Unraveling the interplay between cancer and thrombosis: insights from bench to big data

Anijs, R.J.S.

CHAPTER 1

GENERAL INTRODUCTION

HEMOSTASIS

Hemostasis is the physiological process of the body that prevents (massive) blood loss (1). After the body encounters injury from a damaged vessel, a clot is formed in response (coagulation), while maintaining normal blood flow everywhere else in the circulation (2). Under physiological circumstances, an essential but delicate balance is maintained between the formation and breakdown of blood clots, regulated by pro- and anti-coagulant factors (2). Two main processes of hemostasis are involved; primary and secondary hemostasis, which are initiated simultaneously with primary hemostasis depending on the injury's extent and location (2).

Primary hemostasis includes adhesion of platelets to injury site, platelet aggregation and platelet plug formation (2). Platelet adhesion is triggered by injury, with glycoprotein receptors binding to von Willebrand factor (vWF) and collagen in the sub-endothelial matrix. Thrombin activates platelets through protease-activated receptors (PAR), after which activated platelets change the conformation of integrins, allowing them to interact with ligands like vWF, fibrinogen, collagen, and others, facilitating platelet aggregation and the formation of the primary plug. During secondary hemostasis, insoluble fibrin is formed, generated through the coagulation cascade, which creates a fibrin clot to seal the wound (2). This process involves two pathways: the intrinsic and extrinsic pathway. Tissue Factor (TF) initiates the extrinsic pathway, abundant in various body cells (2). This pathway generates a rapid thrombin release upon vascular tissue injury, involving the TF-VIIa complex, factor X activation, and subsequent fibrin formation for clot stabilization (2). The contact activation or intrinsic pathway is initiated by contact with a damaged surface such as a blood vessel injury. It involves a series of enzymatic reactions, subsequently activating prothrombin into thrombin, after which a fibrin clot can be formed.

Disruption in the balance between pro- and anticoagulant processes can result in various serious events, not necessarily related to vessel injury. In a more pro-coagulant state, thrombosis can occur, which is the process of blood clots developing in a vessel, thereby blocking blood flow. On the other side of the balance, in a more anti-coagulant state, pathological bleeding or hemorrhage can occur.

Venous and Arterial Thromboembolism

Thrombosis or thromboembolism, the formation of a blood clot within the blood vessels, can manifest in both veins and arteries, thereby partially or completely blocking blood flow. In addition, this clot can also break off and block other vessels at distant sites (embolism).

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is the third most common cause of cardiovascular death, with ~3 million

deaths in the general population per year worldwide (3). VTE is a multifactorial disease and is dependent on several environmental and genetic risk factors, including among others, age, obesity, diabetes, fractures, trauma, non-O blood group, factor V Leiden mutation, thrombophilia, oral contraceptives, pregnancy, history of thrombosis and long-haul travel, but none holds as much significance as the occurrence of cancer (3). Currently, it is estimated that the annual incidence of VTE in the general population is around 1-2 per 1000 person years (py) (4). Arterial thromboembolism (ATE) comprises clots in the arterial circulation, leading to (transient) ischemic heart diseases/stroke, and myocardial infarction (MI) among others. Compared to VTE, ATE has incidences of 1.4 per 1000 py (MI), 1.1 per 1000 py (stroke), 15 per 1000 py (ischemic heart disease) (4). Furthermore, it also results in a higher absolute mortality rate (4). The classic acquired risk factors for ATE are mostly different than VTE, such as smoking, diabetes, hypertension, among others. Nevertheless, it is now becoming increasingly more clear that there are also many shared risk factors, such as age, obesity, diabetes and hormone use (5).

Cancer-associated thrombosis

The association between thrombosis and cancer has been recognized for over 200 years (6), but it was not until the French Armand Trousseau lectured on *phlegmasia alba dolens* in 1872, that this phenomenon received recognition. Trousseau warned the population that "spontaneous coagulation is common in cancerous patients", but he was unfortunate to predict his own death in 1867, as he also suffered from cancer-associated thrombosis (CAT) and passed from gastric cancer (7). Over time, more and more researchers verified the observation of thrombotic developments in cancer, showing an increased risk of both VTE and ATE in cancer patients. This severe complication leads to a strong additional burden in cancer patients, and is also the leading cause of non-cancer death in this population (8).

