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Venous Air Embolism and Complement-driven Thromboinflammation

In vitro human whole blood studies and *in vivo* porcine studies on the effect of air emboli on the complement system, cytokine network, and the hemostasis

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A dissertation for the degree of Philosophiae Doctor

April 2022

Norwegian Complement Research Group

Nordland Hospital Trust

Northern Norway Regional Health Authority

“You will not see what you do not look for.”

Stephen King

This work is dedicated to all past, present, and, unfortunately, undoubtedly future patients and relatives Worldwide affected by air embolism.

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Preface

Hysteroscopy is a surgical procedure where a tube is inserted into the cervix, allowing for inspection and surgery inside the uterus. To facilitate surgery, pressurized irrigation fluid is pumped into the uterus through the tube. Hysteroscopy is considered to be a safe procedure, but this routine surgery caused a dramatic and, in one case, fatal air embolism in three women at our hospital. Two common denominators in all three patients were the reinsertion of the hysteroscope into the uterus and the use of a specific fluid infusion pump, and we suspected that both hysteroscope reinsertion and pump malfunction or operator error had played an important role in the development of air embolism. Examination of the pump, initiated by the national health authorities, did not uncover any technical failures, but did not examine possible user errors. These shocking events triggered us to study air embolism in further detail. During our research, it became apparent that although the symptoms and initial treatment of air embolism had been relatively well-studied, these investigations were largely unknown to us. The sub-acute inflammation triggered by air embolism had likewise previously been studied, albeit with some knowledge gaps in the detailed pathophysiological mechanisms.

Air embolism potentially injures many patients worldwide. In respect of the patients and relatives, we wanted to contribute to the awareness and knowledge of air embolism, and thus the outlines of this Ph.D. project were laid.

After finalizing the project, we were contacted by the relatives of a patient who had died due to air embolism in Australia. This woman had undergone the exact same hysteroscopy procedure with the same infusion pump as had been used in our patients. A root cause analysis, including a thorough examination of the infusion pump, concluded with a combination of hysteroscope reinsertion, forcing air into the uterine cavity, and an irrigation pump design flaw, resulting in excessive irrigation fluid pressures, could potentially explain this event, and the pump has now been recalled Worldwide by the manufacturer.

Acknowledgments

First and foremost, I wish to thank my supervisors, Erik Waage Nielsen and Tom Eirik Mollnes, for giving me the opportunity to undertake this research project and for their dedicated support during every step of the process. Without their help, this project would never have been accomplished. I also want to thank the skilled, knowledgeable, and helpful bioengineers at the Research Laboratory, Nordland Hospital, for their engagement and invaluable support. A special thanks to chief bioengineer Dorte Christiansen, who patiently taught me the basics of laboratory practice and immunological analysis, and who helped me keep my spirits up when I had to repeat an analysis over and over again. Also, I want to send a warm thanks to the staff at ANILAB for patiently assisting me through the animal experiments, spending long days at the laboratory. Especially, I want to thank technician Bent Aksel Nielsen for getting up in the middle of the night to drive 100 km with me to retrieve the laboratory animals without question and always in a cheerful mood. A warm thanks to Per Steinar Halvorsen at The Intervention Center, Oslo University Hospital, who opened his laboratory to us in a period when we had no access to animals. I wish to thank Helse Nord Regional Health Authority for making this project possible by sponsoring me with an unrestricted research grant. Without this support, the project would simply have been impossible. Also, thanks to Nord University for hosting the animal experiments. I wish to send a warm thanks to all my colleagues, who willingly and without questions, donated a significant amount of blood to my project. A warm thanks to Corinna, Anne, Kristina, and others, who read my work and provided valuable feedback, and a special thanks to Palli and Paul Alexander Van Buren for thoroughly proofreading my manuscript.

Finally, none of this could have happened without the support of my family. My children, Birk and Isak, have patiently put up with me spending days and nights in front of the computer or stuck in the laboratory on weekdays and weekends. I send my love and gratitude to you.

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Abbreviations

ANOVA = analysis of variance

BALT = bronchial associated lymphoid tissue

βTG = β-thromboglobulin

C1-INH = C1-inhibitor

C3aR = C3a receptor

C5aR = C5a receptor

CD = cluster of differentiation

DAMPS = damage associated molecular patterns

EDTA = ethylenediaminetetraacetic acid

ELISA = enzyme linked immunosorbent assay

F = factor

FB = factor B

FH = factor H

FI = factor I

FP = factor P, properdin

GPRP = Gly-Pro-Arg-Pro peptide

Ig = immunoglobulin

IL = interleukin

MAC = membrane attack complex

MASP = mannose associated serine protease

MBL = mannose binding lectin

MP = micro particle

mRNA = messenger ribonucleic acid

PAMPS = pathogen associated molecular patterns

PAR = protease-activated receptor

PIM = pulmonary intravascular macrophages

PTF1+2 = prothrombin fragment 1+2

qPCR = quantitative (real time) polymerase chain reaction

ROTEM = rotational thromboelastometry

SD = standard deviation

SPF = specific pathogen free

TAT = thrombin-antithrombin complex

TCC = terminal complement complex, sC5b-9

TF = tissue factor

TLR = toll-like receptor

tPA = tissue plasminogen activator

TNF = tumor necrosis factor

Papers included in the thesis

Paper I: Storm, B.S., Christiansen, D., Mollnes, T.E., Nielsen, E.W., 2020. Avoiding ambient air in test tubes during incubations of human whole-blood minimizes complement background activation. Journal of Immunological Methods 487, 112876. https://doi.org/10.1016/j.jim.2020.112876

Paper II: Storm, B.S., Christiansen, D., Fure, H., Ludviksen, J.K., Lau, C., Lambris, J.D., Woodruff, T.M., Braaten, T., Nielsen, E.W., Mollnes, T.E., 2021. Air bubbles activate complement and trigger C3-dependent coagulation and cytokine release ex vivo in human whole blood. J Immunol 207(11): 2828-2840. https://doi.org/10.4049/jimmunol.2100308

Paper III: Storm, B.S., Christiansen, D., Fure, H., Ludviksen, J.K., Pettersen, K., Landsem, A., Nielsen, B.A., Dybwik, K., Braaten, T., Nielsen, E.W., and Mollnes, T.E., 2022. Venous Air Embolism Activate Complement C3 without Corresponding C5 Activation and Trigger Thromboinflammation in Pigs. Frontiers in Immunology 13, 839632. https://doi.org/10.3389/fimmu.2022.839632

Summary

Background: Air embolism may complicate many medical procedures and cause vascular occlusion, organ infarctions, or death. *In vitro*, air triggers the alternative complement pathway and activates platelets. Previous studies of air emboli were conducted in serum, plasma, or heparin anticoagulated whole blood, with ambient air present in tubes, precluding detailed examination of thromboinflammation. The role of complement in air-induced thromboinflammation has not previously been examined *in vivo* in minimally anticoagulated large animal model. Thus, this project aimed to examine the effect of avoiding ambient air during *in vitro* blood incubations and to elucidate the air-induced thromboinflammation both *in vitro* and *in vivo*. **Methods:** *In vitro*, lepirudin anticoagulated human whole blood from 16 donors was either incubated for 180 minutes with air emboli and inhibitors of complement C3 and C5, C5a receptor 1, and the toll-like receptor co-receptor cluster of differentiation 14, or without air. Blood was analyzed for complement activation products, cytokines, tissue factor, β-thromboglobulin, and prothrombin fragment 1+2. *In vivo*, air was infused through an ear vein in 29 pigs for 300 minutes. Hemostasis was monitored using rotational thromboelastometry and thrombin-antithrombin complex. Blood and lung tissue were analyzed for complement activation products and cytokines. **Results:** *In vitro* and *in vivo,* air emboli triggered a C3-driven thromboinflammation without correlating terminal pathway activation. *In vitro,* C3 inhibition, and to a lesser degree, C5 inhibition attenuated cytokine release, and C3 and C5 inhibition equally reduced coagulation, but neither reduced the platelet activation. Avoiding ambient air during *in vitro* incubations reduced complement activation. *In vivo*, air embolism resulted in leukocytosis, hemostasis, increased proinflammatory cytokines and complement activation product C3a, but not TCC in the lung tissue. **Conclusion:** Air embolism triggered a complement C3-driven thromboinflammation *in vitro* and *in vivo*. During *in vitro* blood incubations, avoiding ambient air attenuated and C3 inhibition reduced thromboinflammation.

1 The clinical background for this thesis (introduction)

Three devastating events at our hospital sparked this Ph.D. project into being. During a two-month period, three otherwise healthy middle-aged women suffered severe side effects of routine hysteroscopic surgery; one woman died, one had multiple cerebral infarctions with severe and persistent neurological damage, and one developed a large myocardial infarction. In all women, the initial symptom was a sudden drop in exhaled carbon dioxide followed by circulatory collapse and cardiac arrest. The symptoms were related to inserting a hysteroscope in the uterus and subsequent installment of pressurized fluid distension media. During the resuscitation of the first two patients, none of the involved personnel initially considered air embolism as a possible cause of the circulatory collapse. However, during the third case, peri-arrest echocardiography showed vast amounts of air emboli in the right heart and hepatic circulation. Repeated post-resuscitation echocardiography showed air in the left heart (Figure 1).

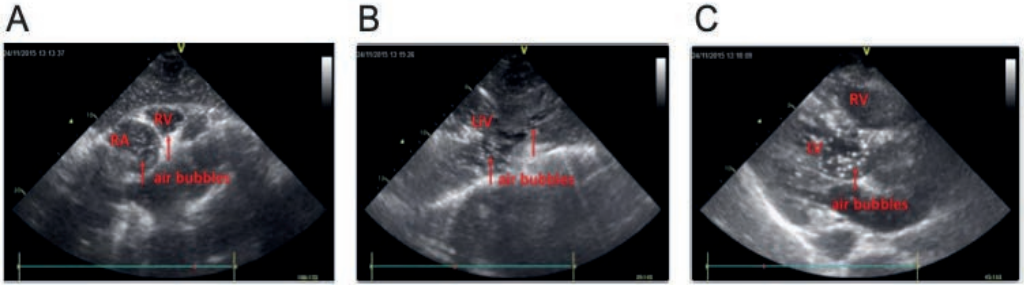

Figure 1 Transthoracic echocardiography of woman with venous air embolism. Panel A: Peri-arrest subxiphoid view with air bubbles (arrows) in the right atrium (RA) and right ventricle (RV) and no air in the left ventricle. Panel B: Peri-arrest echocardiography liver veins (LiV) with air bubbles (arrows). Panel C: Post-resuscitation parasternal long-axis view with air bubbles (arrow) in the left ventricle (LV) and no air in the right ventricle (RV).

This finding led us to consider iatrogenic surgery-related air embolism, possibly linked to the infusion of pressurized fluid into the uterus and re-insertion of the hysteroscope, as a possible cause of the hemodynamic collapse in all three patients. Somewhat to our surprise, iatrogenic air embolism during hysteroscopic surgery and many other surgical and medical procedures was well-described in the medical literature, including specific recommendations for

prevention, diagnosis, and acute treatment of air embolism [1–5]. The Patient Safety Movement Foundation, an international organization with stakeholders from the healthcare sector, the medico-technical industry, and patient organizations, aiming to reduce preventable fatalities in the healthcare system, included air embolism in the "zero preventable deaths by 2020" campaign in 2018 [6]. In Norway, however, air embolism has remained a largely underrecognized condition. After the adverse events at our institution, the Norwegian Directorate of Health released a National warning and gave specific recommendations to reduce and detect air embolism [7]. In line with these recommendations, we made important changes to our surgical and anesthesiologic procedures related to hysteroscopy, and, fortunately, we have had no new events of perioperative air embolism.

After the initial resuscitation, two of our patients developed severe acute inflammation. For decades it has been known from clinical cases, *in vitro* experiments and animals studies, that air emboli and air embolism trigger inflammation, including complement activation, and coagulation [4,8–15]; however, the precise mechanisms have remained not fully understood. As it is impossible to conduct *in vivo* human studies of air embolism, all previous studies are *in vitro* blood or plasma studies, *in vivo* animal studies, or human case reports.

Over the last decades, increased understanding of the immune system, including the complement system, the development of new and refined immunological methods and models, and the development of specific antibodies and immune modulators have made it possible to study the pathophysiological mechanisms in further detail.

Thus, this Ph.D. project aimed to examine the effect of air embolism on thromboinflammation using state-of-the-art immunological methods *in vitro* in human whole blood and *in vivo* in a large mammal model.

1.1 Definition of air emboli and air embolism

An embolus is a foreign particle present in the blood circulation. The word originates from the Greek word “embolus,” meaning “a wedge-shaped object, stopper” [16]. Thus, air emboli are air bubbles in the blood. Air embolism is defined as “the obstruction of the circulation by air that has gained entrance to the veins usually through wounds” [16]. Hence, air embolism is when air bubbles, or emboli, obstruct one or several blood vessels [4]. Depending on location in the circulation, air embolism may be venous (including in the right heart and the pulmonary artery), arterial (in the systemic arterial circulation) [1], or both venous and arterial (Figure 2).

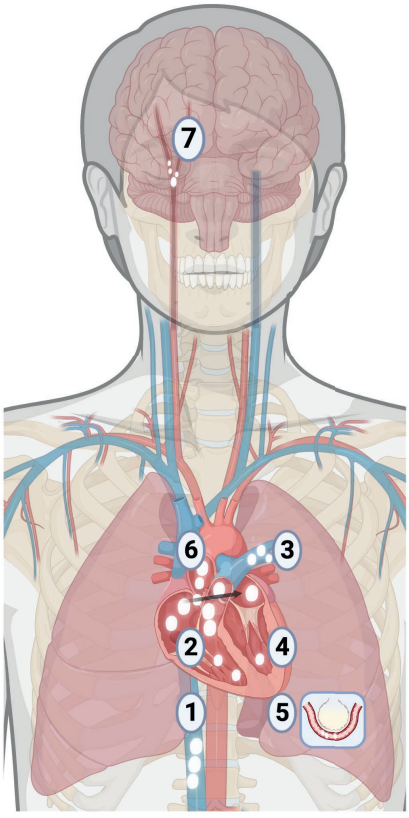

Figure 2 Overview of air embolism. Air emboli (white spheres) may enter the central venous system (1) and reach the right atrium and right ventricle of the heart and the pulmonary artery (2). Air emboli may occlude the pulmonary artery, causing right ventricular failure and left ventricular hypovolemia, resulting in circulatory collapse. Air emboli travel with the blood in the pulmonary artery to the lungs (3), where they may be filtered in the capillaries and exhaled in the alveoli (insert; 5). Air emboli may occlude lung capillaries, resulting in impaired gas exchange with a drop in expired CO_2 and arterial O_2 uptake. Air embolism may overwhelm the pulmonary filtering capacity and enter the left heart (5) and the arterial (systemic) circulation (6). Air emboli may also pass from the venous to the arterial circulation through an open foramen ovale, connecting the right and left atrium (arrow). Systemic air emboli may occlude peripheral arteries (7), causing end-organ ischemia and damage, for example, in the brain and myocardium. Blue vessels are veins and pulmonary arteries. Red vessels are systemic arteries. Created with Biorender.com

1.2 Awareness of air embolism in medicine

Venous air embolism may complicate many surgical and medical procedures [3,4,17] as well as diving, high altitude aviation [18], or even sexual intercourse [19]. Air emboli may cause mild transient and self-resolving symptoms, such as decreased pulmonary gas exchanges or blood pressure. However, air embolism may also lead to hemodynamic collapse, trigger systemic inflammation, cause organ infarctions, or even death [4,20].

Symptomatic air embolism was first described in the 1930s, while the pathophysiological mechanisms were thoroughly investigated for the first time over half a century ago [21]. During the last 80 years, hundreds of case reports, several studies, and many reviews describing the detrimental effects of air embolism in relation to many medical interventions have been published (Figure 2).

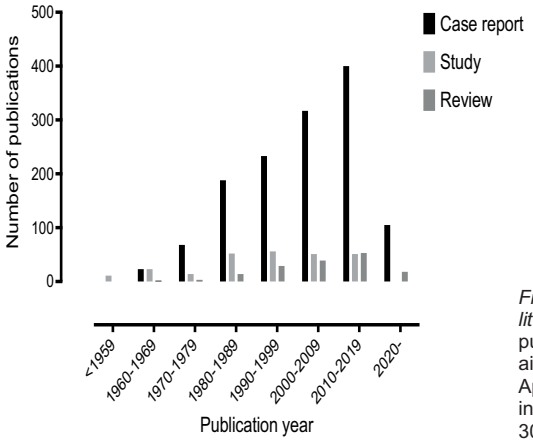

Figure 3 The awareness of air embolism reflected in the literature. This figure shows a Medline search of published case reports, studies, and reviews related to air embolism. The search strategy is described in Appendix I. The search results are divided into ten-year intervals with the last interval spanning 2020 to March 30th 2022.

