei H et al. [94] and subsequently outlined by Brinkmann V et al. [95], it is lytic NETosis, in which there is a rupture of the plasma membrane, a loss of the conventional functions of neutrophils (such as leukocyte recruitment, chemotaxis and phagocytosis) and the death of the cell. In vital NETosis, there is a secretion of the chromatin and the content of the granules without producing a rupture of the cell membrane of the neutrophil. The secreted components assemble extracellularly and the resulting neutrophil, now anucleated, maintains the ability to ingest microorganisms [96]. Leshner M and collaborators [97] studied the process of chromatin decondensation during the NETosis process and the role of the peptidyl-arginine deaminase 4 (PAD4). PAD4 converts arginine or monomethyl arginine from the N-terminus of histones H3 to citrullines. Citrullination of histone H3 is an essential process in the formation of NETs.

Thanks to this mechanism, there is a decrease in the positive charges of the histone residues, producing a weaker binding to the DNA, which is negatively charged, and leading to chromatin decondensation.

NETs have been related to a multitude of different pathologies, such as sepsis, psoriasis, systemic lupus erythematosus, rheumatoid arthritis, type I and type II diabetes mellitus, gout, obesity, thrombosis, etc. [98]. In relation to thrombosis, NETs form a scaffold capable of activating coagulation and are abundant structures in thrombi produced in animal models of DVT [99]. Fuchs T et al. [100] observed NETs that perfused with blood caused platelet adhesion, activation, and aggregation. NETs recruit red blood cells, promote fibrin deposition, and induce a red thrombus, such as that found in veins. NETs bind to important proteins that confer stability to the thrombus, such as von Willebrand factor (VWF), fibrin, and fibrinogen. They also evaluated the effect of heparin, a widely used anticoagulant, on the stability of NETs and observed that NETs were eliminated after perfusion with heparinized blood, and that heparin eliminated platelet aggregates previously attached to NETs as efficiently as DNAse, which digests DNA and therefore the scaffold that can be found in NETs. They also incubated pre-stimulated neutrophils to generate NETs with blood, in order to generate a thrombus composed of DNA and fibrin, and evaluate the effect of thrombolytic treatment. These thrombi were treated with DNase for the digestion of NETs, tissue plasminogen activator (tPA) for the digestion of fibrin, or both. They concluded that the thrombus formed in the presence of activated neutrophils can be completely eliminated using tPA and DNase simultaneously, highlighting the importance of NETs in the formation and stability of the thrombus and its applicability as a possible therapeutic target.

In the particular context of cancer, increased levels of plasma neutrophils [101, 102] and an increased neutrophil/lymphocyte ratio [103-106] have been associated with a worse prognosis in patients with a wide variety of tumors. Thalin C et al.[107] studied a cohort of 60 cancer patients, a cohort of 51 severely ill and hospitalized patients without known active or prior cancer diagnosis and 50 healthy controls. They analyzed plasma levels of citrullinated histone 3 (H3Cit), cell-free DNA (cfDNA), neutrophil elastase and MPO-DNA complexes as NETosis markers. Plasma levels of H3Cit were significantly increased in cancer patients compared to the two control groups. Furthermore, within the group of cancer patients, increased levels of H3Cit were

correlated with higher short-term mortality. Grilz E et al. [108] analyzed various markers of NETs in plasma (H3Cit, cfDNA and nucleosomes) in 957 cancer patients at the time of tumor diagnosis or at the diagnosis of tumor progression after remission. They observed that increased levels of cfDNA and H3Cit were associated with a higher mortality in cancer patients. Nie M et al.[109] studied NETs markers in patients with diffuse large B-cell lymphoma. They analyzed 93 plasma samples by enzyme-linked immunosorbent assay (ELISA) and 233 tissue samples (123 in the screening cohort and 110 in the validation cohort) by immunofluorescence. Those patients with increased plasma levels of MPO-DNA complexes had a worse progression-free survival and overall survival. In tissue, they observed a correlation between increased levels of NETs and a more advanced stage of the disease, greater number of symptoms and worse progression-free survival.

It should be noted that neutrophils induced by tumor cells produce more NETs than those activated by other pathways. In addition to increasing the thrombotic risk in cancer patients, NETs also seem to affect tumor biology by regulating tumor growth, angiogenesis and invasion, favoring metastasis [110]. However, the mechanisms by which NETs can promote metastasis are not well understood. Park J et al. [111] studied the role of NETs in metastasis in a murine model. They observed that metastatic breast cancer tumor cells can induce neutrophils to form NETs in the absence of infection. Furthermore, using intravital imaging techniques, they observed NET-like structures around the metastatic cells that colonized the lungs in mice. Furthermore, the formation of NETs stimulated the invasion and migration of tumor cells *in vitro*. Finally, they analyzed the effect of the inhibition of NETs formation or the digestion of NETs with DNase I and observed a reduction in lung metastasis, which would make NETs an interesting therapeutic strategy.

Recently, Yang L et al. [112] have identified a transmembrane protein present in tumor cells that is a receptor for NET´s DNA. Coiled-Coil Domain Containing 25 (CCDC25) can perceive the presence of extracellular DNA and initiates intracellular pathways that promote cell mobility and, therefore, metastasis. The authors propose CCDC25 as an interesting therapeutic target for the prevention of metastasis. In addition, they identified a high expression of NETs in liver metastases in patients with breast and colon cancer by immunofluorescence of MPO and H3Cit. Finally, they observed that increased levels of serum DNA-MPO complexes, another marker of NETs, can predict

the risk of liver metastasis in patients with breast cancer. To date, only one study has explored the ability of NETs to predict VTE in cancer patients [113]. In this work, Mauracher LM et al. analyzed plasma levels of H3Cit, cfDNA and nucleosomes in 957 cancer patients. They observed that an increase of 100 ng/ml in H3Cit levels was associated with a 13% increase in the thrombotic risk.

2. HYPOTHESIS

Early cancer diagnosis increases patient survival. Particularly in BC, there is a lack of minimally invasive techniques that achieve the reliability of cystoscopy for diagnosis, prognosis or monitoring. Cystoscopy, the gold-standard method of BC diagnosis, is a highly invasive technique and presents a high rate of false positives and negatives, especially in low-grade tumors. Therefore, it is essential to identify novel to improve BC diagnosis, an early detection of relapses and monitoring the response to treatment. For that aim, we explored liquid biopsy as a source of novel diagnostic biomarkers such as miRNAs. Likewise, it is essential to identify a normalizer with stable expression for miRNA studies in BC in order to standardize the methodology and thus facilitate the transfer of these studies to clinical practice.

Thrombosis is the second leading cause of death in cancer patients, increasing its morbidity, as well as health costs as a result of longer hospitalization and the use of additional anticoagulant therapy (overall estimation of $ 20,065/patient). Thus, the identification of a biomarker profile able to identify cancer patients with a higher thrombotic risk would be essential for an early prevention and a tailored thromboprophylaxis in these patients.

miRNAs regulate cancer progression and thrombosis; however, no previous studies had explored the role of miRNAs as biomarkers of cancer-associated thrombosis when this Doctoral Thesis was initiated.

In addition, NETs also promote coagulation and regulate tumorigenesis and metastasis. Accordingly, we aimed to explore neutrophil activation markers as plasma biomarkers for cancer diagnosis and staging and to identify cancer patients with a high thrombotic risk.

3. OBJETIVES

The overall objective of this Doctoral Thesis is to explore the clinical value of novel diagnostic and prognostic methods for cancer and its thrombotic complications. The specific objectives are:

1. To establish the best normalizer for urine miRNA studies in BC with the aim to standardize the inconsistencies among different studies.
2. To identify a new profile of non-invasive urine miRNAs for the diagnosis and stratification of BC patients.
3. To analyze the predictive potential of plasma miRNAs and neutrophil activation markers for thrombotic events in patients with two of the most prothrombotic cancer types, pancreatic cancer and patients with intracranial tumors, namely glioma and meningioma.

4. CHAPTER 1: Identification of miR-29c-3p as a Robust Normalizer for Urine microRNA Studies in Bladder Cancer

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Identification of miR-29c-3p as a Robust Normalizer for Urine microRNA Studies in Bladder Cancer

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Abstract: Bladder cancer (BC) is among the most frequent malignancies worldwide, being the most expensive cancer to treat and monitor and the most lethal urological cancer. Urine microRNAs (miRNAs) have been proposed as novel non-invasive biomarkers to early diagnose and monitor BC patients in order to avoid the performance of current aggressive diagnostic techniques. However, huge discrepancies arise among studies mainly due to the lack of standardization in the normalization, a crucial step in all miRNA studies. Our aim was to identify the best miRNA normalizer for miRNA studies in urine of BC patients. We evaluated the performance of 110 candidate miRNAs in urine of 35 BC patients and 15 healthy controls by Real Time quantitative Polymerase Chain Reaction (RT-qPCR) followed by a stability analysis with RefFinder. In this screening stage, miR-29c-3p arose as the most stably expressed miRNA in BC and controls, with a good expression level. Stability of miR-29c-3p expression was validated in an independent cohort of 153 BC patients and 57 controls. Finally, we evaluated the robustness of miR-29c-3p as normalizer in the expression study of miR-200c-3p, a potential diagnostic marker for BC. We propose miR-29c-3p

as a normalizer for miRNA studies in BC urine. This is the first study that characterizes a reliable normalizer that may allow the comparison of future urine miRNA studies as non-invasive biomarkers for BC diagnosis and monitoring.

Keywords: bladder cancer; urine; miRNA; reference miRNA; normalizer; RT-qPCR

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1. Introduction

Bladder cancer (BC) is among the most frequent malignancies worldwide. Indeed, BC accounts for 3% of all malignant tumors in adults and is the most lethal urological tumor type. According to the GLOBOCAN 2018 study, BC accounts for more than 540,000 new cases worldwide and almost 200,000 deaths annually [1,2]. Moreover, BC largely increases health expenses since it is the most expensive cancer to treat and the one that accounts for the highest monitoring costs. In fact, the cost of the muscle-invasive subtype reaches $150,000 per capita [3,4]and, by the end of the decade, BC is expected to account for >3% of all cancer-related medical expenses [3].

Bladder ultrasound, computerized tomography (CT) scan, cystoscopy or cytology are presently the gold standard techniques for BC diagnosis. However, cystoscopy is a highly invasive procedure that causes a high discomfort in patients and urine cytology, while being non-invasive, is unable to detect low-grade bladder tumors. Furthermore, none of both achieve a high sensitivity and specificity [5]. These last two techniques are routinely used as follow-up methods. As a consequence, novel non-invasive biomarkers are being explored to early and accurately diagnose and monitor BC patients in order to avoid the performance of this aggressive technique while reaching or even exceeding the sensitivity and specificity of cystoscopy [6]. Urine represents the most accessible source of biological markers for the analysis of BC and other urological tumors since it is rapid and easily obtained by the patient, avoids patient discomfort and potential complications from an invasive procedure, is available in copious amounts and re-sampling is easily achievable [7]. Thus, urine represents the ideal sample to develop novel tools to diagnose and monitor the prognosis of BC patients.

microRNAs (miRNAs) are small non-coding RNAs that regulate protein expression. They have been found to play a role in the different processes of tumor development [8,9] and have been proposed as regulatory molecules and biomarkers in virtually all cancer types [10–12]. miRNAs are known to be released from tissues into

biological fluids such as urine and have been proposed as biomarkers for many disorders. Indeed, several profiles of miRNAs have been proposed as diagnostic and prognostic tools for BC [13–24]. However, strikingly only a small number of miRNAs are shared by these studies, what is probably due to the lack of standardization in the protocols used among laboratories. Particularly results are often not reproducible among publications because of different criteria used for selecting patients and controls, the use of different procedures for sample processing, RNA isolation and miRNA quantification but, mainly, it may be caused by different normalization strategies used, what certainly represents a crucial step in any miRNA study. Several RNA species have been proposed as normalizers for miRNAs studies: small nuclear RNAs (snRNAs), nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), miRNAs or even exogenous synthetic RNAs. However, some of these proposed normalizers differ from miRNAs in length (150 nt for snRNAs and 60–200 nt for snoRNAs, compared to 20–24 nt for miRNAs) and also in structure [25]. This disparity in lengths and structure can affect the isolation efficiency, reverse transcription and amplification. Consequently, the use of stably expressed miRNAs for the normalization of miRNAs studies is the best option rather than the use of other small RNA species [26]. Likewise, the addition of exogenous synthetic RNAs is intended to track the isolation and reverse transcription efficiency, not to normalize for amplification. To date, no consensus exists on internal reference miRNAs for BC studies performed in urine samples by Real Time quantitative Polymerase Chain Reaction (RT-qPCR)

In the present study, we aimed for the first time to ascertain the best miRNA normalizer for miRNA studies in BC performed in urine samples. The discovery of a good and reproducible internal miRNA normalizer will eliminate the current inconsistency among studies and will finally allow the comparison of results obtained in urine of BC patients. This is an inexorable requirement in order to apply this technique to clinical practice.

2. Experimental Section

2.1. Study Subjects

BC can be subdivided in two types, superficial or non-muscle-invasive bladder cancer (NMIBC) (70% of total), which comprehends Ta and T1 lesions, and muscle-invasive bladder cancer (MIBC) (30% of total), which comprehends T2, T3 and T4

lesions. Additionally, grading indicates the degree of cellular differentiation, being G1 well differentiated and less likely to spread, G2 moderately differentiated and G3 poorly differentiated and more likely to spread. This can be better envisaged in Figure 1, where the study workflow is also described.

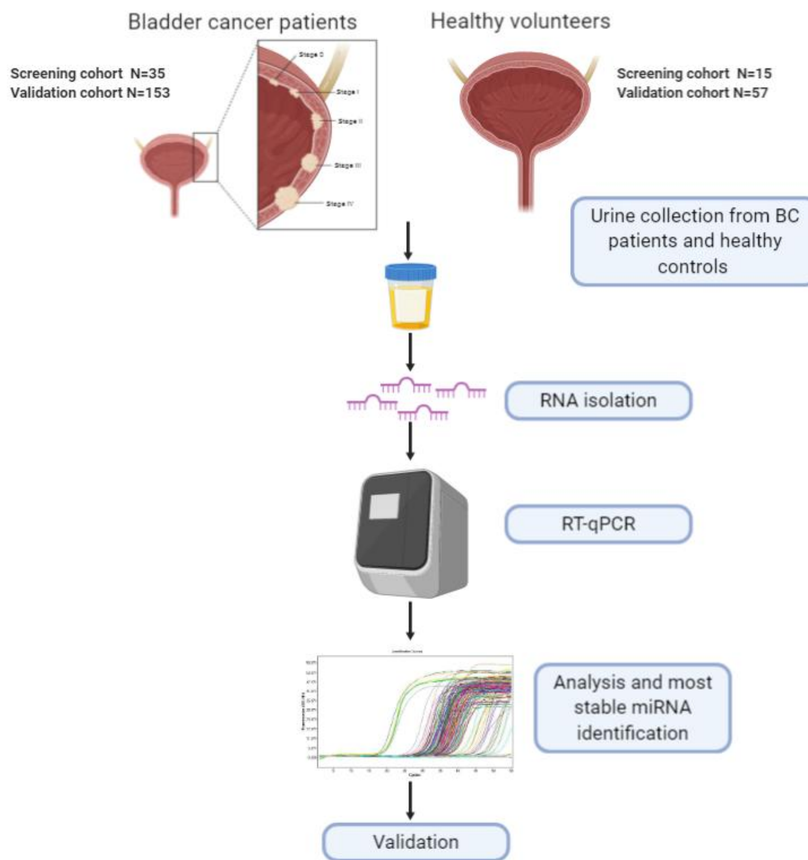

Figure 1. Graphical description of BC subtypes and study workflow.

For the initial screening stage, 35 BC patients (10 TaG1, 8 TaG3, 5 T1G3 and 12 T2G3) were recruited at La Fe University and Polytechnic Hospital (Valencia, Spain). Fifteen healthy volunteers (control group) with similar age and sex who underwent an ultrasound scan to rule out the presence of urological malignancies or other alterations were also recruited. For the validation stage, an independent cohort of 153 BC patients (33 TaG1, 13 TaG3, 29 T1G3, 54 TaG2, 9 T1G2, 4 T2G2, 10 T2G3 and 1 T3G3) and 57 healthy controls were additionally recruited.

Pre-operative clinical staging was performed through physical examination, urine cytology and CT scans of the chest, abdomen and pelvis. The tumor histological classification was done according to both the 1973 and 2004/2016 WHO classifications. Demographic and clinical data were collected.

The exclusion criteria were lack of informed consent, absence of histological confirmation and presence of other malignancies.

Informed consent was obtained from all participants according to protocols approved by the ethics review board at La Fe University and Polytechnic Hospital (2014/0314 and 2017/0474). The study was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

2.2. Urine Collection

A first morning urine sample of 25 to 50 mL was collected in sterile containers from all participants and kept at 4 °C until processing. Urine was centrifuged at 805× *g* for 5 min at 4 °C to remove cellular debris and supernatant was aliquoted and frozen at −80 °C until analyzed. The concentration of creatinine in urine was measured by clinical laboratory standardized methods.

2.3. RNA Isolation and cDNA Synthesis from Urine

Total urine RNA (including miRNAs) was isolated from 600 µL of urine using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with several modifications optimized by our group [27]. Briefly, 200 µL of cell-free urine were transferred to a tube with 1 mL Qiazol (Qiagen) and 1 µL carrier (1 µg/µL yeast RNA, Invitrogen, ThermoFisher Scientific, Waltham, MA, USA). This step was done in three independent tubes for each sample. In one of the three tubes 1 µL spike-in mix (UniSp2/4/5, Exiqon, Vedbaek, Denmark) was also added and each tube was gently mixed. After a 5 min incubation at room temperature, 200 µL chloroform were added to each tube, and centrifuged at 12000× *g*, 15 min at 4 °C to allow phase separation. Ethanol in a proportion of 1.5:1 (volume:volume) was added to the liquid phase. The three tubes containing the urine sample from the same individual were pooled in one single column in order to increase the final RNA yield. Then, 4 cleaning steps with the buffers supplied in the kit were performed. Total RNA was finally eluted in 50 µL of DNase/RNase-free sterile distilled water.

The concentration and purity of the RNA was assessed by spectrophotometric quantification with the NanoDrop ND-1000 (Thermo Fisher Scientific). RNA was stored at −80 °C until used.

In the initial screening stage where predesigned panels were used, cDNA was obtained from 5 µL of urine RNA with the miRCURY LNA RT Kit (Qiagen) according

to the supplied protocol in a final reaction volume of 25 µL. Due to the addition of a RNA carrier during the isolation, the final RNA yield includes the RNA isolated from urine plus the carrier RNA. Therefore, urine RNA retrotranscription was based on volume (µL) rather than RNA quantity (ng), according to the suppliers' recommendations. In the validation stage, where the expression level of selected miRNAs was conducted, cDNA was obtained from 2 µL of urine RNA using the same technology (final reaction volume 10 µL). In all cases, the reaction mix containing RNA, enzyme, buffer, RNAse-free water and UniSp6 RNA spike-in template, was incubated 60 min at 42 °C followed by 5 min at 95 °C for reverse transcriptase inactivation. Reactions were carried out in a thermocycler TC-412 (Techne, Minneapolis, MN, USA).

2.4. miRNAs Quantification

In the screening stage, 35 urine cDNA samples from BC patients and 15 from healthy controls were analyzed. In them, a total of 179 miRNAs were quantified using the commercially predesigned Serum/plasma miRNA PCR Panel V5 (Qiagen). This panel contains 179 miRNAs commonly found in human plasma and serum according to the manufacturer´s in-house analyses of miRNA expression in blood, serum and plasma samples, as well as on the limited number of peer-reviewed published papers available. The list of all the quantified miRNAs is detailed in Table S1. miRCURY LNA SYBR Green Master Mix (Qiagen) was used as a fluorophore, according to manufacturer´s indications. Briefly, cDNA (dilution 1/40), water and PCR master Mix (which includes SYBR Green) were added to a 384-well PCR plate supplied that includes the LNA^{TM} primer sets in a final reaction volume of 10 µL. Furthermore, each panel included the following internal controls: 5 synthetic RNAs of the RNA Spike-in kit aimed to monitor the RNA isolation and cDNA synthesis, and an inter-plate calibrator in triplicate and a negative control to evaluate qPCR performance. qPCR reactions were performed as follows: a polymerase activation/denaturation cycle of 2 min at 95 °C followed by 55 cycles of 10 s at 95 °C and 1 min at 56 °C with a ramp-rate of 2.2 °C/s. All RT-qPCR reactions were conducted in a LightCycler 480 II (Roche, Mannheim, Germany). In the validation stage, selected miRNAs were quantified using specific LNA PCR primer sets (Qiagen) in a total of 153 urine samples from BC patients and 57 healthy controls.

2.5. Selection of Candidate miRNA Normalizers and Analysis of Their Stability

To normalize the expression level of each miRNA, the best candidate with the highest stability and the lowest biological variance over the entire range of samples being investigated (BC and controls) was selected. To that aim, all miRNAs with a mean Ct < 35 in BC and controls were scrutinized. To select the best normalizer, the comprehensive tool RefFinder was employed which integrates the computational programs Genorm[28], BestKeeper[29], the comparative Delta Ct method [30] and NormFinder[31] (https://www.heartcure.com.au/for-researchers/) [32].

2.6. Statistical Analysis

Continuous variables were presented as median and interquartile range, and categorical variables as count and percentage. The analysis of variance (ANOVA) with the Tuckey Post Hoc test and unpaired t-test were used to identify significant differences in miRNA expression levels between BC patients and healthy controls. The statistical analysis was performed using the GraphPad Prism software v.8.0.1 (GraphPad software Inc., La Jolla, CA, USA). The Venn diagram was performed using the online tool available on http://bioinformatics.psb.ugent.be/webtools/Venn/. *p*-Values < 0.05 were considered statistically significant.

3. Results

3.1. Clinical Characteristics of the Study Subjects

A total of 188 BC patients were prospectively recruited together with 72 healthy volunteers (control group) with similar age and sex. The clinical characteristics of the study subjects are depicted in Table 1. The patients studied in the screening stage (n = 35) were: 10 patients (28.57%) with a TaG1 BC, 8 patients (22.86%) with TaG3, 5 patients (14.29%) with T1G3 and 12 patients (34.29%) with T2G3. The patients studied in the validation stage (n = 153) were: 33 patients (21.57%) with TaG1, 13 patients (8.5%) with TaG3, 29 patients (18.95%) with T1G3 and 15 patients (9.80%) with T2G2/T2G3/T3G3. Additionally, in order to validate the proposed normalizer in the whole spectrum of BC patients, 2 more groups of patients were included in the validation stage: TaG2 (54 patients, 35.29%) and T1G2 (9 patients, 5.88%).

Table 1. Clinical characteristics of the BC patients and healthy controls studied.

	BC Patients		**Controls**	
	Screening (*N* = 35)	**Validation (*N* = 153)**	**Screening (*N* = 15)**	**Validation (*N* = 57)**
Age, *y*	67 (61–74)	69 (63–75)	64 (51–76)	64 (56–68)
Male sex, *N (%)*	32 (91.43%)	129 (84.31%)	12 (80.00%)	43 (75.44%)
Urinecreatinine, *mg/dL*	76.5 (37.4–123.3)	77.4 (51.9–118.9)	78.5 (49.0–100.2)	98.2 (69.4–161.6)
Tumor Stage and Grade, *N (%)*				
TaG1	10 (28.57%)	33 (21.57%)	-	-
TaG3	8 (22.86%)	13 (8.50%)	-	-
T1G3	5 (14.29%)	29 (18.95%)	-	-
TaG2	-	54 (35.29%)	-	-
T1G2	-	9 (5.88%)	-	-
T2G2/T2G3/T3G3	12 (34.29%)	15 (9.80%)	-	-

Continuous variables are presented as median and interquartile range and categorical variables are presented as count and percentage.

3.2. Quality Internal Control with Synthetic Spike-in RNAs

To ensure that miRNA quantification was not influenced by technical and interpersonal variability, synthetic non-human spike-in RNAs are frequently used. We assessed the RNA isolation step by adding the synthetic spike-in 2 and spike-in 4 RNAs during all RNA isolations, and the retrotranscription efficiency by adding the spike-in 6 RNA in all retrotranscription reactions. No differences were observed in any spike-in studied among the study groups (Figure 2), thus indicating a proper performance of isolation and retrotranscription steps. To evaluate the qPCR performance of all reactions, the inter-plate calibrator spike-in 3 RNA in triplicate and a negative control were included in each panel. No differences were observed in any comparison made.

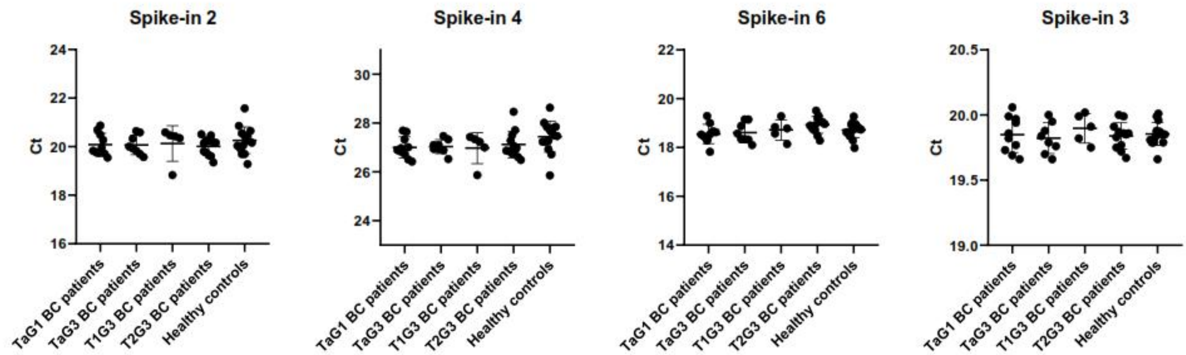

Figure 2. Differences in expression levels of three synthetic spike-in RNAs among BC patients with different stages and grades and healthy controls. Spike-in 2 and spike-in 4 monitor the RNA isolation step, spike-in 6 monitors the retrotranscription efficiency and spike-in 3 functions as inter-plate calibrator. Expression levels are represented as Ct values and error bars represent standard deviation.