Around 20-30% of all first VTE cases is followed by a diagnosis of cancer (8). Compared to the general population, cancer patients are at a 7-11-fold increased risk of developing VTE in the first year after diagnosis, accompanied by worsened morbidity and mortality (9, 10). The exact risk is dependent on several cancer-specific risk factors, such as tumor status (active/non-active), tumor type, stage or grade. High risk tumor categories include pancreatic, brain and stomach cancer and hematological malignancies, whereas lung and gastrointestinal classify as medium risk, compared to breast and prostate tumors in the low risk group (8). A higher and more advanced cancer stage and grade both result in a greater risk of VTE (8). In addition, cancer treatment-related factors greatly influence the VTE risk, such as chemotherapy, immunotherapy and major surgery (8, 10). Furthermore, the combination of cancer and VTE significantly contributes to an increased mortality. A 30-fold increased risk of mortality is seen compared to disease-free subjects and a 4-fold increase compared to patients with cancer alone (11). More recently, tumor-intrinsic

characteristics such as overexpression of pro-thrombotic factors (Tissue Factor (TF) or Podoplanin (PDPN)) or somatic mutations (e.g., JAK, MET, STK11 or KRAS) are known to also contribute to the risk of VTE (9, 12, 13).

In comparison to VTE, ATE in cancer is less extensively studied. So far, a ~2-fold increased risk of ATE in cancer patients in a period of 6-months compared to the general population was only demonstrated in two cohort studies (14, 15). In a similar fashion, patients also have poor prognosis, shown by a 3-fold increased risk of mortality (14).

Prediction and treatment of CAT

According to current ISTH guidelines, anticoagulant treatment in CAT patients is recommended for at least 6 months after the thrombotic event and is to be continued as long as there is an active malignancy present (9, 16). However, this also confers a notable risk of bleeding complications, as cancer patients on anticoagulant treatment have a 2-3 fold increased risk of bleeding compared to non-cancer anticoagulated patients (17). Most often, bleeding occurs from unresected primary tumors, such as those of the GI tract and in gynecological malignancies (17). For accurate treatment of CAT patients, two factors need to be considered, i.e. the risk-benefit ratio translated to the risk of bleeding with anticoagulation or benefit by preventing a recurrent thrombotic occurrence (9). To achieve earlier diagnosis and reduce unnecessary bleeding risks, risk prediction tools have been developed and are constantly updated (9, 18). Despite the fact that CAT has been studied extensively over the years, the biological mechanism is not yet fully understood, complicating accurate risk prediction. Among the pool of risk prediction models, the Khorana score is the currently recommended risk assessment tool for clinical use. It takes into account factors such as cancer type, body mass index, as well as blood parameters like hemoglobin, platelet count, and leukocyte count prior to chemotherapy treatment. However, despite its clinical recommendation, the Khorana score's performance is suboptimal, as evidenced by several external validation studies (18, 19). One reason for this suboptimal performance could be that the Khorana score was initially designed to identify high-risk ambulatory patients undergoing chemotherapy. Nonetheless, many validation studies used different inclusion criteria, such as chemo-naïve patients or included a varied distribution of tumor types (8). Overall, there is still a need for a better understanding of the underlying mechanisms that contribute to VTE risk in cancer patients.

Pathophysiological mechanisms behind CAT

As thrombosis and thus CAT is a multifactorial disease, the exact mechanism is dependent on many different players, however, there is still a lot of uncertainty about the pathophysiological mechanism (9, 19). It is clear that these patients usually show abnormalities in each component of Virchow's triad (9, 19), encompassing stasis of