Despite a wealth of medical literature related to air embolism, the awareness of air embolism as a clinically relevant problem varies significantly in the medical community worldwide. Failure to recognize air emboli can seriously jeopardize patient safety, and in the United States, preventive and diagnostic measures are implemented in official recommendations and clinical

guidelines [3,22,23]. In contrast, in Norway until recently, there has been little awareness of air emboli, and no national recommendations regarding preventive measures, perioperative surveillance, or treatment existed before the three catastrophic events of procedure-related air emboli at Nordlandssykehuset in 2015 [24]. After these events, the Norwegian Directorate of Health released a warning to the Norwegian medical community [7] to increase awareness of the problem.

1.2.1 A short historical perspective on air embolism

At the beginning of the 19th-century, vascular air embolism was identified as a potential adverse event during medical procedures such as neurosurgery and thoracic surgical procedures. However, little research elaborating on air embolism had been published. In the 1930s, several case reports described intravenous air emboli in conjunction with pneumothorax [25], and in 1931 the first experimental studies that examined the effects of air emboli in the coronary arteries were published [26]. In the 1940s, intravenous and intra-arterial infusion of ambient air or CO_2-gas was commonly used as an x-ray contrast media. Also, it was common to insufflate gas into the vagina and uterus to aid the surgeon during gynecological procedures, and there was a general perception that these procedures were safe. However, during the 1940s, several case reports describing fatal vascular air emboli during medical gas insufflation were published, leading to increased concern regarding intra-uterine gas infusion [27]. The exact mechanisms by which air emboli caused fatalities were largely unknown until 1947, when the first large-scale animal study elaborating on the pathophysiological mechanisms and consequences of venous air embolism was published by Durant et al. [28].

Despite several human case reports and animal studies describing fatalities and complications related to iatrogenic air embolism throughout the late 1940s and early 1950s, only a few

diagnostic methods and no therapeutic methods were available. In 1953, Oppenheimer et al. showed that mortality in dogs could be decreased significantly if dogs with iatrogenic air embolism were positioned in a left lateral steep Trendelenburg position [29]; this intervention was later named "Durant's position."

In the 1960s, with the increasing use of extracorporeal perfusion systems, such as dialysis machines and heart-lung machines, came renewed awareness of and interest in the effects of intravascular gas emboli [30–32]. However, apart from "Durant's position," few treatment options were available. During the 1970s, recompression of air emboli by hyperbaric treatment of vascular air emboli was introduced as a definitive treatment [33]. During the 1980s and 1990s, with the advancement and increasing availability of ultrasound equipment, extensive research was carried out into *in vivo* diagnosis of vascular air embolism. In the same period, it was increasingly recognized that air embolism not only caused a mechanical occlusion of vessels but also triggered a multitude of immunological reactions [8,34–36].

Based on the established research and case reports, air embolisms were recognized by the American medical community as relatively frequent and potentially severe complications related to many medical procedures [17,22] with specific symptoms and treatment options. From 2000 until the present several medico-technical companies have implemented air bubble detector and removal systems into their products, and monitoring and treatment strategies have been implemented into many clinical guidelines [22,23,37–39].

1.2.2 Pathophysiology of acute air embolism

In several animal models of venous air embolism (dogs and pigs), it has been recognized that air embolism causes fatal effects by obstructing the blood circulation, especially by obstructing

the right ventricular outlet tract (Figure 2) [28,36]. Air emboli in the pulmonary circulation trigger immediate pulmonary hypertension. The combined effect of pulmonary hypertension and obstruction of blood flow in the ventricular outlet tract causes right ventricular dilation, hindering perfusion of the ventricular wall and leading to myocardial ischemia and right ventricular failure [28,36]. In animal experiments, the lethality related to venous air embolism depended on the amount and speed of air injection and the position of the experimental animal [28,33,35].

Venous air emboli are normally filtered in the lung capillaries and gradually exhaled (Figure 2). However, the filtering capacity may be overwhelmed and air emboli may pass through the lungs to the arterial (systemic) circulation [17,35,40,41]. Air may also pass through an open foramen ovale, connecting the right and left atrium of the heart (seen in 10-25% of the general population [42,43]).

In the systemic arterial circulation, air emboli may occlude arteries in several organs and cause severe organ ischemia (Figure 2). The most severe complication of arterial air embolism is acute occlusion of coronary or cerebral vessels resulting in myocardial or cerebral ischemia [39]. Air triggers complement and coagulation cascades [44,45], and the obstruction of blood flow may be aggravated by platelet activation and thrombi formation on air bubbles, as shown in animal studies of decompressions sickness [46].

1.2.3 Symptoms of acute air embolism

Symptoms of air embolism relate to where the emboli become lodged in the circulation. Venous air emboli are carried with the blood to the pulmonary circulation, and the initial symptoms arise from the obstruction of the pulmonary circulation. Choking, a sudden drop in end-tidal

expired CO_2, or acute bronchospasm are the most frequent initial symptoms of air emboli [17,24]. The symptoms may be discrete or unspecific and are probably often misinterpreted as, for example, anaphylactic reactions, bronchospasms, or myocardial ischemia. A drop in blood pressure and a compensating tachycardia, likely due to the occlusion of the right ventricular outlet tract, is observed with more severe emboli [36]. Myocardial ischemia may complicate air emboli due to right ventricular dilatation and pressure overload and is observed as ST-segment changes (especially in the right precordial leads) on an electrocardiogram [28]. If air passes to the arterial circulation, acute myocardial infarction, hemodynamic collapse, or acute stroke may arise [2,14,17].

To date, no specific biomarker of air emboli has been identified. Suspected air emboli can be confirmed with high sensitivity and specificity by computed tomography (CT) or magnetic resonance imaging (MRI) examination [17,47], transesophageal or transthoracic echocardiography, or precordial blood flow Doppler monitoring of the right heart. On echocardiographic examination, air emboli present as hyperechoic structures, and on Doppler, the air produces a distinct "windmill murmur" [3].

1.2.4 Surgical procedures with a high risk of air embolism

It is known that some surgical and medical procedures carry an inherently higher risk of air embolism. All procedures involving the opening of the vasculature, especially the opening of the large veins of the brain and the central venous system, carry a risk of air being "sucked" into the bloodstream [1]. This is mainly due to the low or even negative pressure in the venous system relative to the ambient pressure [48]. Positioning the surgical field above heart level further aggravates the negative venous pressure, increasing the risk of air entering the vessels [4,49,50].

On the contrary, positioning the surgical field below heart level increases intravenous pressure and thus reduces the likelihood of air entering the veins [5].

Insufflation of gas into body cavities, for example, during laparoscopy, carries an inherent risk of gas entering the circulation [51]. Keeping the insufflation pressure as low as possible and using gasses with high solubilities in blood, such as CO_2, instead of ambient air or nitrogen, decreases the risk of bubble formation in the bloodstream if air enters [3,52].

In all procedures involving the infusion of pressurized fluids into the bloodstream or body cavities and in the extracorporeal circulation of the blood, there is a risk of circuit malfunction and air unintentionally entering the bloodstream. Ensuring that no air is present in tubing and paying meticulous attention to infusion systems reduces this risk [1,5,22,53], and many medical devices marketed in the United States are equipped with bubble detectors and removal systems to reduce the risk of iatrogenic air embolism.

1.2.5 Emergency treatment of acute air embolism

When symptomatic air emboli occur, only few treatment measures exist. As mentioned above, based on animal studies, it is recommended that patients with air emboli are placed in Durant's position to "capture" air in the liver veins and thus reduce the rate of air returning to the heart (Figure 4).

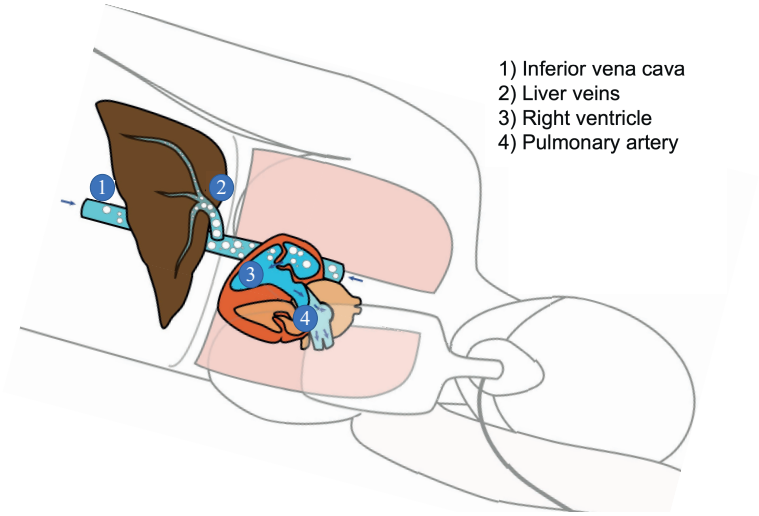

Figure 4 Durant's position may reduce the hemodynamic effects of massive air embolism. By placing the patient (or experimental animal) in the left lateral decubitus position, head tilted down, the air emboli are retained in the inferior vena cava (1) and the liver veins (2) rather than in the right ventricle (3), the ventricular outlet tract and pulmonary artery (4), thus avoiding massive occlusion of the pulmonary circulation. Illustration copyright Storm BS.

This position decreased mortality and morbidity in dog studies [28,29], but it has not been verified in human studies. However, it seems reasonable to assume the same pathophysiology is valid in humans.

Definitive treatment of intravascular air embolism consists of breathing 100% oxygen, which accelerates nitrogen washout from the blood through the lungs by creating a steep concentration gradient of nitrogen between blood and alveoli. However, as air emboli may obstruct the flow of blood in the lung capillaries, eliminating nitrogen from the air bubbles through the exhaled air may not be sufficient to remove bubbles rapidly. In such cases, acute hyperbaric treatment,

which is advocated by some authors [33], will effectively reduce bubble size and increase blood flow, allowing for the exhalation of nitrogen.

1.2.6 Air embolism causes thromboinflammation

In addition to the immediate hemodynamic derangement described above, *in vitro* human studies, *in vivo* human case reports, and *ex vivo* and *in vivo* animal studies have shown that air emboli and air embolism trigger a complex thromboinflammation, involving both the complement system, leukocytes [8,9,54], platelets [55,56] and coagulation [10]. The inflammation may cause a severe inflammatory response syndrome with hypotension, capillary leakage, lung edema, reduced cardiac contractility, and gas exchange [8,12,24]. In line with these findings, a recent human study of divers found that decompression sickness, where air bubbles form in the blood, triggers the inflammation system with induction of the leukocyte transcriptome [54]. Animal studies have shown that decompression sickness triggers endothelial dysfunction [57]. Contrary to these findings, however, human studies have not shown that decompression sickness activates complement [58,59].

1.3 Thromboinflammation

Traditionally, hemostasis and inflammation have been viewed as two distinct processes, the one involving the coagulation cascade system, including tissue factor (TF), platelets, endothelial cells, and the other involving complement, antibodies, and leukocytes. However, it is now known that the complement and coagulation cascades, which have coexisted for over 500 million years, are closely related [60]. Extensive crosstalk exists between complement and hemostasis, and the activation of either inevitably affects the other [61,62]. The platelet, previously

thought to play a role only in hemostasis, is now known to form an important link between coagulation, complement, and the inflammatory cells [63], as it may be activated by both TF, complement activation products, and cytokines released from leukocytes [61,64,65]. Thus, with evolving insight into coagulation and inflammation, the term “thromboinflammation” is increasingly used to describe the joint activation and interaction between the inflammation system and the hemostatic system [62,66].

The thromboinflammation may be offset by several mechanisms, summarized in Figure 5. As shown in the figure, thromboinflammation can be initiated through several initial mechanisms, including complement activation, upregulation, and release of TF from activated leukocytes and endothelial cells, blood contact with subendothelial TF, e.g., in a traumatized vessel, or through activation of the platelets. The complement and hemostatic systems will be briefly described in the following sections.

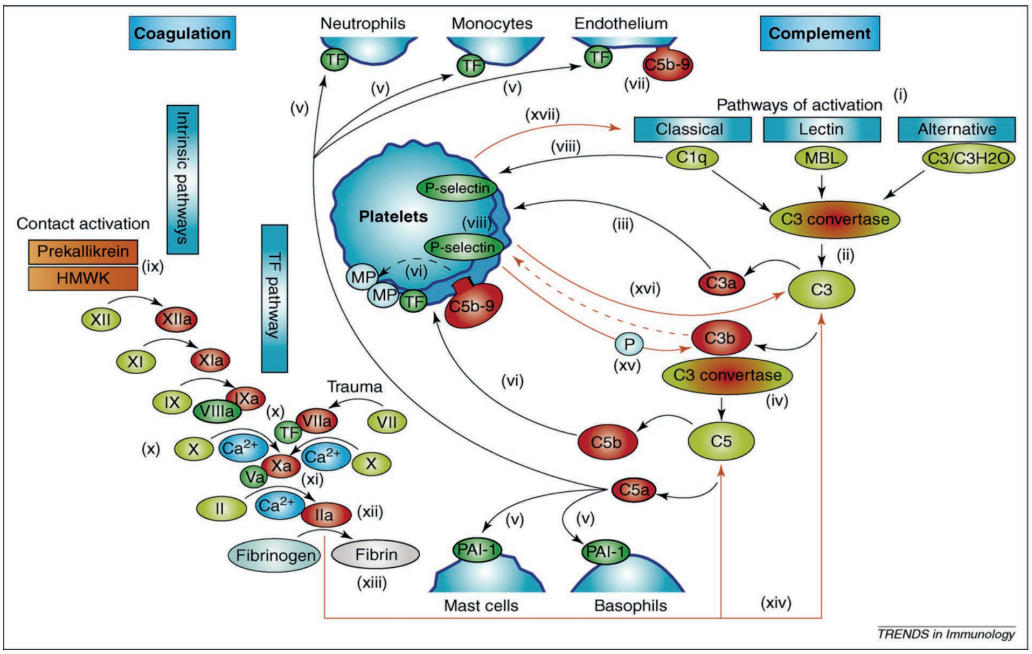

Figure 5 Complement–coagulation interactions. Complement and coagulation cascades are serine proteases activated through partial cleavage by an upstream enzyme. Zymogens are marked in light green, and active components are red. Complement is activated through the classical, lectin, or alternative pathways (i) that converge at the central molecule of the complement system, C3 (ii). The C3 convertases, generated through various pathways, cleave C3 to C3a and C3b (ii). C3a anaphylatoxin activates platelets, enhancing their aggregation and adhesion (iii). C3b contributes to the formation of C5 convertase, which cleaves C5 to C5a and C5b (iv). In addition to its well-established role in inflammation, C5a enhances blood thrombogenicity, mainly through the upregulation of TF and PAI-1 expression on various cell types (v). C5b contributes to the formation of the TCC (also known as C5b-9), which is incorporated into the cellular membrane of platelets, inducing an alteration in membrane polarization and, thus, increasing the surface area on which clotting can occur (vi). TCC also induces the release of MPs bearing TF on their surface (vi) and affects procoagulant properties of the endothelium (vii). C3b binds to P-selectin, the expression of which is induced on platelets by C1q (viii), an initiator of the classical pathway of complement activation. Black arrows illustrate the interactions of complement with coagulation, increasing the propensity of blood to clot. Coagulation launched through the contact (intrinsic) pathway begins with contact activation of HMWK, prekallikrein, and factor XII (ix). TF expressed on various cells or released from injured cells initiates the physiologically more important TF (extrinsic) pathway (x). Both pathways merge at the level of factor X (xi), which, following activation, converts prothrombin (II) to thrombin (IIa) (xii). The final step of the coagulation process, catalyzed by thrombin, requires partial cleavage of soluble fibrinogen and polymerization to insoluble fibrin (xiii). Thrombin cleaves C3 to C3a and C3b and C5 to C5a and C5b, thus amplifying the activation of complement (xiv). Platelets, the central cells in hemostasis, also contribute to the amplification of complement through the phosphorylation of C3b (xv), which prolongs the life span of this molecule. Activated platelets are also involved in C3 cleavage (xvi) and initiation of the classical pathway of complement activation (xvii). The amplification of complement activation exerted through the components of the coagulation system is shown as red arrows. Abbreviation: P, P-selectin. Reproduced from Markiewski M et. al. Complement and coagulation: strangers or partners in crime? *TRENDS Immunol.* 2007 with permission from Trends in Immunology/Elsevier.

1.3.1 The complement system

The complement system is a biological proteolytic cascade system with many functions, including important roles in the innate and adaptive immune response. The existence of the system has been known for over a century, but only in the last four decades has the function been extensively elaborated, as summarized in [67,68]. The complement system constitutes an integral part of the innate immune system in all vertebrae species. In contrast to the adaptive immune system, complement factors have even been shown in non-vertebrae species, such as jawless fish and deuterostomes [69]. Over 40 proteins, including regulatory proteins and designated cellular receptors, have been identified as a part of the complement system [67,68]. Most plasma complement proteins are produced and secreted from hepatocytes, but several other cell types can produce and secrete complement proteins [68]. Phylogenetically the complement and coagulation systems originated from a common ancestor, and the two systems work in close synergy with extensive crosstalk occurring [62], with the activation of one cascade system inevitably leading to the activation of the other cascade system. As mentioned above, complement peptides interact with platelets and leukocytes, in sum triggering a joint activation of inflammation, hemostasis, and coagulation, termed thromboinflammation [62,68,70].