3.3. Selection of Candidate miRNA Normalizers and Analysis of Their Stability

Of the 179 miRNAs quantified in each sample of the screening stage, we obtained high quality signals in 110 miRNAs (mean of the Ct < 35) both in BC patients and controls, thus they were included in the analysis with RefFinder. This tool comprehends the computational algorithms Genorm, BestKeeper, Delta Ct and NormFinder. Figure 3 shows the best 10 reference miRNAs selected by each algorithm. The stability analysis conducted with Genorm revealed that the greatest stability was reached by the combination of let-7e-5p and let-7a-5p (Figure 3a). BestKeeper revealed that the most stable miRNA was miR-2110 (Figure 3b). The Delta Ct method and NormFinder agreed with the most stable miRNA being miR-29c-3p (Figure 3c,d). Finally, the recommended comprehensive ranking that integrates all the previous analyses, rendered miR-29c-3p as the most stable miRNA (Figure 3e), being aligned with the results of the Delta Ct method and NormFinder. Next, we represented in a Venn diagram the overlap among the best 10 reference miRNAs selected by each algorithm (Figure 4). Three miRNAs were shared by the Delta Ct method, Genorm and NormFinder: miR-29c-3p, miR-26a-5p and miR-361-5p. Likewise, 7 miRNAs were shared by Delta Ct Method and NormFinder. In contrast, none of the 10 most stable miRNAs rendered by BestKeeper were selected by any other algorithm, thus none of them were included in the comprehensive ranking provided by RefFinder and consequently discarded as potential normalizers in our study.

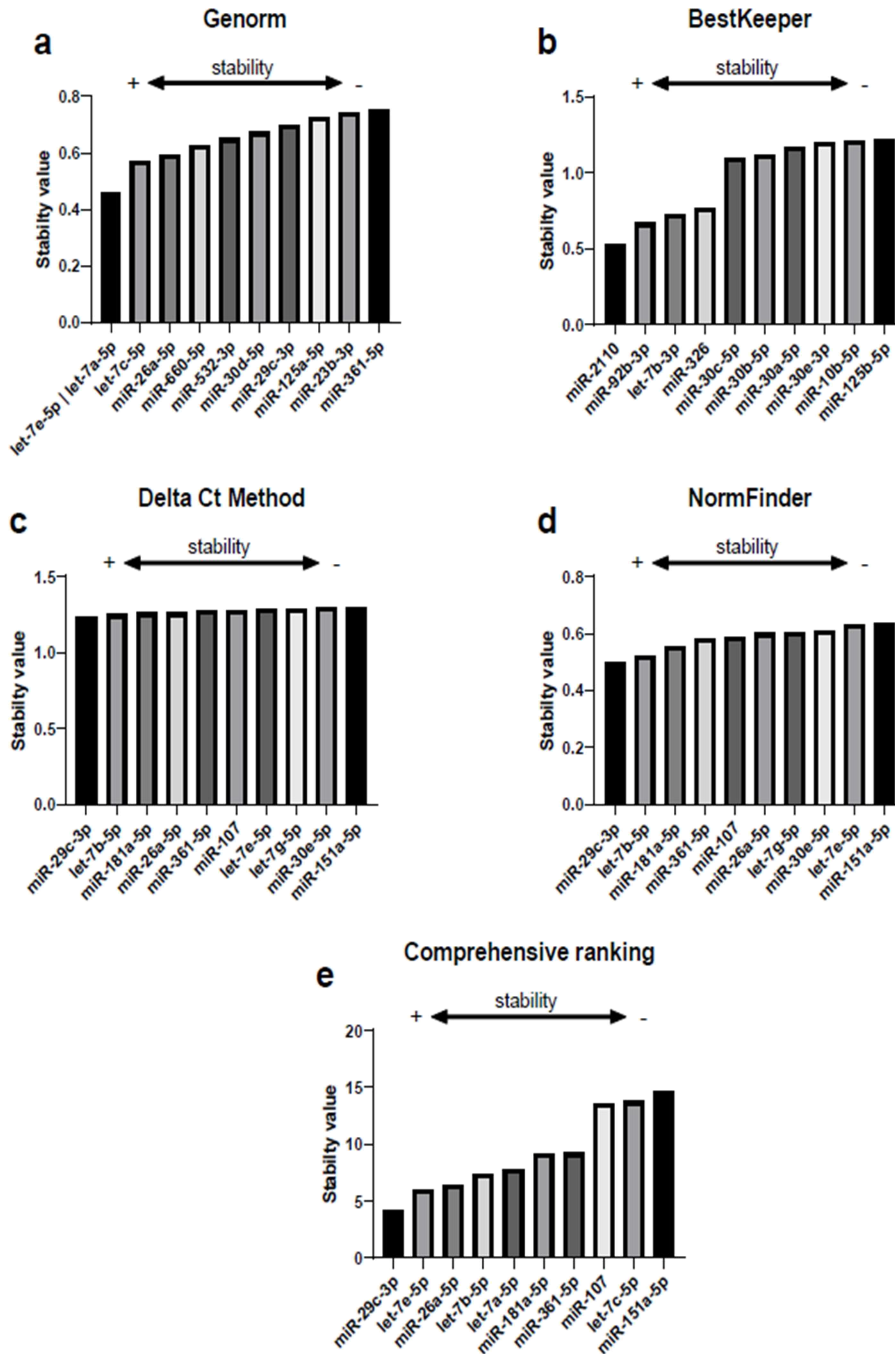

Figure 3. Selection of candidate miRNA normalizers and analysis of their stability conducted with the comprehensive tool RefFinder. Each graph represents the best 10 reference miRNAs selected by each algorithm: (**a**) Genorm, (**b**) BestKeeper, (**c**) Delta Ct method, (**d**) NormFinder and (**e**) Comprehensive ranking. The lower the stability value, the higher the stability of each miRNA.

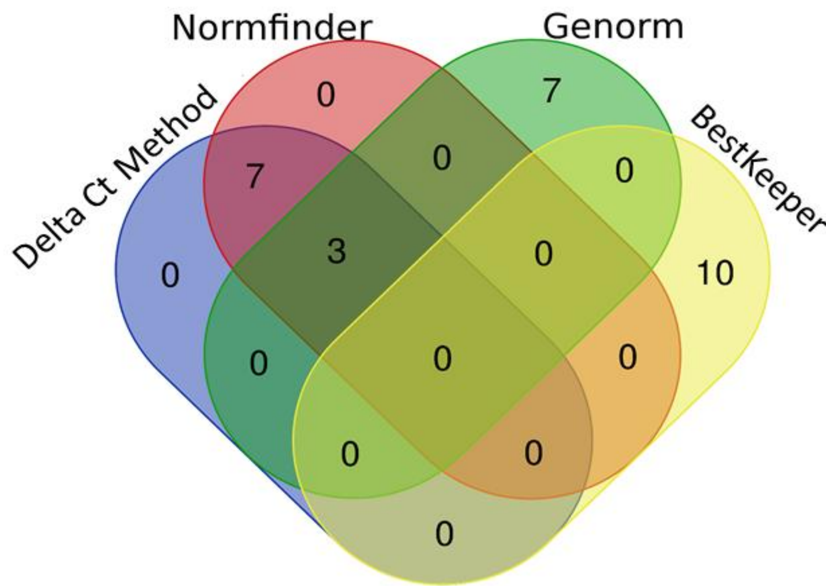

Figure 4. Venn diagram presenting the overlap among the best 10 reference miRNAs selected by each algorithm. Delta Ct method, NormFinder, Genorm and BestKeeper.

3.4. Differences in Expression Levels of Candidate miRNA Normalizers between BC Patients and Controls

A crucial characteristic of a good normalizer in miRNAs studies is the stable expression among the samples analyzed. Thus, we compared the mean Ct values of the best 10 reference miRNAs selected by the comprehensive ranking of RefFinder between BC patients and controls. No significant differences were observed in the expression of miR-29c-3p, let-7e-5p, miR-26a-5p, let-7a-5p and let-7c-5p (unpaired t-test, $p > 0.05$). In contrast, we found significant differences in the expression levels of let-7b-5p ($p = 0.029$), miR-181-5p ($p = 0.026$), miR-361-5p ($p = 0.025$), miR-107 ($p = 0.012$) and miR-151-5p ($p = 0.015$) (Figure 5). As expected, those miRNAs with the highest stability value had similar expression levels between BC and controls.

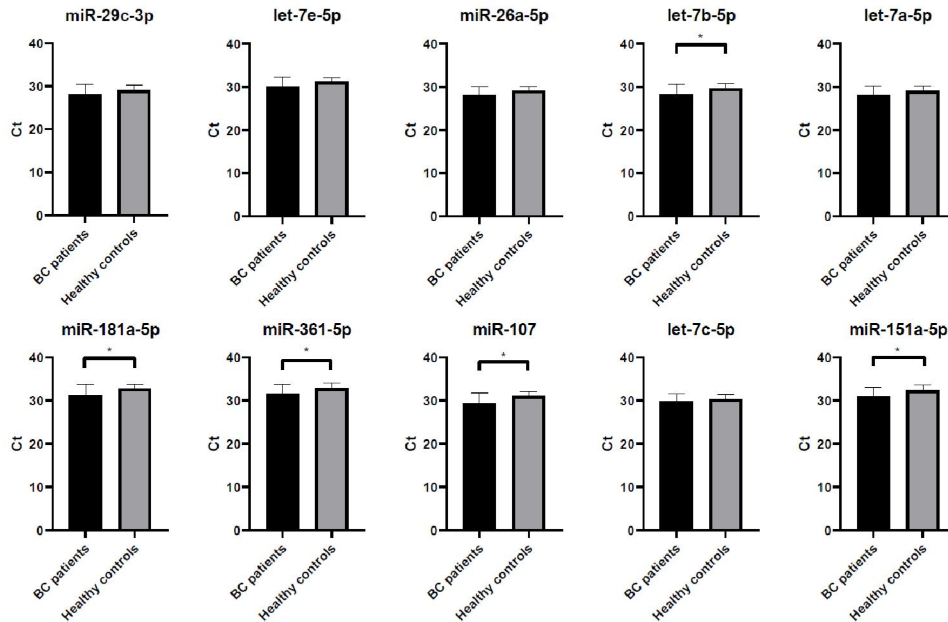

Figure 5. Differences in expression levels of the candidate miRNA normalizers selected by the comprehensive ranking of RefFinder between BC patients and controls. Expression levels are represented as Ct values and error bars represent standard deviation. Unpaired T-test: *, $p < 0.05$.

3.5. Effect of Different Normalization Strategies on the Relative Quantification of a miRNA Closely Related with BC

miR-200c-3p has been previously proposed as urinary diagnostic biomarker for BC [24,33]. Thus, we evaluated the performance of the 10 most stable miRNAs, selected by the comprehensive ranking of RefFinder, as normalizers for miR-200c-3p quantification in the screening cohort. As seen in Figure 6, significant differences among the BC groups studied and controls were observed when any of the normalizers were used, although the results obtained were not always comparable in magnitude and trend.

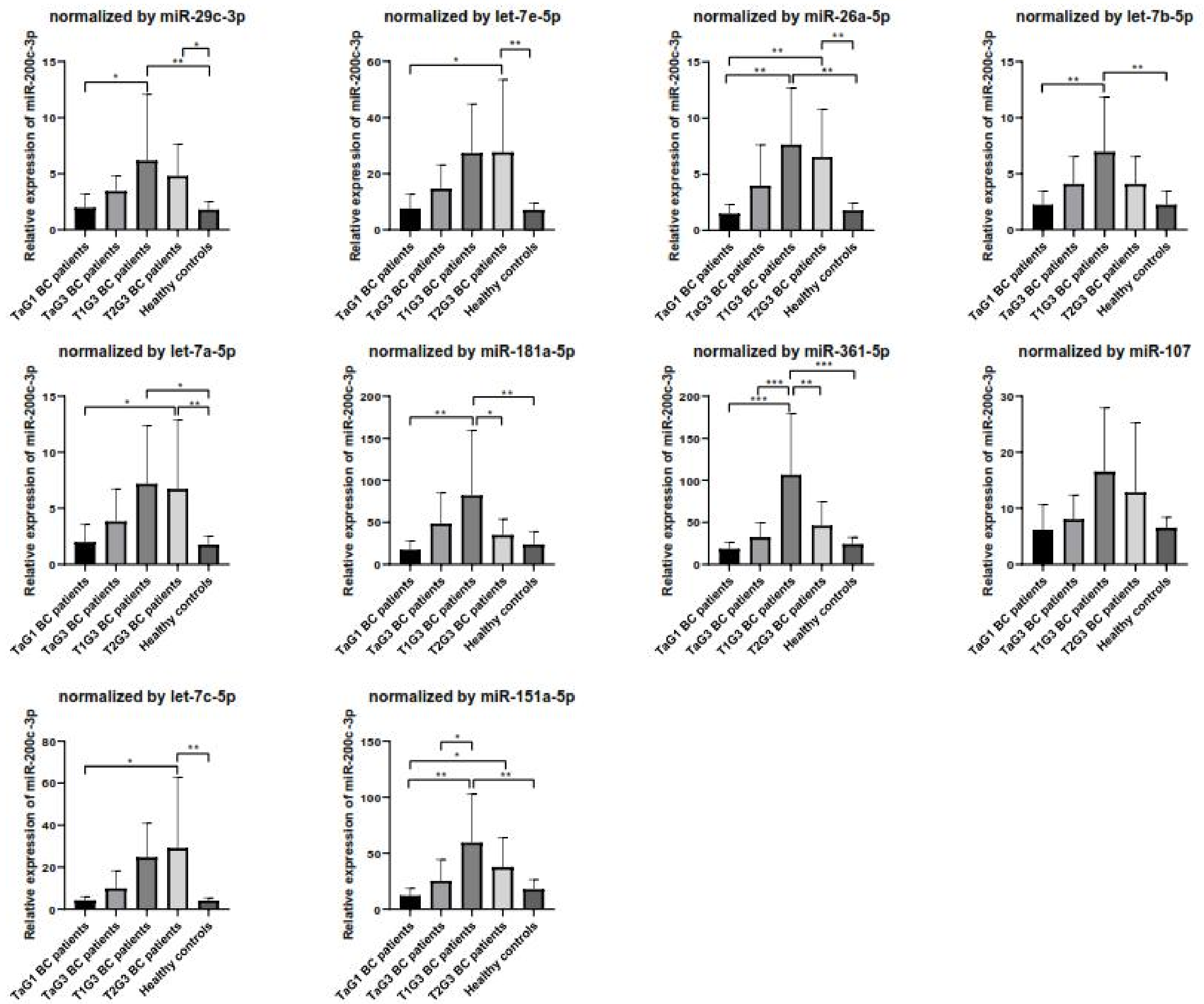

Figure 6. Relative expression of miR-200c-3p normalized by each candidate miRNA selected by the comprehensive ranking of RefFinder. Normalization was performed by the $2^{-\Delta\Delta Ct}$ method. Error bars represent the standard error of the mean. ANOVA with the Tuckey Post Hoc test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

In consideration of the aforementioned results, we selected miR-29c-3p as the best normalizer for the following reasons: (1) It was proposed as the most stable miRNA in the comprehensive analysis of RefFinder, and in two of the main algorithms independently tested (Figure 2); (2) Its expression level was optimal for a proper quantification (mean Ct = 28.4) (data not shown); and (3) Significant differences in the expression of miR-200c-3p, a miRNA related to BC, were observed when it was used as normalizer (Figure 6).

3.6. Validation of the Performance of miR-29c-3p as Normalizer in an Independent Cohort of BC Patients and Controls

We verified the stability of miR-29c-3p in an independent cohort of BC patients and healthy controls. As in the screening cohort, no significant differences were observed in the expression of miR-29c-3p between BC patients (mean Ct = 27.65) and healthy controls (mean Ct = 28.13) ($P = 0.37$). Next we validated the robustness and efficacy of miR-29c-3p as endogenous control for BC in the validation cohort using the $2^{-\Delta\Delta Ct}$method by analyzing the expression level of the BC-related miR-200c-3p in every group of BC patients and controls. As occurred in the samples studied in the screening stage, we observed significant differences in the expression of miR-200c-3p among the different clinical groups studied in the validation cohort ($p < 0.001$), with an increase of miR-200c-3p with the increase in BC stage (Figure 7). As occurred in the screening cohort, the biggest differences were found in the T1G3 BC group. As the patients with mildest stage of BC seem to have a lower expression level of miR-200c-3p, we grouped all the patients with the Ta stage and repeated the analysis. Figure S1a confirms that the expression of miR-200c-3p significantly increases with the severity of NMIBC, being T1G3 the BC type with the highest expression. Next, we grouped all NMIBC patients (TaG1+TaG2+TaG3+T1G2+T1G3) and compared the expression of miR-200c-3p to that of to MIBC patients (T2G2+T2G3+T3G3) and healthy controls. While NMIBC patients still showed the highest expression level of miR-200c-3p, no significant differences were observed when compared to MIBC or healthy controls (Figure S1b), probably because the highest difference occurs within the NMIBC group.

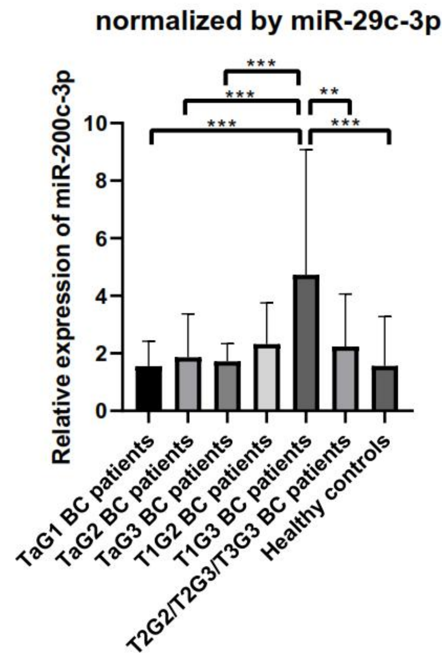

Figure 7. Relative expression of miR-200c-3p normalized by miR-29c-3p in the validation cohort. Normalization was performed by the $2^{-\Delta\Delta Ct}$ method. Error bars represent the standard error of the mean. ANOVA with the Tuckey Post Hoc test: **, $p < 0.01$; ***, $p < 0.001$.

4. Discussion

New non-invasive markers are presently being under study to circumvent several drawbacks in BC diagnosis, monitoring and prognosis. Urine miRNAs are non-invasive promising biomarkers that have been previously proposed for BC diagnosis [13–17,19–21,23,24]. However, huge discrepancies arise among miRNA studies, to a great extent due to nonexistence of standardized procedures. Although the populations studied, sample processing, and RNA isolation and miRNA quantification methods are partly responsible for these inconsistencies; the normalization strategy used may represent the main hurdle. In fact, to minimize the effect occasioned by methodology-related factors on miRNA expression levels, an accurate data analysis ought to be performed using appropriate normalizers for external and internal variation correction [26]. These normalizers should be chosen from a selection of candidates that are expected to be stably expressed over the entire range of samples being investigated, since miRNAs can be affected by the condition under study. As an alternative, the mean expression value of all commonly expressed microRNAs in a given sample has been proposed for normalization [34]. Although this strategy presents good and robust results it implies that a large number of miRNAs have to be always profiled, which may not be possible

or cost-effective in all studies [26,35]. Thus, as a general guideline suggested by the manufacturer, the use of the global mean for normalization is limited to the use of PCR panels that contain a larger number of microRNA assays, and it cannot be used for studies analyzing less than 20–50 different miRNAs.

Different RNA species have been proposed as normalizers (snRNAs, snoRNAs, rRNAs, miRNAs or exogenous synthetic RNAs); however, substantial differences in length and structure to that of miRNAs generate a high variability in the results [26]. Regarding the use of exogenous synthetic RNAs as normalizers, these are meant to track isolation and reverse transcription efficiency in order to eliminate deviations in the experimental process and make the results more reliable. However, it is important to remark that their use would never correct for deviations in sampling, in the quality of samples or in the amplification process. In fact, age, sample collection, preparation or storing can modify miRNA expression levels, which may be caused by cell lysis or miRNA degradation [26].

Several molecules have been proposed as normalizers for miRNAs studies in different disorders, mainly in blood [36], plasma [37–39], cell cultures studies [25,40], tissue [41] and urine [35,42–44]. However, there is no consensus, even in the same sample type, regarding which is the best normalizer for miRNA studies. Indeed, no consensus exists on a robust normalizer for urine studies. A recent study proposed miRNA miR-193a and miR-448 as normalizers [35]. However, in this study, only urine from healthy donors was analyzed and these results could vary when urine from cancer patients is investigated. In prostate cancer, miR-191-5p showed the lowest degree of variation and the highest stability value [42]. In our study, miR-191-5p ranked 29 out of 110 according to the comprehensive ranking of RefFinder, thus it is not a reliable normalizer for BC studies in urine. miR-16 was identified as the most stable endogenous reference miRNA in chronic kidney disease, making it a suitable normalizer for urinary exosome-derived miRNA [43]. Conversely, in our data set, miR-16-5p ranked 106 out of 110 according to the comprehensive ranking of RefFinder, turning it an ineffectual normalizer for urine BC studies. Finally, U6 has been proposed as normalizer for miRNA studies in urinary sediment of IgA nephropathy [44]. Although U6 was widely used as normalizer in countless studies at the origins of miRNA investigation, it is a member of the larger small RNA species which have a different biogenesis pathway (originate from the nucleus), may not be secreted or

protected in cell-free biofluids in the same way that microRNAs are and may also behave differently during RNA isolation. Thus, U6 is an unreliable normalizer for urine miRNA studies. Other studies have employed the combination of two stable miRNAs as normalizers however, this strategy presents several drawbacks: it may increase technical variability, it implies a higher economic cost since the number of miRNAs to be quantified by RT-qPCR increases and it is more time consuming. Altogether, the use of a combination of miRNAs as normalizers hampers the direct translation of miRNA studies to daily clinical practice with diagnostic/staging purposes.

In the present study, we set for the first time the aim to ascertain the best miRNA normalizer for miRNA studies in urine of BC patients in order to avoid future inconsistencies among studies. We evaluated the performance of 110 candidate miRNAs with the comprehensive tool RefFinder that integrates 4 programs (Genorm, BestKeeper, Delta Ct method and NormFinder) in 35 BC patients and 15 healthy controls. We selected miR-29c-3p as the best normalizer for miRNA studies in urine of BC. It was the most stable miRNA according to the comprehensive analysis of RefFinder among the 110 studied, and also according to the Delta Ct method and NormFinder. Moreover, it had a good expression level in urine (mean Ct value in BC patients and healthy controls = 28.4) and no differences were observed between BC patients and controls, both in the screening and the validation cohorts.

Urine miR-200c-3p has been previously related to BC and it has been proposed as diagnostic and staging marker [24,33]. miR-200c-3p appears to control the epithelial-to-mesenchymal transition process through BMI-1 in BC cells, and it inhibits their proliferation by down-regulating E2F3 [45]. Thus, we selected this miRNA to test the robustness of miR-29c-3p as endogenous normalizer. We found significant differences in miR-200c-3p among the different clinical groups studied both in the screening and validation cohorts, with a trend in the increase of miR-200c-3p with the severity of NMIBC.The evaluation of additional stably expressed miRNAs proposed by RefFinder may have rendered other potential normalizers for urine miRNA studies in the context of BC, what represents a limitation of our study. Nonetheless, our results confirm previous findings and reinforce the use of miR-29c-3p as normalizer.

5. Conclusions

In summary, our study is the first report characterizing a reliable normalizer for the analysis of urine miRNAs in BC patients. miR-29c-3p, being one of the most stably expressed miRNAs in urine of BC patients and healthy individuals, arises as an optimal reference miRNA that may allow the comparison of future urine miRNA studies as non-invasive biomarkers for BC diagnosis and monitoring.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: miRNAs and internal controls included in the Serum/plasma miRNA PCR Panel V5 (Qiagen); Figure S1. Relative expression of miR-200c-3p normalized by miR-29c-3p in the validation cohort. (a) Comparison of the mildest stage of NMIBC patients (TaG1+TaG2+TaG3) and the other clinical groups. (b) Comparison of NMIBC patients (TaG1+TaG2+TaG3+T1G2+T1G3), MIBC (T2G2+T2G3+T3G3) and healthy controls. Normalization was performed by the $2^{-\Delta\Delta Ct}$ method. Error bars represent the standard error of the mean. ANOVA with the Tuckey Post Hoc test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Author Contributions: J.O. processed samples, performed the research, analyzed the data and wrote the manuscript. E.P. analyzed the data and wrote the manuscript. Á.F.-P. performed the research and critically revised the manuscript. F.C. processed the samples and prepared the databases. M.M.-S. recruited the patients, revised the clinical records and critically revised the manuscript. C.D.V.-D. recruited the patients, revised the clinical records and critically revised the manuscript. F.E. designed and supervised the study and critically revised the manuscript. P.M. designed and supervised the study, analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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5. CHAPTER 2: Validation of a microRNA profile in urine liquid biopsy with diagnostic and stratification value for bladder cancer subtypes, usage through the open app BladdermiRaCan

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Author Contributions: J.O. processed samples, performed the research, analysed the data and wrote the manuscript.

Validation of a microRNA profile in urine liquid biopsy with diagnostic and stratification value for bladder cancer subtypes, usage through the open app BladdermiRaCan

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KEYWORDS

Biomarker, bladder cancer, diagnosis, liquid biopsy, miRNAs, prognosis, stratification, urine

ABSTRACT

Background: Bladder cancer (BC) is the most lethal urological malignancy. Non-invasive markers are needed to diagnose and stratify BC more accurately than current invasive procedures. microRNAs (miRNAs) are small non-coding RNAs that regulate protein expression, cancer progression and represent potential diagnostic and prognostic biomarkers.

Objective: We aimed to identify a profile of urine miRNAs with diagnostic and stratification potential in the whole range of BC subtypes.

Design, Setting, and Participants: For the screening stage, we collected a first morning urine sample from 35 BC patients and 15 age- and gender-matched healthy controls with normal renal and bladder ultrasound. For the validation stage, we recruited an independent cohort of 172 patients and 94 controls.

Outcome Measurements and Statistical Analysis: In the screening stage we analyzed the expression level of 179 miRNAs. We performed an ordinal regression for each miRNA with FDR adjustment and an ordinal Elastic Net logistic regression model for the diagnosis and stratification of BC with R (v3.5.1). Next, we validated the most dysregulated miRNAs.

Results and Limitations: With the expression level of 7 miRNAs in urine (miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p, miR-21-5p) we could stratify BC patients and healthy subjects. Moreover, we identified 70 dysregulated miRNAs in BC patients. We also identified miR-21-5p, miR-425-5p and miR-99a-5p as potential follow-up markers for BC relapse and miR-21-5p and miR-221-3p as potential markers for tumor metastasis. Among the dysregulated miRNAs, miR-93-5p, miR-425-5p, miR-21-5p and miR-200c-3p could be markers of in situ BC subtype. These miRNAs were also dysregulated in paired BC tissue sections. All miRNAs target proteins along the bladder cancer pathway.

Conclusions: We have validated a urine profile of 7 miRNAs able to diagnose and stratify BC patients and BladdermiRaCan will enable the global use of our model for BC diagnosis and stratification. Urine, as liquid biopsy, may reflect miRNA tumor microenvironment.

Patient summary: Current diagnostic techniques for BC are expensive and harmful for the patient. We have identified and validated a profile of 7 miRNAs in urine able to

classify BC patients. We have also identified potential markers of tumor relapse, metastasis and cellular subtypes. Finally, we have created an open app to enable any researcher the use of our model for BC diagnosis and stratification.