blood flow, endothelial and vessel wall injury and activation of coagulation, each one contributing to thrombosis (9, 19). The prothrombotic function of cancerous tissue, the inflammatory response as a defense mechanism against the tumor, but also the use of catheters and anti-cancer therapy (chemotherapy, radiation and immunotherapy) damage the endothelial wall and contribute to a pro-thrombotic state (9). Furthermore, cancer patients are often hospitalized during treatment/surgery, with long bed rest, resulting in extrinsic compression of the blood vessels and stasis of blood flow (20). However, it is unclear what the exact mechanisms are underlying the abnormalities in Virchow's triad in cancer patients leading to CAT. In addition, as there are large risk differences between cancer types, it is thought that a tumor-specific mechanism is in play. Several hypotheses have been generated over the years; tumor cells are known to express an increased amount of pro-coagulant factors. For example, Tissue Factor (TF), the initiator of the extrinsic coagulation cascade is expressed by the tumor and released into the blood stream on the surface of extracellular vesicles and is also linked to enhanced tumor growth and progression (19, 21). Another important factor expressed by cancerous cells is polyphosphate (Poly-P), which can bind and activate factor XII, and makes fibrin more resistant to fibrinolysis (9, 22). In a similar matter, phosphatidyl serine (PS) and plasminogen activation inhibitor-1 (PAI-1) are expressed in high amounts, resulting in a more pro-coagulant state (9, 23). Tumor cells are also able to induce platelet activation, after which the activated platelets stimulate the secretion of pro-coagulant factors (e.g. TF) (23). The release of podoplanin (PDPN) directly results in platelet activation and aggregation through the calcium dependent lectin-like receptor 2 (CLEC-2) on platelets, often seen in brain cancer (9, 23). In a more indirect setting, the tumor releases inflammatory and angiogenic factors, which are able to induce a more immunothrombosis-like phenotype (9, 24). For example, neutrophils are affected and produce more neutrophil extracellular traps (NETs), which can activate the coagulation cascade, and act as a scaffold for platelet adhesion, activation and aggregation (25). Furthermore, proinflammatory cytokines are released by the tumor, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) which have an established thrombogenic role (19).

Outline

Overall, several hypotheses have been proposed to explain the mechanism behind CAT, but there is still a knowledge gap, complicating accurate prediction and treatment. Therefore, a better understanding of underlying mechanisms contributing to CAT, specifically tumor-specific ones, may result in identification of novel and improved biomarkers and hence improved identification of high-risk individuals, as well as new insights for developing therapeutic targets. In **part I** of this thesis, we aim to gain insight into the pathophysiology underlying (colorectal) cancer associated thrombosis. In **chapter 2**, we look into the functional validation of a protein target (REG4) recently identified by next generation

RNA sequencing of colorectal cancer samples from patients with CAT. By using several *in vivo* and microfluidic methods, we aimed to identify the possible involvement of REG4 in thrombosis development. Furthermore, in **chapter 3, 4 and 5**, we explore the promising role for microRNAs (miRNAs), a novel class of biomarkers in health and disease, which only recently started to unfold and may also be extended to CAT. Firstly, in **chapter 3**, we extensively summarize the current literature on the role of miRNAs in VTE, cancer, and also CAT studies. Of interest, we looked into overlapping miRNAs identified in VTE and CAT studies, giving insight into new possible biomarkers. In **chapter 4**, a nested case-control miRNA sequencing study was performed, to identify new miRNA targets associated with colorectal cancer associated CAT. Lastly, in **chapter 5**, we highlight the role of biomarkers as well as the recently published Atlas of the Hemostatic miRNA Targetome to unravel function and start validation studies.

In **part II** of this thesis, we looked into epidemiological perspectives of CAT in Scandinavia (**chapter 6**) and the Netherlands (**chapter 7 and 8**), using large nation-wide data. **Chapter 6** reports the mortality rates after CAT in a Scandinavian population, where we compare different cancer types and stages. In **chapter 7**, we provide an extensive overview of incidences, risk factors and prognosis concerning colorectal cancer associated CAT in the Netherlands. Chapter 8 reports the risk of a cancer diagnosis after incident venous/arterial thromboembolism (VTE/ATE) on both absolute and relative scales. Both these chapters give detailed information to help guide thrombotic prophylactic management decisions in CAT patients, as well as future research perspectives.

Lastly, **chapter 9** is a general summary and discussion and provides an overview of future perspectives.

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