1.3.1.1 Activation of the complement system

The complement system identifies and binds to pathogen-associated molecular patterns (PAMPS), damaged own proteins (damaged associated molecular patterns; DAMPS), or foreign surfaces by three distinct different mechanisms (Figure 6) [71].

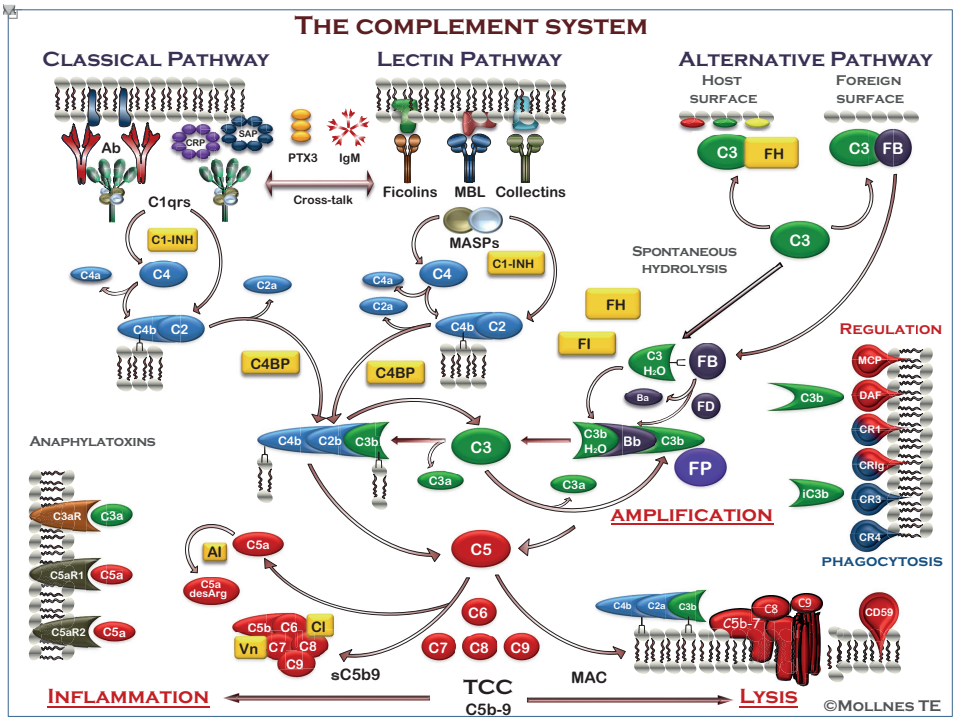

Figure 6 The complement system. The complement system can be activated through 3 pathways (top), all converging to the cleavage of C3 to generate C3a and C3b (middle). The classic pathway (CP) is typically activated by antibodies, but also, pentraxins (PTX), including C-reactive protein (CRP), serum amyloid P component (SAP), and PTX3 can activate C1q. The lectin pathway (LP) is activated through recognition of carbohydrates by mannose-binding lectin (MBL), ficolins, and collectins. Furthermore, LP activation may be mediated through IgM antibodies, e.g., directed against damaged self-antigens. The alternative pathway (AP) is activated by foreign or damaged own cells, facilitated by the continuous spontaneous hydrolysis of C3. AP also has an important function in the complement system, providing an amplification loop that enhances C3 activation independent of which pathway is initially activated. This effect is mainly a result of properdin (FP), the only positive regulator in the complement system, which stabilizes the C3 convertase. Activation of C3 leads to formation of a C5 convertase, cleaving C5 into C5a and C5b. The anaphylatoxins C3a and C5a bind to the receptors C3aR, C5aR1, and C5aR2, leading to downstream production of inflammatory mediators (bottom). C5b initiates the formation of the TCC, which forms the membrane attack complex (MAC) if inserted into a membrane (bottom). This may lead to lysis of bacteria and cells or in sublytic doses to activation of cells. The cleavage and inactivation of C3b generate iC3b, bind to complement receptors 3 (CR3; CD11b/CD18) and 4 (CR4; CD11c/CD18), facilitating phagocytosis, oxidative burst, and downstream inflammation (right). The complement system is tightly regulated by soluble inhibitors (yellow), including C1 inhibitor (C1-INH), factor H (FH), factor I (FI), C4binding protein (C4BP), anaphylatoxin inhibitor (AI) inactivating the anafylatoxins (e.g., C5a to C5adesArg), vitronectin (Vn), and clusterin (Cl), keeping the continuous low-grade activation in the fluid phase in check. Host cell membranes are equipped with a number of inhibitors to protect them against attack by complement (right), including membrane cofactor protein (MCP; CD46), CR1 (CD35), decay accelerating factor (DAF; CD55), controlling C4 and C3 activation, and CD59 protecting against final assembly of the C5b-9 complex. Some attractive targets for therapeutic inhibition are indicated with black arrowheads, e.g., specific CP activation by one of the C1qrs components, specific LP activation by MBL and MASP intervention, and specific AP activation by neutralizing factor D (FD), which will attenuate the amplification of the system induced by all initial activation mechanisms. The inhibition of C3 is the broadest possible strategy, whereas inhibition of C5 cleavage will clock both the inflammatory potent C5a fragment and formation of the inflammatory and lytic C5b-9 complex. Alternatively, C5a can be inhibited, preserving the C5b-9 pathway, or the anaphylatoxin receptors can be blocked to prevent signaling. In particular, the blocking of C5aR1 will attenuate inflammation, whereas the effect of blocking C3aR and C5aR2 receptors is to be studied in more detail, as they might have more anti-inflammatory effects. Reproduced from Barratt-Due A et. al. Dual inhibition of complement and Toll-like receptors as a novel approach to treat inflammatory diseases—C3 or C5 emerge together with CD14 as promising targets. *Jour Leukocyte Biol 2017* under CC BY4.0 license and with permission from Mollnes TE.

In the “classical pathway,” complement is typically activated by factor C1q binding to the fragment crystallizable region (Fc region) of immunoglobulin (Ig) IgG or IgM, or acute phase reactants such as C reactive protein (CRP) [68]. IgA may also activate complement, however through another yet not fully understood mechanism, possibly involving the lectin pathway [72–74]. When activated, C1q attaches to proenzyme serine proteases C1r and C1s, forming an active serine protease, $C1qr_2s_2$, which cleaves C4 into C4a and C4b. C4b attaches to C2, and C2a is cleaved off. C4bC2b is a C3-convertase, binding and cleaving C3 to C3a and C3b. C4bC2b may bind C3b and form the C5-convertase, C4bC2bC3b. When complement is activated through the “lectin pathway,” mannose-binding lectin (MBL), ficolins, or collectins bind to specific carbohydrate structures present on the surface of certain bacteria, fungi, or viruses. When MBL, ficolin, or collectin has bound to a carbohydrate trait, mannose-associated serine proteases (MASP) attach and are activated. Activated MASP cleaves C4 into C4a and C4b. As in the classical pathway, C4b binds C2 to form the C3-convertase, C4bC2b, and the C5-convertase, C4bC2bC3b. The “alternative pathway” function is somewhat different from the classical and lectin pathways. The key peptide of the pathway, C3, contains a thioester bond, prone to spontaneous hydrolysis and subsequent activation. Spontaneous hydrolyzed C3 is immediately inactivated to $iC3(H_2O)$ by the regulatory proteins factor H (FH) and factor I (FI), present in ubiquitous amounts in blood and on the surface of own cells [67,75]. When C3 comes in contact with foreign surfaces, be it pathogens, biomaterials, or even gas bubbles [76,77], on which regulatory factors are not present, immediate hydrolysis of the internal thioester bond occurs, and $C3(H_2O)$ is formed. $C3(H_2O)$ binds factor B, and Ba is then cleaved off by factor D, forming the initial C3-convertase $C3(H_2O)Bb$. $C3(H_2O)Bb$ cleaves C3 into C3a, and C3b, where C3b binds to factor B and Ba is cleaved off by factor D to form the C3-convertase C3bBb. This step is known as the “amplification loop” of the alternative pathway, as the formation of one $C3(H_2O)Bb$ or one C4bC2b may lead to the subsequent synthesis of several C3bBb

molecules. C3bBb is further stabilized by binding to properdin, forming C3 convertase, C3bBbP, enabling more C3 cleavage. Finally, C3bBbP may combine with factor B to form the C5-convertase, $C3b_nBbP$ [68]. In addition to the inactivation of $C3(H_2O)$ to $iC3(H_2O)$, FH obstructs the formation of the C3 convertases by binding to C3b and acts as a co-factor for FI, cleaving C3b to iC3b, thus accelerating the decay of formed convertases, thus downregulating complement activation [78].

Once formed, the C5-convertases, C3b2BbP, and C4bC2bC3b cleave C5 into C5a and C5b. C5b attaches to C6, C7, C8, and C9 to form C5b-9, also termed the "terminal complement complex" (TCC). If the assembly of the TCC occurs on the surface of a membrane, the C5b-9 complex forms a large ring structure, termed "the membrane attack complex" (MAC), which integrates into the membrane, causing sublytic attack, cell activation, and in some instances, such as *Neisseria* species infections, cell lysis [79]. If the assembly of the C5b-9 occurs in plasma, a soluble TCC, also termed sC5b-9, is formed. Thus, under most conditions, activation of complement results in the generation of both C3a and TCC, as shown in Figure 6.

1.3.1.2 Fluid phase regulation of complement activation

The complement system is tightly regulated by several plasma- and membrane-bound proteins (Figure 6), including the plasma-proteins factor H (FH), factor I (FI), and C1-inhibitor (C1-INH) [68]. The C1-INH is continuously released into plasma by hepatocytes [67]. Further, C1-INH has historically been considered an acute phase reactant, where inflammation or tissue injury was thought to stimulate C1-INH release from extrahepatic cells, including monocytes and macrophages [67]. However, recent studies suggest that the role of C1-INH as an acute phase reactant is neglectable [80,81]. The C1-INH binds irreversibly to C1r, C1s, and MASP2, attenuating further classical or lectin pathway activation [67]. As mentioned above, the C3

molecule is prone to spontaneous hydrolysis and subsequent alternative pathway activation. In addition to complement regulation, the C1-INH plays a very important role in the bradykinin-forming cascades, inhibiting FXII activation and bradykinin formation [82]. C1-INH deficiency results in excessive bradykinin formation and subsequent profuse capillary leakage – a condition known as hereditary angioedema [83]. To avoid inappropriate complement activation, the alternative pathway is down-regulated by two plasma proteins, FH and FI [84]. Both factors are synthesized in the liver and several extracellular tissues and circulate in plasma in ubiquitous amounts. IFN-γ increases the synthesis. FH is present on the surface of own cells but not on foreign surfaces, preventing C3 from binding to own tissue. As mentioned above, FH inhibits factor B binding and thus C3b amplification and acts as a cofactor to FI, thereby attenuating the C3 convertase [78]. The activated FI cleaves C3b (and C4b) to an inactive form. Foreign surfaces, for example, bacteria, viruses, plastic materials, or air bubbles, are not coated with FH; thus, when C3 meets these surfaces, spontaneous hydrolysis and alternative pathway activation occur. In the fluid phase, the C3a and C5a anaphylatoxins are degraded by the anaphylatoxin inhibitor carboxypeptidase N [85,86]. TCC formation is regulated in the fluid phase by vitronectin and clusterin binding lipophilic sites, thereby rendering the TCC water-soluble [67].

1.3.1.3 Complement anaphylatoxins

Numerous C4a, C3a, and C5a molecules are split off during the complement cascade activation. These small peptides, termed “anaphylatoxins,” have distinct biological properties, including activation of inflammation and hemostasis. The most important proinflammatory anaphylatoxin is C5a [68]. C5a receptors 1 and 2 (C5aR1 and C5aR2) have been found on all cells of the myeloid lineage [67], including neutrophil leukocytes [71,87]. Activation of C5aR1 trigger a strong

proinflammatory response, where activation of C5aR2 may have both pro- and anti-inflammatory effects [88]. C3a is perceived to be a less potent anaphylatoxin than C5a, possessing pro- and anti-inflammatory effects [67,68]. C3a receptor (C3aR) has been found on leukocytes, mast cells, and macrophages, as well as on erythrocytes and platelets [67,89]. Some studies suggest that C3a may activate human platelets and play an important role in thrombus formation [89,90], although this finding has been questioned by others [91]. C4a has traditionally been considered not to play an important role as an anaphylatoxin [67]. However, C4a has recently been shown to interact with protease-activated receptors 1 and 4 found on endothelial cells, thereby increasing endothelial permeability [92]. PAR1 and PAR4 are also present on platelets, and it is speculated that C4a might activate platelets [64], although this remains to be shown.

1.3.1.4 Complement in disease and inflammation

The complement system plays a role in many – if not all – inflammatory diseases. Pathogens, damaged cells, and foreign materials may activate complement, and the cascade may also be triggered by crosstalk with the coagulation cascade [44]. Complement is a major driver of pathology in diseases like C3 glomerulopathies, atypical hemolytic uremic syndrome, paroxysmal nocturnal hematuria, anti-phospholipid syndrome, age-related macular degeneration, neurodegenerative disorders, hematological diseases, lung diseases, ischemic diseases, graft rejections, cancer, bacterial infections and sepsis, and Covid 19 to name but a few [68,93–98].

Interestingly, C5 activation seems to be the major driver of pathology in most complement-mediated diseases, and C5 inhibition using the C5 blocking antibody eculizumab (Alexion, Boston, MA) is currently used to treat paroxysmal nocturnal hematuria, atypical hemolytic uremic syndrome, myasthenia gravis, and neuromyelitis optica specrum disorder under

approval of the Federal Drug Administration and the European Medicines Agency [68]. Additionally, eculizumab is used off-label to treat several of the other diseases [99]. Notably, in C3-driven diseases, such as alternative pathway dysfunction, for example C3 glomerulopathies, the pathophysiology is not due the C3 activation, but rather due to the secondary C5 activation, and thus C5 inhibitions is sufficient treatment [68].

In vivo, the complement system does not exert its proinflammatory effects in isolation, and although the complement anaphylatoxins C3a and C5a may trigger a robust inflammatory response alone, the leukocytes may also be activated through other non-complement mechanisms, for example, by bindings specific DAMPS or PAMPS to the toll-like receptors (TLR) and the co-receptor cluster of differentiation (CD)-14 [71]

1.3.1.5 Experimental and clinical complement inhibition

Complement plays an essential role in killing invading pathogens by C3 fragment deposition on the foreign cell membranes, subsequent opsonization by phagocytes, C5 binding and MAC assembly in the cell membrane, and subsequent lysis. Several human complement inhibitors have been developed for research use or are under development [68]. Although complement is an ancient and well-preserved system, many of the inhibitors are species-specific. Thus human reagents might not work reliably in swine [101], thus limiting the possibilities of *in vivo* porcine complement inhibition experiments. Clinically, C5 inhibition with the C5 inhibitors eculizumab has been used for approximately 20 years, with the caveat that long-term C5 inhibition increases the risk of infection with encapsulated bacteria such as *Neisseria meningitides* [102]. However, this risk is mitigated through vaccination of the patients [103]. Due to the risk of severe bacterial infections, C3 inhibition has until recently only been used sparsely, although a primate study

found C3 inhibition to be safe in healthy monkeys [104], and short-term inhibition of C3 in conjunction with acute sterile inflammation is considered feasible and safe [103].

As mentioned above, leukocytes may be activated by both complement and TLR stimulation, and previous experimental studies of bacterial sepsis have shown a synergistic anti-inflammatory effect of dual inhibition of blocking the C5a/C5aR1 axis and the TLR co-receptor CD14 [71,98].

1.3.2 Hemostasis

Hemostasis is a complex process involving platelet activation, coagulation, and fibrinolysis. In summary, the purpose of hemostasis is to stop bleeding from damaged vessels by the formation of a fibrin plug. Hemostasis may be divided into three phases; In primary hemostasis, the platelets are activated and adhere to the injury site. The secondary hemostasis, also termed "the coagulation cascade," involves assembling several circulating coagulation factors to form a cascade of serine proteases, the activation of thrombin, and the formation of the fibrin mesh and a blood clot. Tertiary hemostasis, also termed "the fibrinolysis," involves the degradation of the fibrin mesh [105]. Traditionally, the secondary hemostasis is considered isolated and divided into an intrinsic and extrinsic pathway, depending on the initial activation of the coagulation cascade. However, this does not reflect *in vivo* conditions, where the primary and secondary hemostasis are intertwined and coincide, and several steps in the coagulation cascade are catalyzed on the surface of the activated platelet [105–107]. In the "cell-based coagulation model," coagulation is divided into three phases; an initiation phase, occurring on TF-bearing cells, including monocytes, activated platelets, and subendothelial tissue, an amplification phase, and a propagation phase, both occurring mainly on the surface of activated platelets [106,108–110] (Figure 7).