INTRODUCTION

Bladder cancer (BC) occasions 3% of all malignant tumors in adults worldwide and stands as the most lethal urological malignancy. Indeed, according to the GLOBOCAN 2020 study, BC accounts for more than 573,278 new cancer cases worldwide and almost 212,536 deaths annually (1). In addition, BC highly increases medical expenditure as it is the tumor with the highest monitoring costs and the most expensive cancer to treat, with the cost of the muscle-invasive subtype approaching $150,000 per capita (2, 3). The main risk factors for BC are smoking, occupational and environmental exposure to chemicals (4).

Presently, bladder ultrasound, cytology and cystoscopy are the gold standards for BC diagnosis and monitoring despite its low sensitivity or high invasiveness, respectively (5). Thus, several urine markers for BC have recently emerged, as the UroVysion test, ImmunoCyt or the nuclear matrix protein (NMP-22) test; although, none of them achieve a high sensitivity and specificity (5). Consequently, novel non-invasive markers are being ascertained in liquid biopsies, such as microRNAs (miRNAs) (6, 7) or cell-free DNA (8).

miRNAs are short, noncoding RNA molecules of 20-22 nucleotides that regulate gene expression. miRNAs are involved in various processes of tumorigenesis like tumor initiation, growth and progression (9) and have been emerged as novel tumor biomarker candidates (10, 11). One of the most promising aspect of miRNAs as biomarkers is that they can be reproducibly isolated from a broad spectrum of biological samples (such as blood plasma/serum, urine, saliva or feces) (12) and are stable when properly stored and handled (13) .

Previous studies evidence that miRNAs are involved in the development and progression of BC. miRNAs have been found dysregulated in several biofluids like plasma/serum (14, 15), urine (16, 17) and BC tissue (18, 19) . However, these studies have not been conducted in the whole range of BC subtypes, but have only studied one BC subtype like non-muscle invasive BC (NMIBC) (20-22) , muscle invasive BC (MIBC) (23) or low grade BC (24).

In our study we aimed to identify and validate a profile of non-invasive urine miRNAs with diagnostic and stratification potential in the whole range of BC subtypes. We also aimed to identify dysregulated miRNAs associated to BC recurrence, metastasis and different cellular subtypes. We verified the dysregulation of miRNAs in formalin-fixed paraffin-embedded (FFPE) BC tissue sections and in silico identified the targets of these dysregulated miRNAs implicated in tumor progression. Finally, we developed the easy-to-use free web application BladdermiRaCan to enable any researcher worldwide the use of our predictive model to aid in the diagnosis and stratification of their patients.

MATERIALS AND METHODS

Patients and control subjects

Two-hundred and seven BC patients (43 TaG1, 22 TaG3, 35 T1G3, 70 TaG2, 8 T1G2, 29 T2G2/T2G3/T3G3) were recruited between April 2016 and January 2020 at La Fe University and Polytechnic Hospital (Valencia, Spain). One-hundred and nine age- and sex-matched healthy volunteers (control group) who underwent an ultrasound scan to rule out the presence of urological malignancies or other alterations were also recruited. Patients and controls were clinically followed-up until May 2021.

Additionally, 51 serial samples were processed from a selection of 17 BC patients in order to longitudinally study miRNAs expression over time depending on BC relapse.

Pre-operative clinical staging was performed through physical examination, urine cytology and CT scans of the chest, abdomen and pelvis (in case of invasive bladder cancer). The tumor histological classification was done according to grade in the WHO 1973 and 2004 classifications. Demographic and clinical data were collected.

The exclusion criteria were lack of informed consent, absence of histological confirmation and presence of other malignancies.

Written informed consent was obtained from all participants according to protocols approved by the ethics review board at La Fe University and Polytechnic Hospital. The study was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

Urine collection

A first morning urine sample of approximately 25 to 50 ml was collected in sterile containers from all participants. Urine was kept at 4 ºC until processing. Urine was centrifuged at 805 x g for 5 min at 4 ºC to remove cellular debris and supernatant was aliquoted and frozen at -80 ºC until analyzed. The concentration of creatinine in urine was measured by clinical laboratory standardized methods.

miRNA studies

RNA isolation

Total RNA (including miRNAs) from 600 ul of urine was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following manufacturer´s instructions with several modifications optimized by our group (25). During the isolation, an RNA carrier (tRNA, Ambion, Bleiswijk, TheNetherlands) was included to enhance the yield, and a mixture of synthetic miRNAs (Spike-in kit UniRT, Qiagen) was included to control for RNA isolation efficiency, cDNA synthesis and inter-plate quantitative PCR (qPCR) performance.

Total RNA from ten 20 µm sections was isolated per patient with the miRNeasy FFPE kit (Qiagen) following manufacturer´s instructions.

The concentration and purity of the RNA was assessed by spectrophotometric quantification with the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was stored at -80 ºC until used.

Quantification of the expression level of miRNAs

The expression level of miRNAs was quantified by real-time reverse transcription qPCR (RT-qPCR) in two stages:

Screening stage

Based on the quality of the isolated RNA, 35 BC patients and 15 controls were selected and miRNAs expression level was studied. The Universal cDNA Synthesis Kit II (Qiagen) was used for the retrotranscription and the expression level of 179 miRNAs was quantified with the Serum/Plasma Focus microRNA PCR Panel V5 (Qiagen) with the ExiLENT SYBR Green Master Mix (Qiagen) in a LightCycler 480 II (Roche, Mannheim, Germany) as previously reported (26-29) . Furthermore, each panel includes the following internal controls: 5 synthetic RNAs of the RNA Spike-in-kit

aimed to monitor the RNA isolation and cDNA synthesis, and an inter-plate calibrator in triplicate and a negative control to evaluate qPCR performance.

To normalize the expression level of each miRNA studied, we employed the expression of miR-29c-3p as it has been recently proposed as a robust normalizer for urine miRNA studies in BC (28) .

Next, an ordinal regression was performed to stratify BC patients and controls patients according to miRNAs levels before surgery.

Validation stage

Once identified a miRNA profile potentially able to diagnose and stratify BC patients, their expression level was quantified in an independent and larger cohort of 172 BC patients and 94 controls at inclusion in duplicate. For that aim, specific miRNA miRCURY LNA miRNA PCR Assays (Exiqon) were used. Each miRNA was measured in duplicate and a standard deviation (SD) <0.5 was considered satisfactory.

We also analyzed the variation of urine miRNA expression over time depending on BC relapse in 51 serial samples from 17 BC patients of whom 6 patients had BC recurrence. In addition, we analyzed the expression of the dysregulated miRNAs according to the presence of metastasis and cellular subtype.

Furthermore, the expression of the dysregulated miRNAs was quantified in FFPE tissue sections from 30 BC patients of the screening stage from whom tumor tissue sections were available. In addition, adjacent healthy bladder urothelium was available from two of these patients. miRNA expression levels were quantified as aforementioned. The selection of the most stable normalizer among all samples was performed with the comprehensive tool RefFinder that comprehends the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method (https://www.heartcure.com.au/for-researchers/). The candidate normalizers evaluated were those proposed by the manufacturer for tissue samples (miR-103a-3p, miR-423-5p and miR-423-3p) and miR-29c-3p, the urine normalizer.

Identification of miRNAs´ targets

Once we selected a miRNA profile with diagnostic and staging potential, we identified their validated and predicted target proteins related to BC using the database miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) that comprehends 12

miRNA-target prediction programs. Next, these targets were integrated within the bladder cancer pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/).

Statistical analysis

Data were summarized using mean (standard deviation) and median (1st, 3rd quartile) in the case of continuous variables and relative and absolute frequencies in the case of categorical variables. miRNA values were normalized by substracting from the raw Ct values for each sample the corresponding Ct values of the normalizer miRNA: miR-29c-3p for urine samples and miR-103a-3p for FFPE tissue sections. An elastic net penalized ordinal regression model was adjusted to the screening cohort for discriminating between the different stages and grades using as potential predictors the miRNAs from the panel. Additionally, univariate ordinal regression models were adjusted for each miRNA. P-values were adjusted for multiple comparisons using false discovery rate (FDR). Results from the screening cohort were validated in an independent cohort of patients. Predictions were made using the ordinal regression model fitted on the screening cohort and the discriminative power of the model was assessed by estimating the rank correlation coefficient (rho) between predicted and observed values. Additionally, goodness of fit was determined by a Bangdiwala's agreement plot (30). Perfect matches were weighted as 1 and adjacent categories were weighted as 0.5 in the estimation of the Bangdiwala's statistic. Differences between metastatic and non-metastatic patients and between tumor types (papillary vs. in situ) were assessed using the Wilcoxon-Mann-Whitney test. P-values < 0.05 were considered statistically significant. All statistical analyses were performed using R (version 4.0.2) and R packages clickR (version 0.4.47), glmnetcr (version 1.06), ordinal (version 2019.12-10) and vcd (version 1.4-7).

Shiny app development

To enable any researcher worldwide to diagnose and stage their patients using our model, we created the open web page app BladdermiRaCan. The interface was created with Shiny (v1.4) implemented with a graphical user interface (GUI), thus it can be used by researchers without knowledge of the R language.

RESULTS

Clinical characteristics of the study subjects

A total of 207 BC patients were prospectively recruited together with 109 healthy volunteers (control group) with similar age and sex. The clinical characteristics of the study subjects are depicted in Table 1. The patients studied in the screening stage (n = 35) were: 10 patients (28.6%) with a TaG1 BC, 8 patients (22.9%) with TaG3, 5 patients (14.3%) with T1G3 and 12 patients (34.3%) with T2G3. The patients studied in the validation stage (n = 172) were: 33 patients (19.3%) with TaG1, 14 patients (8.1%) with TaG3, 30 patients (17.4%) with T1G3, 70 patients (40.7%) with TaG2, 8 patients (8.7%) with T1G2 and 17 patients (9.9%) with T2G2/T2G3/T3G3. BC patients with grade 2 tumors (TaG2 and T1G2) were included in the validation cohort in order to validate the proposed miRNAs in the whole spectrum of BC patients. Regarding the control groups, in the screening stage 15 controls were analyzed (median age 64 years, 80% men) and 94 in the validation stage (mean age 64 years, 85% men).

Table 1. Clinical characteristics of the BC patients and healthy controls studied.

	BC patients		**Controls**	
	Screening (N=35)	**Validation (N=172)**	**Screening (N=15)**	**Validation (N=94)**
Age, *y*	67 (61-74)	68.5 (63-74)	64 (51-76)	63.5 (56-68)
Male sex, *N (%)*	32 (91%)	145 (84%)	12 (80%)	80 (85%)
Urine creatinine, *mg/dL*	76.5 (37.4-123.3)	77.1 (53.1, 118.7)	78.5 (49.6-100.2)	110.9 (70.6, 160.0)
Tumor Stage and Grade, *N (%)*				
TaG1	10 (28.6%)	33 (19.2%)	-	-
TaG3	8 (22.9%)	14 (8.1%)	-	-
T1G3	5 (14.3%)	30 (17.4%)	-	-
TaG2	-	70 (40.7%)	-	-
T1G2	-	8 (4.6%)	-	-
T2G2/T2G3/T3G3	12 (34.3%)	17 (9.9%)	-	-
Surgical approach, *N (%)*				
TURBT	35 (100%)	160 (93%)	-	-
Partial cystectomy	-	2 (1%)	-	-
Radical cystectomy	-	10 (6%)	-	-
Hystology				
Papillary	35 (100%)	158 (91.9%)	-	-
In situ	-	1 (0.6%)	-	-
Papillary + *In situ*	-	13 (7.5%)	-	-
Sampling				
At first BC diagnosis	35 (100%)	89 (51.7%)		
At BC recurrence	0 (0%)	83 (48.3%)		
Sampling				
At first BC diagnosis	35 (100%)	89 (51.7%)		
At BC recurrence	0 (0%)	83 (48.3%)		

Continuous variables are presented as median and interquartile range. Categorical variables are presented as count and percentage. BC, bladder cancer; TURBT, transurethral resection of bladder tumor.

Identification of dysregulated miRNAs in the different categories of BC patients and controls

At the screening stage, we were able to successfully quantify 157 miRNAs in urine (Ct<36 in at least one clinical group) of the 35 BC patients and 15 controls studied.

To identify dysregulated miRNAs between the different subgroups of BC patients and controls, we performed a univariate ordinal regression for each miRNA and, after FDR adjustment, we obtained 70 miRNAs with $P<0.05$ (Supplementary Figure 1).

Supplementary Table 1 details the sequence, mean Ct value and *P*-value of the 70 dysregulated miRNAs among the clinical groups studied.

Next, we adjusted an ordinal Elastic Net logistic regression model for BC diagnosis and staging using the expression level of the 179 miRNA comprised in the predesigned panel in urine of patients and controls. The model included 7 miRNAs as predictors: miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p, miR-21-5p and miR-221-3p (Figure 1).

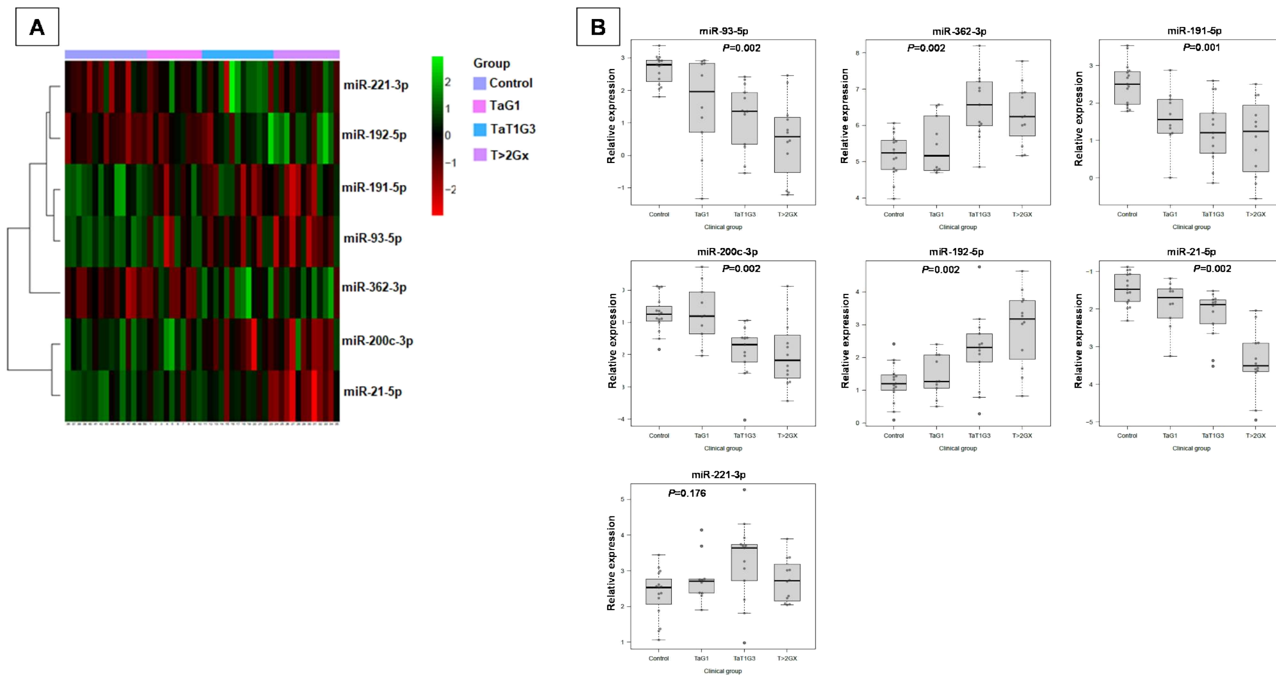

Figure 1. Expression of the 7 miRNAs comprised in the Elastic Net logistic regression model for BC diagnosis and staging. **A**. Heatmap, green represents miRNA overexpression and red represents underexpression. **B**. Relative expression in the different BC subtypes and controls.

The formula to calculate the probability of belonging to a given category is as follows:

$$\text{logit}_i = -4.061045 + \text{intercept}_i + 0.15 * \text{miR-221-3p} - 0.055 * \text{miR93-5p} + 0.1086 * \text{miR362-3p} - 0.181 * \text{miR_191-5p} - 0.11 * \text{miR_200c-3p} + 0.2154 * \text{miR_192-5p} - 0.7692 * \text{miR_21-5p}$$

Intercept_1: 1.9428

Intercept_2: 0.7839

Intercept_3: -0.28

Prob(T>2GX) = $\frac{e^{logit_3}}{1+e^{logit_3}}$

Prob(TAT1G3) = $\frac{e^{logit_2}}{1+e^{logit_2}} * 1 - \frac{e^{logit_3}}{1+e^{logit_3}}$

Prob(TAG1) = $\frac{e^{logit_1}}{1+e^{logit_1}} * \left(1 - \frac{e^{logit_2}}{1+e^{logit_2}}\right) * \left(1 - \frac{e^{logit_3}}{1+e^{logit_3}}\right)$

Prob(Control) = 1 – (Prob(T>2GX) + Prob(TAT1G3) + Prob(TAG1))

Next, we validated in an independent cohort of BC patients and controls those miRNAs included in the Elastic Net logistic regression model (miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p, miR-21-5p and miR-221-3p,) and the most significantly dysregulated miRNAs identified in the ordinal regression approach with FDR adjustment: miR-30a-5p, miR-425-5p, miR-99a-5p,, miR-23a-3p, miR-215-5p and miR-10b-5p.

Validation of the dysregulated miRNAs in the different categories of BC patients and controls

All the miRNAs proposed for validation had an optimal expression value (Ct <36) in an independent cohort of 172 BC patients and 94 healthy controls. Unlike the screening stage, here we included BC patients with grade 2 tumors to analyze the whole spectrum of BC patients. The differences in expression levels could be validated using univariate ordinal regression models (Figure 2).

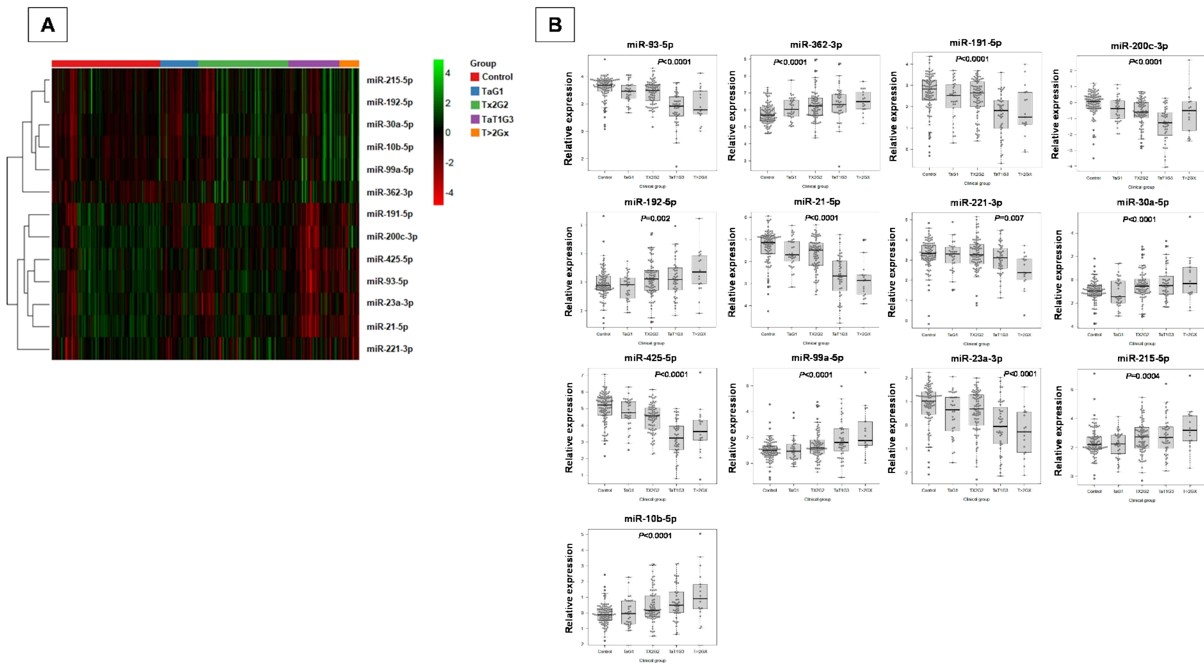

Figure 2. Expression of the 13 dysregulated miRNAs in the validation cohort. **A**. Heatmap, green represents miRNA overexpression and red represents underexpression. **B**. Relative expression in the different BC subtypes and controls.

We could also validate the predictive ability of our elastic net model in the cohort of 172 BC patients and 94 healthy controls. The discriminative power of the model was assessed by estimating the rho values between predicted and observed values and the rho value of the validated model was 0.61. The goodness of fit was determined by a Bangdiwala's agreement plot (Figure 3). Perfect matches were weighted as 1 (represented as black squares) and adjacent categories were weighted as 0.5 (represented as grey squares) in the estimation of the Bangdiwala's statistic. Lack of match between predicted and observed values were represented as white squares. The bangdiwala weighted value was 0.62 and the unweighted was 0.41, reflecting a high coincidence between predicted and observed values for each individual studied.

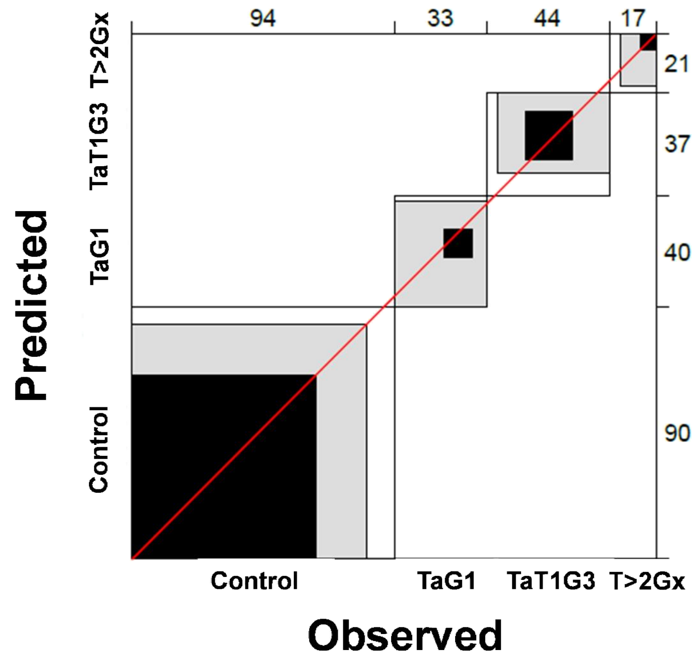

Figure 3. Bangdiwala agreement plot that represents the agreement between predicted and observed values obtained in the validation cohort with our ordinal regression model. Perfect matches between predicted and observed values are resented as black squares, adjacent categories are represented as grey squares and lack of match are represented as white squares.

Dysregulation of miRNAs according to BC relapse, metastasis and cellular subtypes

We analyzed the variation of the validated miRNAs over time depending on BC relapse in 51 serial urine samples from 17 BC patients. 6 patients had BC recurrence while 11 patients had tumor remission. We performed lineal mixed models with random slope and intercept of all the miRNAs quantified in the validation stage. We observed an increase in miR-21-5p and miR-425-5p and a decrease in miR-99-5p in patients with tumor relapse compared to those patients with tumor remission and controls (Figure 4A). We analyzed the variation of their expression over time and observed that miR-21-5p and miR-425-5p increased over time in those patients with tumor relapse, while their expression normalized (come close to that of controls) in patients with tumor remission (P=0.013 and P=0.044, respectively). Finally, miR-99a-5p decreased over time in those patients with tumor relapse, while it normalized in patients with tumor remission (P=0.036) (Figure 4B). To evaluate whether the expression of these 3 miRNAs normalized to the levels of controls in those patients with tumor remission but not in those with tumor relapse, we compared two samples collected from each patient during

follow-up. In contrast to what occurs to patients with tumor relapse, the expression of miR-21-5p, miR-425-5p and miR-99a-5p in patients with tumor remission reached the level of the controls in sample 2 (Figure 4C).

We also studied the variation of the validated miRNAs depending on the presence of tumor metastasis in the validation cohort, among whom 15 BC patients had metastasis. In line with the results obtained in the validation cohort, the expression of miR-21-5p (*P*=0.0008) and miR-221-3p (*P*=0.0016) was higher in BC patients with metastasis (Figure 4D) (the lowest the relative expression which means lower normalized Ct values, the highest miRNA expression).

Finally, we analyzed the variation of the validated miRNAs depending on the BC cellular subtype, comparing the 155 patients with papillary BC and those 14 patients with *in situ* BC. We evidenced a significant increase in miR-93-5p (*P*=0.01), miR-425-5p (*P*=0.007), miR-21-5p (*P*=0.006) and miR-200c-3p (*P*=0.031) in those BC patients with *in situ* BC (Figure 4E) (the lowest the relative expression which means lower normalized Ct values, the highest miRNA expression).

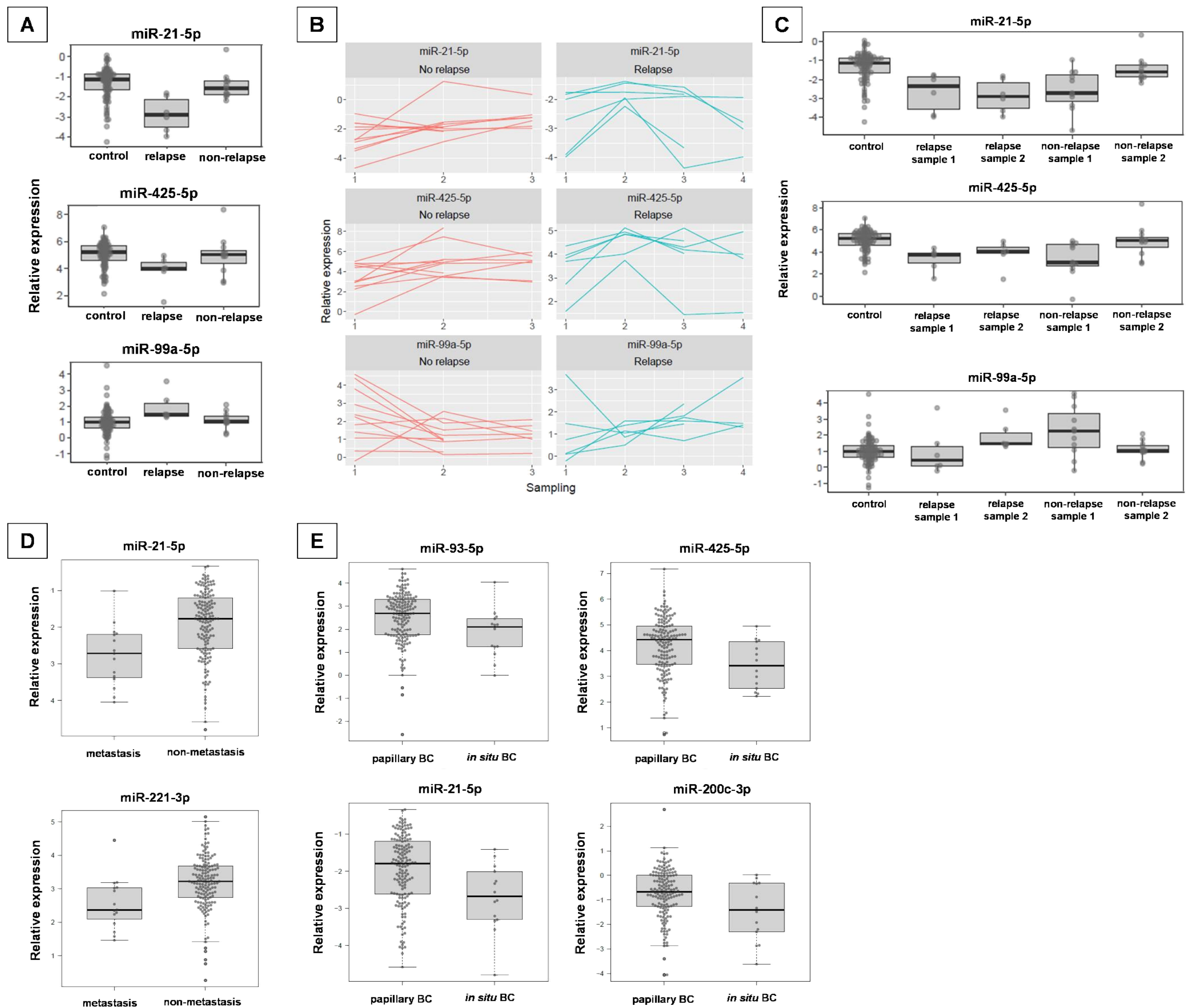

Figure 4. Relative expression of dysregulated miRNAs according to tumor relapse, metastasis and BC cellular subtype in BC patients of the validation cohort. A. Relative expression of miR-21-5p, miR-425-5p and miR-99a-5p according to tumor relapse. **B.** Relative expression of miR-21-5p, miR-425-5p and miR-99a-5p over time according to tumor relapse. **C.** Relative expression of miR-21-5p, miR-425-5p and miR-99a-5p in two samples collected during follow-up according to tumor relapse. **D.** Relative expression of miR-21-5p and miR-221-3p depending on the presence of tumor metastasis. **E.** Relative expression of miR-93-5p, miR-425-5p, miR-21-5p and miR-200c-3p depending on the BC cellular subtype.

Analysis of the dysregulated miRNAs in FFPE BC tissue sections

Next, we analyzed the expression level of the 13 dysregulated miRNAs in tissue specimens from 30 BC patients of the screening stage from which tumor tissue sections were available together with two adjacent healthy bladder urothelium samples. The Comprehensive Ranking of RefFinder rendered miR-103a-3p as the one with the highest stability and the lowest biological variance among all samples. Thus, we normalized the expression level of each miRNA studied in FFPE tissue by that of miR-103a-3p as previously mentioned.

All 13 miRNAs had an optimal expression value in tissue sections (Ct <36). The differences and trend in expression levels in tissue could be verified using univariate ordinal regression models. The results obtained in tissue are in the same direction to those obtained in urine, being the following miRNAs significantly dysregulated in tissue: miR-93-5p (Odds ratio, OR =0.114; 95% Confidence Interval, CI [0.026, 0.400]; *P*=0.002), miR-21-5p (OR =0.474; 95% CI [0.225, 0.929]; *P*=0.036), miR-30a-5p (OR =1.922; 95% CI [1.124, 3.443]; *P*=0.019) and miR-10b-5p (OR =2.546; 95% CI [1.356, 5.231]; *P*=0.006) (Figure 5). These results suggest that urine might reflect the expression of miRNAs in the tumor microenvironment, thus possibly acting as liquid biopsy for BC.

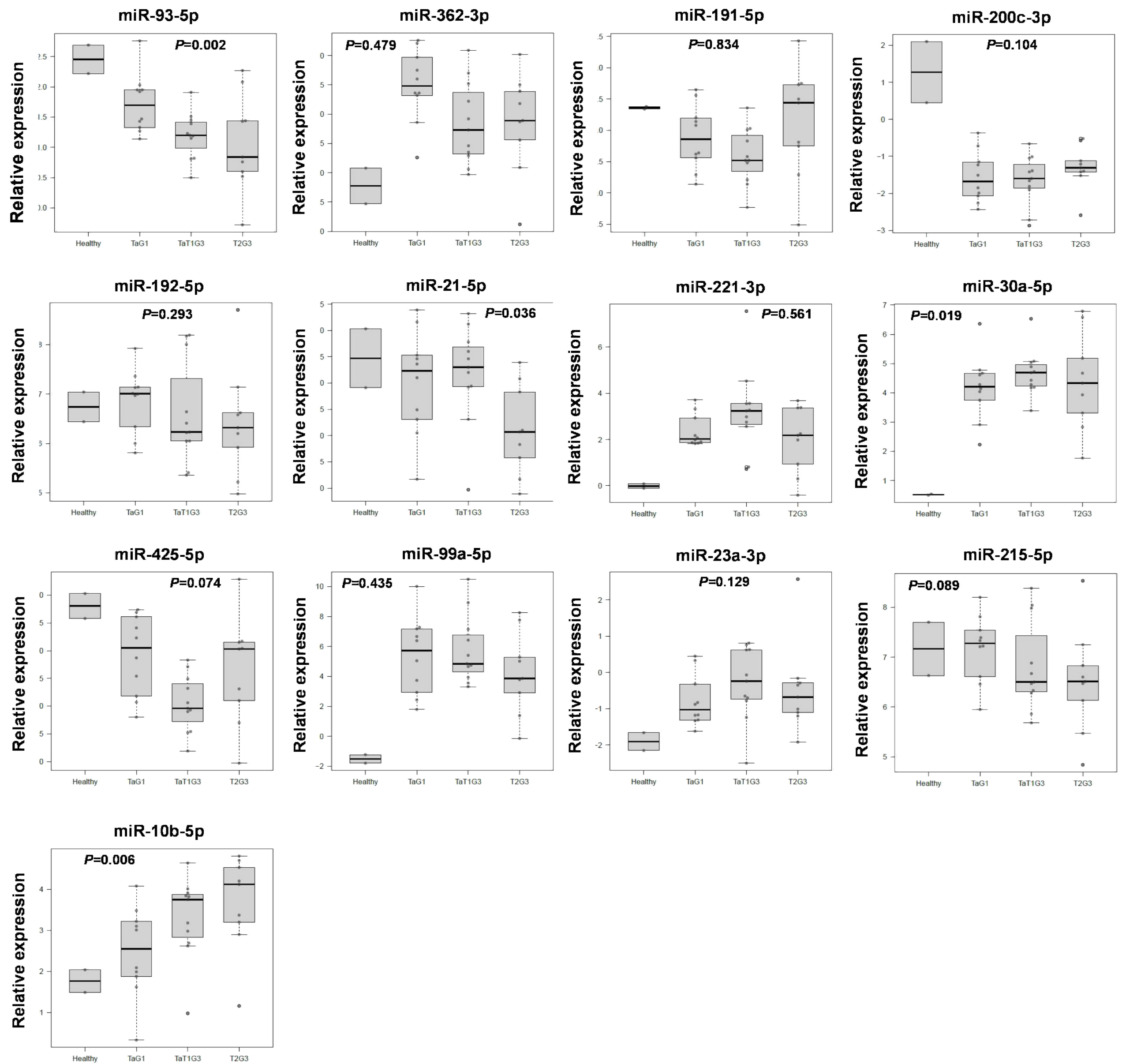

Figure 5. Relative expression in FFPE tissue sections of the dysregulated miRNAs found in urine of the same BC patients.

miRNAs target analysis

Once we validated the dysregulation of the selected miRNAs, we identified their validated and predicted target proteins related to BC using the databases miRWalk 2.0 and KEGG. The targets obtained with miRWalk 2.0 were integrated within the *bladder cancer* pathway from KEGG. Remarkably, all the studied miRNAs have validated targets related to BC. Additionally, 10 of the 13 studied miRNAs have predicted targets along the pathway (Table 2). Interestingly, a significant correlation was observed

between several of these miRNAs (data not shown), reinforcing the common regulation of the *bladder cancer* pathway by these miRNAs.

Table 2. Validated and predicted targets of the 13 dysregulated miRNAs in BC. These target proteins were identified using miRWalk 2.0 and were further integrated within the *bladder cancer* pathway. Validated targets are defined as those that have been empirically proven to be regulated by a miRNA. Predicted targets are defined as those that have been theoretically estimated based on the free binding energy between the miRNA and the presumed target mRNA sequence.

	***Bladder cancer* pathway**	
miRNA	**Validated targets**	**Predicted targets**
hsa-miR-221-3p	E2F3, MDM2, RB1, TP53	THBS1, EGF, FIGF
hsa-miR-93-5p	CCND1, CDKN1A, DAPK3, E2F1, E2F2, E2F3, IL-8, MAPK1, MDM2, MYC, RB1, RPS6KA5, VEGFA	EGFR, DAPK2, EGF, MMP2, MAPK3, THBS1, VEGFB, EGF, ERBB2, MAP2K2, RASSF1, TP53, CDH1, FIGF
hsa-miR-362-3p	E2F1, VEGFA	DAPK1
hsa-miR-191-5p	BRAF	DAPK1, E2F3
hsa-miR-200c-3p	E2F3, KRAS, VEGFA	MYC
hsa-miR-192-5p	CDKN2A, RB1	CDK4, RASSF1, DAPK2
hsa-miR-21-5p	E2F1, E2F2, E2F3, EGFR, ERBB2, MMP2, MMP9, MYC, RB1, VEGFA	-
hsa-miR-30a-5p	CDH1, EGFR, MAPK1, THBS1, TP53	RPS6KA5
hsa-miR-425-5p	CCND1, E2F3, FGFR3, MDM2, NRAS	FIGF
hsa-miR-99a-5p	FGFR3, RB1	-
hsa-miR-23a-3p	CDH1, IL-8, MYC	E2F2, CCND1, RPS6KA5
hsa-miR-215-5p	CDKN2A, RB1	-
hsa-miR-10b-5p	CDKN1A, CDKN2A, TP53	RAF1

Shiny app

By using our open shiny app BladdermiRaCan (https://remote.iislafe.san.gva.es/sample-apps/bladder_miracan/), any researcher can use our predictive model to easily diagnose and stage their BC patients. The steps in the process are easy to follow: 1) Quantify in your study subjects by RT-qPCR the expression level of 7 miRNAs (miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p and miR-21-5p) and the normalizer (miR-29c-3p). 2) Upload the raw Ct values of these 8 miRNAs using a simple interface. 3) Download a file with the predictions of belonging to the different

categories analyzed (different BC stages-grades or healthy controls) for each subject studied (Supplementary Figure 2).

DISCUSSION

Although various studies have previously aimed to identify urine miRNAs as novel non-invasive biomarkers to improve BC diagnosis and monitoring (16, 17, 20, 22), to the best of our knowledge none has included BC patients with the whole broad spectrum of grades and stages. Moreover, none has aimed to identify a miRNA profile to stratify BC patients.

In the present study, we have created and validated a model comprising 7 urine miRNAs as predictors (i.e. miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p and miR-21-5p) able to identify and stratify BC patients from healthy controls. In addition, after FDR adjustment, we identified other 63 dysregulated miRNAs among the clinical categories studied. We also proved in the validation cohort the dysregulation of the 6 most significantly dysregulated miRNAs (miR-30a-5p, miR-425-5p, miR-99a-5p, miR-23a-3p, miR-215-5p and miR-10b-5p) identified in the screening cohort.

Henceforth, we will discuss previous findings about the role of miRNAs in BC. Armstrong DA et al (31) analyzed the miRNA pattern of paired biological samples from BC patients and identified seven miRNAs (miR-4454, miR-21, miR-720/3007a, miR-205, miR-200c-3p and miR-29b-3p) dysregulated in both urine exosomes and FFPE tissue specimens. Later, Pardini B et al (32) proposed urine miR-200c-3p as a diagnostic and staging marker for BC. Finally, Liu L et al (33) described that miR-200c-3p controls the epithelial-to-mesenchymal transition process in BC cells through BMI-1, and it inhibits their proliferation by down-regulating E2F3. In our study, miR-200c-3p is a key miRNA able to diagnose and stratify BC, as it is comprised in the validated Elastic Net model. Andreu Z et al (34) studied urinary miRNAs contained in extracellular vesicles from 34 BC patients and 9 healthy volunteers. They found that miR-375 was downregulated in high-grade BC patients and miR-146a was upregulated in low-grade BC patients. In our study, we also observed that miR-375 was downregulated in BC patients (P=0.0087) and miR-146a-5p (P=0.0052) was upregulated in BC patients, confirming the previous results presented by Andreu Z et al. Sapre N et al(16) identified and validated a urinary profile of six miRNAs, namely

miR-16, miR-200c, miR-205, miR-21, miR-221 and miR-34a, able to predict the presence of BC with an AUC = 0.85. All these miRNAs were overexpressed in a subgroup with BC recurrence (tumor present) compared with patients with a previous history of BC but no recurrence (tumor absent). In our study, we also found an increased expression in BC patients in 5 of the 6 miRNAs: miR-16-5p (P=0.0029), miR-200c-3p (P=0.0003), miR-205-5p (P=0.0018), miR-21-5p (P=0.0002), miR-34a (P=0.0019). Remarkably, 3 of the 6 miRNAs proposed by Sapre N et al were included in our Elastic Net logistic regression model (miR-221-3p, miR-200c-3p, miR-21-5p), which highlights the potential role of these 3 miRNAs in BC. Erdmann K et al (35) analyzed 9 miRNAs previously related to BC in urine sediments from 104 patients with primary BC and 46 control subjects. In their analysis, Bonferroni's correction, revealed a diagnostic potential for miR-125b, miR-126, miR-145, miR-183, and miR-221. While miR-126 and miR-183 displayed a higher relative expression in BC-derived urine sediments, miR-125b, miR-145, and miR-221 were downregulated in these specimens compared to the control group. In our study, taking into account the miRNAs we both studied, we found the same trend in miR-125b-5p (P=0.0004) and miR-126-3p (P=0.0059), but we observed an upregulation of miR-145-5p in BC (P=0.026) and no differences in miR-221 (P=0.11). However, it is worth noting that we analyzed urine samples while Erdmann K et al analyzed its sediment.

We also analyzed the variation of miRNA expression levels over time according to BC relapse. We found an increase in miR-21-5p and miR-425-5p and a decrease in miR-99a-5p over time in BC patients with tumor relapse. In fact, the expression of these three miRNAs normalized to the levels of controls only in BC patients with tumor remission. All in all, these three miRNAs may be valuable as follow-up markers to identify BC recurrence.

We also evidenced an increase of miR-21-5p and miR-221-3p in urine of BC patients with metastasis compared to those without metastasis, turning them potential non-invasive biomarkers for tumor metastasis in BC patients. In fact, we observed an increase in the expression of these two miRNAs with the degree of BC severity in the validation cohort. This result is consistent with previous findings. In cervical carcinoma, miR-21-5p promotes a metastatic phenotype, while its silencing suppressed proliferation, migration and invasion of CaSki human cervical carcinoma cells (36) . Similarly, miR-221-3p has been shown to promote metastasis in cervical cancer by

facilitating the epithelial–mesenchymal transition and promoting cell migration and invasion in vitro and lymphatic metastasis in vivo (37).

We analyzed the variation of miRNA expression according to the BC cellular subtype. We observed a significant increase in miR-93-5p, miR-425-5p, miR-21-5p, miR-200c-3p in BC patients with in situ BC compared to papillary BC. Our results suggest that these miRNAs may be involved in the development of one of this specific BC tumor subtype.

Finally, to unravel the origin of the dysregulation of these 13 miRNAs, we analyzed their expression in FFPE tissue specimens from most patients of the screening cohort. The expression of these miRNAs according to BC severity in cancer tissue specimens follow the trend observed in urine samples from the same patients. These results propose the study of urine as liquid biopsy for BC as it may apprise the expression of miRNAs in the tumor microenvironment.

To delve into the biological mechanism(s) regulated by these miRNAs, we evidenced that all the miRNAs have validated targets related to BC development and progression. Additionally, ten of the thirteen miRNAs studied had predicted targets along the BC pathway. It is very interesting that each miRNA targets different proteins along the bladder cancer pathway and that many of these proteins are simultaneously targeted by several miRNAs (i.e. E2F3, which promotes cancer progression, is regulated by miR-221-3p, miR-93-5p, miR-200c-3p, miR-21-5p and miR-425-5p). TP53, a tumor suppressor that is mutated in most human cancer types, is one of the most popular genes in cancer research (38). Interestingly, TP53 is regulated by several miRNAs dysregulated in BC patients (miR-221-3p, miR-30a-5p, miR-10b-5p) and is a predicted target of miR-93-5p. c-MYC is another well-known oncogene that contributes to the genesis of many human cancers (39). In our in silico analysis we found that this gene is regulated by several dysregulated miRNAs in BC (miR-93-5p, miR-21-5p, miR-23a-3p) and is a predicted target of miR-200c-3p. IL-8, regulated by miR-93-5p and miR-23a-3p, enhances angiogenesis and regulates the tumorigenesis and production of spontaneous metastases in human BC (40).

In the past few years, research has faced up new challenges derived from novel technologies. In the field of miRNAs studies, researchers deal with an enormous quantity of data, standardization is urgently needed, and the application of previous

findings becomes somewhat challenging. For these reasons, we decided to step forward and generate an open tool for BC diagnosis and stratification, BladdermiRaCan. We have developed a user-friendly and easy-to-use web application to enable any researcher worldwide to diagnose and stage their patients using our model. Knowledge generated in the basic bench side has to be applied in the clinic side and, in this way little by little, improve the diagnosis and prognosis implementing novel approaches.

A limitation of our study is the reduced number of healthy bladder urothelium specimens analyzed. However, the tissue surrounding a bladder tumor cannot be considered healthy as it contains genetic alterations, and bladder urothelium from healthy individuals is almost impossible to obtain. Strengths of our study are the validation of our findings in an independent large cohort of BC patients and controls including all BC subtypes, a thorough evaluation of patients at inclusion and during follow-up and the analysis of paired urine and tissue samples. Future studies using BC cell lines could improve the understanding of these miRNAs in the development and progression of BC.

CONCLUSIONS

In summary, we have identified and validated a urine profile of 7 miRNAs (miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p and miR-21-5p) with potential diagnostic and stratification value for BC, together with 6 other dysregulated miRNAs (miR-30a-5p, miR-425-5p, miR-99a-5p, miR-23a-3p, miR-215-5p, miR-10b-5p), in a wide cohort of BC patients and controls. We also identified miR-21-5p, miR-425-5p and miR-99a-5p as potential follow-up markers for BC relapse and miR-21-5p and miR-221-3p as potential markers for tumor metastasis. Among the dysregulated miRNAs, miR-93-5p, miR-425-5p, miR-21-5p and miR-200c-3p could be markers of in situ BC subtype. The analysis of PPFE tumor tissue specimens revealed that miRNA content in urine may reflect that of tumor microenvironment, thus becoming valuable as liquid biopsy. Finally, as an added value to our results, we created the open app BladdermiRaCan to enable the global use of our model for BC diagnosis and stratification. The use of these novel non-invasive urine biomarkers may improve BC management thus reducing the use of current invasive diagnostic techniques.

TAKE HOME MESSAGE

Current diagnostic techniques for BC are expensive and harmful for the patient. We have identified and validated a profile of 7 miRNAs in urine able to stratify BC patients. We have also identified potential markers of tumor relapse, metastasis and cellular subtypes. Finally, we have created an open app to enable any researcher the use of our model for BC diagnosis and stratification.

CONFLICTS OF INTEREST

The authors state they have no conflict of interest.

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AUTHOR CONTRIBUTIONS

JO processed samples, performed the research, analysed the data and wrote the manuscript. EP supervised the analysis and critically revised the manuscript. AF-P supervised the analysis and critically revised the manuscript. JP-A recruited the patients and revised the clinical records. DH performed the statistical analysis and critically revised the manuscript. AC performed the statistical analysis and critically revised the manuscript. FC processed the samples and prepared the databases. RH performed the research and critically revised the manuscript. DR-S performed the anatomopathological diagnosis of BC from tissue specimens and critically revised the manuscript. MM-S performed the patients´ follow-up and critically revised the manuscript. CDV-D performed the patients´ follow-up and critically revised the manuscript. PM designed and supervised the study, analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

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6. CHAPTER 3: microRNAs and Neutrophil Activation Markers Predict Venous Thrombosis in Pancreatic Ductal Adenocarcinoma and Distal Extrahepatic Cholangiocarcinoma

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microRNAs and Neutrophil Activation Markers Predict Venous Thrombosis in Pancreatic Ductal Adenocarcinoma and Distal Extrahepatic Cholangiocarcinoma

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ABSTRACT: Cancer-associated venous thrombosis (VTE) increases mortality and morbidity. However, limited tools are available to identify high risk patients. Upon activation, neutrophils release their content through different mechanisms, thereby prompting thrombosis. We explored plasma microRNAs (miRNAs) and neutrophil activation markers to predict VTE in pancreatic ductal adenocarcinoma (PDAC) and distal extrahepatic cholangiocarcinoma (DECC). Twenty-six PDAC and 6 DECC patients recruited at cancer diagnosis, were examined for deep vein thrombosis and pulmonary embolisms, and were then followed-up with clinical examinations, blood collections, and biCUS. Ten patients developed VTE and were compared with 22 age- and sex-matched controls. miRNA expression levels were measured at diagnosis and right before VTE, and neutrophil activation markers (cell-free DNA, nucleosomes,

calprotectin, and myeloperoxidase) were measured in every sample obtained during follow-up. We obtained a profile of 7 miRNAs able to estimate the risk of future VTE at diagnosis (AUC = 0.95; 95%Confidence Interval [CI] [0.87, 1]) with targets involved in the *pancreatic cancer* and *complement and coagulation cascades* pathways. Seven miRNAs were up- or down-regulated before VTE compared with diagnosis. We obtained a predictive model of VTE with calprotectin as predictor (AUC = 0.77; 95% CI [0.57, 0.95]). This is the first study that addresses the ability of plasma miRNAs and neutrophil activation markers to predict VTE in PDAC and DECC.

Keywords: Pancreatic ductal adenocarcinoma; distal extrahepatic cholangiocarcinoma; microRNA; neutrophil; venous thromboembolism; calprotectin; biomarker; thrombosis; NETosis

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1. Introduction

Venous thromboembolism (VTE) is a condition in which blood clots form most often in the deep veins of the leg, known as deep vein thrombosis (DVT). When this blood clot is disrupted from the vessel wall, it can travel in the circulation and lodge in the lungs, thereby causing a pulmonary embolism (PE). VTE is a common complication of cancer patients often leading to a reduced survival [1], and treatment of cancer patients with VTE results in significantly increased treatment costs and reduced quality of life [2].

Epidemiologic studies have confirmed that pancreatic cancer patients have a high incidence of VTE [3], with 4.3 VTE events per 100 hospitalizations [4]. In pancreatic cancer, VTE is associated with a median overall survival of 5.8 months in patients with VTE *vs.* 10.3 months in patients without VTE (p =0.031). The overall survival is even worse when the VTE event occurs during chemotherapy [5]. Furthermore, metastatic pancreatic cancer patients have a 2.1-fold higher risk for recurrent VTE than other metastatic cancer patients.

Distal extrahepatic cholangiocarcinoma (DECC) is anatomically closely related to pancreatic ductal adenocarcinoma (PDAC) [6]. DECC can often not be separated from PDAC unless the patients undergo radical surgery and even then the differentiation can be difficult [7,8]. Furthermore, the risk of VTE in patients with cholangiocarcinoma is almost as high as in pancreatic cancer patients with a comparable low survival [9].

Several clinical assessment scores have been proposed for thrombotic risk stratification in cancer patients [10–15]. The Khorana score is commonly used in predicting the risk of VTE in chemotherapy treatment [10]. As for all clinical assessment scores there are limitations [12,16–20] and current tools for predicting and monitoring the risk of VTE are inadequate, especially in pancreatic cancer [21]. The discovery of novel and precise biomarkers to identify cancer patients with a high risk of VTE could either, replace or strengthen a clinical risk score.

microRNAs (miRNAs) are small non-coding RNAs that regulate protein expression. They are identified as regulatory molecules and biomarkers in virtually all cancer types, and in pancreatic cancer, several miRNAs could turn out to be valuable biomarkers [22–26]. Despite an extensive bibliographic revision, there is, to our knowledge, no literature on miRNA and risk of VTE in PDAC or DECC patients.

Neutrophil granulocytes are the most abundant type of white blood cells in the immune system. Upon activation, they play a prominent role in defense mechanisms by phagocytosis, degranulation and by neutrophil extracellular trap (NET) formation. NETs are extracellular networks of DNA, histones and granule proteins (calprotectin, myeloperoxidase, elastase, etc.) released by neutrophils in response to an inflammatory stimulus [27] or to the presence of pathogens, in a process called NETosis[28]. NETs may trigger coagulation and, in turn, increase the risk of VTE. In cancer-associated thrombosis, cancer cells activate neutrophils to produce more NETs than those activated by other means [29,30]. Pancreatic cancer cells can stimulate the rapid release of NETs, which promote thrombus formation under venous shear stress ex vivo[31]. Boone et al. demonstrated in a murine model of pancreatic cancer that NETs promote hypercoagulability, which is diminished by chloroquine [32].

In a prospective study, which included patients with a suspected upper gastrointestinal cancer, the patients with PDAC and DECC were examined at time of cancer diagnosis and followed for two years with blood samples and VTE examination every third month. We aimed to identify a profile of plasma miRNAs and markers of neutrophil activation, in order to predict a VTE event in PDAC and DECC. In addition, plasma was investigated for up- or down-regulated miRNAs in the last blood sample before the VTE event, and then compared with the analysis of the blood sample at inclusion in an attempt to identify one or more mechanisms triggering VTE in PDAC and DECC.

2. Results

2.1. Clinical Characteristics of the Study Subjects

Among the 121 cancer patients recruited in the original study [33], a VTE event was objectively diagnosed in 15 patients (12.4%) at the time of cancer diagnosis and, due to the study protocol of the present study, excluded. During follow-up 10 patients (8.3%) developed a VTE event. The characteristics of patients and age- and sex-matched controls are listed in Table 1, and those of the original cohort of 121 cancer patients can be found in the original study [33]. Three patients developed DVT and two developed pulmonary embolism. Five patients developed DVT and PE simultaneously during follow-up.

Table 1. Characteristics of the 32 patients with pancreatic ductal adenocarcinoma (PDAC) and distal extrahepatic cholangiocarcinoma (DECC) patients studied.