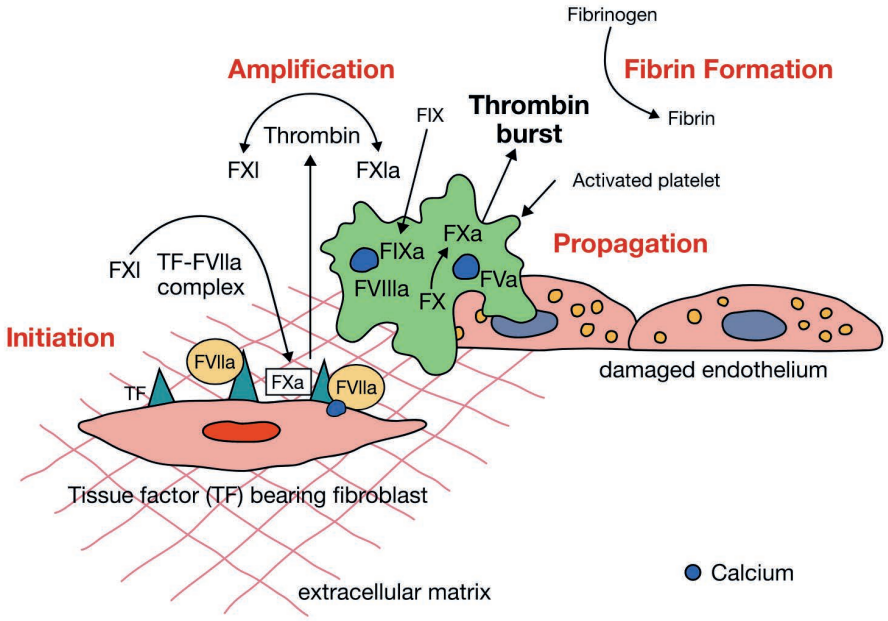

Figure 7 The cell-based model of coagulation. The three steps of coagulation; initiation, activation, and propagation, all occur on the surface of activated platelets. Initiation occurs when tissue factor (TF) is exposed and combines with FVII. TF-FVIIa cleaves FX. FXa, which anchors in the platelet membrane, catalyzes the formation of thrombin, subsequently activating multiple platelets. In the amplification phase, FXa anchored in the membrane of activated platelets catalyzes more thrombin formation, thus activating more platelets. This process is termed "the thrombin burst." In the propagation phase, prothrombin is cleaved to thrombin by the tenase, FXaFVIIIa, on the surface of the activated platelets. Thrombin cleaves the fibrinogen to fibrin, ultimately forming the blood clot. Reproduced from Ho KM, Pavey W. Applying the Cell-Based Coagulation Model in the Management of Critical Bleeding. *Anaesth Intensive Care.* 2017 with permission from SAGE Publishing.

1.3.2.1 Primary hemostasis – platelet activation

The platelet activation involves numerous receptors on the cell surface binding to molecules such as subendothelial collagen, which is exposed when the endothelium is damaged, von Willebrand factor, present on endothelial cells and in plasma, fibronectin, and vitronectin, present in plasma and extracellular matrix [111]. Further, thromboxane A2, synthesized by activated platelets and thrombin, may activate the platelet [111]. Importantly, the platelets have numerous complement receptors (Figure 8), and the binding of several activated complement

components, including C1q, C3a, and C5a, or integration of TCC in the cell membrane activates the platelets [62,112].

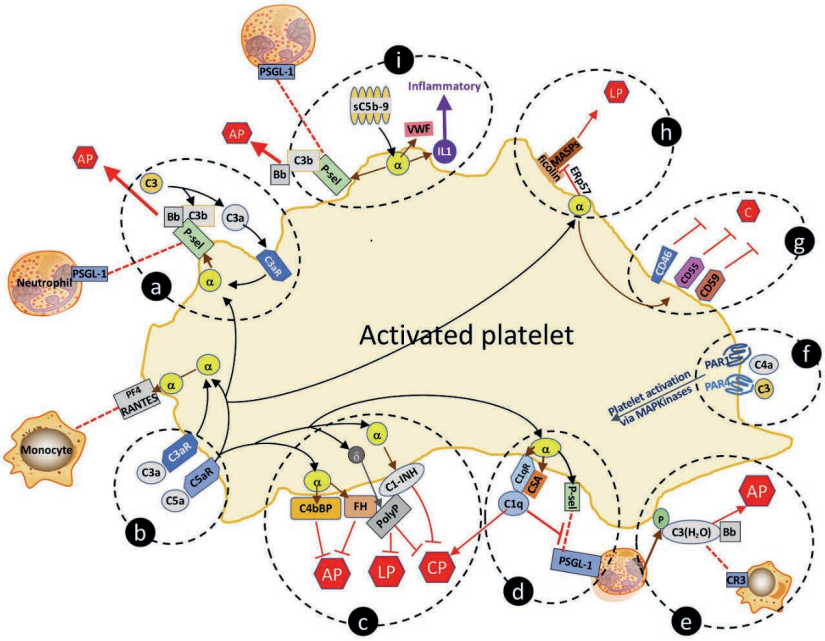

Figure 8 Activation of the platelets by complement. Complement-platelet interactions that can facilitate an inflammatory response that favors atheroma formation. Selected interactions between complement and platelets, as described in the manuscript, are highlighted. (a,b) C3a and C5a bind to their cognate receptors to trigger release of factors from α-granules. P-selectin localizes to the platelet surface and is a receptor for leukocyte expressed PSGL-1 and for C3b, the latter which allows for initiation of the AP and amplification of C3a-triggered platelet activation. (c) Complement activation on the platelet surface is dampened by α-granule release of cofactors C4bBP, FH and C1-INH, and δ-granule release of the anti-complement, prothrombotic polyphosphate. Polyphosphate binds to FH and C1-INH, and downregulates complement activation via the CP and the terminal pathway. (d) By binding to C1qR, C1q also triggers α-granule release of P-selectin and chondroitin sulfate (CSA), the latter which enhances the C1q-C1qR interaction, initiating the CP but negatively regulates leukocyte recruitment by interfering with P-selectin-PSGL-1 interactions. (e) Neutrophil-released properdin (P) stabilizes the convertases and is also a receptor for C3(H2O) which complexes with Bb to form the AP C3-convertase, and is a ligand for leukocyte-expressed CR3, thereby facilitating leukocyte cell migration to the site of inflammation. (f) C4a and C3 can activate platelets via distinct interactions with PAR1 and PAR4. (g) Activated platelets are also protected against complement-mediated destruction by granule release of negative regulators of complement, cell surface expressed CD46, CD55, and CD59. (h) Sublytic C5b-9 (sC5b-9) triggers platelet activation with release of VWF, P-selectin and inflammatory cytokines (e.g., IL1), the latter which further promotes inflammation. (i) Ficolins on the surface of activated platelets are receptors for MASPs which can trigger the LP. Release of the isomerase ERp57 modifies the ficolin to limit its functional capacity to trigger complement. C, complement activation pathways; AP, alternative pathway; LP, lectin pathway; CP, classical pathway; PF4, platelet factor 4; C3aR, C3a receptor; C5aR, C5a receptor; C1qR, C1q receptor; P-sel, P-selectin; PSGL-1, P-selectin glycoprotein ligand-1; sC5b-9, sublytic C5b-9; VWF, von Willebrand factor; α, α-granule; δ, δ-granule; ERp57, endoplasmic reticulum protein 57; PAR, protease activated receptor; P, properdin; CR3, complement receptor 3; PolyP, polyphosphate; IL1, interleukin 1. Reproduced from Kim H, Conway EM. Platelets and Complement Cross-Talk in Early Atherogenesis. *Front Cardiovasc Med.* 2019 under the CC-BY4.0 license.

1.3.2.2 Secondary hemostasis – the coagulation cascade

The secondary hemostasis, also termed "the coagulation cascade," involves the assembly of several circulating coagulation factors to form a cascade of serine proteases. The final protease, thrombin, also termed factor IIa, converts fibrinogen to fibrin (Figure 5). The cascade can occur in plasma at slow rates but is significantly catalyzed on the surface of activated platelets (Figure 7) [106,108,109], and it is now widely accepted that the platelet plays a pivotal role in the coagulation cascade.

The primary activator of coagulation is the binding of TF to factor VII, forming factor TF-VIIa (Figure 5). Factor VIIa catalyzes the activation of factors IX and X [105]. TF is present in vast amounts in the subendothelial tissue and is exposed to the coagulation cascade if endothelial damage occurs. Additionally, TF may also be released by various cells, including monocytes, neutrophils, and possibly activated platelets [110], thus triggering coagulation even in the absence of endothelial damage. The secondary hemostasis is kept in check by anticoagulants such as thrombomodulin, TF pathway inhibitor, protein C, and heparan sulfate, present on the surface of intact endothelium [113]. Heparin enhances the activity of these coagulation inhibitors; most importantly, heparin sulfate combines with antithrombin III to form an inhibitory peptide blocking several enzymes in the coagulation cascade, including factor Xa and thrombin [113].

1.3.2.3 Tertiary hemostasis – the fibrinolysis

The tertiary hemostasis, also termed "the fibrinolysis," involves the release of tissue plasminogen activator (tPA) from damaged endothelium. tPA combines with fibrin and catalyzes the conversion of plasminogen to plasmin. Plasmin catalyzes fibrin degradation, thus preventing the overgrowth and resolution of the blood clot [105].

1.3.3 Interactions between complement and hemostasis

As mentioned previously, the coagulation and complement cascades are ancestral homeostatic systems with many similarities, and extensive crosstalk happens between the two cascades. Many of the peptides involved in the coagulation cascade also exert effects in the complement system, and vice versa [44] and the systems indirectly interact through platelet activation and stimulation of cytokine-releasing cells [112]. For example, complement indirectly activates hemostasis by stimulating TF-releasing cells, resulting in TF release, thrombin generation, and subsequently platelet activation, and C3a and C5a bind to fibrin and augment clot stability [44]. Conversely, hemostasis may activate complement, for example, through the platelet mediated classical pathway or alternative pathway activation [62], or possibly thrombin-mediated cleavage of C3 and C5 [44,62], although a recent study has shown that the thrombin only cleaves C5 under very acidic conditions, seldomly occurring *in vivo* [114]. Additionally, heparin may inhibit or activate complement in a dosage-dependent fashion [115], and C1-INH works as an inhibitor both in the complement and the coagulation systems [44].

The complement and coagulation systems undoubtedly play a pivotal role in the host defense. However, these cascades interact with immunoglobulins, cells, and other cascade systems, including the kallikrein-kinin and the renin-angiotensin systems, both known to activate inflammation and coagulation [116].

2 The candidate's role in the project

The candidate conceptualized the study and wrote the protocol, including the application to the Research Animal Committee (Forsøksdyrutvalget) in close collaboration with the supervisors,

Professor immunologist Tom Eirik Mollnes and Professor anesthesiologist Erik Waage Nilsen. The candidate planned and conducted all *in vitro* human experiments in close collaboration with chief bioengineer Dorte Christiansen, Research Laboratory, Nordland Hospital, and all *in vivo* pig experiments, including animal retrieval, anesthesia, surgery, intervention, resuscitation, and data collection, in close collaboration with Professor Erik Waage Nielsen and the staff at ANILAB, Nord University, Bodø, and in close collaboration with Dr. Per Steinar Halvorsen and the staff at The Intervention Center, Oslo University Hospital, Oslo. The candidate participated in sample collection, preparation, and analysis, including RNA extraction, enzyme-linked immunosorbent assay (ELISA), quantitative (real-time) polymerase chain reaction (qPCR) analysis, blood gasses, rotational thromboelastometry (ROTEM), and multiplate analysis in close collaboration with the staff bioengineers at the Research laboratory, Nordland Hospital. The candidate independently performed several ELISA analyses, but not the multiplex analysis. The candidate independently organized and charted all data and conducted the statistical analysis in collaboration with the supervisors and statistician Tonje Braathen. In collaboration with all coworkers, the candidate wrote all articles as the first author.

3 Aim of the thesis

There exists a paucity of research into air emboli and air embolism. However, most *in vitro* studies are somewhat limited by their design, as they were conducted either in plasma, whole blood anticoagulated with heparin, or whole blood with antifoam additives. Further, ambient air was present in most experimental setups as per standard laboratory practice. Experimenting in plasma or heparin anticoagulated blood precluded the holistic examination of complement, leukocytes, and hemostasis. Ambient air and antifoam could potentially have influenced the experiments. The *in vivo* large animal studies of air embolism date back to the early 1990s, when many modern immunological and hematological methods such as multiplex technology, ELISA, qPCR, and ROTEM were not widely available. Further, these studies were not focused on thromboembolism. Thus, the exact mechanism by which air emboli activate the thromboinflammation and the effect of complement inhibition on the thromboinflammation remains to be examined in detail both *in vitro* in human whole blood and *in vivo* in an animal model without the limitations mentioned above.

The overall aim of this thesis is to elaborate on the thromboinflammation induced by air emboli and air embolism using state-of-the-art immunological techniques. To achieve this overall aim, a series of specific aims were laid out for each paper:

Paper I: This study aimed to examine whether complement "background activation," i.e., unavoidable and undesired complement activation due to properties of the model system, could be reduced by eliminating ambient air from *in vitro* incubations of human whole blood and if incubation with air emboli would increase complement activation compared to incubations with ambient air only.

Paper II: Using the model described in Paper I, this study aimed to examine *in vitro* in human whole blood the effects of air emboli on the thromboinflammation, including the effect on the

complement system, the cytokine network, the platelets, and the coagulation, and the effect of specific complement inhibition on the thromboinflammation. Further, to study the effects of incubation in blood-filled tubes on blood gasses and the effect of antifoaming agents on complement and coagulation. Finally, to compare the effect of incubations in plasma and whole blood on complement and coagulation.

Paper III: This study aimed to examine the effects of venous air embolism on the complement, inflammation, and hemostasis *in vivo* in a porcine model.

4 Main results of the studies

We discovered that ambient air in test tubes, present under normal laboratory conditions, activated complement. Therefore, we developed an *in vitro* model of air emboli using lepirudin anticoagulated human whole blood without ambient air. Using this model and an *in vivo* porcine model, we showed that air emboli *in vitro* and air embolism *in vivo* activated complement C3 and triggered thromboinflammation. *In vitro,* we showed how air activated the complement system through the alternative pathway and that this activation did not correlate with terminal pathway activation. Further, we showed how C3 activation triggered the release of proinflammatory cytokines and TF and that air activated the coagulation cascade through a complement-dependent mechanism and a complement-independent mechanism. We also showed that the platelets were activated through a complement independent mechanism and theoretically through a complement-dependent TF mediated mechanism. Finally, we showed that selective C3 inhibition and, to a lesser degree, selective C5 inhibition attenuated the inflammatory response. Both C3 and C5 inhibition equally reduced coagulation, but neither reduced the platelet activation. *In vivo*, we showed that air embolism triggered thromboinflammation with leukocyte and cytokine release, hemostasis, and C3a and cytokine

deposition in the lungs. Our findings were published in three papers. The main findings from each paper are shortly presented in the following sections.

4.1 Paper I

This paper showed that ambient air and air emboli incubated in human whole blood activated the alternative complement pathway, quantified by measuring C3a, C3bc, and C3bBbP (Figure 9A-C), with only a minor increase in terminal pathway activation, quantified by measuring C5a and TCC (Figure 9D and E). Incubations without ambient air in the tubes reduced, and incubations with air emboli increased the complement "background" activation compared to incubations with ambient air in the tubes, as per standard laboratory practice.