	VTE Patients	**Non-VTE Patients**	**Statistical significance *P***
N (% of total)	10 (31.3)	22 (68.8)	-
Age, y, median (range)	64 (50–79)	66 (51–84)	0.57¤
Female sex, N (%)	4 (40)	9 (40.4)	0.64 *
Time to VTE, months, median (range)	3 (1–24)		
Tumor location:			
PDAC N (%)	9 (90)	17 (77.3)	
DECC N (%)	1 (10)	5 (22.7)	0.64 *
Leukocyte count	8.6 ± 3.2 x 10^9/L	8.7 ± 2.5 x 10^9/L	0.92
Neutrophil count (Mean ± SD)	6.6 ± 3.6 x 10^9/L	6.1 ± 2.5 x 10^9/L	0.65
Treatment:			
Curative intended surgery	2	11	
Neoadjuvant treatment	1	0	
Postop. Chemotherapy	0	3	
Palliative gemcitabine	7	11	0.12 *
UICC stage:			
I	1	4	
II	2	6	
III	2	5	
IV	5	7	0.83 *
WHO performance score:			
0	3 (30)	18 (81.8)	
1	5 (50)	3 (13.6)	
2	2 (20)	1 (4.6)	0.012 *

CCI score:			
0	5 (50)	18 (81.8)	
1	2 (20)	3 (13.6)	
2	2 (20)	1 (4.6)	
4	1 (10)	0	0.14 *
Khorana score:			
2	3 (30)	9 (40.9)	
3	4 (40)	8 (36.4)	
4	3 (30)	4 (18.2)	
5	0	1 (4.6)	0.92 *

UICC, Union for International Cancer Control, 6th Edition; WHO, World Health Organization; CCI score, Charlson Comorbidity Index score. Mann-Whitney. * Fisher's exact test.

2.2. miRNA Expression Levels: Screening Stage

Based on the quality of the isolated RNA, we selected five patients who suffered a VTE event during follow-up (VTE group) and 5 who did not. These selected patients were matched on age and sex. In them, the expression level of 179 miRNAs commonly found in human plasma was studied, at inclusion and also right before the VTE event for the VTE patients. We obtained high quality signals (Ct < 36) in 123 of the 179 miRNAs included in the panel in at least one study group. Regarding the synthetic RNA controls for RNA isolation, cDNA synthesis and inter-plate performance we did not observe differences between both clinical groups (data not shown).

With respect to the best endogenous reference to normalize the expression level of each miRNA, the comprehensive tool RefFinder rendered miR-93-5p as the one with the highest stability and the lowest biological variance among all samples. As a result, we normalized every miRNA expression level using the miR-93-5p as endogenous reference by the $2^{-\Delta\Delta CT}$ method.

Our main goal was to identify a miRNA profile at diagnosis able to predict the occurrence of a VTE event in PDAC and DECC cancer patients during follow-up. To that end, in this screening stage, we adjusted an elastic net logistic regression model for VTE risk using the miRNA expression levels at inclusion. It comprised the expression level of 11 miRNAs as predictors: miR-486-5p, miR-32-5p, miR-106b-5p, miR-326, let-7i-5p, let-7g-5p, miR-144-5p, miR-144-3p, miR-19a-3p, miR-103a-3p and miR-30e-3p (Table 2). These miRNAs had a fold-change ranging from -2.58 to 4.28.

Table 2. miRNAs comprised in the predictive model of VTE in PDAC and DECC patients obtained in the screening stage. miRNA sequences according to miRBase 22.1. Fold-change is defined as the ratio of the average expression level of a miR in PDAC and DECC patients who suffered a VTE event during follow-up and those who did not.

miRNA	Sequence	Fold-change	Coefficient
hsa-miR-486-5p	uccuguacugagcugccccgag	1.82	0.041
hsa-miR-32-5p	uauugcacauuacuaaguugca	2.60	0.082
hsa-miR-106b-5p	uaaagugcugacagugcagau	1.96	1.235
hsa-miR-326	ccucugggcccuuccuccag	−2.58	−0.761
hsa-let-7i-5p	ugagguaguaguuugugcuguu	1.87	0.668
hsa-let-7g-5p	ugagguaguaguuuguacaguu	1.74	0.066
hsa-miR-144-5p	ggauaucaucauauacuguaag	3.57	2.509
hsa-miR-144-3p	uacaguauagaugauguacu	4.28	0.166
hsa-miR-19a-3p	ugugcaaaucuaugcaaaacuga	1.51	0.201
hsa-miR-103a-3p	agcagcauuguacagggcuauga	1.73	0.284
hsa-miR-30e-3p	cuuucagucggauguuuacagc	2.63	1.820

Furthermore, we aimed to identify up- or down-regulated miRNAs in the sample right before the VTE event compared with that obtained at inclusion, since this could shed light on the mechanism triggering a VTE event in PDAC and DECC patients. We identified a profile of 7 down-regulated miRNAs (miR-30e-3p, let-7i-5p, let-7g-5p, miR-144-3p, miR-199a-3p, miR-101-3p and miR-15a-5p) that might prompt the VTE event in these patients during follow-up (Table 3). Provided that this statistical analysis corresponds to paired samples, a delta ought to be calculated instead of a fold-change. Delta represents the difference in the expression level of a given miRNA between the sample at inclusion and the one right before the VTE event. The greatest difference in the delta values were found in miR-144-3p (delta: -0.8) and let-7g-5p (delta: -0.34). It could indicate a greater involvement of these two miRNAs in triggering the VTE event in PDAC and DECC patients.

Table 3. Dysregulated miRNAs in PDAC and DECC patients who develop a VTE during follow-up, comparing the sample at inclusion and that right before the VTE event. miRNA sequences according to miRBase 22.1. Delta is defined as the difference of the average expression level of a miRNA between the samples at inclusion and the ones right before the VTE event. The negative values of delta represent down-regulation of miRNAs in the samples right before the VTE event.

miRNA	Sequence	*p* (T-Test)	Delta
hsa-miR-30e-3p	cuuucagucggauguuuacagc	0.015	−0.035
hsa-let-7i-5p	ugagguaguaguuugugcuguu	0.026	−0.062
hsa-let-7g-5p	ugagguaguaguuuguacaguu	0.03	−0.34
hsa-miR-144-3p	uacaguauagaugauguacu	0.03	−0.8
hsa-miR-199a-3p	acaguagucugcacauugguua	0.025	−0.11
hsa-miR-101-3p	uacaguacugugauaacugaa	0.029	−0.26
hsa-miR-15a-5p	uagcagcacauaaugguuugug	0.031	−0.07

2.3. miRNA Expression Levels: Confirmation Stage

Next, we aimed to confirm the predictive ability of our model in a larger cohort of PDAC and DECC patients at inclusion, 10 who developed a VTE during follow-up and 22 who did not. In these, we could quantify by RT-qPCR the expression level of 7 of the 11 miRNAs comprised in the predictive model: miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p. The remaining 4 miRNAs had a very low expression level and did not achieve a suitable qPCR criteria (Ct < 35 and SD between duplicates <0.5). In this confirmation stage, we obtained a receiver operator characteristic ROC curve AUC of 0.95 in this cohort of 32 patients (95% Confidence Interval [CI] [0.87, 1], *p* <0.001) (Figure 1). The formula for estimating the thrombotic risk in PDAC and DECC patients with our model is as follows (Formula 1):

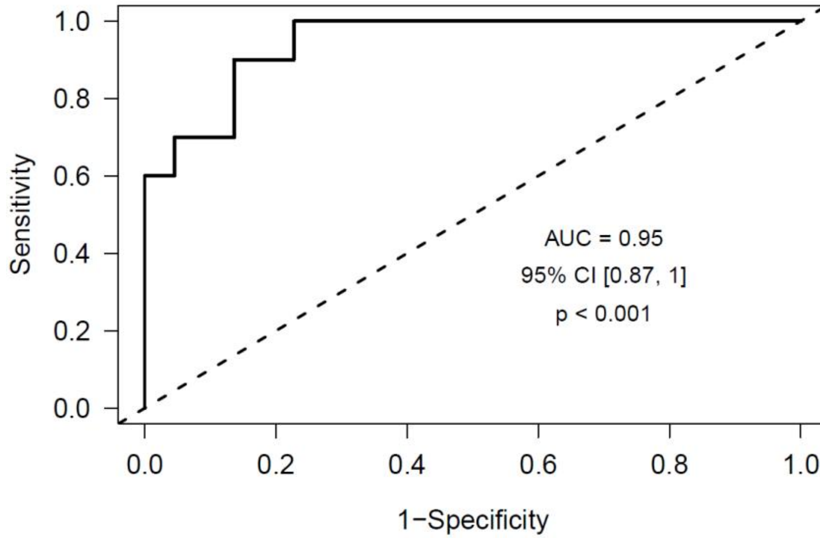

Figure 1. Receiver operator characteristic ROC curve obtained from the confirmation data set using the Elastic Net model that includes 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) as risk predictors of future cancer-associated venous thrombosis (VTE) in pancreatic ductal adenocarcinoma (PDAC) and distal extrahepatic cholangiocarcinoma (DECC) patients at diagnosis.

$$\Pr(Thrombosis) = \frac{e^{LP}}{1 + e^{LP}} \quad (1)$$

Where LP is:

$$LP = -0.65 + 0.28 * miR486_5p + 1.97 * miR106b_5p - 1.12 * miR19a_3p - 5.23 * miR103a_3p - 9.67 * let7i_5p + 1.8 * let7g_5p + 8.61 * mir144_3p$$

Furthermore, we estimated the thrombotic risk of each patient at inclusion by applying the aforementioned formula of our predictive model. The median thrombotic risk at inclusion of the group of patients who suffered a VTE during follow-up was 0.72, while it was only 0.13 in the group of patients who did not suffer a VTE during follow-up (p <0.0001).

2.4. Identification of the miRNAs' Targets

We identified a profile of 7 miRNAs able to accurately predict at inclusion a VTE event in PDAC and DECC patients during follow-up. Next, we identified their validated and predicted target proteins related to cancer and VTE by using the database miRWalk 2.0. This database integrates computational algorithms for target identification

rendering two types of targets: Predicted targets were those being theoretically estimated based on the free binding energy between a miRNA and a putative target mRNA sequence; and validated targets were those empirically validated to be regulated by a miRNA. Subsequently, we integrated these targets within the *pancreatic cancer* pathway and the *complement and coagulation cascades* pathway from KEGG (Table 4). Noticeably, we identified an important number of validated targets of these miRNAs in both pathways clearly related to PDAC, DECC and VTE. Moreover, we further identified a group of predicted targets whose regulation by these miRNAs could be experimentally demonstrated in future studies.

Table 4. Validated and predicted targets of the 11 miRNAs included in the predictive model of VTE in PDAC and DECC patients at inclusion. These target proteins were identified using miRWalk 2.0 and were further integrated within the *pancreatic cancer* pathway and the *complement and coagulation cascades* pathway from KEGG. Validated targets are those that have been empirically validated to be regulated by a miRNA. Predicted targets are those that have been theoretically estimated based on the free binding energy between a miRNA and a putative target mRNA sequence.

	***Pancreatic cancer* pathway**		***Complement and coagulation cascades* pathways**	
miRNA	**Validated target**	**Predicted target**	**Validated target**	**Predicted target**
hsa-miR-486-5p	-	CDK4	SERPINE1	F2R, F9, C6, C8A, PLAT, C5AR1, SERPING1
hsa-miR-32-5p	-	MAPK8, PIK3CB, BRAF, CASP9, PLD1, CDC42	-	-
hsa-miR-106b-5p	ACVR1B, CCND1, CDC42, E2F1, E2F2, E2F3, JAK1, MAPK1, MAPK9, RB1, SMAD4, STAT3, TGFBR2, TP53, VEGFA	BRAF, KRAS	F2R, F3	CD46, C5
hsa-miR-326	AKT1, CCND1, ERBB2, KRAS	TGFA, PGF, CDKN2A, RAC2, MAPK10	C1R, F9	BDKRB2, C8G, C2, SERPINF2, C8B, C1S, MASP1
hsa-let-7i-5p	CCND1	MAPK8, AKT2, BCL2L1, TP53	CD59	-
hsa-let-7g-5p	AKT2, BCL2L1, CCND1, CDKN2A, KRAS, SMAD2, TGFBR1	MAPK8, TP53	CD59	-

hsa-miR-144-5p	-	STAT1, STAT3, E2F3	-	F2R
hsa-miR-144-3p	RAC1, TGFB1	STAT1, E2F3, MAPK9, CDC42, AKT2, PIK3CG	FGA, FGB, FGG	F13B, PLAT, PLG, CR1, CR2
hsa-miR-19a-3p	AKT1, CCND1, MAPK1, PIK3R3, RAF1, SMAD4, TGFBR2, TP53	CCND1, RAF1, PIK3CA, PIK3R1	PLAU	TFPI, CR2, C7, F3, PLAU, THBD, C6, CD55, SERPIND1, BDKRB2
hsa-miR-103a-3p	CDK6, PIK3R1, RAD51	SMAD4, PLD1, FIGF, RALBP1, CDC42, MAPK3, IKBKG, RALGDS	-	C1QB, MASP1, SERPING1, VWF, C1S, SERPINC1, CR2
hsa-miR-30e-3p	KRAS	MAPK10, RALBP1, ERBB2, RALB, CASP9, RAD51	C6	C1S, FGG

Similarly, we identified the targets of the 7 miRNAs that were down-regulated in the VTE group in a sample right before the VTE event, and compared it with the sample obtained at inclusion (Table 5). Again, we identified a great number of validated and predicted targets of these down-regulated miRNAs that could shed light on the mechanisms triggering the VTE event in these patients.

Table 5. Validated and predicted target genes of the 7 miRNAs down-regulated in the VTE group of patients in a sample right before the VTE event compared with the sample obtained at inclusion. These target proteins were identified using miRWalk 2.0 and were further integrated within the *pancreatic cancer* pathway and the *complement and coagulation cascades* pathway from KEGG. Validated targets are those that have been empirically validated to be regulated by a miRNA. Predicted targets are those that have been theoretically estimated based the free binding energy between a miRNA and a putative target mRNA sequence.

	***Pancreaticcancer*pathway**		***Complement and coagulation cascades* pathways**	
miRNA	**Validatedtarget**	**Predictedtarget**	**Validatedtarget**	**Predictedtarget**
hsa-miR-30e-3p	KRAS	MAPK10, RALBP1, ERBB2, RALB, CASP9, RAD51	C6	C1S, FGG
hsa-let-7i-5p	CCND1	MAPK8, AKT2, BCL2L1, TP53	CD59	-
hsa-let-7g-5p	AKT2, BCL2L1, CCND1, CDKN2A, KRAS, SMAD2, TGFBR1	MAPK8, TP53	CD59	-

hsa-miR-144-3p	RAC1, TGFB1	STAT1, E2F3, MAPK9, CDC42, AKT2, PIK3CG	FGA, FGB, FGG	F13B, PLAT, PLG, CR1, CR2
hsa-miR-199a-3p	AKT1, E2F2, MAPK1, MAPK8, MAPK9	CDC42	-	C4BPA, PLG, C3AR1
hsa-miR-101-3p	E2F3, MAP2K1, RAC1, TGFBR1, TGFBR2, VEGFA	ACVR1C, BRAF, EGFR, PLD1, CDC42, AKT2, PIK3CG	CD46	FGA, CR2, F13B, PLAT, PLG
hsa-miR-15a-5p	ACVR1B, AKT3, CCND1, CDK6, CHUK, E2F3, IKBKG, NFKB1, PIK3R1, SMAD3, TP53, VEGFA	SMAD4, IKBKB, MAP2K1, RAF1, ARHGEF6	-	-

2.5. Neutrophil Activation Markers and Risk of Thrombosis

Using the Wilcoxon-Mann-Whitney test, we analyzed the differences in the levels of each marker of neutrophil activation between patients who developed VTE and those who did not . The neutrophil counts at inclusion were similar in the two groups (Table 1). We observed an increase in calprotectin levels in those patients who developed VTE (1374 ng/mL) compared with those who did not (427 ng/mL) (p =0.017) and also in plasma myeloperoxidase (MPO) levels (98 *vs.* 87 ng/mL, respectively; p =0.059). No substantial differences were observed between groups in the plasma levels of nucleosomes (0.12 *vs.* 0.096 U, respectively; p =0.25) or cfDNA (2048 *vs.* 1688 ng/mL, respectively; p =0.41). Furthermore, to evaluate whether the different markers of neutrophil activation measured in plasma have the same cellular origin, we evaluated their correlation. A significant correlation was observed between calprotectin and MPO levels (Spearman r = 0.648, p <0.001), between calprotectin and cfDNA levels (Spearman r = 0.594, p <0.001), between MPO and cfDNA levels (Spearman r = 0.587, p =0.001), between calprotectin and nucleosomes levels (Spearman r = 0.467, p =0.009) and between cfDNA and nucleosomes levels (Spearman r = 0.359, p =0.044).

To evaluate the ability of neutrophil activation markers to identify PDAC and DECC patients at high risk of developing VTE during follow-up, we conducted a Cox regression survival model with a time-dependent covariate, including the markers of neutrophil activation measured in each sample collected from the patients in rank form. We observed that, for each unit that the logarithm of the calprotectin concentration increases, the VTE risk in PDAC and DECC patients increases 6 times (p =0.009). Next, we adjusted an Elastic Net logistic regression model and obtained a predictive

model of VTE with calprotectin as predictor (AUC = 0.77, 95% CI [0.57, 0.95], *p* =0.008), optimism-corrected AUC = 0.76 (Figure 2). The other neutrophil activation markers showed no significant predictive capacity of VTE.

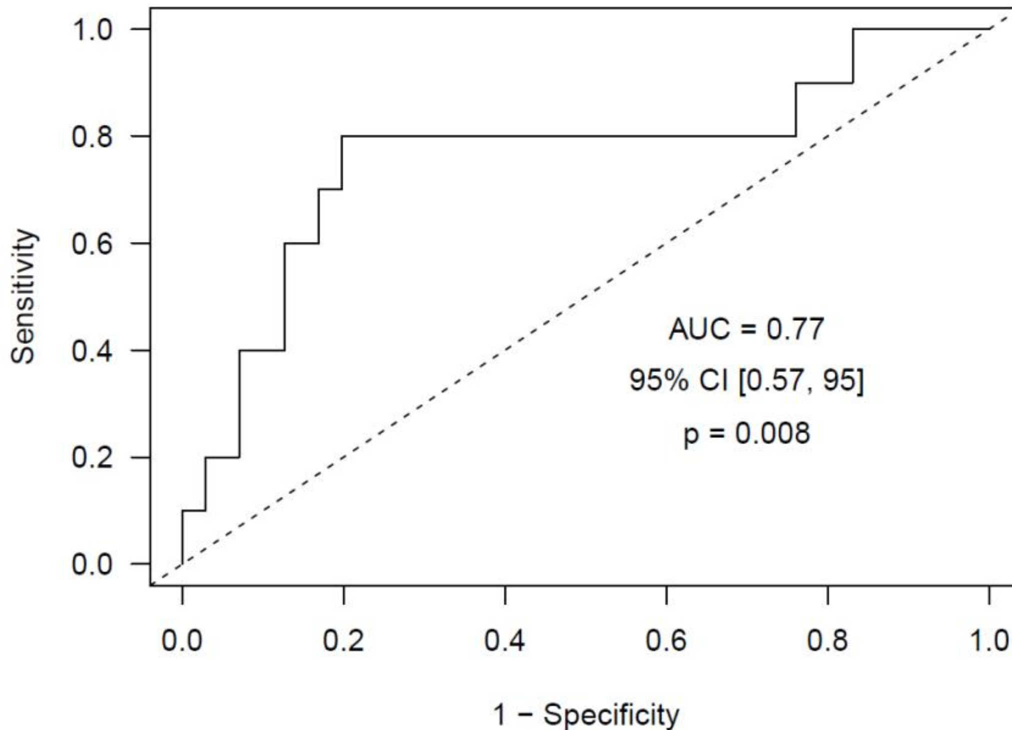

Figure 2. ROC curve obtained using the Elastic Net model that includes calprotectin as risk predictor of future VTE in PDAC and DECC patients at diagnosis.

3. Discussion

Pancreatic cancer induces a hypercoagulable state mainly mediated by a high tumoral expression of tissue factor, the activation of leukocytes with the release of NETs, the dissemination of tumor-derived microvesicles that promote hypercoagulability and an increased platelet activation [3]. In fact, pancreatic cancer bears the highest incidence of VTE complications [4], while cholangiocarcinoma is almost the same [9]. The appearance of disabling co-morbidities and a potential increase in future vascular thromboembolic events [1,5,34] brings about a reduction in the overall survival. Several scores have been proposed to evaluate the thrombotic risk of cancer patients [10–15], like the widely used Khorana score [10]. However, several limitations have been raised [12,16–20], thus creating the need to develop novel tools to predict and monitor the thrombotic risk [21].

Numerous studies conducted over the past decade have revealed that aberrantly expressed miRNAs are a hallmark of cancer and many other diseases. Thereby, the expression profile of miRNAs has been associated with tumor development, progression and response to therapy, suggesting their possible use as diagnostic, prognostic and predictive biomarkers [35]. Moreover, previous evidence shows that miRNAs can function as potential oncogenes or oncosuppressor genes by targeting each of the essential features described of cancer progression: Self-sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis evasion, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis [35]. Furthermore, cancer metastasis is facilitated by miRNAs encapsulated in extracellular vesicles, released by primary tumor cells in order to create a cancer microenvironment for their progression [36].

In the present study we have identified a miRNA profile at diagnosis able to predict the occurrence of a VTE event in PDAC and DECC patients during follow-up. This model includes 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) and achieves a high predictive capacity (ROC curve AUC = 0.95, 95% CI [0.87, 1], p <0.001). Furthermore, applying this predictive model, we estimated the thrombotic risk of each PDAC and DECC patient of our study at inclusion, and the median thrombotic risk of the group of patients who suffered a VTE during follow-up was 0.72, while it was only 0.13 in the group of patients who did not suffer a VTE during follow-up (p <0.0001), thus confirming the predictive ability of our model. Our promising results support the accomplishment of validation studies in an independent cohort of prospectively recruited biliopancreatic cancer patients.

Additionally, we aimed to identify up- or down-regulated miRNAs that could be involved in triggering the VTE event in PDAC and DECC cancer patients. In that respect, we studied the expression profile of miRNAs in the sample right before the VTE event compared with that obtained at inclusion. We identified a profile of 7 down-regulated miRNAs (miR-30e-3p, let-7i-5p, let-7g-5p, miR-144-3p, miR-199a-3p, miR-101-3p and miR-15a-5p) that might prompt the VTE event in these patients during follow-up. Among these, miR-144-3p and let-7g-5p showed the greatest differences in expression levels between both samples studied, what could indicate a greater involvement of these two miRNAs in triggering the VTE event in PDAC and DECC patients.

In order to further understand the biological mechanism potentially dysregulated by these miRNAs, we identified their targets and those pathways where they participate. Remarkably, most miRNAs were comprised in the predictive model or were down-regulated in the VTE patients, before the event, have targets involved in the *pancreatic cancer* pathway and in the *complement and coagulationcascades* that have been validated in previous studies. This fact reinforces the potential regulatory role of these miRNAs in PDAC and DECC patients. Henceforth, we will discuss several of these regulations in detail.

KRAS, *TP53* and *SMAD* act as oncogenes in pancreatic cancer. In fact, pancreatic adenocarcinoma is characterized by several germline or acquired genetic mutations, the most common being *KRAS* (90%), *CDK2NA* (90%), *TP53* (75%–90%), *SMAD4*/*DPC4* (50%). Thus, the diagnostic and prognostic value of the mutational status is currently under evaluation,[37] and may represent a future therapeutic target [38]. Furthermore, this tumor type holds epigenetic alterations that could guide personalized cancer therapies. In addition, the tumor microenvironment, the chemo-resistant cancer stem cells, and the desmoplastic stroma have been the target of recent clinical investigations [39–42]. Two of the miRNAs included in our VTE predictive model and down-regulated in VTE patients, let-7g-5p and let-7i-5p, belong to the let-7 miRNA family that regulates *RAS*. Gain-of-function approaches have shown that miRNAs of the let-7 miRNA family act as tumor suppressors by targeting oncoproteins with crucial roles in various cancer pathways, such as *RAS* [35]. Moreover, the combination of let-7i, miR-142, miR-26a and miR-141 has been proposed as prognostic model to robustly stratify nasopharyngeal carcinoma patients into high- and low- risk groups of distant metastasis [43].

A common characteristic of many cancer cells is the mutational status of the tumor suppressor gene *TP53*, with almost half of human malignancies harboring an altered form of this gene [44]. Four miRNAs comprised in our VTE predictive model (miR-106b-5p, let-7i-5p, let-7g-5p and miR-19a-3p) have *TP53* as validated or predicted target.

As observed, the regulation of the human biological pathways is very complex since one miRNA usually targets many mRNAs in the same pathway and every mRNA is targeted by many miRNAs to ensure a fine-tuned global regulation. Furthermore, the targets regulated by each miRNA may have opposite functions, which represents a

controversy on whether the final outcome of a miRNA would then be oncogenic or tumor suppressive. It is now known that the miRNA may produce an overall net oncogenic or net tumor suppressive effect, depending on the balance between miRNA-mediated upregulation or downregulation of oncogenic and tumor suppressive pathways, as well as the effects of the miRNA on cancer-immune system interactions and various other tumor-modifying extrinsic factors [45].

Additionally, in vitro studies have demonstrated that several miRNAs comprised in our VTE predictive model and those down-regulated miRNAs that could be involved in triggering the VTE event in PDAC and DECC patients, target proteins involved in coagulation, such as serpins (plasminogen activator inhibitor-1, miR-486-5p; urokinase, miR-19a-3p) and coagulation factors (tissue factor, miR-106b-5p; fibrinogen alpha and beta and gamma, miR-144-3p) [46–50]. Noticeably, other predicted targets involved in coagulation may be regulated by these miRNAs, such as serpins, thrombomodulin, von Willebrand factor, other coagulation factors, etc. Future studies conducted in vitro in cell cultures and in vivo in animal models would verify the regulation of these predicted targets and would elucidate the degree of participation of each miRNA in the final complex regulatory mechanisms exerted by these miRNAs in PDAC and DECC patients. To the best of our knowledge, this is the first study in which the predictive role of miRNAs for VTE in PDAC and DECC patients is addressed.

Upon activation, neutrophils release their content through different mechanisms like degranulation and NETosis, thus prompting thrombosis. Hence, in our study we also explored the ability of several markers of neutrophil activation, in order to identify PDAC and DECC patients at high risk of developing VTE during follow-up. To that end, we measured different plasma markers of neutrophil activation following the strategy addressed in previous studies [51–57]. We have observed an increase in calprotectin and MPO plasma levels in those patients who developed VTE compared with those who did not. Nucleosomes and cfDNA levels were also slightly increased in these patients. The neutrophil activation markers studied, herein, could have a different cellular origin other than neutrophils. Calprotectin and MPO could be released by monocytes, macrophages or eosinophils, but only to a lesser extent. In fact, calprotectin accounts for approximately 60% of total soluble proteins in the cytosolic fraction of neutrophils [58] and, although, low levels are found in other phagocytic cells, it is clinically considered to be neutrophil-specific and higher levels in plasma or feces are

found in diseases associated with increased neutrophil activity. cfDNA and nucleosomes could be released into plasma by apoptotic or necrotic cells which, not least, may be present in these cancer patients. However, all these four markers were significantly correlated pairwise, indicating that they all had, to some extent, the same origin, probably an increased activation of neutrophils or NETs formation. Although only calprotectin and MPO were significantly increased. It could be speculated that the level of neutrophils may affect the levels of plasma activation markers, but no differences in neutrophil counts were observed between VTE and non-VTE patients (Table 1). Several patients were treated with gemcitabine, a cytostatic agent that may lower the neutrophil count and, by that, interfere with the extent of neutrophil activation markers. However, no correlation was observed between changes in neutrophil counts during treatment and the level of neutrophil activation markers, thus discarding this effect in our study. Furthermore, a greater proportion of VTE patients (7 of 10) than non-VTE patients (11 of 22) received this treatment, so it would tend to reduce the difference in plasma markers between the two groups.

Regarding the predictive ability of these neutrophil activation markers, we observed that for each unit that the logarithm of the calprotectin concentration increased, the VTE risk in PDAC and DECC patients increased six times. Finally, we obtained a predictive model for VTE with calprotectin as a predictor, which achieved an AUC = 0.77 (95% CI [0.57, 0.95], p =0.008). To date, only one study has explored the ability of markers of NETs to predict a VTE event in pancreatic cancer patients [57], where an increase in these markers was associated with the occurrence of VTE. Additionally, Jin et al. [59] observed that NETs markers predicted poor postsurgical survival of patients with PDAC. Furthermore, the incorporation of these markers with the standard TNM stating system refined the risk stratification and predicted survival in PDAC with improved accuracy.

A limitation of our study is the rather small sample size studied. However, the thorough clinical assessment of cancer patients for VTE every three months during two years hinders the recruitment and management of a high number of patients for this type of prospective studies. Nevertheless, consistent with the frequency of VTE events in biliopancreatic cancer patients, only 10 patients developed a VTE during follow-up. Thus, following common practice [60], a ratio of 2 controls per 1 patient was established in our case-control study. The validation of our results in an independent

external cohort of biliopancreatic cancer patients prospectively recruited and followed would definitively reinforce our findings. The strengths of our study is that we conducted a thorough evaluation of patients at inclusion and during follow-up.

In conclusion, our study has highlighted the ability of plasma miRNAs and calprotectin as biomarkers for predicting a VTE event in PDAC and DECC patients. We have obtained and confirmed a profile of 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) able to estimate the risk of future VTE in PDAC and DECC patients at diagnosis. These miRNAs are deeply involved in the *pancreatic cancer* pathway and the *complement and coagulation and cascades* pathways. Similarly, plasma calprotectin may be a valuable tool to estimate the VTE risk in these patients. Personalized medicine and targeted therapy have presently become the cornerstone in medicine. Thus, once validated in a larger independent cohort of patients, our predictive models may be implemented into daily clinical practice. The estimation of the thrombotic risk of each PDAC and DECC patient at diagnosis might promote a closer follow-up and a personalized thromboprophylaxis in high-risk patients.

Moreover, we have observed that 7 miRNAs (miR-30e-3p, let-7i-5p, let-7g-5p, miR-144-3p, miR-199a-3p, miR-101-3p and miR-15a-5p) are significantly down-regulated in PDAC and DECC patients right before the VTE event compared with inclusion at diagnosis. These miRNAs have highlighted the aforementioned target proteins and the mechanism that might be triggering a VTE in PDAC and DECC patients. Interestingly, four of these seven miRNAs are dysregulated in both analyses (miR-30e-3p, let-7i-5p, let-7g-5p and miR-144-3p), upregulated in VTE patients at inclusion compared with non-VTE patients and down-regulated right before the VTE event compared with inclusion. Particularly, miR-144-3p and let-7g-5p showed the greatest differences in the expression level right before the VTE event, what reinforces the idea of these miRNAs being strong candidates for prompting a thrombotic complication in PDAC and DECC patients. Future studies in different thrombotic scenarios would shed light in shared dysregulated mechanisms between pathologies of different etiology but with a common outcome.

4. Materials and Methods

4.1. Study Subjects

A total of 121 patients admitted on suspicion of an upper gastrointestinal cancer were prospectively recruited and followed for two years, between February 2008 and February 2011, at the Department of Gastrointestinal Surgery of the Aalborg University Hospital (Aalborg, Denmark) [33]. Cancer was confirmed histologically and staged according to the UICC6 system by means of diagnostic computer tomography (CT) of the thorax and abdomen, or positron emission tomography-CT (PET-CT). During follow-up, patients were objectively assessed for DVT by bilateral compression ultrasound and PE by an arterial-stage scan covering the pulmonary arteries (a CT pulmonary angiogram) as previously described [33] every three months, beginning at the time of cancer diagnosis. Patients who underwent curatively intended surgery were examined preoperatively and postoperatively.

The exclusion criteria were: Previous (within the past three years) or concomitant cancer of any origin; known immunologic connective tissue disease; mental disorder; previous episodes of VTE; and treatment with heparin, low molecular weight heparin (LMWH) or vitamin K antagonists at the time of inclusion in the study. Patients who did not provide consent because of debilitation, advanced age or refusal to participate in the study were also excluded. All patients provided written and oral informed consent (Clinical Trials.gov: NCT00660205; approval of local ethics committee of Region North Jutland, Denmark: N-20080002). The study was performed according to the declaration of Helsinki, as amended in Edinburgh in 2000.

4.2. Blood Collection

Blood was drawn from all patients at diagnosis and every three months during follow-up. Blood was collected in Monovette tubes (Sarstedt, North Rhine-Westphalia, Germany) containing 0.109 M trisodium citrate and then centrifuged at 2600x *g* for 20 min at 4 °C. Plasma was collected ensuring the absence of platelet and leukocyte contamination, by only taking the 2/3 of upper plasma, and stored in aliquots at −80 °C until used.

4.3. RNA Isolation

Total plasma RNA (including miRNAs) was isolated using the miRNeasy Mini Kit (Qiagen, Hiden, Germany), following manufacturer´s instructions with several modifications optimized by our group [61]. During the isolation, an RNA carrier (tRNA, Ambion, Bleiswijk, The Netherlands) was included to enhance the yield, and a

mixture of synthetic miRNAs (Spike-in kit UniRT, Exiqon, Vedbaek, Denmark) was included to control for RNA isolation efficiency and cDNA synthesis. The concentration and purity of the RNA was assessed by spectrophotometric quantification with the NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was stored at −80 °C until used. The haemolysis of plasma was assessed by measuring the absorbance of the haemoglobin at 412 nm.

4.4. Quantification of the Expression Level of miRNAs

The expression level of miRNAs was quantified by real-time quantitative reverse transcription PCR (RT-qPCR) in two stages:

4.4.1. Screening Stage

Based on the quality of the isolated RNA, 5 patients who suffered a VTE event during follow-up (VTE group) and five who did not were selected and miRNAs expression level was studied at inclusion. Additionally, in the VTE patients, the expression level of miRNAs was studied in the last plasma sample obtained right before the VTE event to identify dysregulated miRNAs that may prompt the VTE event. The Universal cDNA Synthesis Kit II (Exiqon, Vedbaek, Denmark) was used for the retrotranscription and the expression level of 179 candidate miRNAs mostly present in plasma was quantified with the Serum/Plasma Focus microRNA PCR Panel V4 (Exiqon, Vedbaek, Denmark) and the ExiLENT SYBR Green Master Mix (Exiqon, Vedbaek, Denmark) in a LightCycler 480 II (Roche, Mannheim, Germany). Furthermore, each panel includes the following internal controls: five synthetic RNAs of the RNA Spike-in-kit aimed to monitor the RNA isolation and cDNA synthesis, and an inter-plate calibrator in triplicate and a negative control to evaluate qPCR performance.

To normalize the expression level of each miRNA, the best endogenous reference with the highest stability and the lowest biological variance among all samples was selected. The candidate normalization miRNAs proposed by the Serum/plasma Focus microRNA PCR Panel V4 were miR-423-5p, miR-425-5p, miR-93-5p, miR-191-5p and miR-103a-3p. To select the best reference, the comprehensive tool RefFinder was employed which integrates the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method (https://www.heartcure.com.au/for-researchers/). The miRNA expression levels were normalized by the 2-ΔΔCT method.

Next, a multivariable logistic regression model was generated, able to accurately predict a VTE event during follow-up using the data of both patient groups at inclusion. Additionally, those miRNAs dysregulated in a sample right before the VTE event, compared with the sample obtained at inclusion in the VTE group were identified.

4.4.2. Confirmation stage

Once selected a miRNA profile potentially able to predict a VTE event in PDAC and DECC patients during follow-up, their expression level was quantified in a larger cohort of patients (26 patients with PDAC and 6 with DECC, 10 who developed a VTE during follow-up [9 PDAC and 1 DECC], and 22 age- and sex-matched who did not) at inclusion by RT-qPCR in duplicate. For that aim, specific primers for each miRNA, miRCURY LNA miRNA PCR Assay (Exiqon, Vedbaek, Denmark) were used. Each measurement in duplicate was considered suitable when the standard deviation (SD) was <0.5.

4.5. Identification of the miRNAs' Targets

Once the dysregulated miRNAs were detected, the identification of their predicted and validated target proteins related to cancer and VTE was conducted by using the databases miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, Kyoto, Japan) (https://www.genome.jp/kegg/). miRWalk 2.0 (Mannheim, Germany) combines information from 12 existing miRNA-target prediction programs (DIANA-microTv4.0, DIANA-microT-CDS, miRanda-rel2010, mirBridge, miRDB4.0, miRmap, miRNAMap, doRiNAi.e.,PicTar2, PITA, RNA22v2, RNAhybrid2.1 and Targetscan6.2). Finally, the targets obtained with miRWalk 2.0 were integrated within the *pancreatic cancer* pathway and the *complement and coagulation cascades* pathway from KEGG.

4.6. Quantification of Neutrophil Activation Markers

Different markers of neutrophil activation were measured in every plasma sample obtained during follow-up from the 32 patients studied herein, following the strategy addressed in previous studies [51–57]. Cell-free DNA (cfDNA; Quant-iTPicoGreen dsDNA kit, Life Technologies, Eugene, OR, USA) and nucleosomes (Cell Death Detection ELISA^{PLUS} kit, Roche, Mannheim, Germany) were measured as markers of the nuclear content of neutrophils released by neutrophils upon NETosis. Calprotectin

(Human Calprotectin ELISA kit, Hycult Biotech, Uden, The Netherlands) was measured as a marker of cytoplasmic content and MPO (Human MPO ELISA kit, Abnova, Taoyuan, Taiwan) as a marker of the content of neutrophil granules, were both released upon neutrophil activation by different mechanisms. In all cases the experiments were performed following the manufacturer´s instructions.

4.7. Statistical Analysis

All statistical analyses were performed using R (version v3.5.1; Vienna, Austria). Continuous variables were presented as median and interquartile range, and categorical variables as count and percentage. In the screening stage, an elastic net logistic regression model for VTE risk was adjusted using the miRNA expression levels at inclusion of 10 selected patients (five who developed VTE and five without VTE). The predictive ability of the model was assessed by estimating an optimism-corrected area under the curve (AUC) for the receiver operator characteristic (ROC) analysis, and using 1000 bootstrap replicates in the screening stage. Next, this AUC was verified in the confirmation stage. The formula to calculate the risk of VTE in each patient was built with the coefficients rendered by the model for each predictive variable. Furthermore, a paired T-test was applied to identify dysregulated miRNAs in the sample right before the VTE event compared with that obtained at inclusion. The association of neutrophil activation markerswith VTE was assessed by comparing the levels of each marker in both clinical groups (with and without VTE) by using the Wilcoxon-Mann-Whitney test. The ability of neutrophil activation markers to identify PDAC and DECC patients, at high risk of developing VTE during follow-up, was evaluated by means of a Cox regression survival model with a time-dependent covariate, including the neutrophil activation markers measured in each sample of the patients in rank form. The results were considered statistically significant at $p < 0.05$. Additionally, an elastic net logistic regression model for VTE risk was adjusted using the levels of neutrophil activation markers in every sample obtained from the 32 patients at inclusion and during follow-up. For this case-control study, a ratio of two controls per one case was established. Accordingly, the 10 patients who developed VTE during follow-up were selected, along with a selection of 22 patients who did not develop VTE, age- and sex-matched (two extra controls to make the control group more comparable to the VTE-group) and representative of the whole sample set.

Authors Contributions: J.O. performed the experiments, analyzed the data and critically revised the manuscript. S.N. performed the experiments, analyzed the data and critically revised the manuscript. A.C.L. included the patient, collected the samples, and made the data analysis of the initial paper and wrote it, and critically revised this manuscript. M.J.S. processed the samples and performed the experiments. E.P. performed the experiments and critically revised the manuscript. D.H. analyzed the data and critically revised the manuscript. A.F.-P. performed the experiments. F.E. designed and supervised the experiments, analyzed the data and critically revised the manuscript. S.R.K. and O.T.-U. designed the initial study and contributed to the data analysis and preparation of this manuscript and critically revised the present manuscript. P.M. designed and performed the experiments, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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7. CHAPTER 4: microRNAs and Markers of Neutrophil Activation as Predictors of Early Incidental Post-Surgical Pulmonary Embolism in Patients with Intracranial Tumors

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microRNAs and Markers of Neutrophil Activation as Predictors of Early Incidental Post-Surgical Pulmonary Embolism in Patients with Intracranial Tumors

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Abstract: Venous thromboembolism (VTE) is a common complication of cancer that severely increases morbidity and mortality. Patients with intracranial tumors are more likely to develop VTE than patients with cancers at other sites. Conversely, limited tools exist to identify patients with high thrombotic risk. Upon activation, neutrophils release their content through different mechanisms triggering thrombosis. We explored the ability of microRNAs (miRNAs) and plasma markers of neutrophil activation measured before surgery to predict the risk of early post-surgical pulmonary embolism (PE) in glioma and meningioma patients. We recruited and prospectively followed 50

patients with glioma and 50 with meningioma, 34% of whom in each group developed an early objectively-diagnosed post-surgical PE. We measured miRNA expression and neutrophil markers (cell-free DNA, nucleosomes, calprotectin and myeloperoxidase) before surgery. In glioma patients, we adjusted and validated a predictive model for post-surgical PE with 6 miRNAs: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p (AUC = 0.78; 95% Confidence Interval (CI) [0.63, 0.94]) and another with cfDNA and myeloperoxidase as predictors (AUC = 0.71; 95%CI [0.52, 0.90]). Furthermore, we combined both types of markers and obtained a model with myeloperoxidase and miR-140-3p as predictors (AUC = 0.79; 95%CI [0.64, 0.94]). In meningioma patients we fitted and validated a predictive model with 6 miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p (AUC = 0.69; 95%CI [0.52, 0.87]). All our models outperformed the Khorana score. This is the first study that analyzes the capability of plasma miRNAs and neutrophil activation markers to predict early post-surgical PE in glioma and meningioma patients. The estimation of the thrombotic risk before surgery may promote a tailored thromboprophylaxis in a selected group of high-risk patients, in order to minimize the incidence of PE and avoid bleedings.

Keywords: microRNA; neutrophil activation; pulmonary embolism; glioma; meningioma; cancer; venous thromboembolism

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1. Introduction

Cancer patients have a higher risk of venous thromboembolism (VTE) than non-cancer patients. As a consequence, the prognosis of cancer patients is worsened, with an increase in morbidity and mortality that exacerbates health costs [1]. Patients with intracranial tumors are more likely to develop VTE than patients who have cancers at other sites [2]. Accordingly, high-grade brain tumors correlate with a higher rate of VTE, including both deep vein thrombosis and pulmonary embolism (PE), being glioma one of the most thrombogenic among intracranial tumors [2]. The frequency of VTE after brain surgery is further increased [3]. However, it is different in malignant brain tumors such as glioma than in benign tumors such as meningioma, where operated patients have a risk of VTE up to 26% [4] and 30% [5,6], respectively. The underlying mechanisms responsible for the increased VTE risk seem to be heterogeneous,

including factors related to the tumor itself, to the patient, to other physiopathological mechanisms (e.g., inflammation) as well as iatrogenic factors [7,8]. Tumor cells seem to induce hypercoagulability through multiple mechanisms, such as the production of procoagulant and proaggregating molecules (e.g., tissue factor), and the release of pro-inflammatory cytokines that activate endothelial cells, platelets and leukocytes. A direct effect of haemostasis in enhancing angiogenesis, cell survival and metastasis has also been observed [9,10].

Because VTE is a frequent complication of cancer that strongly increases morbidity and mortality in these patients, novel biomarkers are needed to identify cancer patients with high VTE risk. Furthermore, the postoperative diagnosis of VTE in neurosurgical patients is of paramount importance provided that the use of anticoagulant therapy increases the risk of intracranial hemorrhagic complications [5].

microRNAs (miRNAs) are small non-coding RNAs that regulate protein expression, and have been suggested as regulatory molecules and biomarkers in practically all cancer types [11]. In the particular scenario of intracranial tumors, several miRNAs have been proposed as biomarkers in glioma [12–16] and meningioma [17–19], and even these molecules have been proposed to have therapeutic benefits in these tumors [20,21]. However, to date, the role of miRNAs in the stratification of VTE risk in patients with intracranial tumors has never been explored.

Once activated, neutrophils a crucial role in host defense by phagocytosis, degranulation and also by neutrophil extracellular trap (NET) formation. NETs are extracellular networks of DNA, histones and proteins (calprotectin, myeloperoxidase, elastase, etc.) released by neutrophils in response to an inflammatory stimulus [22] or to the presence of pathogens, in a process called NETosis [22]. Moreover, NETs prompt coagulation [23], thus inducing cancer-associated thrombosis, since neutrophils activated by cancer cells produce more NETs than those activated by other mechanisms [24]. The neutrophil-lymphocyte ratio has been widely explored as predictor for overall survival in intracranial tumor patients [25,26], however no previous studies have addressed the role of neutrophil activation markers in these tumors or their thrombotic complications.

Our main goal was to evaluate the ability of miRNAs and neutrophil activation markers, measured before surgery, to predict the risk of early post-surgical PE in glioma

and meningioma patients, in order to identify a subgroup of patients at a higher risk of PE who may deserve a tailored thromboprophylaxis.

2. Results

2.1. Clinical Characteristics of the Study Subjects

In the original cohort, 59 consecutive patients with glioma and 93 consecutive patients with meningioma were recruited and prospectively followed. In 18 patients with glioma (31%) and in 36 with meningioma (39%) an early post-surgical incidental PE was objectively diagnosed. From the original cohort, we selected for this study 50 patients from each tumor type, 34% of which (17 patients for each tumor type) had developed an early post-surgical PE. These patients were all the available glioma patients with follow-up completed at the time of the validation stage commencement, and a random selection of meningioma patients with an incidence of PE similar to that of the whole original cohort. The clinical characteristics of the study subjects studied herein are depicted in Table 1 and raw data from each individual can be consulted in Table S1.

Table 1. Baseline clinical characteristics of the study subjects.

Clinical characteristic	**Glioma Patients (*n* =50)**	**Meningioma Patients (*n* =50)**
PE events, *n* (%)	17 (34)	17 (34)
Age, y	61 (51–70)	64 (50–71)
Female sex, *n* (%)	22 (44)	33 (66)
BMI, Kg/m^2	24.7 (22.2–27.4)	25.9 (21.1–29.7)
Comorbidities, *n* (%)		
Cardiovascular	20 (40)	18 (36)
Respiratory	1 (2)	2 (4)
Metabolic *	8 (16)	5 (10)
Miscellanea †	5 (10)	5 (10)
Pre-operative KPS ≥ 80, *n* (%)	47 (94)	49 (98)
Post-operative KPS ≥ 80, *n* (%)	44 (88)	47 (94)
WHO classification, *n* (%)		
Grade I	0 (--)	45 (90)
Grade II	6 (12)	5 (10)
Grade III	8 (16)	0 (--)
Grade IV	36 (72)	0 (--)

Tumor location, *n* (%)		
Skull base	0 (--)	15 (30)
Cerebral convexity-falx	0 (--)	35 (70)
Superficial	12 (24)	0 (--)
Deep-seated	38 (76)	0 (--)
Tumor dimension, cm^3	24 (12.3–50.2)	16 (5,8–35.2)
Duration of surgery, min	240 (210–240)	215 (176.3–277.5)
Khorana score, *n* (%)		
0	27 (54)	40 (80)
1	23 (46)	10 (20)
Hemoglobin, g/dL	13.7 (12.7–14.9)	12.9 (11.9–13.3)
WBC, $\times 10^3$/mmc	10.04 (6.64–12.10)	6.10 (4.92–7.69)
Neutrophils, $\times 10^3$/mmc	6.03 (3.99–7.26)	3.66 (2.95–4.62)
Platelets, $\times 10^3$/mmc	226 (188–248)	216 (189–278)
PT ratio	0.99 (0.93–1.09)	1.02 (0.97–1.08)
APTT ratio	0.81 (0.73–0.89)	0.94 (0.86–1.01)
Fibrinogen, mg/dL	229 (196–279)	257 (222–304)
D-dimer, ng/mL	218 (125–565)	167 (100–210)
CRP, mg/L	0.07 (0.03–0.18)	0.11(0.06–0.26)
eGFR, mL/min/1.73 m^3	93.6 (79.8–113.9)	90.8 (78.3–107.6)

Continuous variables are displayed as median and interquartile range. Categorical variables are displayed as count and percentage. *Diabetes mellitus, hypercholesterolemia, obesity, hyper- or hypothyroidism, chronic liver or renal disease. † Previous surgeries, other neoplasms, psychiatric disorders. PE, pulmonary embolism; BMI, body mass index; KPS, Karnofsky Performance Status; WHO, World Health Organization Classification of brain tumors; WBC, white blood cells; PT, prothrombin time; APTT, activated partial thromboplastin time; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate.

No differences in age were observed between both clinical groups. Female sex was more frequent in meningioma than in glioma patients (p =0.0439), consistent with the higher prevalence of this tumor type in women. All patients had negative baseline lung perfusion scans, thus indicating that none had pre-surgical asymptomatic PE. Those patients who developed an early post-surgical PE diagnosed with perfusion lung scan and confirmed with CT scan, underwent a color-Doppler compression ultrasound of the lower limbs to check for deep vein thrombosis. None of them had a positive result, thus discarding the presence of deep vein thrombosis. An intracranial hemorrhagic complication occurred in three patients with glioma (6%; two during surgery and one after surgery), and in two patients with meningioma (4%, both during surgery). Thirty-four of the 50 patients with glioma (68%) and 30 of the 50 patients with meningioma

(60%) had one or more comorbidities, mostly cardiovascular. These selected patients were representative of the whole sample set in terms of comorbidities. In accordance with meningioma being a benign tumor and glioma a malignant tumor, a higher proportion of grade III and IV tumors were observed in glioma patients and a higher proportion of grade I and II tumors were observed in meningioma patients. Additionally, a higher proportion of patients with score 1 of Khorana were found in the glioma group (46%) compared to meningioma (20%), while most meningioma patients (80%) had a Khorana score of 0 (p =0.0099). No overrepresentation of score 1 of Khorana occurred among PE patients.

Regarding tumor location, 12 gliomas (24%) were superficial and 38 were deep-seated (76%), while 15 meningiomas (30%) were located in the base of the skull and 35 (70%) were located in the cerebral convexity or falx. To rule out the possibility that the surgery performed had an impact on the occurrence of post-surgical PE, we compared the tumor size and the duration of the surgery (Table 1). Glioma tumors were bigger than meningiomas (24 and 16 cm^3, respectively; p =0.0481), while the duration of the surgery was similar between both tumor types (240 and 215 min, respectively; p =0.606).

Regarding other clinical variables registered, significant differences were observed between glioma and meningioma patients in hemoglobin levels (median 13.7 and 12.9 g/dL, respectively; p =0.0131), white blood cells (median 10.04 and 6.10 × 10^3/mmc, respectively; p <0.0001), neutrophil counts (median 6.03 and 3.66 × 10^3/mmc, respectively; p <0.0001) and activated partial thromboplastin time (APTT) ratio (median 0.81 and 0.94, respectively; p <0.0001).

2.2. miRNA Expression Levels and Risk of Incidental Post-Surgical PE

At the initial screening stage, we studied before surgery the expression level of 179 miRNAs frequently present in human plasma in 10 glioma and 10 meningioma patients, five with post-surgical PE in each group (cases and controls) by using predefined panels. Of the 179 miRNAs comprised in the panel, 130 showed high quality signals (Ct < 36) in at least one study group (Table S1). Concerning the RNA controls used for RNA isolation (spike-in 2), cDNA synthesis (spike-in 6), and qPCR performance (spike-in 3), no differences were observed in any comparison made (Figure S1 and Table S1).

Regarding the best normalizer for the expression level of each miRNA studied, the Comprehensive Ranking of RefFinder rendered miR-93-5p as the one with the highest stability and the lowest biological variance among all glioma and meningioma samples (Figure S2A and Table S1). Additionally, miR-93-5p was the candidate normalizer with the best expression value (lowest Ct) (median Ct = 29.17 [28.48–30.10]), compared to miR-423-5p (median Ct = 32.85 [32.32–33.99]), miR-425-5p (median Ct = 31.06 [30.30–31.54]), miR-191-5p (median Ct = 32.10 [31.35–33.03]) and miR-103a-3p (median Ct = 30.18 [29.56–31.49]) (Figure S2B). Thus, we normalized the expression level of each miRNA studied by that of miR-93-5p using the $2^{-\Delta\Delta CT}$ method. Next, we validated the predictive models obtained in the independent cohort of 40 glioma and 40 meningioma patients.

2.2.1. Glioma

In the screening stage, we adjusted a multivariable elastic net logistic regression model for PE risk with the miRNA expression levels before surgery. The model included eight miRNAs as predictors: miR-363-3p, miR-93-3p, miR-22-5p, miR-130b-3p, miR-885-5p, miR-451a, miR-222-3p and miR-140-3p. The effect of each miRNA was stated as standardized odds ratio (OR), reporting an increase of one standard deviation in the miRNA expression level. Accordingly, these miRNAs had standardized ORs ranging from 0.77 to 1.1 (Table 2). With this predictive model of PE we obtained an optimism-corrected area under the curve (AUC) of 0.78 (p =0.003). Importantly, there was a strong correlation among the different miRNAs included in our predictive model (Figure 1).