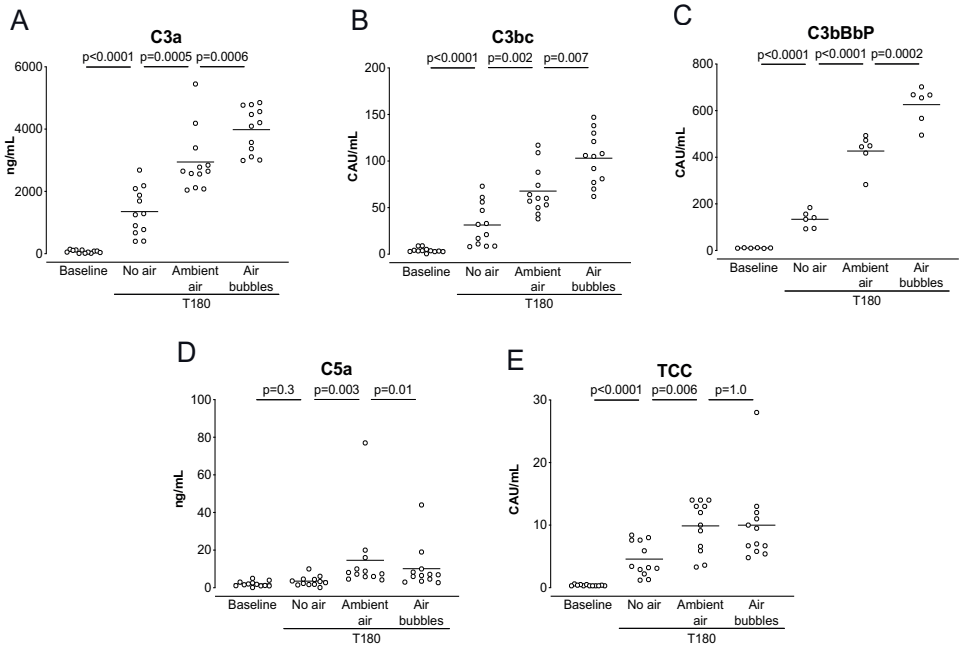

Figure 9 The effect of ambient air on complement. In incubations of human whole blood, ambient air and air bubbles activated the alternative complement pathway and increased C3 activation products C3a (Panel A), C3bc (Panel B), and C3bBbP (Panel C). C5a (Panel D) and TCC (Panel E) were also increased but lesser than C3 activation products. Adapted from Storm BS et al. Avoiding ambient air in test tubes during incubations of human whole-blood minimizes complement background activation. *J Immonol. Methods* 2020 under CC-BY 4.0 license.

4.2 Paper II

In this study, we elucidated the effect of air emboli on the human thromboinflammatory system and the effect of specific complement inhibition. We developed an *in vitro* human whole blood model with lepirudin anticoagulation, incubating air bubbles rather than constant bubbling. Using this model and inhibitors of complement C3, C5, complement receptor C5aR1, and the central TLR co-factor CD14, we showed that air emboli activated the alternative complement cascade without a corresponding terminal pathway activation. Air emboli triggered TF synthesis and release, measured as TF mRNA and TF-bearing microparticles (MP-TF), respectively, activated platelets, measured as β-thromboglobulin (βTG), and triggered coagulation, measured as prothrombin fragment 1+2 (PTF1+2). C3 and C5 inhibition equally reduced coagulation but neither reduced platelet activation. Further, air emboli triggered a complement C3-dependent release of 24 of 27 measured cytokines (Table 1). C3-inhibition reduced the release of all 24 cytokines significantly, and this reduction was further increased by the combined inhibition of C3 and CD14. In contrast, C5 inhibition only reduced 3 of 24 cytokines, and C5 receptor inhibition only 2 of 24 cytokines (Table 1).

Table 1. Cytokine response to incubation without or with air bubbles or with air and inhibitors of either C3, C3 and CD14 combined, or C5

Cytokine	No air vs. Air bubbles		Air bubbles vs. air bubbles and Cp40		Air bubbles vs. air bubbles and Cp40/r18D11		Air bubbles vs. air bubbles and Euclizumab	
	Fold increase	FDR adjusted p^1	Fold decrease	FDR adjusted p^1	Fold decrease	FDR adjusted p^1	Fold decrease	FDR adjusted p^1
PDGF-BB	78	***<0.0001***	2.9	***<0.0001***	5.6	***<0.0001***	1.1	0.5
IL-8	29	***<0.0001***	4.9	***<0.0001***	8.6	***<0.0001***	2	***0.001***
IFN-γ	20	***<0.0001***	5	***0.0001***	9.5	***<0.0001***	1.4	0.2
RANTES	20	***<0.0001***	1.8	***<0.0001***	2.1	***0.0001***	1.2	0.2
IL-1ra	14	***<0.0001***	4.2	***<0.0001***	3.5	***<0.0001***	1.3	*0.3*
G-CSF	14	***<0.0001***	3.3	***0.0005***	3.4	***0.0004***	1	0.7
VEGF	11	***<0.0001***	4.2	***<0.0001***	4.4	***<0.0001***	1.2	0.4
MCP1	11	***<0.0001***	3.9	***<0.0001***	2.4	***<0.0001***	2	***0.005***
MIP-1α	9.6	***<0.0001***	4.3	***<0.0001***	5.2	***0.0001***	1.1	0.4
IL-1β[3]	8	***<0.0001***	3.2	***<0.0001***	6.4	***<0.0001***	1.5	***0.02***
IL-17α	7.2	***<0.0001***	2.1	***<0.0001***	2.8	***<0.0001***	1.1	0.4
IL-15	5.8	***<0.0001***	2.1	***0.0004***	3.6	***<0.0001***	1	0.8
IL-10	5.2	***<0.0001***	2.7	***<0.0001***	4	***<0.0001***	1.3	0.2
IL-6[3]	5	***<0.0001***	2.5	***0.0004***	9.3	***<0.0001***	1.2	0.2
GM-CSF	4.9	***<0.0001***	1.5	***0.04***	4.4	***0.0001***	0.9	0.8
IL-9	4.7	***<0.0001***	1.4	***0.02***	1.3	***0.002***	1	0.5
IL-5[2]	4.3	***<0.0001***	2.9	***0.0001***	3	***0.0001***	1	0.7
Basic FGF[3]	4.1	***<0.0001***	1.5	***0.0008***	2.4	***<0.0001***	1.1	0.3
IL-2	4	***<0.0001***	1.9	***0.0001***	3.4	***0.0001***	1.1	0.3
MIP-1β	3.8	***<0.0001***	2	***0.002***	3	***0.0009***	0.8	0.8
IL-4	3.5	***<0.0001***	2.2	***0.0005***	1.9	***0.0002***	1.1	0.4
TNF	3.4	***<0.0001***	2.4	***0.0008***	3	***<0.0001***	1.3	0.3
IL-7	2.3	***<0.0001***	1.7	***0.003***	1.6	***0.0002***	1.2	0.1
IL-13	1.8	***<0.0001***	1.5	***0.0003***	1.5	***0.0002***	1.1	0.3
Eotaxin	1.5	***<0.0001***	1.7	***<0.0001***	1.7	***<0.0001***	1.1	0.2

[1] Log transformed data. Linear mixed-effects model with random intercept. Multiple comparisons corrected using the False discovery rate (FDR) method with Q=0.05. Discoveries (FDR adjusted p<0.05) in italic and bold print.
[2] Due to low bead number, only nine incubations were analyzed for IL-5.
[3] Compared to incubations with air bubbles only, incubations with air bubbles and r18D11 reduced IL-1β 6.4-fold (p=0.03[1]), IL-6 9.3-fold (p=0.02[1]), and Basic FGF 2.4-fold (p=0.05[1]).

Table 1 Cytokine response to incubation without or with air bubbles or with air and inhibitors of either C3, C3 and CD14 combined, or C5. Adapted from BS Storm et al. Air bubbles activate complement and trigger C3-dependent hemostasis and cytokine release ex vivo in human blood. *J Immunol* 2021;207(11):2828-2840 under a CC-BY4.0 license

4.3 Paper III

In this study, we showed in pigs that air embolism triggered a thromboinflammatory response with an increase in plasma C3a, leukocytosis, and hemostasis, quantified using ROTEM and TAT. Further, air embolism triggered the synthesis and release of proinflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, and complement C3a (Figure 10A and B) in lung tissue of pigs receiving air infusion, confirming that the lungs play an important role in the pathology of

venous air embolism. In line with the human *in vitro* findings in Paper II, C3a in the lung tissue was not associated with increased TCC (Figure 10C).

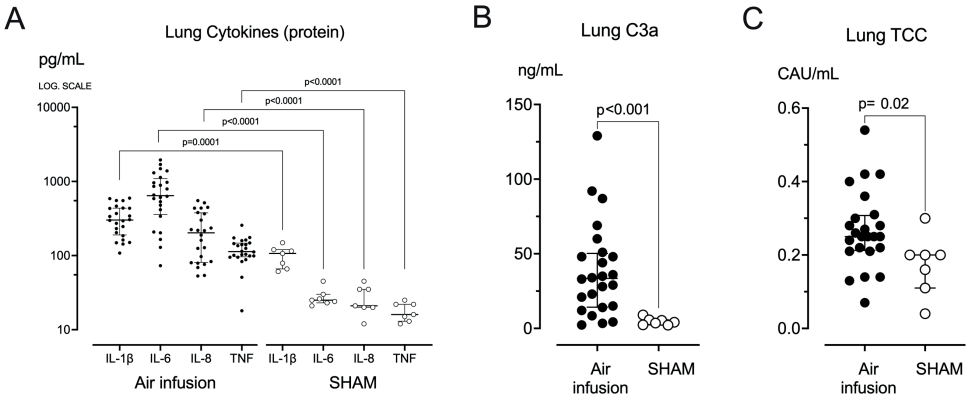

Figure 10 Air embolism trigger pulmonary inflammation, increasing lung C3 and proinflammatory cytokines. Pigs were exposed to 300 or 360 minutes of intravenous air infusion (n=24) or served as sham animals (n=7). Post-mortem lung tissue was analyzed for cytokines using ELISA or multiplex (A) and for complement C3a (B) and TCC (C) using ELISA. Horizontal lines are medians, and error bars span the interquartile range. Groups were compared using two-tailed Mann-Whitney U-test. Adapted from Storm BS et al. Venous Air Embolism Activates Complement C3 Without Corresponding C5 Activation and Trigger Thromboinflammation in Pigs. *Frontiers Immunol* 2022(13):839632 under a CC-BY license.

5 Methods and materials

5.1 Paper I

This study examined the effect of ambient air present in test tubes on the complement system. We drew blood from 12 healthy human donors and added lepirudin anticoagulation as detailed in [115]. A baseline sample was drawn, and the blood was split into three aliquots. Ambient air emboli were added to one tube using a syringe and needle, one tube was filled with blood, and one was half-filled with ambient air present inside the tube. The samples were incubated for 180 minutes at 37 °C on a roller mixer. After incubation, ethylenediaminetetraacetic acid (EDTA) was added to the samples and plasma isolated. The plasma was analyzed for complement activation products C4d, C4bc, C3a, C3bc, C3bBbP, C5a, and TCC using a combination of commercially available and in-house ELISA assays.

5.2 Paper II

Based on the findings in Paper I, we developed a whole blood model of air embolism. We drew blood from 16 healthy donors and added lepirudin as detailed in [115]. The experiments are summarized in (Paper II, Figure 1). A baseline was drawn, the blood was split into seven aliquots and incubated in tubes without ambient air, tubes with air emboli, or tubes with air emboli and one of the following: the C3 inhibitor compstatin (Cp40), the C5 blocking antibody eculizumab, the C5aR1 blocking peptide PMX53, the CD14 blocking antibody r18D11, a combination of r18D11 and Cp40 or PMX53, respectively. The tubes were incubated for 180 minutes at 37 °C on a roller mixer. After incubation, EDTA was added to the samples and plasma isolated. Additionally, we prepared and incubated blood from six donors as described above. After incubation, we added PAXgene solution to the blood for subsequent RNA extraction. We also drew and incubated blood from as six donors as described above in aliquots without air or with air emboli. Blood gasses were sampled every sixty minutes throughout the incubation. Blood from another six donors was obtained as described above, split into three aliquots, and incubated as described above with antifoam A, antifoam B, or no additive. After incubation, EDTA was added to the samples and plasma isolated. Finally, we collected blood from six donors as described above, isolated plasma immediately after blood collection, and incubated these under the same conditions as described above with either no air in the tubes, with air emboli, or for three of the donors, with air emboli and either Cp40 or eculizumab.

Plasma from the whole blood and the plasma incubations was analyzed using a combination of commercial and in-house ELISA assays for C4d, C3a, C3bc, C3bBbP, C5a, and TCC.

Plasma from 13 whole blood incubations was analyzed using a multiplex kit for the 27 cytokines: IL-1b, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-

12, IL-13, IL-15, IL-17, MCP-1, MIP-1a, MIP-1b, eotaxin-1, IP-10, basic fibroblast growth factor 6, G-CSF, GM-CSF, IFN-g, platelet-derived growth factor-BB, RANTES, TNF, and vascular endothelial growth factor. From the PAXgene treated blood, mRNA was extracted and analyzed for TF mRNA using qPCR and the ΔΔCt method.

5.3 Paper III

In this study, we used a porcine model of venous air embolism, developed at our laboratory as described [40], based on previous models by Durant et al. [28], Oppenheimer et al. [29], and Vik et al. [35]. Forty-five pigs were allocated to undergo 300 minutes of air infusion through an ear vein or serve as sham, as detailed in (Paper III, Figure 1). The air infusion was titrated based on pilot experiments and the findings by Vik et al. [35] to cause hemodynamic instability but not the death of the animals. Twelve pigs were excluded from the study for various reasons, including infections and unforeseen adverse events. To avoid coagulation of infusion lines, low dosage heparin (approximately 30 IU/hour) was continuously administered through the lines. Arterial blood was sampled before starting air infusion, after 30, 60, 180, 240, and 300 minutes into EDTA tubes, citrate tubes, PAXgene tubes, and heparinized blood gas syringes. EDTA plasma was analyzed for TAT and complement C3a and TCC using ELISA assays known to work reliably with pigs. White cells were counted at the central hospital laboratory. Blood gasses were analyzed using the heparinized blood and a bedside blood gas analyzer. The citrated samples were recalcified, and the hemostasis was analyzed using ROTEM. Postmortem lung tissue was sampled, homogenized, and split into triplicates. One triplicate was analyzed for complement C3a and TCC using ELISA assays known to work well in pigs. One triplicate was analyzed for cytokines TNF, IL-1β, IL-6, IL-8, and IL-10, using a combination of multiplex and ELISA assays known to work reliably in pigs. From the last triplicate, RNA was extracted

using TRIzol reagent and cleaned using RNeasy and DNase treatment. The samples were then analyzed for cytokine IL-1β, IL-6, and IL-8, TNF mRNA using qPCR and the ΔΔCt method.

5.4 Statistical methods

All acquired data was organized in Microsoft Excel for Mac ver. 16.16.5 to 16.16.9 (Microsoft Inc., Redmond, CA). Statistical analysis and charting were done in Prism for Mac ver. 8.4.2 to 9.3.1 (GraphPad Software, La Jolla, CA) or STATA for Windows ver. 16 (StataCorp LLC, College Station, TX). The studies included in this ph.d. project had different designs, and thus different statistics were used. *In vitro*, blood samples from individual donors were compared, yielding paired data. *In vivo*, tissue and blood from two groups of pigs were compared, yielding unpaired data, or repeated samples from each animal were compared, yielding paired data. Most data were either normally distributed or log-transformed to fit a normal distribution; thus, where possible parametric tests were used. Single comparisons were analyzed using Sudents' t-tests, and repeated measures were analyzed using analysis of variance (ANOVA). Missing data were imputed using an appropriate method, or datasets were analyzed using a mixed-effects model handling missing data. Some data was not normally distributed, nor transformed, and analyzed using Wilcoxon matched pair or Mann-Whitney test. The choice of statistics and statistical considerations are further discussed in Section 6.3.

6 Methodological Considerations

In the project, we examined thromboinflammation *in vitro* in human whole blood and *in vivo* in pigs. To detect complement activation products and cytokines *in vitro* and *in vivo*, we isolated plasma and obtained lung tissue. We analyzed the samples using standard immunological

assays, including ELISA, multiplex, and qPCR. To study hemostasis *in vitro*, we used ROTEM. The design of the *in vitro* and *in vivo* models, the sample acquisition, and the analysis are detailed in papers I, II, and III. The following sections will briefly discuss some important methodological considerations related to the *in vitro* studies (Section 6.1), the *in vivo* study (Section 6.2), and general statistical considerations (Section 6.3).

6.1 Development of a human *in vitro* model of air emboli

With the development of advanced cardiac surgery and the introduction of *ex vivo* blood oxygenators, it became evident that *ex vivo* circulation activated a complement-driven inflammatory response in patients [117]. This was partly explained by the plastic material's complement activating properties [118] and partly by complement activation in the blood oxygenator [77,119]. In the early 1990s, Ekdahl's group found a complement C3 activation during cardiac surgery with *ex vivo* blood circulation and oxygenation and during *in vitro* circulation of human serum and heparin anticoagulated human whole blood in an oxygenator system [77,119]. These studies showed that when blood was circulated through a bubble oxygenator, where oxygen was bubbled through the blood, C3 was hydrolyzed to iC3, also named C3(H_2O). In contrast, iC3 was not formed when blood was circulated through a membrane oxygenator, where blood was not in direct contact with oxygen bubbles. The group also demonstrated that the C3 hydrolysis occurred equally when blood came in contact with oxygen, nitrogen, and ambient air bubbles. These findings align with Shastri et al., showing that bubbling nitrogen through human serum activated complement [120], and Ward et al., showing that ambient air bubbles incubated in plasma activated the complement system [121].

We based our *in vitro* model of air emboli on the model described by Ekdahl et al. [77]. Briefly, in the model, oxygen (or ambient air) is bubbled through either anticoagulated whole blood or serum incubated at 37 ℃. An antifoaming agent is added to the samples to avoid excessive foam buildup due to the constant bubbling (Figure 11).