Table 2. miRNAs included in the multivariable elastic net logistic regression predictive model of post-surgical PE in glioma patients attained in the screening stage. miRNA sequences detailed in accordance with miRBase 22.1. Fold-change expresses the ratio of the average expression level of a miRNA in glioma patients who suffered a post-surgical PE event and those who did not.

miRNA	Sequence	Standardized OR	Fold-Change
miR-363-3p	aauugcacgguauccaucugua	0.85	-1.79
miR-93-3p	acugcugagcuagcacuucccg	0.91	-1.75
miR-22-5p	aguucuucaguggcaagcuuua	0.99	-2.50
miR-130b-3p	cagugcaaugaugaaagggcau	1.08	2.78
miR-885-5p	uccauuacacuacccugccucu	0.99	-2.94
miR-451a	aaaccguuaccauuacugaguu	0.94	-1.61
miR-222-3p	agcuacaucuggcuacugggu	0.88	-1.54
miR-140-3p	uaccacaggguagaaccacgg	0.77	-2.04

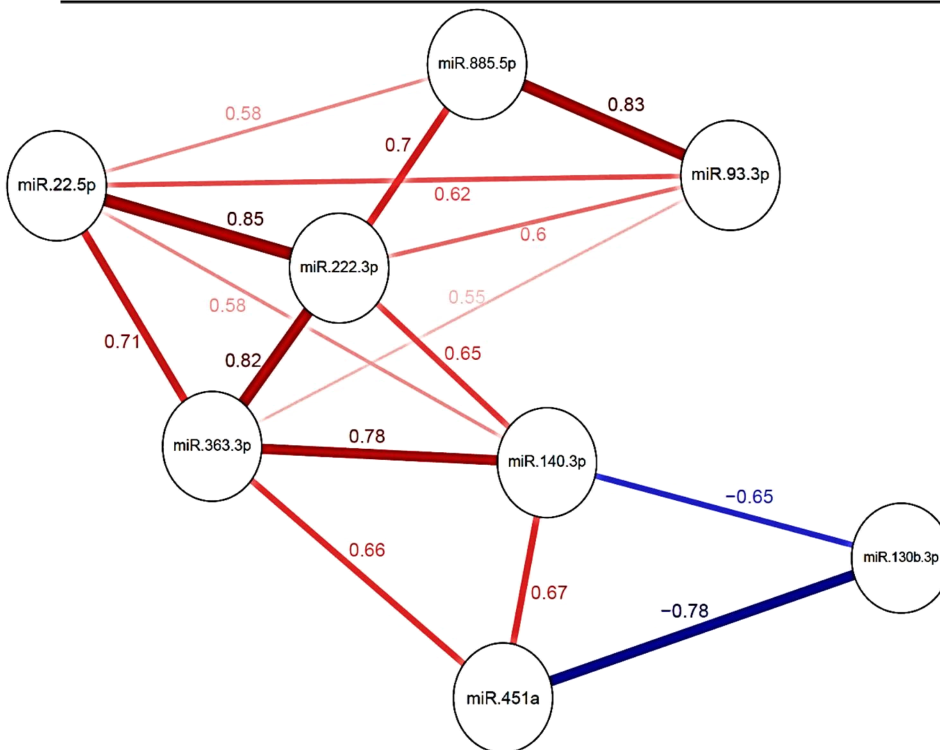

Figure 1. Correlation among the miRNAs included in the predictive model of post-surgical PE in glioma patients. Spearman correlation coefficients between two miRNAs are depicted next to the lines. Positive correlations are represented by red positive correlation values and red lines; negative correlations are represented by blue negative correlation values and blue lines. The degree of correlation is represented by the intensity of the number and the line and by the thickness of the lines, the more intense and thicker the line between two miRNAs, the strongest the correlation.

Next, we validated the predictive ability of our model in the cohort of 40 glioma patients. In them, we could quantify by real-time quantitative reverse transcription PCR (RT-qPCR) using specific primers the expression level of six of the eight miRNAs included in the model: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p. The remaining two miRNAs had a very low expression level thus not achieving an appropriate qPCR criteria (Ct < 35 and standard deviation between duplicates < 0.5) (Table S1). At this validation stage we obtained a receiver operator characteristic (ROC) curve AUC of 0.78 (95% Confidence Interval (CI) [0.63, 0.94], *p* =0.003) (Figure 2).

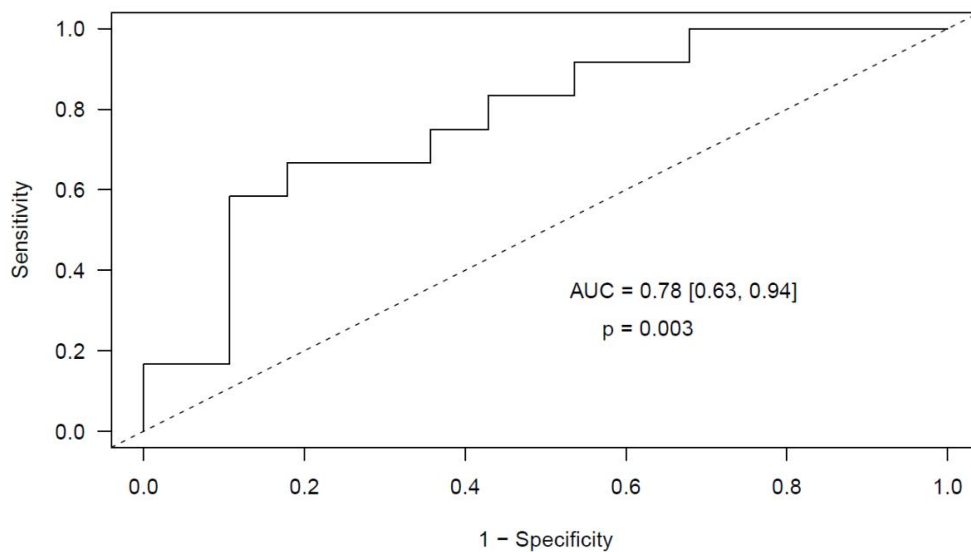

Figure 2. Validated ROC curve obtained from the multivariable elastic net logistic regression predictive model that contains six miRNAs measured before surgery (miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p) as predictors of post-surgical PE in glioma patients.

Regarding the clinical variables studied, the APTT ratio was the only variable significantly increased in glioma patients who developed PE (*p* =0.0139) (Table S2). Additionally, a higher proportion of grade IV tumors occurred in glioma patients who developed PE (*p* =0.0183). However, the inclusion of the clinical variables registered did not improve the predictive model obtained. The formula for estimating the risk of post-surgical PE in glioma patients with this model (Equation 1) was built with the coefficients provided by the model for each predictive variable, and is as follows:

$$\Pr(PE) = \frac{e^{LP}}{1 + e^{LP}} \tag{1}$$

where LP is given by the expression LP = −1.25 + 0.70 × miR-363-3p − 0.89 × miR-93-3p + 0.58 × miR-22-5p − 1.02 × miR-451a − 0.39 × miR-222-3p + 1.66 × miR-140-3p

The sensitivity and specificity of our model can be calculated for different thresholds from a plot (Figure S3). Furthermore, applying the abovementioned formula of our predictive model, we estimated the thrombotic risk of each glioma patient before surgery. The median thrombotic risk before surgery of those patients who suffered a post-surgical PE was 0.46, while it was only 0.2 in the group of patients who did not suffer post-surgical PE.

Next, we ascertained the validated and predicted target proteins related to VTE of these miRNAs in the database miRWalk 2.0. Next, we combined these targets with the complement and coagulation cascades pathway from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Table 3).

Table 3. Targets of the eight miRNAs contained in the predictive model of post-surgical PE in glioma patients. Target proteins were identified in miRWalk 2.0 and were combined with the *complement and coagulation cascades* pathway from KEGG. Validated targets are defined as those that have been experimentally proven to be regulated by a miRNA. Predicted targets are defined as those that have been theoretically identified based on the free binding energy between the miRNA and the presumed target mRNA sequence.

	***Complement and Coagulation Cascades* Pathway**	
miRNA	**Predicted target**	**Validated target**
miR-363-3p	C4BPA, CR2, CD55, KNG1	-
miR-93-3p	PROS1, TFPI, MASP1, F9, C6, C8B, MBL2	-
miR-22-5p	TFPI, F11, C8B	-
miR-130b-3p	SERPINA1, SERPING1, C3, MBL2, SERPINE1, C8A	F3
miR-885-5p	CD59, CFI, KNG1	-
miR-451a	-	-
miR-222-3p	-	-
miR-140-3p	CD59, SERPINA1, MASP1	-

2.2.2. Meningioma

In the screening stage, we adjusted a multivariable elastic net logistic regression model for post-surgical PE with miR-660-5p as predictor. Furthermore, with the

Random Forest regression test we obtained a predictive model of post-surgical PE with six miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p (Table 4 and Figure S4). These miRNAs had a fold-change ranging from −1.59 to 2.20.

Table 4. miRNAs included in the Random Forest regression predictive model of post-surgical PE in meningioma patients attained in the screening stage. miRNA sequences detailed in accordance with miRBase 22.1. Fold-change expresses the ratio of the average expression level of a miRNA in meningioma patients who suffered a post-surgical PE event and those who did not.

miRNA	Sequence	Fold-Change
miR-29a-3p	uagcaccaucugaaaucgguua	1.57
miR-660-5p	uacccauugcauaucggaguug	−1.59
miR-331-3p	gccccugggccuauccuagaa	2.20
miR-126-5p	cauuauuacuuuugguacgcg	1.99
miR-23a-3p	aucacauugccagggauuucc	1.91
miR-23b-3p	aucacauugccagggauuaccac	1.95

Next, we validated the predictive ability of our model in the cohort of 40 meningioma patients. In them, we could quantify by RT-qPCR using specific primers the expression level of all 6 miRNAs comprised in the model. In this validation stage, we obtained a ROC curve AUC of 0.69 (95% CI [0.52, 0.87], p =0.03) (Figure 3).

Regarding the clinical variables studied, the duration of surgery and platelet count were significantly increased in those meningioma patients who developed PE (p =0.00258 and p =0.0029, respectively). Similarly, a higher proportion of comorbidities catalogued as miscellanea (previous surgeries, other neoplasms, psychiatric disorders) occurred in meningioma patients with PE (p =0.0400). However, the inclusion of the clinical variables registered did not improve the predictive model obtained.

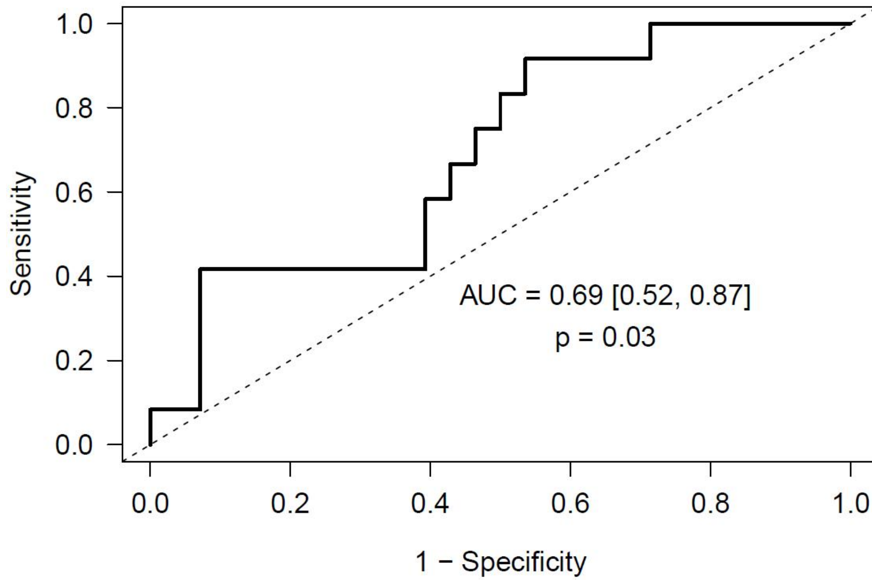

Figure 3. Validated ROC curve obtained from the Random Forest regression predictive model that includes 6 miRNAs measured before surgery (miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p, miR-23b-3p) as predictors of post-surgical PE in meningioma patients.

The formula for estimating the risk of post-surgical PE in meningioma patients with this model (Equation 2) was built with the coefficients provided by the model for each predictive variable, and is as follows:

$$\Pr(PE) = \frac{e^{LP}}{1 + e^{LP}} \tag{2}$$

Where LP is given by LP = −1.32 − 0.90 × miR-660-5p + 0.88*miR-23b-3p − 1.3 × miR-23a-3p − 0.03 × miR-29a-3p + 1.27 × miR-331-3p − 1.57 × miR-126-5p

Subsequently, we ascertained the validated and predicted target proteins related to VTE of these miRNAs in the database miRWalk 2.0. Finally, we combined these targets with the complement and coagulation cascades pathway from KEGG (Table 5).

Table 5. Targets of the six miRNAs contained in the predictive model of post-surgical PE in meningioma patients before surgery. Target proteins were identified in miRWalk 2.0 and were combined with the *complement and coagulation cascades* pathway from KEGG. Validated targets are defined as those that have been experimentally proven to be regulated by a miRNA. Predicted targets are defined as those that have been theoretically identified based on the free binding energy between the miRNA and the presumed target mRNA sequence.

	***Complement and Coagulation Cascades* Pathway**	
miRNA	**Predicted target**	**Validated target**
miR-29a-3p	BDKRB1, CR1, KNG1, BDKRB2, C8G	FGA, FGB, FGG
miR-660-5p	C9, KNG1	-
miR-331-3p	C3AR1, CFB, F10, F11, KLKB1, SERPINF2, F7	-
miR-126-5p	CR2, F8, F9	-
miR-23a-3p	CR1, F11, F2R, F8, MBL2, PLAU, PLAUR, PROS1, C1S, SERPINC1	-
miR-23b-3p	CR1, F11, F2R, F8, MBL2, PLAUR, PROS1, C1S, SERPINC1	PLAU

2.3. Markers of Neutrophil Activation and Risk of Incidental Post-Surgical PE

2.3.1. Glioma

To explore the ability of neutrophil activation markers, measured before surgery, to predict the risk of early post-surgical PE in our patients, we quantified cfDNA, nucleosomes, MPO and calprotectin using specific assays and we compared their levels with the Wilcoxon-Mann-Whitney test between those patients who suffered post-surgical PE and those who did not (cases and controls). The neutrophil counts before surgery were similar in the two groups (median 6.52 and 5.6 × 10^3/mmc, respectively; *p* =0.1345) (Table S2). The values of these markers obtained for each individual studied can be consulted in Table S1. We found an increase in myeloperoxidase (MPO) levels in patients who suffered PE (118.5 ng/mL) compared to those who did not (75.7 ng/mL) (*p* =0.028) as occurred with calprotectin levels (2051 vs. 1368 ng/mL, respectively; *p* =0.06). We did not observe considerable differences in plasma cell-free DNA (cfDNA) levels (1180 vs. 1146 ng/mL, respectively; *p* =0.31) or nucleosomes levels (0.35 vs. 0.22 U, respectively; *p* =0.23). We found a significant correlation between cfDNA and MPO levels (Spearman r = 0.323, *p* =0.022), suggesting that neutrophils may be the source of these makers in plasma of glioma patients.

At the screening stage (10 glioma patients) we attained a reliable multivariable elastic net logistic regression model of post-surgical PE with cfDNA and MPO as predictors (AUC = 0.88, 95% CI [0.63, 1.00], p =0.028). We validated this predictive model in the independent cohort of 40 glioma patients and obtained a validated AUC of 0.71 (95% CI [0.52, 0.90], p =0.02) (Figure 4).

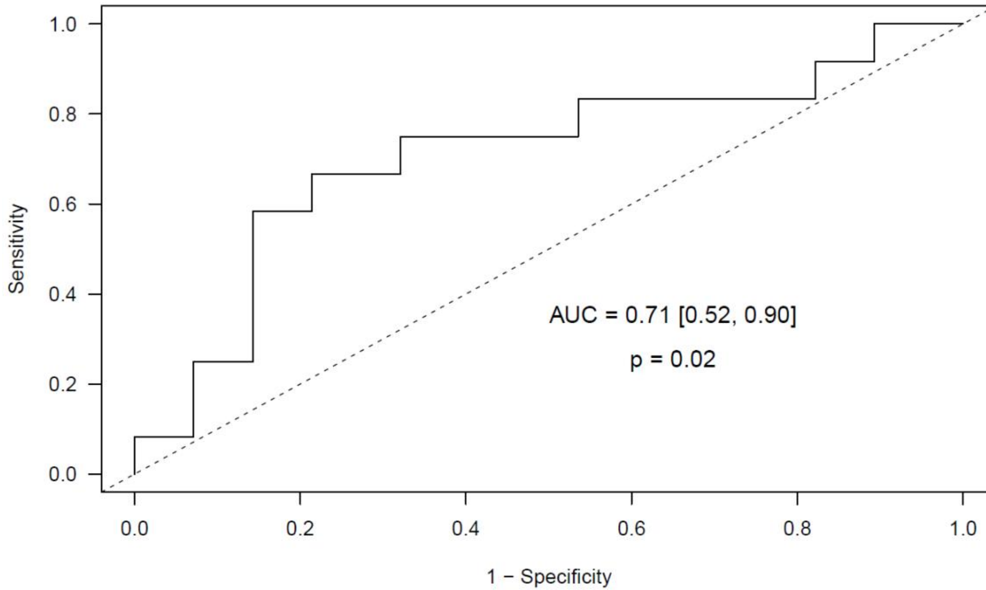

Figure 4. Validated ROC curve obtained from the multivariable elastic net logistic regression predictive model that includes MPO and cfDNA measured before surgery as predictors of post-surgical PE in glioma patients.

The inclusion of the clinical variables registered did not improve the predictive model obtained. The formula for estimating the risk of post-surgical PE in glioma patients with our model (Equation 3) was built with the coefficients provided by the model for each predictive variable, and is the following:

$$\Pr(PE) = \frac{e^{-1.96 \times 0.021 \times \mathrm{MPO} - 0.001 \times DNA}}{1 + e^{-1.96 \times 0.021 \times MPO - 0.001 \times DNA}} \tag{3}$$

To evaluate whether markers of neutrophil activation combined with miRNAs improved the predictive capability of the individual models, we adjusted a new multivariable elastic net logistic regression model that included MPO and miR-140-3p as predictors, achieving an AUC of 0.79 (95% CI [0.64, 0.94], p =0.002) (Figure 5), which became 0.76 after correction for optimism.

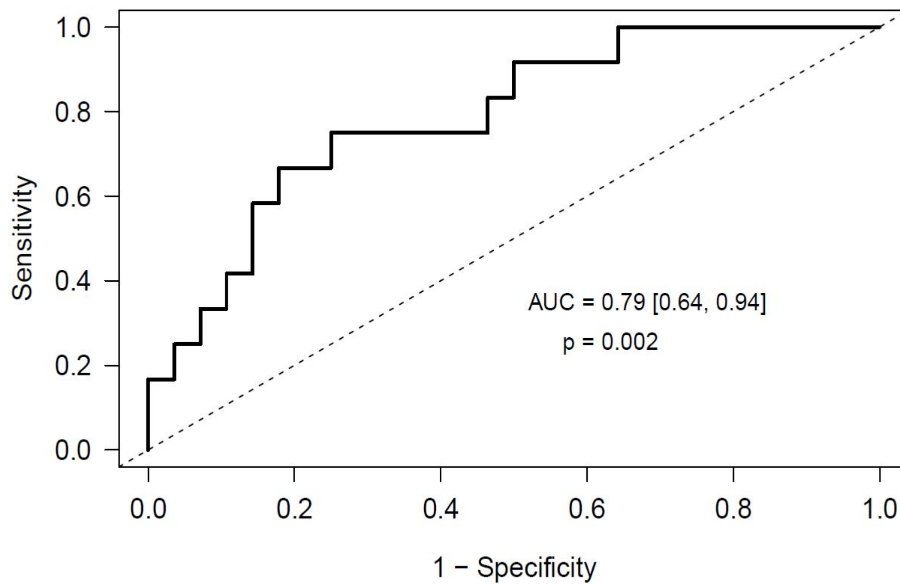

Figure 5. ROC curve obtained from the multivariable elastic net logistic regression predictive model that includes MPO and miR-140-3p measured before surgery as predictors of post-surgical PE in glioma patients.

Finally, we wanted to compare the performance of our predictive models with the Khorana score in our samples. The ROC curve of the Khorana score in our samples achieved an AUC of 0.52 (95% CI [0.31, 0.72], p =0.72) (Figure S5). Therefore, our predictive models outperformed the Khorana score in the prediction of post-surgical PE in glioma patients.

2.3.2. Meningioma

We compared the levels of the neutrophil activation markers studied between meningioma patients who suffered post-surgical PE and those who did not with the Wilcoxon-Mann-Whitney test, and we did not observe any difference in these parameters (data not shown). The neutrophil counts before surgery were similar in both groups (median 3.64 and 3.66 × 10^3/mmc, respectively; p =0.4203) (Table S3). The values of these markers obtained for each individual studied can be consulted in Table S1. Moreover, in meningioma patients the markers of neutrophil activation were not predictors of early incidental post-surgical PE.

3. Discussion

The occurrence of VTE in cancer patients increases morbidity, mortality, and medical expenses. The poor prognosis and the increase in disease burden related to VTE in cancer patients urges the need for the elaboration of risk stratification models to

identify those patients at the highest risk to suffer VTE [27]. Despite the use of thromboprophylaxis in glioma and meningioma patients, a subgroup still develops post-surgical PE [2,3]. This clearly indicates that standard thromboprophylaxis is not adequate for all patients and those at a higher thrombotic risk are in need of a tailored dose and/or duration. Moreover, the bleeding risk of a standard thromboprophylaxis dose in low-risk patients reinforces the need to tailor thromboprophylaxis, especially in neurosurgical patients undergoing a craniotomy for brain tumor resection where the bleeding risk is approximately 3% [5]. For this purpose, biomarkers like D-dimer, sP-selectin and parameters of the thrombin generation test have been implemented in the existing risk stratification models that comprehend clinical variables such as cancer location, blood count parameters, and body mass index [1]. Indeed, efforts have been made to discover specific risk factors for thrombosis in patients with glioma and meningioma [28]. However, the degree of specificity of these models is low and cannot identify most of the cancer patients that will undergo a VTE during the progress of their disease. Although several clinical variables in our study increased with PE occurrence in glioma patients (APTT ratio and grade IV tumors) and meningioma patients (duration of surgery, platelet count and miscellanea comorbidities), none of the clinical variables registered were able to predict post-surgical PE in our patients. Thus, additional improvement of risk appraisal is needed to identify those cancer patients at high or low VTE risk to tailor thromboprophylaxis.

In our study we have firstly proved the utility of miRNAs and neutrophil activation markers as biomarkers for predicting early post-surgical PE in patients with intracranial tumors, namely glioma and meningioma. We have obtained a predictive model for early post-surgical PE in glioma patients that includes a profile of 6 miRNAs as predictors, identified in samples collected before surgery: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p. This validated model achieves an AUC of 0.78 (95% CI [0.63, 0.94], p =0.003). These miRNAs have been previously studied in intracranial tumors and in other malignancies. miR-363-3p has been related to lung adenocarcinoma [29] and glioblastoma [30]. miR-93-3p has been postulated as diagnostic biomarker for triple negative breast cancer [31] and renal cell carcinoma [32]. miR-22-5p seems implicated in tumorigenesis in breast cancer cells [33] and has been postulated to repress cancer progression by inducing cellular senescence [34]. miR-451a has been related to proliferation, invasion, apoptosis and stemness in glioma

[35] and other types of cancer, like renal cell carcinoma [36] and colorectal [37]. Several reports have implicated miR-222 in glioma [38,39] and miR-140 has also been reported in intracranial tumors [40].

In meningioma, we have obtained a predictive model for post-surgical PE that includes a profile of 6 miRNAs as predictors: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p, miR-23b-3p. This validated model achieves an AUC of 0.69 (95% CI [0.52, 0.87], p =0.03). miR-29a-3p has been related to many different types of cancer, like gastric cancer [41] and hepatocellular carcinoma [42]. miR-660-5p seems to regulate the proliferation, migration, and invasion of human breast cancer cells [43] and it has been related to cell migration, invasion, proliferation and apoptosis in renal cell carcinoma [44]. miR-331-3p has been recently postulated as predictor of recurrence in esophageal adenocarcinoma [45] and as regulator in pancreatic cancer [46]. miR-126-5p has been studied in cervical cancer [47] and renal cell carcinoma [48]. miR-23a-3p and miR-23b-3p regulate the expression of proapoptotic proteins in human pancreatic beta-cells [49].

To delve into the biological mechanism(s) regulated by these miRNAs, we identified their targets and the pathways where they are involved. We found that most miRNAs are potential regulators of several components along the *complement and coagulation cascades* pathway. Interestingly, 12 targets may be regulated by at least two different miRNAs as for example miR-93-3p and miR-22-5p that may both regulate the expression of tissue factor pathway inhibitor. Indeed, the human TFPI-2 has been previously related with cell invasion in glioma [50]. Additionally, six targets are regulated by our miRNAs both in glioma and meningioma: CR2, KNG1, PROS1, F9, MBL2 and F11, while different miRNAs are dysregulated in each cancer type. Our results support the complexity in the regulation of human biological pathways exercised by miRNAs, given that one miRNA targets many mRNAs within the same pathway and each mRNA is targeted by several miRNAs to guarantee a fine-tuned global regulation. In addition, the targets regulated by one miRNA can have opposite functions, and the final outcome of the miRNA could then be either pro-thrombotic or anti-thrombotic. Additional in vitro studies in cell cultures and in vivo in animal models will prove the final regulation of each predicted target and will shed light on the degree of participation of each miRNA in the global regulatory mechanisms in glioma and meningioma patients. To the best of our knowledge, ours is the first study that addresses

the predictive role of plasma miRNAs for PE in glioma and meningioma patients. The study of these miRNAs in paired cancer tissue specimens may unravel the origin of the dysregulation.

Regarding the role of neutrophil activation markers, we observed an increase in MPO and calprotectin levels in glioma patients who underwent post-surgical PE. cfDNA and nucleosomes levels also were slightly increased in these patients. Differences in neutrophil counts may be the source of variation in plasma activation markers between both groups, however no differences in neutrophil counts were observed between glioma patients who suffered post-surgical PE and those who did not. Although the origin of these neutrophil activation markers could be other than neutrophils, we observed a significant correlation between cfDNA and MPO levels, suggesting that neutrophils may be the source of these makers in plasma of glioma patients. Furthermore, we attained a predictive model for post-surgical PE in glioma that includes cfDNA and MPO as predictors. This validated model achieves an AUC of 0.71 (95% CI [0.52, 0.90], p =0.02). On the contrary, neutrophil activation markers were not good predictors of thrombotic complications in meningioma patients. In a similar strategy as that conducted in the present study, several neutrophil activation markers like DNA and MPO have been associated to other prothrombotic states [51–56] however, to date, this is the first study in which the predictive role of neutrophil activation markers for PE in glioma and meningioma patients is addressed. To further evaluate whether markers of neutrophil activation combined with miRNAs improved the predictive ability of the individual models in glioma patients, we adjusted a new comprehensive elastic net model that included MPO and miR-140-3p as predictors, achieving an AUC of 0.79 (95% CI [0.64, 0.94], p =0.002).