Figure 11 In vitro air emboli model using continuous bubbling. Air is bubbled continuously through anticoagulated whole blood incubated at 37 ℃ using a needle connected to an air-filled syringe and a syringe driver with a set rate. To avoid foam formation, antifoam is added to the blood. In the figure, different concentrations of antifoam were tested in the four test tubes. Private photo.

During our pilot experiments, it became evident that the model developed by Ekdahl et al. had some important drawbacks, potentially affecting our results; First, heparin was used as an anticoagulant. Heparin interferes with the complement system, acting as an activator in low concentrations and as an inhibitor at higher concentrations [115]. Second, experiments were carried out in serum, which is depleted of both platelets, leukocytes, and coagulation factors, precluding any studies of crosstalk between the cascades. Further, the isolation of serum from whole blood partly activates the complement system [122]. Third, antifoam was used to avoid foam formation. The effect of antifoam on thromboinflammation has, to our knowledge, not been thoroughly tested. Fourth, as gas bubbles are known to activate complement, ambient air present in the test tubes could potentially have a similar effect. Therefore, we decided to develop a new *in vitro* model of air emboli to avoid these drawbacks.

6.1.1 Lepirudin anticoagulation allows for detailed studies of thromboinflammation

Traditionally, *in vitro* immunological experiments have been conducted in either serum, plasma, or heparin anticoagulated whole blood. However, as mentioned above, plasma and serum are depleted of platelets, leukocytes, and erythrocytes, and serum is also depleted of coagulation factors. Heparin binds to and activates the enzyme antithrombin III, attenuating the coagulation cascade by inactivating coagulation factor Xa and thrombin (factor IIa) [113]. Additionally, heparin has been shown to interfere with the complement system, activating complement in low dosages and inhibiting at high dosages [115,123]. EDTA and citrate block several plasma cascades and cannot be used for *in vitro* experiments. Therefore, plasma, serum, and EDTA, citrate, or heparin anticoagulated blood are unsuitable for studying the coagulation, platelet function, inflammation, and crosstalk between the humoral systems and the cellular systems in the blood. To study these systems, it is necessary to use whole blood and, if possible, avoid the use of heparin. However, *in vitro* handling of whole blood inevitably activates coagulation, and the use of anticoagulation is considered unavoidable when experimenting in whole blood. Synthetic or animal analogs of the naturally occurring human heparin are the most used anticoagulation *in vivo* and *in vitro*. In 2002 Mollnes et al. developed an *in vitro* whole blood model, replacing heparin with the direct thrombin inhibitor lepirudin [115]. In contrast to heparin, lepirudin does not interfere with the complement system, and it inhibits only thrombin, leaving the rest of the upstream coagulation cascade uninhibited. Thus, lepirudin anticoagulated whole blood is a good choice when studying the complement, inflammation, and coagulation systems, albeit lepirudin inhibits thrombin, making this model less suitable for studying the specific effects of thrombin, e.g., the thrombin-induced platelet activation.

6.1.2 The effect of antifoam on complement and coagulation

An inherent problem when bubbling gases through blood is the formation of a protein foam on the surface of the blood sample (Figure 11) and, ultimately, loss of the sample material out of the test tube. In the original *in vitro* model of air emboli, Ekdahl et al. used an antifoam additive in the samples to avoid this problem [77] (the specific type of antifoam was not stated).

Antifoams are a group of chemicals used in industrial fermenting and other foam generating procedures to reduce surface tension and foam generation [124]. In the era of bubble oxygenators, various antifoams were also used clinically in these devices to reduce blood foam formation [125–127]. However, this was shown to come at a high risk of cerebral antifoam micro-emboli. Many organic and inorganic antifoams exist. In *in vitro* experiments, inorganic silicone-based compounds and organic siloxane-based compounds are frequently used. The effect of antifoam on the complement and coagulation systems has to our knowledge, never been examined. As neither silicone nor siloxane molecules are native to blood, we speculated that they could interfere with complement and coagulation. Additionally, the proteins on the surface of air bubbles might activate platelets [56], and bubbles themselves may activate complement [121], thus, eliminating air bubbles using antifoam could interfere with the study of thromboinflammation.

To assess the potential effect of antifoam on our *in vitro* model, we conducted a series of experiments, incubating two commonly used antifoam substances, antifoam A and antifoam B, in whole blood. Subsequently, we measured complement and coagulation activation. These studies showed that antifoam B activated both complement and coagulation and was best avoided during experiments (Figure 12). Antifoam A did not activate the systems. To avoid potential interactions, we chose to omit antifoam from our model.

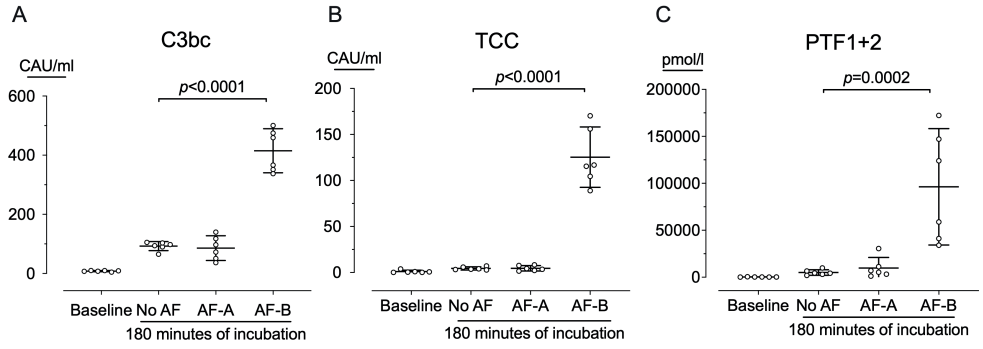

Figure 12 Incubation of antifoam in whole blood. Antifoam B activated C3bc (Panel A), TCC (Panel B), and TCC (Panel C). Antifoam A did not affect the readouts. Reproduced from Storm BS et al. Air bubbles activate complement and trigger C3-dependent hemostasis and cytokine release ex vivo in human whole blood. *J Immunol.* 2021 with permission from the American Association of Immunologists.

6.1.3 Continuous bubbling versus incubations with air bubbles

Avoiding antifoam in our model meant we had to abandon experimental setups where the air was bubbled continuously through blood due to excess foam formation. In the 1980s, Ward et al. described incubation of bubbles in plasma compared to tubes completely filled with plasma [121]. In this model, test tubes with plasma isolated from heparinized blood were rigorously shaken until the plasma was filled with bubbles and then incubated on a roller mixer. Inspired by this model, we conducted pilot experiments using lepirudin anticoagulated whole blood rather than plasma. Instead of vigorously shaking the tubes, which may unwantedly activate the platelets [128], we added ambient air bubbles with a needle and syringe before capping the tubes.

To avoid erythrocyte sedimentation, we incubated the samples on a roller mixer. Using this approach, the bubbles maintained their integrity throughout 180 minutes of incubation (Figure 13).

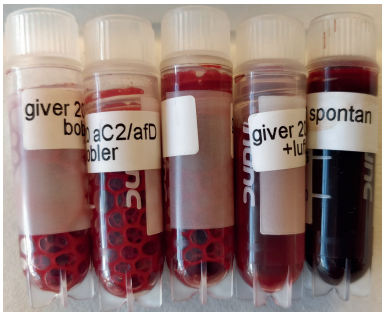

Figure 13 Blood incubated with air emboli. Whole blood incubated 180 minutes on a roller mixer with air emboli (sample 1 to 4) and control sample without air (sample 5). The air emboli maintained their integrity throughout the incubation period. The oxygen tension in the control sample was lower than in samples incubated with air emboli. Private photo.

6.1.4 The effect of ambient air on thromboinflammation

As mentioned above, studies have demonstrated how the alternative complement cascade is activated on the surface of gas bubbles [77,121] and that air bubbles activate platelets [56]. Ward et al. showed in plasma that the alternative complement pathway was activated when incubated with air bubbles [121] and that the activation was significantly reduced when the plasma was incubated without ambient air. However, the study did not examine the effect of incubating plasma with ambient air.

We speculated that the ambient air present in half-filled test tubes (Figure 14A) would have similar complement- and platelet-activating properties as air bubbles. This was important to examine as such activation could interfere with our experiments and trigger thromboinflammation even in control samples if ambient air was present in the tubes. Thus, we developed a method of filling the tubes and tube caps completely, eliminating ambient air inside the tubes (Figure 14B). Further, instead of introducing air emboli by continuously bubbling air or vigorously shaking and thumbing the tubes, air was introduced by a needle and syringe before the incubation (Figure 14C). These modifications to the *in vitro* model kept complement

activation products (Paper I, Figure 2 and Paper II, Figure 2), platelet activation and coagulation (Paper II, Figure 5), and cytokine release (Paper II, Figure 6 and 7) near baseline values in the control samples.

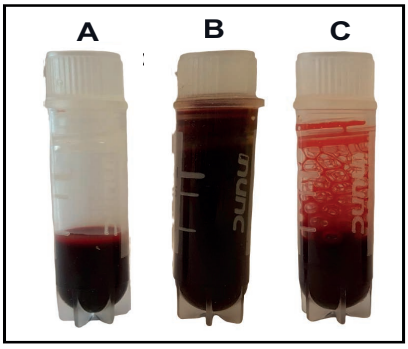

Figure 14 Development of in vitro whole blood model of air emboli. Samples were incubated with ambient air in the tube as per standard laboratory practice (A), in tubes without ambient air (B), or with air emboli introduced to the sample using a syringe and needle before incubation (C). Adapted from Storm BS et al. Avoiding ambient air in test tubes during incubations of human whole-blood minimizes complement background activation. *J Immunol. Methods* 2020 under CC-BY 4.0 license.

6.1.5 The effect of ambient air on pH

Eliminating ambient air from the test tubes as described above could potentially alter the partial pressure of oxygen and CO_2 due to the ongoing metabolism in the blood during incubation. Hypoxia and lowered pH due to increased CO_2 tension may affect the function of the biological cascade systems and thus the results of the experiments. To assess the effect of air-free incubations on blood gasses, we conducted a series of 180-minute incubations with or without air (as shown in Figures 14A and 14B) and measured blood gasses every hour. We found lower but constant oxygen tension (Figure 15A), higher and increasing carbon dioxide tension (Figure 15B), and a slightly lower pH (Figure 15C) in samples incubated without air compared to samples incubated with air. However, both oxygen and carbon dioxide levels in samples incubated without air remained within normal venous levels, and the pH differed only slightly between the samples, thus likely not affecting our results.

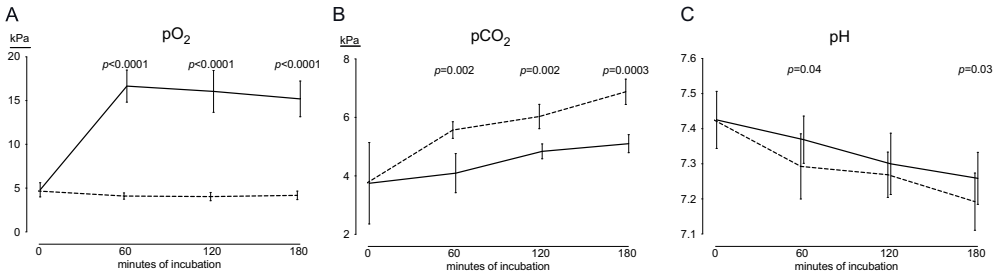

Figure 15 Effect of air emboli on blood gasses. Blood was incubated with and without air for 180 minutes. O_2 tension (pO_2) increased significantly (Panel A) in samples incubated with ambient air and remained at baseline level in samples incubated without air. CO_2 tension (pCO_2) increased (Panel B), and pH decreased (Panel C) slightly in samples incubated without air compared to samples incubated with air. Solid lines are samples incubated with air emboli. Dotted lines are samples incubated without air. Reproduced from Storm BS et al. Air bubbles activate complement and trigger C3-dependent hemostasis and cytokine release ex vivo in human whole blood. *J Immunol.* 2021 with permission from the American Association of Immunologists.

6.2 Development of a porcine *in vivo* model of air embolism

A serious shortcoming of our (and most other) *in vitro* model is the absence of the vascular endothelium, the glycocalyx, and any organs. The endothelium interacts with and regulates both the complement and coagulation systems [129], the glycocalyx modulates both coagulation, platelets, and leukocytes [130], and the lungs play a pivotal role in the pathophysiology of air embolism [9,11,131]. Thus, the absence of endothelium, glycocalyx, and lungs means that our *in vitro* findings had to be verified *in vivo* to make robust conclusions that might transfer to the clinical patient setting. Obviously, we could not conduct *in vivo* studies on human subjects. As an alternative to human experiments, pigs are often used as a model when studying the immune system [132]. In contrast to, for example, rodents, there are many similarities between the human and pig anatomy and immune system. It has been estimated that the porcine immune system has more than 80% genetic resemblance to the human system, making the species a preferred choice when studying these systems [132]. In contrast, the immune system in mice, another often-used species, has only a 10% resemblance to humans. However, there are important differences between humans and pigs, which one must bear in mind when using pig models to study human

pathophysiology. Most relevant to this project are the different lung histology of pigs and humans, differences in the molecular composition of the complement system, and differences in the coagulation cascade.

6.2.1 Hemodynamic and immunological differences between human and pig lungs

Through porcine studies of air embolism, we and others have shown that dose- and rate-dependent pulmonary hypertension and potentially fatal right heart failure develop immediately after starting an intravenous air infusion [28,36,40]. Several animal studies and human case series have shown that air emboli trigger inflammation [8,11,54]. This pulmonary hypertension occurs after only a few milliliters of air are infused. Larger amounts of air are needed to occlude the pulmonary arteries (own, unpublished data). Thus, the pathophysiological mechanism for pulmonary hypertension must involve more than a mechanical occlusion of the vasculature. Interestingly, Skjeflo et al. found a similar increase in pulmonary arterial pressure during intravenous infusion of *E. coli* in pigs [98]. This acute pulmonary hypertension was attenuated by simultaneous infusion of a complement C5 inhibitor and a CD14 inhibitor. In sum, these findings suggest that pulmonary hypertension in pigs is, at least partly, driven by a local inflammatory mechanism involving leukocytes and complement. These findings align with the pathophysiology of chronic pulmonary hypertension, where inflammation with the release of cytokines, chemokines, migration, and activation of lymphoid cells are known to play an important role [133].

Pigs exposed to air embolism, endotoxins, or other inflammation triggers develop rapid onsetting and dramatic acute pulmonary hypertension [40,134], exceeding that of humans. This may, in part, be attributed to the abundance of lymphoid tissue in porcine lungs, which in contrast to adult humans, have specific follicles-like aggregations of bronchial associated

lymphoid tissue (BALT) [135,136] and abundant pulmonary intravascular macrophages (PIM) [134]. The high concentration of leukocytes in BALT and the PIM explains why inflammatory processes affect the swine lungs more than the human lungs. Cytokines released from activated leukocytes trigger pulmonary hypertension [137]. Of note, one study indicates that BALT is equally absent in healthy pigs and humans [138], and further that BALT may form in human lungs during infection [136]. Summarized, the pig lungs play an important role in inflammation, including pulmonary hypertension. The magnitude of pulmonary hypertension and the extent of tissue inflammation might not be directly comparable to humans.

6.2.2 Titration of the air infusion in the porcine model

An essential part of this research project was to verify the *in vitro* thromboinflammatory findings in a relevant *in vivo* model. Several groups have studied other aspects of air embolism *in vivo* in large animal models through the last century. After a thorough literature search, we found the dog models by Durant et al. and Oppenheimer et al. [28,29] and the swine model by Vik et al. [35] both to be extremely relevant. Oppenheimer et al. showed that the clinical effects of air embolism were largely dependent on the amount and rate of the air infusion and the position of the animals [29] and that repeated injections of 100 ml of air were well tolerated in dogs (weight of animals not stated) with intact thorax [28], however, that injection of 5-7.5 ml air/kg body weight over 1.5 seconds was lethal [29]. Durant et al. and Oppenheimer et al. also showed in dogs that opening the thorax decreased the lungs filtering capacity and increased the lethal effects of air infusion [28], and that placing the dogs in the left lateral decubitus position, the so-called "Durant's position" (Figure 4) to retain the air emboli in the liver, increased the amount of air

required to cause mortality [29]. Some authorities advocate the same position [2,5] to treat clinical venous air embolism in humans.

Vik et al. showed that pigs receiving an infusion of 0.05 ml air/kg body weight/minute tolerated this well, and systemic passage of air did not occur, but when the rate was increased to 0.2 ml air/kg body weight/minute, all pigs had a systemic passage of air after a median time of 8 minutes [35]. Recently, in a porcine study of air embolism in pigs with closed and open thorax, based on the air infusion protocol developed by Vik et al., we examined the filtering capacity of the lungs and the aorta, the lethality of air infusion, and the effects of opening the thorax using ultrasound to detect air in the pulmonary artery and the aorta [40]. As Vik showed in pigs and Durant and Oppenheimer in dogs, we found that the filtering capacity of lungs was dependent on the rate and amount of air infusion. Also, as Durant and Oppenheimer showed in dogs, we found and described for the first time in pigs that opening the thorax reduced the filtering capacity significantly and increased the lethality of air infusion (Figure 16A, B, and C).

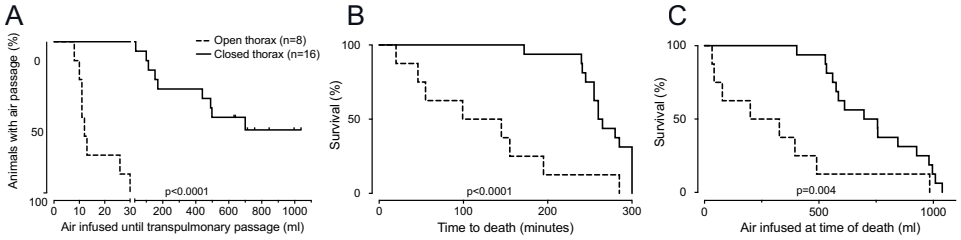

Figure 16 Open thorax reduces air embolism tolerance. In pigs with open thorax compared to closed thorax, the amount of air infused before systemic passage (A), the time to death (B), and the amount of air infused at death (C) were all significantly reduced. Reproduced from Storm BS at al. Open chest and pericardium facilitate transpulmonary passage of venous air emboli. *Acta Anest. Scand.* 2021 under CC-BY 4.0 license.

This finding is relevant to the porcine animal model since many experimental setups involve opening the thorax and pericardium to instrument the heart, e.g., to attach ultrasound sensors

or accelerometers to the myocardium. Due to the reduced tolerance to air, an open thorax is best avoided when studying venous air embolism.

6.2.3 Echocardiographic bubble detection

Air emboli can be detected by many methods [39], including echocardiography, which allows for real-time bedside detection and localization of air emboli with very high sensitivity (0.02 mL air/kg body weight) and stands out as the gold standard. A major drawback to echocardiography is its dependence on operator skills. Vik et al. described an automated bubble detection system using a transesophageal echo probe visualizing the pulmonary artery and the aorta in the same window [35]. This window, however, may be challenging to obtain and maintain in focus, and the automated system developed by Vik et al. is no longer available. We have enhanced the method described by Vik et al. using a slightly different and easier to obtain window of the pulmonary artery. Further, we used M-mode with a low sweep rate rather than 2D echocardiography, allowing us to detect with very high sensitivity both air emboli in the pulmonary artery and the aorta [40] (Figure 17).

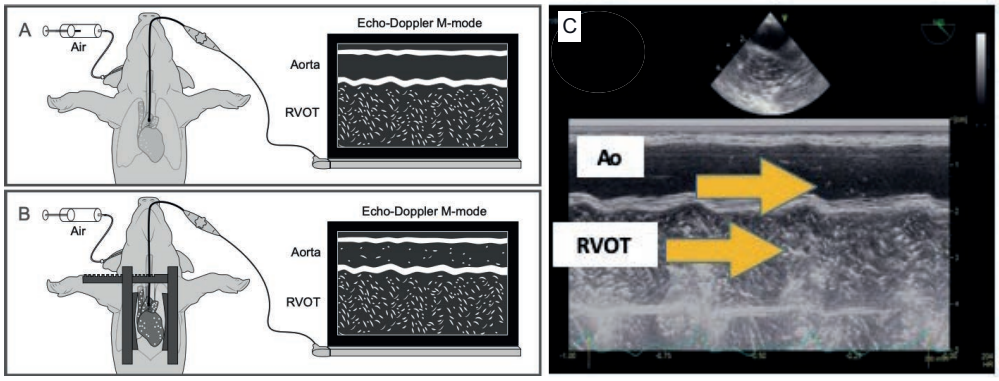

Figure 17 Detection of air embolism by transesophageal echocardiography. Schematic setup in animals with closed (Panel A) and open (Panel B) thorax. The probe is positioned to visualize the aorta (Ao) and the right ventricular outflow tract (RVOT). The air emboli are visualized as white streaks using M-mode with a low horizontal sweep rate. Echocardiographic recording of a pig with air embolism /yellow arrows) in the RVOT and Ao (Panel C). Reprint from Storm BS at al. Open chest and pericardium facilitate transpulmonary passage of venous air emboli. *Acta Anest. Scand.* 2021 under CC-BY 4.0 license.

6.2.4 Duration of the experiments.

The duration of the animal experiments depends mainly on the desired readouts. We aimed to study air-induced thromboinflammation, including complement, cytokines, and hemostasis. Complement- and coagulation factors circulate in plasma, and both cascades are rapidly activated. In contrast to complement activation and hemostasis, cytokine synthesis is a time-demanding process involving leukocyte activation, and cytokine messenger ribonucleic acid (mRNA) upregulation. Cytokine peptide production and the proinflammatory cytokines tumor necrosis factor (TNF), IL-6, and IL-1β peaks after 1.5 to two hours, and then gradually decrease, and leukocyte count peaks after six hours [139]. Thus, we titrated the intravenous air infusion to cause maximal hemodynamic instability, but not the death of the animals and without overwhelming the lung filtering capacity and systemic passage of air as described above, aiming for a duration of the experiments of 300-360 minutes.

6.2.5 Immunological readouts

As mentioned above, pigs and humans have many similarities and some differences in the composition of their immune system and coagulation cascade. Thus, assays developed for human diagnostics may not work reliably in porcine models, and where available, porcine assays should be preferred. However, a recent study by Ueland et al. found that porcine-specific cytokine immunoassays showed a significant variation in sensitivity for detecting specific cytokines [140], meaning that one multiplex assay might not reliably detect all cytokines measured. Unfortunately, the findings by Ueland et al. had not been published when we conducted our study, and we analyzed plasma and tissue for cytokines using a validated porcine multiplex kit (Invitrogen Cytokine & Chemokine 9-Plex Porcine ProcartaPlex Panel 1 kit; Thermo Fischer Scientific). Unfortunately, this kit did not reliably detect IL-1β, IL-6, IL-8, and

TNF, and we had to repeat the analysis using a combination of ELISA and plex methods as suggested by Ueland et al. (Table 2).

Table 1
Recommended method for quantification of porcine cytokines in plasma and tissue.

Cytokine	Plasma[a]			Tissue		
	ELISA	9-plex	13-plex	ELISA	9-plex	13-plex
TNF	X			X		
IL-1β	X[b]		X[b]	X		
IL-6	X					X
IL-8			X			X
IL-10		X			X	

[a] For the ELISA, IL-1β and IL-8 were quantified in serum, according to kit instructions.
[b] ELISA and 13-plex were comparable for IL-1β in blood samples.

Table 2 Recommended method for quantification of porcine cytokines in plasma and tissue. Reprinted from Ueland et al. Choice of immunoassay to evaluate porcine cytokine level. *Vet. Immunol. Immunopatholol.* Vol. 12 (2020), Copyright 2020, with permission from Elsevier B.V.

For complement C3a detection, we used a porcine-specific ELISA assay developed at our laboratory [141]. As no porcine-specific C5a ELISA exists, we used a standard TCC (sC5b9) ELISA with an anti-human capture antibody shown to cross-react with swine [142,143]. This is feasible as C5a and TCC are synthesized in equimolar amounts. The half-life of C5a is approximately one minute [144] compared to TCC, with a half-life of approximately fifty minutes [117,145]. To detect thrombin-antithrombin complex (TAT), we used a human ELISA kit, shown to work well in swine [146]. We used qPCR with primers specifically targeting porcine genes for cytokine mRNA detection, and leukocytes were measured using a flow cytometer with a validated porcine-specific protocol.

To measure complement in lung tissue, we homogenized lung tissue and treated it with a protein extraction reagent. This preparation may have affected the integrity of the peptides, and receptor-bound peptides, not available for capture antibody detection in intact tissue, may have

dissociated from the receptors, possibly yielding higher concentrations than in plasma samples. Thus, a direct comparison of complement levels in plasma and tissue is impossible.

We measured both the cytokine mRNA and the cytokine peptide concentration. Early in the inflammation, cytokine-producing cells may have been stimulated, and mRNA detected without concomitant detection of cytokine peptide. Later, the cytokine production may have been downregulated, and cytokine mRNA was no longer detected, but the cytokine peptide was still detectable.

6.2.6 Coagulation cascade and hemostasis

The hemostatic system is nearly identical in humans and pigs; however, the important difference is that pigs are hypercoagulable compared to humans, with a considerably higher concentration of several plasma coagulation factors [147]. Many human hemostasis assays can be used in pigs, albeit with a different reference range. In our study, we monitored the coagulation cascade and hemostasis using human TAT and ROTEM assays. Both are documented to work well in swine [146]. The porcine hypercoagulability is reflected in ROTEM readouts and TAT; pigs have a shorter clot formation time, a steeper α-angle, a higher maximum clot firmness, and a higher TAT than humans [146].

Surgical preparation and the experimental air embolism and inflammation all activate hemostasis. To avoid clogging of cannulas and sensors due to hypercoagulability, anticoagulation unavoidably must be used when experimenting in porcine models. The most used anticoagulation is low molecular heparin infused at a low rate through the indwelling cannulas and catheters. Additionally, intermittent heparin boluses may be needed to clear lines of thrombi. Heparin increases the effect of antithrombin and inhibits factor Xa, thus affecting

both the TAT formation and the ROTEM readouts. Therefore, due to the differences in the amount of coagulation factors and the use of heparin, findings in the porcine model might not be directly comparable with human results. However, a direct comparison can be made between pigs receiving air infusion and sham animals exposed to the same surgery and anesthesia but not receiving air infusion, considering that heparin boluses may interfere with results.

6.2.7 Correction for hemoconcentration / hemodilution

Interpretation of results from animal studies may be complicated by changes to the blood composition by hemodilution (a fall in fraction of red blood cells in the whole blood due to an increase in plasma volume) due to repeated blood sampling and simultaneous administration of intravenous fluids and by hemoconcentration (an increase in the fraction of red blood cells in the whole blood due to decrease in the plasma volume) due to capillary leakage of plasma fluid and smaller proteins [148], associated with acute inflammation.

To minimize the effects of hemoconcentration and maintain hemodynamic stability during the experiments, crystalloid fluid is often administered according to a protocol or as needed. To reduce the effects of hemoconcentration, no more than 10% of the calculated blood volume is usually sampled (institutional laboratory practice). Capillary leakage with loss of smaller molecules and plasma water to the extravascular tissue is triggered by inflammation [149], whereby plasma is concentrated. Larger blood components such as erythrocytes remain in the circulatory system; thus, hematocrit is increased. Judicious fluid therapy may dilute the blood, thus lowering the hematocrit and the concentration of plasma molecules. During porcine inflammatory studies, pronounced shifts in hematocrit often occur, as exemplified in a study by Nielsen et al. [150]. Logically, changes in the concentration of plasma proteins occur concomitantly through capillary leakage of smaller molecules and dilution of the plasma. Shifts

in plasma volume due to fluid therapy or blood sampling may mask biological findings. Thus, some researchers compensate for the change in plasma volume when interpreting results. Several compensation methods can be used; however, none have, to my knowledge, been validated in inflammatory studies. Correcting the plasma volume utilizing the change in hematocrit or hemoglobin concentration using the formula developed by Van Beaumont [151] or Dill and Costill [152] is often used in porcine inflammatory studies. Both formulas have been developed based on studies of the effect of severe excise or dehydration, not severe inflammation. The formulas show that during dehydration or rehydration, the change in hematocrit or hemoglobin concentration is not linearly correlated to the change in plasma volume. This is explained by the concomitant changes in plasma volume and electrolytes, loss of proteins, and thus osmotic shift, affecting the size of the red blood cells. Other methods of correction for hemoconcentration or hemodilution based on the change in the plasma volume exist [153]. An important prerequisite for using these formulas is that the amount of red blood cells remains constant throughout the experiments. This prerequisite is voided during repeated blood sampling experiments, and using these correction formulas may potentially over-amplify results, leading to wrong conclusions. An ideal correction method should be unaffected by blood sampling and thus change the number of red blood cells. However, such a correction formula has to my knowledge, not been developed, and we did not use any correction formula on our results.

6.2.8 Experimental animal health and consequences of infections

The use of animals in medical research is controversial and should be avoided or substituted with *in vitro* experiments whenever possible. However, as discussed, *in vitro* experiments are of limited value in studies of complex interactions between organ systems, endothelium, and

blood components. Animal experiments are subject to strict ethical regulations and must adhere to the "3 R's" principle; that is, replace animal studies with other methods whenever possible, reduce the number of animals used, and refine the studies to achieve maximum information from each animal [154]. In Europe, animal experiments must be conducted in accordance with the EU regulative 2010/63/EU, ensuring animal welfare [155].

When conducting animal experiments, either specially bred laboratory animals or animals from domestic herds can be used. We use swine from nearby domestic herds, as they are less costly and easy to acquire. However, several diseases affect swineherds, and sick animals must be avoided when conducting inflammation studies. Of special concern is infection with the bacteria *actinobacillus pleuropneumoniae*, a highly contagious respiratory tract pathogen with a high prevalence in swine herds worldwide [156] including in Norway [157]. Acute *actinobacillus pleupneumoniae* infection may cause fever, heart failure, and death. However, the bacteria may also cause a chronic infection, lung abscesses, pleuritis, pericarditis, and elevated inflammatory markers, but few clinical symptoms. Animals with chronic *actinobacillus pleupneumoniae* are challenging to identify and avoid, but the infection is discovered at lung autopsy or when analyzing inflammatory readouts. Such animals must be excluded from inflammation studies. If available, animals may be purchased from herds documented as "specific pathogen-free" (SPF) to reduce the risk of using infected animals for experiments. Animals from SPF herds are bred under strict hygienic measures and are certified free of *actinobacillus pleupneumoniae*. Thus, although SPF animals are more costly to purchase, the number of animals excluded from studies is likely lower, reducing costs and animals needed for repeat experiments, in line with the "3 R" principle "reduce."

During my experiments, we initially retrieved animals from two non-SPF-herds, as these were the only available suppliers. Unfortunately, many of the animals from one herd had suspected *actinobacillus pleupneumoniae* infection and had to be excluded. In the last series of my

experiments, one of the herds had been certified SPF, and we only used animals from this herd, thus reducing the number of excluded sick animals.

6.3 Statistical considerations

Modern statistical software offers many "on the fly" statistics, including complex analyses, and "getting some p-values" from statistical software is straightforward. However, getting "the right p-values" requires statistical insight and planning. Before experiments are conducted, a hypothesis, a power analysis, and a tentative choice of statistical method should be made. This facilitates designing experiments with a large enough sample size to yield statistical power to draw robust conclusions but small enough to limit unnecessary resource use. When data has been collected, pre-analytical adjustment of the dataset, e.g., imputation of missing values, log-transformation of non-normally distributed data, etc., must be made before calculating the statistics. Finally, the data should be analyzed and interpreted using the correct statistic. Mistakes in any one of these steps may void the estimates, leading to invalid conclusions. Thus, a statistician should always be consulted before planning experiments and analyzing data.

This chapter will briefly highlight some important aspects of statistics with relevance to my project.

6.3.1 Sample size and power calculation

Biological experiments are often resource-demanding. In *in vitro* experiments, donor blood is required, and in *in vivo* experiments, pigs are sacrificed. Thus, to limit unnecessary resource use and animal sacrifice, a tradeoff between large experiments with many subjects and strong statistical power and as few as possible, but enough subjects for adequate statistical power.

Therefore, the required sample size must be calculated before starting the experiments. To calculate the required sample size, we defined the study endpoints, e.g., a change in C3a, TCC, PTF1+2, or cytokine levels, and estimated the presumed effect through a series of pilot experiments; *In vitro*, we did six incubations with air emboli, and *in vivo*, we did six experiments with air embolism and one sham. To limit the use of animals, we also used historical shams from other experiments conducted at our laboratory.

In vivo, based on these pilot experiments, we made a conservative estimate of approximately 25% difference in the estimates between the groups. We used an online power calculator (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html), aiming for a power of 80% and a significance level (Type I error) of 5% in all studies, assuming normal distribution with a common standard deviation (SD), and found that 13 observations were needed to detect a difference between the groups reliably. We chose to include animals in a 3:1 ratio; thus, we estimated that 24 pigs receiving air infusion and seven shams would be needed.

In vitro, we did not conduct formal statistical planning but based on the pilot studies, we estimated that approximately six to twelve donors would be needed to detect a difference, with slight variations between readouts.

6.3.2 Choice of statistics

The choice of statistical test depends on the study design from which the data was acquired and the measurement level and distribution of the data. Some statistical tests have more power than others (Figure 18) and are thus preferred when the nature of the data permits. “Paired data” arises when each subject is compared to itself, for example, when comparing in vitro incubations of aliquots of blood from a donor. “Unpaired data” arises where different subjects

are compared, for example, when comparing pigs receiving air infusion to sham animals. Paired data can be analyzed with statistics tests with more power than unpaired data. Likewise, parametric tests have more power than non-parametric tests and are thus preferred. A prerequisite for using parametric tests is that data follow a Gaussian distribution. If this is not the case, data may be transformed, or a non-parametric test used.