Presently, the most used risk stratification tool for thrombosis in cancer patients is the Khorana score [57]. However, its limitations reveal that current tools to predict and monitor the risk of VTE are inadequate [58,59]. The Khorana score might not be appropriate for brain tumors, as the original study only included a few patients with these types of cancers. However, since the Khorana score is one of the most popular predictive tools used in clinical practice, we decided to include this score in our study, in order to compare it with our novel predictive tools for the occurrence of post-surgical PE in patients with brain tumors. All our predictive models described herein outperformed the widely used Khorana score.

Strengths of this study are the validation of our findings in an independent cohort of patients prospectively followed, and the exhaustive evaluation of patients at inclusion and during follow-up. A limitation of our study could be the sample size studied. However, the incidence of early post-surgical PE was sufficiently high to guarantee a reliable prediction model. Indeed, consistent with the high frequency of PE events in glioma and meningioma patients, 31% of patients with glioma and 39% with meningioma in the original cohort developed a post-surgical PE. The 50 patients with glioma and 50 with meningioma, who formed the study sample, represent almost the entire cohort of patients with glioma and a random selection of patients with meningioma, with an incidence of PE as high as 34%, similar to that of the original cohort.

4. Materials and Methods

4.1. Study Subjects

Fifty-nine consecutive patients with glioma and 93 with meningioma, candidates to removal of the intracranial tumor (primary or relapse) were recruited and prospectively followed between 2012 and 2016 at the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan (Italy). For the purpose of this study, 50 patients with glioma and 50 with meningioma were selected, who had similar demographic and clinical characteristics to those of the original cohort. This selection was conducted as follows: the 50 glioma patients studied herein represent all the available patients with follow-up completed at the time of the validation stage commencing (out of 59 glioma patients from the original cohort), while the 50 meningioma patients represent a random selection of patients with an incidence of PE similar to the whole original cohort. Cancer was objectively diagnosed with brain CT scan or magnetic resonance. The tumor histological classification was done according to the 2007 World Health Organization (WHO) Classification of brain tumors in grade I, II, III and IV [60], being grade I the less severe and IV the most severe.

PE was objectively diagnosed by means of a perfusion lung scan. To rule out the existence of pre-surgical asymptomatic PE, patients were scanned at the visit before surgery. To diagnose post-surgical PE, all patients underwent a second perfusion lung scan within 2–7 days after surgery. In case of a positive result, a chest CT scan was performed to confirm the diagnosis. Only PE diagnosed with perfusion lung scan and

confirmed with CT scan were considered. A color-Doppler compression ultrasound of the lower limbs was performed only in patients with a PE diagnosis to check deep vein thrombosis.

Clinical and demographic data were collected from all patients. Pre-operative co-morbidities were categorized in cardiovascular, respiratory, metabolic (diabetes mellitus, hypercholesterolemia, obesity, hyper- or hypothyroidism, chronic liver or renal disease) and miscellanea (previous surgeries, other neoplasms and psychiatric disorders). Karnofsky Performance Status (KPS) was documented before surgery and at patient's discharge. Post-operative risk factors comprised the presence of neurological aggravation and walking complications. As standard clinical practice, antithrombotic prophylaxis with low molecular weight heparin (enoxaparin) was daily administered at a single dose of 4000 IU subcutaneously without any adjustment for body weight or renal function (since neither overweight patients nor patients with renal disease were present) beginning 24 h after surgery or later when intracranial bleeding was revealed at the post-operative brain CT scan. All patients wore elastic stockings in the perioperative period and none were treated with chemotherapy during the duration of the present study since it was limited to the first week after surgery. When VTE occurred, anti-coagulation was initiated with low molecular weight heparin personalized to each patient's clinico-radiological picture (mainly 4000 IU twice a day).

The exclusion criteria were: patient's refusal to participate in the study, previous history of VTE, need for anticoagulant or antiplatelet therapy for other reasons, and known blood coagulation disorders that contraindicated the use of antithrombotic prophylaxis.

All participants provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan.

4.2. Blood Collection

Blood was obtained from all patients before surgery, collected in Vacutainer tubes (BD Diagnostics, Franklin Lakes, NJ, USA) containing 0.109 M trisodium citrate and then centrifuged at 1500 ×*g* for 30 min at 4 °C. Plasma was stored in aliquots at -72 °C until used. The following blood tests were also performed: complete blood count,

kidney and hepatic function, prothrombin time (PT), APTT, fibrinogen and D-dimer plasma levels.

4.3. miRNA Studies

4.3.1. RNA Isolation

Total plasma RNA (including miRNAs) was isolated with the miRNeasy Mini Kit (Qiagen, Hiden, Germany) according to manufacturer´s instructions with slight optimizations reported by our group [61]. An RNA carrier (tRNA, Ambion, Bleiswijk, The Netherlands) was added during the isolation to increase the final yield. Several synthetic RNAs (Spike-in kit UniRT, Exiqon, Vedbaek, Denmark) were included in different steps of the whole procedure to track the RNA isolation efficiency, cDNA synthesis and inter-plate qPCR performance. The concentration and purity of the RNA was spectrophotometrically evaluated in a NanoDrop ND-1000 system (Thermo Fisher Scientific, Wilmington, DE, USA). RNA was stored at -80 °C until used. No plasma sample was haemolized, determined at the absorbance of haemoglobin (412 nm).

4.3.2. Quantification of the Expression Level of miRNAs

The expression level of miRNAs was quantified by RT-qPCR in two stages:

Screening Stage

Based on the quality of the isolated RNA, 10 glioma and 10 meningioma patients were selected (five with and five without post-surgical PE in each clinical group, representative of the whole cohort, cases and controls) and the miRNAs expression level was studied before surgery. For that aim, we used the Universal cDNA Synthesis Kit II (Exiqon, Vedbaek, Denmark) and the Serum/Plasma Focus microRNA PCR Panel V4 (Exiqon, Vedbaek, Denmark) with the ExiLENT SYBR Green Master Mix (Exiqon, Vedbaek, Denmark) were used in a LightCycler 480 II (Roche, Mannheim, Germany), as previously reported [56]. To ensure that miRNA quantification was not influenced by technical and interpersonal variability, we included the following internal controls: we assessed the RNA isolation step by adding the synthetic spike-in 2 RNA in every RNA isolation, the retrotranscription efficiency by adding the spike-in 6 RNA in every cDNA synthesis reaction, and the qPCR performance by measuring the inter-plate calibrator spike-in 3 RNA included in triplicate in every panel and a negative control in every qPCR reaction.

The selection of the most stable normalizer among all samples was performed with the comprehensive tool RefFinder that comprehends the computational programs geNorm, Normfinder, BestKeeper and the comparative delta-Ct method (https://www.heartcure.com.au/for-researchers/). The candidate normalizers evaluated were those miRNAs proposed by the Serum/plasma Focus microRNA PCR Panel V4: miR-423-5p, miR-425-5p, miR-93-5p, miR-191-5p and miR-103a-3p. Once selected, the expression level of each miRNA was normalized by the $2^{-\Delta\Delta CT}$ method.

Next, a multivariable logistic regression model was adjusted to predict post-surgical PE in glioma and meningioma patients according to miRNAs levels before surgery.

Validation Stage

Once we identified a miRNA profile potentially capable of predicting a post-surgical PE event in glioma and meningioma patients, their expression level was measured in an independent and larger cohort of patients (40 patients with glioma and 40 with meningioma; 12 cases of PE in each group) at inclusion in duplicate. For each miRNA, specific miRCURY LNA miRNA PCR Assay (Exiqon, Vedbaek, Denmark) were employed. Each miRNA was measured in duplicate and a standard deviation <0.5 was considered satisfactory.

4.3.3. Identification of miRNAs´ Targets

Once we selected a miRNA profile capable of predicting a PE event in these patients, we ascertained their validated and predicted target proteins related to VTE in the databases miRWalk 2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) that comprehends 12 miRNA-target prediction programs. Next, these targets were combined with the *complement and coagulation cascades* pathway from KEGG (https://www.genome.jp/kegg/).

4.4. Quantification of Neutrophil Activation Markers

Different markers of neutrophil activation were quantified in the plasma sample obtained before surgery, following the strategy addressed in previous studies [51–56,62] and following manufacturer´s instructions. These markers were: cfDNA (Quant-iTPicoGreen dsDNA kit, Life Technologies, Eugene, OR, USA) and nucleosomes (Cell Death Detection ELISA^{PLUS} kit, Roche, Mannheim, Germany) as markers of the neutrophil nuclear content released upon NETosis; calprotectin (Human Calprotectin ELISA kit, Hycult Biotech, Uden, The Netherlands) as marker of cytoplasmic content;

and MPO (Human MPO ELISA kit, Abnova, Taoyuan, Taiwan) as marker of granules content, both released upon neutrophil activation by different mechanisms.

4.5. Statistical Analysis

All statistical analyses were conducted in R (version v3.5.1). Continuous variables were expressed as median and interquartile range, and categorical variables as count and percentage. In the screening stage (10 patients with glioma and 10 with meningioma; 5 cases of PE in each group), elastic net logistic regression models for PE risk were adjusted for each tumor type using the miRNA expression levels and the levels of neutrophil activation markers before surgery. The formulas to calculate the risk of PE in each individual patient were built with the coefficients provided by the model for each predictive variable. Additionally, a random forest analysis was performed in meningioma. The predictive ability of the models was evaluated by estimating the optimism-corrected AUC for the ROC analysis, using 1000 bootstrap replicates. Next, this AUC was verified in the validation stage (40 patients with glioma and 40 with meningioma; 12 cases of PE in each group). For all the estimates, 95% CIs were calculated. The association of neutrophil activation markers with PE was evaluated by comparing the levels of every marker in both clinical groups (with and without post-surgical PE, cases and controls), and for each tumor type, with the Wilcoxon-Mann-Whitney test. Results were considered statistically significant at $p < 0.05$. The 50 patients for each tumor type of the whole study group (10 for the screening stage and 40 for the validation stage) represented almost the entire cohort of the 59 patients with glioma and a random selection of the 93 patients with meningioma, with an incidence of PE similar to that of the original cohort.

5. Conclusions

In our study we reveal that plasma miRNAs and markers of neutrophil activation, measured before surgery, may be suitable predictors of early incidental post-surgical PE in patients with intracranial tumors, namely glioma and meningioma. A good prediction could be obtained with the expression level of 6 miRNAs in glioma (miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p) and in meningioma (miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p) patients, respectively. Furthermore, a good prediction could also be obtained in glioma patients with markers of neutrophil activation, cfDNA and MPO, measured before surgery.

Remarkably, we explored the predictive ability of both regulatory mechanisms in combination, and a good prediction could be obtained with MPO and miR-140-3p before surgery.

In recent years, targeted therapy to achieve a personalized medicine has become a crucial goal in medicine. Certainly, in patients with intracranial tumors an uncomplicated brain surgery is essential to prevent the occurrence of VTE influenced by a deterioration of neurological and/or clinical conditions [5]. Therefore, once confirmed in a large independent cohort of prospectively recruited patients, the plausible implementation of our risk stratification tools may provide physicians the possibility to tailor the thromboprophylaxis in the high-risk subgroup of patients during the perioperative period and promote a closer follow-up to minimize the incidence and morbidity of VTE.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Differences in the expression level of three synthetic spike-in RNAs among glioma and meningioma patients who suffered post-surgical PE and those who did not. A) Spike-in 2 monitors the RNA isolation step. B) Spike-in 6 monitors the retrotranscription efficiency. C) Spike-in 3 functions as inter-plate calibrator to evaluate qPCR performance. Expression levels are represented as Ct values. Figure S2: Selection of candidate miRNA normalizers and analysis of their stability conducted with the comprehensive tool RefFinder. A) Stability value of the 5 candidate miRNAs normalizers proposed, rendered by the Comprehensive Ranking of RefFinder. The lower the stability value, the higher the stability of each miRNA. B) Ct values of the 5 candidate miRNAs normalizers proposed arranged from the most stable miRNA (miR-93-5p) to the less stable miRNA (miR-103-3p). The lower the Ct value, the higher the expression level of a miRNA. Figure S3: Sensitivity and Specificity Profile Plot of the multivariable elastic net logistic regression predictive model that includes 6 miRNAs measured before surgery (miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p) as predictors of post-surgical PE in glioma patients. Figure S4: Predictive model obtained with the Random Forest regression with miRNAs measured before surgery as predictors of post-surgical PE in meningioma patients. Figure S5: ROC curve of the Khorana score as predictor of post-surgical PE in glioma patients. Table S1: Database including the clinical variables and markers studied in glioma and meningioma patients. Table S2: Differences in the baseline clinical

characteristics in glioma patients according to the occurrence of PE. Table S3: Differences in the baseline clinical characteristics in meningioma patients according to the occurrence of PE.

Author Contributions: J.O. performed the experiments, analyzed the data and wrote the manuscript. E.P. performed the experiments and critically revised the manuscript. M.J.S. processed the samples and performed the experiments. A.F.-P. performed the experiments. D.H. analyzed the data and critically revised the manuscript. F.C. processed the samples and prepared the databases. F.E. designed and supervised the experiments, analyzed the data and critically revised the manuscript. AA designed the study and contributed to data acquisition and interpretation. P.B. designed the study, contributed to data acquisition and interpretation and critically contributed to the final draft of the manuscript. G.C. designed the study, included the patients and performed the clinical follow-up. S.N. performed the experiments, analyzed the data and critically revised the manuscript. G.M. designed the study and contributed to data acquisition and interpretation. P.M. designed and performed the experiments, analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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8. DISCUSSION

Data from the Spanish National Institute of Statistics of December 19, 2019 reveal that cancer represents the second cause of death in Spain (28.3%) after disorders of the circulatory system (28.3%). According to the report of the Spanish Society of Medical Oncology 2019, BC is the 4th most frequent in Spain (23,819 new diagnoses in 2019) and the 10th in number of deaths. In BC diagnostic methods or complementary to cystoscopy are missing, what is performed quarterly, semiannually or annually for 10-15 years per patient. Considering that BC is highly prevalent, it causes huge healthcare expenses; in fact, BC is the tumor with the highest monitoring costs. Furthermore, current diagnostic and follow-up methods for BC are harmful (CT scan radiation) and detrimental (cystoscopy) for the patient. Although several biomarkers have been described, none have succeeded in replacing cystoscopy as a diagnostic and follow-up method. Thus, novel minimally invasive innocuous markers, as a liquid biopsy, could prevent frequent performance of this detrimental technique for BC diagnosis and monitoring, would reduce the resulting high rate of false positives and negatives, would improve the therapeutic impact and would also increase patient survival, directly linked to an early diagnosis.

Accordingly, in this Doctoral Thesis we have overcome the aim to evaluate miRNAs as novel non-invasive biomarkers in urine as a liquid biopsy for BC diagnosis and staging.

Thrombosis is the second leading cause of death in cancer patients due to the patient´s high hypercoagulability, and the occurrence of venous thromboembolism (VTE) is strongly related to a lower survival. Furthermore, the strong association between cancer and VTE imply that approximately 20% of new VTE diagnoses are associated with a cancer, often hidden. It is currently known that 20% of cancer patients develop VTE, often changing or postponing therapeutic patterns. In addition, chemotherapy increases the thrombotic risk by 47% and the presence of metastases further raises it.

Patients with cancer and VTE have a higher morbidity due to bleeding complications and VTE recurrence. Consequently, Spanish health costs due to thrombotic complications in cancer patients shoot up 30% per patient in additional treatments, hospitalizations and bleeding management. It should be noted that the number of new

cancer diagnoses worldwide keeps growing every year, and the gradual increase in life expectancy of cancer patients implies that those with a greater number of comorbidities will receive antitumor therapy for longer periods, with the consequent increase in thrombotic risk.

Despite the low cost of anticoagulant prophylaxis, it is blatant that a subgroup of cancer patients is currently dying as a result of thrombotic complications because the individual risk of each patient is unknown. Moreover, according to the Cancer-Associated Thrombosis Awareness Survey of the European Cancer Patient Coalition, 72% of cancer patients are unaware of their increased thrombotic risk and, consequently, do not pay attention to symptoms and don´t discuss with their oncologists about the need of thromboprophylaxis in high-risk situations. Several clinical assessment scores have been proposed to this aim, like the Khorana score during chemotherapy treatment, but all have limitations thus urging the need to develop novel predictive tools. In fact, there is a growing concern among oncologists regarding the early diagnosis of VTE in cancer patients, the management of primary thromboprophylaxis, and anticoagulant therapy once the thrombotic event has occurred. All these data highlight the urge for biomarkers able to identify patients at high thrombotic risk in order to tailor thromboprophylaxis (type and duration) with the ultimate goal of avoiding thrombotic complications and improving their life expectancy and quality of life.

Accordingly, in this Doctoral Thesis we have overcome the aim to evaluate miRNAs and neutrophil activation markers as novel biomarkers to estimate the thrombotic risk of two of the most prothrombotic tumor types, pancreatic cancer and glioma.

8.1. NEW NON-INVASIVE BIOMARKERS FOR THE DIAGNOSIS AND STRATIFICATION OF BC PATIENTS

Provided that the lack of consensus among miRNA studies in BC are in part caused by divergent normalization strategies employed, in the present Doctoral Thesis, we set for the first time the aim to ascertain the best miRNA normalizer for miRNA studies in urine of BC patients with the ultimate goal of avoiding future inconsistencies among studies. We evaluated the performance of 110 candidate miRNAs with the comprehensive tool RefFinder in 35 BC patients and 15 healthy controls. We selected miR-29c-3p as the best normalizer for miRNA studies in urine of BC and validated its stability in an independent cohort of 153 BC patients and 57 controls. Moreover, miR-

29c-3p displayed a good expression level in urine and no differences were observed between BC patients and controls, both in the screening and the validation cohorts. To test the robustness of miR-29c-3p as endogenous normalizer, we quantified the expression of miR-200c-3p, a miRNA previously proposed as diagnostic and staging marker of BC [114, 115]. We found significant differences in miR-200c-3p among the different clinical groups studied both in the screening and validation cohorts, with a trend in the increase of miR-200c-3p with the severity of NMIBC. All in all, we propose miR-29c-3p as an optimal reference miRNA that may allow the comparison of future urine miRNA studies as non-invasive biomarkers for BC diagnosis and monitoring.

Next, we aimed to find a profile of miRNAs in urine with diagnostic potential and capable to stratify BC patients using miR-29c-3p as normalizer. First, in a screening stage, we prospectively recruited 35 BC patients and 15 age- and gender-matched healthy controls, from whom we collected a first morning urine sample. We evaluated the expression level of 179 miRNAs, among which we were able to successfully quantify 157 miRNAs. We performed an ordinal regression for each miRNA with false discovery rate (FDR) adjustment and we obtained 70 significantly dysregulated miRNAs in BC patients compared to controls. Next, we performed an ordinal Elastic Net logistic regression model to identify a miRNA profile with diagnostic and stratification purposes. With the expression level of 7 miRNAs (miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p, miR-21-5p) we could stratify BC patients and healthy subjects. Furthermore, we could validate our model in and independent cohort of 172 patients and 94 controls. Moreover, we searched the validated and predicted targets of these miRNAs and found that all miRNA had targets related to bladder cancer pathway. Remarkably, these results are protected under a patent under development, which will also enhance the technological development of our country.

Furthermore, to enable the widespread application of our diagnostic and stratification model, we developed a Shiny App called Bladder-miRaCan. This is a free available online tool where any researcher worldwide can upload their miRNA expression data and obtain a predictive value of BC diagnosis and stating for each subject. We hope that the knowledge generated in the basic bench side combining the statistical model and the web interface is applied in the clinic side, thus little by little improving BC diagnosis and stratification with the implementation of novel non-invasive approaches.

8.2. NEW BIOMARKERS FOR CANCER-ASSOCIATED THROMBOSIS

As far as the study of new biomarkers for cancer-associated thrombosis is concerned, in this Doctoral Thesis we evaluated miRNAs and neutrophil activation markers as novel biomarkers to estimate the thrombotic risk in pancreatic cancer patients and in patients with intracranial tumors like glioma and meningioma.

8.2.1. Pancreatic cancer

Focusing on cancer-associated thrombosis in pancreatic cancer, we studied 6 distal extrahepatic cholangiocarcinoma (DECC) patients and 26 pancreatic ductal adenocarcinoma (PDAC) patients. Ten patients developed VTE and were compared with 22 age- and sex-matched controls. miRNA expression levels were measured at diagnosis and right before VTE event, and neutrophil activation markers (cfDNA, nucleosomes, calprotectin, and myeloperoxidase) were measured in every sample obtained during follow-up. We have identified a miRNA profile at diagnosis able to predict the occurrence of a VTE event in PDAC and DECC patients during follow-up. This model includes 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p and miR-103a-3p) and achieves a high predictive capacity (ROC curve AUC=0.95, 95% CI [0.87, 1], $P<0.001$). Additionally, we aimed to identify up- or down-regulated miRNAs that could be involved in triggering the VTE event in PDAC and DECC cancer patients. In that respect, we studied the expression profile of miRNAs in the sample right before the VTE event compared with that obtained at inclusion. We identified a profile of 7 down-regulated miRNAs (miR-30e-3p, let-7i-5p, let-7g-5p, miR-144-3p, miR-199a-3p, miR-101-3p and miR-15a-5p) that might prompt the VTE event in these patients during follow-up. Interestingly, four of these seven miRNAs are dysregulated in both analyses (miR-30e-3p, let-7i-5p, let-7g-5p and miR-144-3p), upregulated in VTE patients at inclusion compared with non-VTE patients and down-regulated right before the VTE event compared with inclusion. Particularly, miR-144-3p and let-7g-5p showed the greatest differences in the expression level right before the VTE event, what reinforces the idea of these miRNAs being strong candidates for prompting a thrombotic complication in PDAC and DECC patients. Additionally, we obtained a predictive model of VTE with calprotectin as predictor (AUC=0.77, 95% CI [0.57, 0.95], $P<0.008$). Ours was the first study that addressed the ability of plasma miRNAs and neutrophil activation markers to predict VTE in PDAC and DECC. Thus, once validated in a larger independent cohort of patients, our predictive models may be

implemented into daily clinical practice. The estimation of the thrombotic risk of each PDAC and DECC patient at diagnosis might promote a closer follow-up and a personalized thromboprophylaxis in high-risk patients.

8.2.2. Intracranial tumors

As previously mentioned, patients with intracranial tumors are more likely to develop VTE than those with cancers at other sites. In addition, brain surgery further increases the frequency of VTE as, despite the use of thromboprophylaxis in glioma and meningioma patients, a subgroup still develops post-surgical PE. This clearly indicates that standard thromboprophylaxis is not adequate for all patients and those at a higher thrombotic risk are in need of a tailored dose and/or duration, also to prevent intracranial bleedings. All in all, we focused our research in studying new biomarkers, plasma miRNAs and markers of neutrophil activation, measured before surgery, as suitable predictors of early incidental post-surgical PE in patients with intracranial tumors, namely glioma and meningioma. We recruited and prospectively followed 50 patients with glioma and 50 with meningioma, 34% of whom in each group developed an early objectively diagnosed post-surgical PE. We measured miRNA expression and neutrophil markers (cfDNA, nucleosomes, calprotectin and myeloperoxidase) before surgery. In glioma patients, we adjusted and validated a predictive model for post-surgical PE with 6 miRNAs: miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p (AUC=0.78, 95% [0.63, 0.94], *P*=0.003) and another with cfDNA and myeloperoxidase as predictors (AUC=0.71, 95% CI [0.52, 0.90], *P*=0.02). Furthermore, we combined both types of markers and obtained a model with myeloperoxidase and miR-140-3p as predictors (AUC=0.79, 95% CI [0.64, 0.94], *P*=0.002). In meningioma patients we fitted and validated a predictive model with 6 miRNAs: miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p (AUC=0.69, 95% CI [0.52, 0.87], *P*=0.03). Interestingly, all our models outperformed the Khorana score in our patients (AUC=0.52, 95% CI [0.31, 0.72], *P*=0.72). This was the first study that analyzed the capability of plasma miRNAs and neutrophil activation markers to predict early post-surgical PE in glioma and meningioma patients. The estimation of the thrombotic risk before surgery may promote a tailored thromboprophylaxis in a selected group of high-risk patients, in order to minimize the incidence of PE and avoid bleedings.

9. CONCLUSIONS

1. The identification of new biomarkers is necessary for the stratification of cancer patients according to their thrombotic risk to improve current available tools. Neutrophil activation markers and miRNAs seem to be good biomarkers of thrombotic events in patients with pancreatic cancer and in glioma and meningioma patients.
2. miR-29c-3p represents a good normalizer for miRNAs studies in urine of BC patients.
3. In bladder cancer patients, we have identified and validated a profile of 7 miRNAs in urine (miR-221-3p, miR-93-5p, miR-362-3p, miR-191-5p, miR-200c-3p, miR-192-5p and miR-21 -5p) with diagnostic and stratification value. We also identified miR-21-5p, miR-425-5p and miR-99a-5p as potential follow-up markers for BC relapse and miR-21-5p and miR-221-3p as potential markers for tumor metastasis. Furthermore, the miRNA content in urine may reflect that of tumor microenvironment, thus becoming valuable as liquid biopsy. All these miRNAs have validated or predicted proteins that participate in the development and progression of BC.
4. In addition, we have developed the open app Bladder-miRaCan, to enable the global use of our model for BC diagnosis and stratification.
5. In pancreatic cancer patients, we have identified and validated a profile of 7 miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a- 3p and miR-103a-3p) able to estimate the future risk of VTE in these patients at diagnosis. These miRNAs are closely related to the *complement and coagulation* pathway and the *pancreatic cancer* pathway. Also in pancreatic cancer, we have generated a predictive model of VTE with calprotectin, a marker of neutrophil activation, as a predictor variable.
6. In patients with glioma, we have identified and validated a profile of 6 miRNAs (miR-363-3p, miR-93-3p, miR-22-5p, miR-451a, miR-222-3p and miR-140-3p) that, quantified before surgery, can predict the risk of early incidental post-surgical PE. These miRNAs are closely related to the complement and coagulation pathway. Additionally, we have identified a PE prediction model with cfDNA and MPO as predictor variables. Combining both types of biomarkers, we obtained a predictive model with MPO and miR-140-3p that

slightly improves the predictive ability of the model including the miRNA profile.

7. In meningioma, we have identified and validated a profile of 6 miRNAs (miR-29a-3p, miR-660-5p, miR-331-3p, miR-126-5p, miR-23a-3p and miR-23b-3p) with the ability to predict incidental early post-surgical PE. These miRNAs are closely related to the *complement and coagulation* pathway. Neutrophil activation markers do not appear to be good biomarkers of PE in these patients.

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