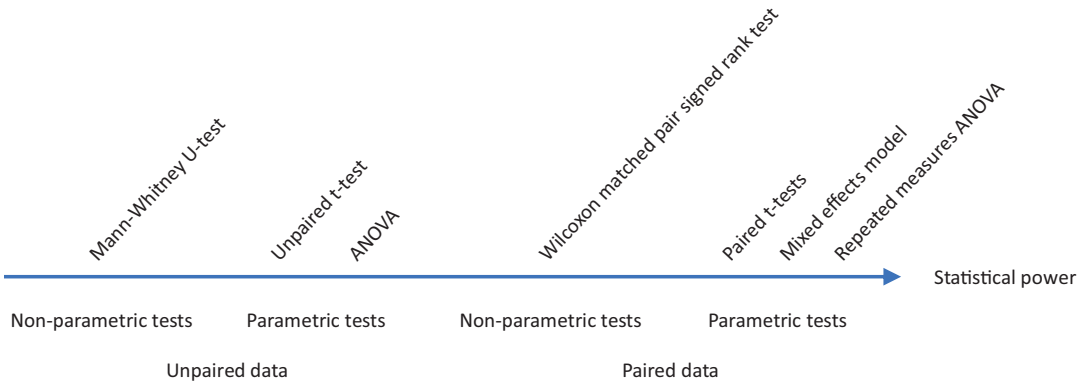

Figure 18 Choice of statistics. The statistical power varies between tests. The choice of statistical test depends on study design and data distribution. Parametric tests of data from studies with paired designs have the highest statistical power and are preferred. The studies in this Ph.D. were both paired and unpaired. Most data were normally distributed or transformed to fit a normal distribution and thus analyzed using parametric tests. Only statistics used in the Ph.D. project are shown.

6.3.3 Handling missing values in datasets

Paired statistical analyses require complete datasets. Missing values are a common problem in biomedical research, despite careful planning and thorough conducting of experiments. One approach to missing variables is only to analyze subjects with complete datasets or use less powerful unpaired statistics. As the likelihood of missing variables increases with the number of subjects and readouts in an experiment, only analyzing subjects with complete datasets potentially reduces the statistical power significantly. Instead of excluding subjects, missing values can be replaced with a calculated but plausible value in an imputation process [158]. In a

simple imputation, the missing variable may be replaced with the average of this variable from the other subjects. This approach, however, artificially reduces the variance and should be avoided if possible. Instead of a simple imputation, if a sample is "missing completely at random," e.g., lost or damaged due to unforeseen events such as little sample material, wrong sample handling, malfunctioning reagents, failed analysis, or failed data recording, the missing variables can be imputed using multiple imputations [158].

The datasets in our studies varied in size from six to over twenty subjects, and different variables were missing for various reasons; some analyses were corrupted due to technical issues, such as loss of sample material or failed analysis, and some baseline variables, such as complement activation products or cytokines were below the lower detection limit. Low baseline values of inflammatory readouts are biologically plausible, as healthy human donors and pigs are not expected to have ongoing inflammation. We imputed these missing variables with a random value between the lowest and the highest values from the other subjects, thus avoiding underestimation of the SD, as would have been the case had we used the average of the other subjects. For other missing variables, we used a combination of listwise exclusions, where this would have minimal impact on the statistical power and imputations, including multiple chained equations under the missing at random assumption, or we used a mixed-model, working with missing variables [159]. In the *in vivo* experiments, where a substantial number of the animals died close to, but before the end of the experiments, we used the "last observation carried forward" imputation.

6.3.4 Multiple comparisons – correcting and losing power

We registered and subsequently compared several variables for each subject in our experiments. For example, in incubations of air emboli, we measured C3a, C3bc, C3bBbP, TCC, PTF1+2, and 27 cytokines and compared these to control incubations without air emboli using analysis of variance (ANOVA) and mixed-effects models. Thus, for each variable, we made approximately 32 comparisons. We regarded a per-comparison p-value < 0.05 as significant, meaning that for each comparison, we accepted a 5% likelihood of making a false discovery (Type I error). Thus, if we made only one comparison, we would have a 5% risk of making a false discovery, but with 32 comparisons, the cumulative risk of making a false discovery increased to 80% [160]. To reduce or avoid such an unacceptably high risk of a Type I error, p-values may be corrected for multiple comparisons. However, in most mathematical correction methods, every additional comparison reduces statistical power. Thus, keeping multiple comparisons to a minimum increases statistical power. To reduce or avoid multiple comparisons, some researchers choose not to include all readouts in analysis, or split and present results in several papers – a method known as "salami-slicing" [161]. Obviously, this method makes no biological sense and is not recommended. Another approach to multiple comparisons, advocated by some statisticians, is not to correct for the multiple comparisons at all, as this mathematical operation precludes statistical significance in complex biological studies [162,163].

Several methods of correcting for multiple comparisons exist, with perhaps the Bonferroni correction being the most conservative as opposed to the Fischer Least Significant Difference (LSD) being the less conservative. In this Ph.D. project, we used the Fischer LSD and the Benjamini-Hochberg false discovery rate (FDR). Bonferroni correction tests the error rate

across all comparisons. If more than a few comparisons are made, or some non-significant, all are rejected (thus increasing Type II error), making it less suitable and thus not recommended for more than a few comparisons [160,161]. Fischer LSD is not a true correction for multiple comparisons but basically a set of individual t-tests, one for each comparison. When the p-values are calculated, instead of using the SD for each variable, the pooled SD for the entire dataset is used. Thus the p-value for each compared value depends on the scatter of all other variables [160]. Benjamini-Hochberg's method for controlling the false discovery rate (FDR) is another method of adjusting p-values in multiple comparisons. In this method, an acceptable false discovery rate, for example 5%, is chosen. The test then adjusts the p-values, taking the distribution of all p-values into account. The FDR adjusted p-values correspond to a test-wide false discovery (Type I error) of 5% [160].

In repeated-measures ANOVA, where each subject is compared to itself, the within-subject variations must also be considered. Such variations may occur randomly and affect several variables at one or more sample times. This may affect the statistics when conducting multiple comparisons. In the ANOVA analysis, we used the Greenhouse-Geisser correction to compensate for such within-subject correlations [160]. As an alternative to ANOVA, a linear mixed-effect model may be used. These models handle missing data, thus avoiding imputation or listwise exclusions with potential bias. The mixed-effects models are also more flexible than ANOVA, allowing for both within-subject and between-subject variations [164]. Thus, with large datasets containing both within and between-subject variations and missing data, linear mixed-effect models outperform ANOVA, and the use of these models is rapidly increasing within medical sciences [164]. The drawback of the linear mixed effect models is the complexity, complicating a thorough understanding of the models, thus potentially resulting in a wrong application of the models.

7 Discussion

This Ph.D. project has elaborated extensively on thromboinflammation triggered by air emboli and air embolism. We constructed our *in vitro* and *in vivo* models based on previously published works [77,119,121,165]. However, we made several very important modifications to the existing models and extended the analysis to cover all parts of thromboinflammation, thus adding novel insight into the pathophysiological understanding of air embolism. We showed *in vitro* how air triggered thromboinflammation and verified these findings *in vivo*. We tested the effect of complement inhibition *in vitro*, laying the theoretical foundation for the potential future treatment of air embolism.

We conducted our *in vitro* experiments in human lepirudin anticoagulated whole blood, which, except for the endothelial cells and glycocalyx, contains all components necessary to trigger thromboinflammation [122]. This is a major advantage over heparinized blood, where coagulation is either inhibited or plasma and serum, which are both depleted of platelets and leukocytes. However, the model does come with a caveat; thrombin activates platelets [111]. Thus, inhibition of thrombin formation might have attenuated platelet activation, and our findings may not precisely mirror *in vivo* conditions. As an alternative to lepirudin anticoagulation, we could have used the Gly-Pro-Arg-Pro (GPRP) synthetic peptide as an anticoagulant as described by Nilsson et al. [114]. GPRP inhibits fibrin polymerization but does not inhibit thrombin or the rest of the coagulation cascade upstream of the fibrin polymerization. The complement system is equally activated in lepirudin and GPRP anticoagulated whole blood [114].

In the *in vivo* study, we used low-rate heparin infusion to keep lines open throughout the experiments, likely thereby interfering with coagulation readouts. Theoretically, we could alternatively have used a low-rate infusion of citrate instead of heparin. Citrate preparations for

use with hemodialysis are commercially available. In the lines, citrate binds calcium, inhibiting coagulation and other calcium-dependent cascades. As citrate is an endogenous metabolite of pyruvate, it is rapidly metabolized in the Krebs cycle when it enters the circulation. Thus, if administered in low doses, citrate should not interfere with the experiments. Before performing hemostasis analysis, such as ROTEM, blood is re-calcified. Thus, any residual citrate present in the lines at sampling would not impact the ROTEM readout. However, these are theoretical speculations, and further studies are needed to verify this.

We performed six air emboli incubations in plasma to compare our findings with previous plasma and serum models [77,120,121]. In these six experiments, air emboli did not trigger the coagulation (as evidenced by the absence of an increase in PTF1+2), but interestingly, C3bc and C3bBbP were approximately two-fold higher than in whole blood incubations [166]. These differences could be explained by the absence of membrane-bound complement regulating molecules on blood cells. Thus, findings may not mirror findings in whole blood. Thus, at least regarding air emboli, complement activation effect is overestimated in plasma models compared to whole blood models. Further, plasma models are not suited for studying coagulation activation, most likely due to the absence of platelets. Aligning with the plasma studies by Ward et al. [121], we found the terminal pathway was activated (quantified as TCC release) in incubations with air emboli, albeit to a lesser degree than in the studies by Ward (quantified as C5a). Ward et al. introduced air emboli by vigorously shaking the tubes and repeatedly thumbing the tubes during incubations. In contrast, we introduced the air emboli using a syringe and needle. Thus, the terminal pathway activation might be caused by the mechanical stimulation of the whole blood during plasma production and the exact manner in which air emboli were introduced.

To mimic air embolism, where air enters the circulation during medical procedures or bubbles form during decompression, we incubated air emboli on a roller mixer instead of continuously bubbling air through blood. It is known that a protein layer forms on the surface of air emboli and that platelets are activated on the bubble surface [56]. Air emboli possibly remain there for a prolonged time [24]. Antifoam, which was used in the previous studies of air and complement [77], reduces bubble size and accelerates bubble break-down [124]. Therefore, the use of this chemical precludes the study of stable air emboli. When designing our model, we examined two antifoam chemicals and found that one of these triggered both complement and coagulation [166]. Thus, we avoided bubble breakdown throughout the experiments by avoiding antifoam, allowing us to study the effect of long-lasting air emboli on both complement and hemostasis.

When designing our model, we made an interesting discovery. Avoiding ambient air in tubes during incubations significantly reduced the complement and coagulation activation, TF and βTG release, and cytokine production fell to near baseline levels [166,167]. This discovery is very important, as activation of complement, coagulation, platelets, and leukocytes caused by contact of blood and the plastic material [118] has been considered to cause inevitable background activation in *in vitro* experiments. We suggest that our discovery is not limited to air emboli experiments and therefore speculate that avoiding ambient air in test tubes during various *in vitro* incubations reduces unwanted background activation and thus significantly increases the sensitivity of the experiments. We suggest that other researchers examine whether avoiding ambient air in their *in vitro* experiments reduces "background" cascade activation. We have proposed a principal test tube design that allows air-free experiments [167], which, when produced, will facilitate experimentation in air-free environments.

In our studies, air emboli triggered profound C3 driven thromboinflammation. Based on our findings, using several complement inhibitors and a wide array of analyses, we propose a possible pathophysiological mechanism for the air-induced complement-driven thromboinflammation, summarized in Figure 19 and detailed here. We found that air emboli led to a substantial C3 activation. Five decades ago, Tack et al. and Pangburn et al. showed that C3 contains an unstable internal thioester bond, continuously hydrolyzed to form $C3(H_2O)$ [168,169]. Ekdahl et al., Pekna et al., Gong et al., and others have shown that when gas bubbles contact plasma or blood, the C3 thioester bond is hydrolyzed to $C3(H_2O)$, thus triggering the alternative complement pathway [77,119,170]. These findings were recently confirmed by Lachman et al. [171]. We suggest that the air-induced complement activation starts with C3 contacting an air bubble, whereby the internal thioester bond is broken, and C3 hydrolyzed to $C3(H_2O)$ as described above. In plasma, $C3(H_2O)$ is immediately inactivated to $iC3b(H_2O)$, catalyzed by factors H and I, present in ubiquitous amounts in the plasma and on the surface of own cells [67,75], but not on foreign surfaces. Thus when C3 comes in contact with foreign surfaces, e.g., air emboli, $C3(H_2O)$ is not inactivated and may bind to factor B, cleave off C3a and Ba, bind factor P, and eventually form the C3-convertase $C3b(H_2O)BbP$. The C3-convertase splits further C3 molecules to C3a and C3b, thus activating the alternative pathway. Ekdahl et al. demonstrated that the C3 activation occurs to an equal extent on oxygen, nitrogen, and air bubbles, suggesting that the C3 activation depends on the gas-blood interface rather than the actual gas composition [77], supporting the theory that the absence of FH and FI on the gas bubble is, in fact, responsible for the C3 activation.

Usually, the C3 convertase would merge with one or several C3b to form the C5 convertase, $C3b_nBbP$. However, in contrast to what would be expected under normal conditions, we found that when air emboli activated the alternative pathway, only minor amounts of terminal complement complex (TCC) were formed, and neither *in vitro* nor *in vivo* did we find a

correlation between C3 activation products and TCC. In sum, these findings suggest that a less-effective C5 convertase is formed when complement is activated by air, somewhat mimicking the situation with C3 nephritic factors (C3NeF), where the active C3 convertase is stabilized by the C3NeF, and the terminal pathway is not activated [172].

Air emboli triggered a robust release of proinflammatory cytokines and chemokines *in vitro* and *in vivo*. In addition to the cytokine release, *in vivo* air embolism led to an increased number of leukocytes and an up-regulation of TF mRNA and release of MP-TF. Both C3a and C5a are known anaphylatoxins, stimulating the leukocytes and other cytokine and TF releasing cells [71]. Interestingly, the air emboli-induced cytokine release was significantly reduced under C3 inhibition, and combined C3 and CD14 inhibition further enhanced this reduction. In contrast, CD14 inhibition alone reduced only three cytokines, and C5 and C5aR1 inhibition had almost no effect. This finding shows that the leukocytes were activated primarily through the C3 activation. This contrasts with other inflammatory conditions, for example, sepsis, where the C5a-C5aR axis is the important link between complement and the cellular immune system [98]. The MP-TF release was only reduced, but not attenuated, by C3 inhibition. In contrast, TF mRNA expression was inhibited by both C3 and C5 inhibition, suggesting a predominantly C3-driven release of ready synthesized TF and a C5- and possibly C3-driven up-regulation and de novo synthesis of TF. As we lacked a C3 inhibitor working in pigs, we could not verify the effect of specific inhibition *in vivo* in our animal model.

The hemostasis was triggered by air, *in vivo* measured as changes in ROTEM readouts, and *in vitro* as PTF1+2, reflecting coagulation and βTG reflecting platelet activation. The platelets interact with the complement system and the leukocytes through many different mechanisms. Importantly, the platelets are activated by thrombin binding to the protease-activated receptor (PAR) and possibly also directly by various complement components, including C3a, C4a, C5a, and C5b [62,64,89,112]. As mentioned above, air emboli trigger the release of MP-TF, which

combines with FVIIa to form the TF-FVIIa complex, subsequently catalyzing FXa formation. FXa cleaves prothrombin to thrombin, which may activate the platelets. The FXa-FVa complex assembles in the membrane of activated platelets. The FXa-FVa complex cleaves prothrombin to thrombin in a process termed the thrombin burst [173]. This thrombin burst, and thus the platelets, are necessary for effective hemostasis. In line with this, complement activation was only accompanied by coagulation activation in our whole blood experiments and not in our plasma experiments. Despite the use of lepirudin in our model, and thus blocking the thrombin-mediated platelet activation, air emboli triggered a significant βTG release, not reduced by either C3 or C5 inhibition. However, C3 inhibition significantly reduced but did not abolish MP-TF. Both C3 and C5 inhibition significantly reduced but did not abolish PTF1+2. These findings show that the platelets were activated through a complement-independent mechanism, possibly by direct interaction with the air emboli as suggested by Thorsen et al. [56], and that the coagulation was activated partly through a complement-dependent and partly through a complement-independent mechanism. Had we not used lepirudin but rather GPRP, as mentioned above, we would have been able to elaborate in further detail on the role of thrombin on platelet activation. However, due to the design of our model, we cannot pinpoint the exact mechanism for the platelet activation.

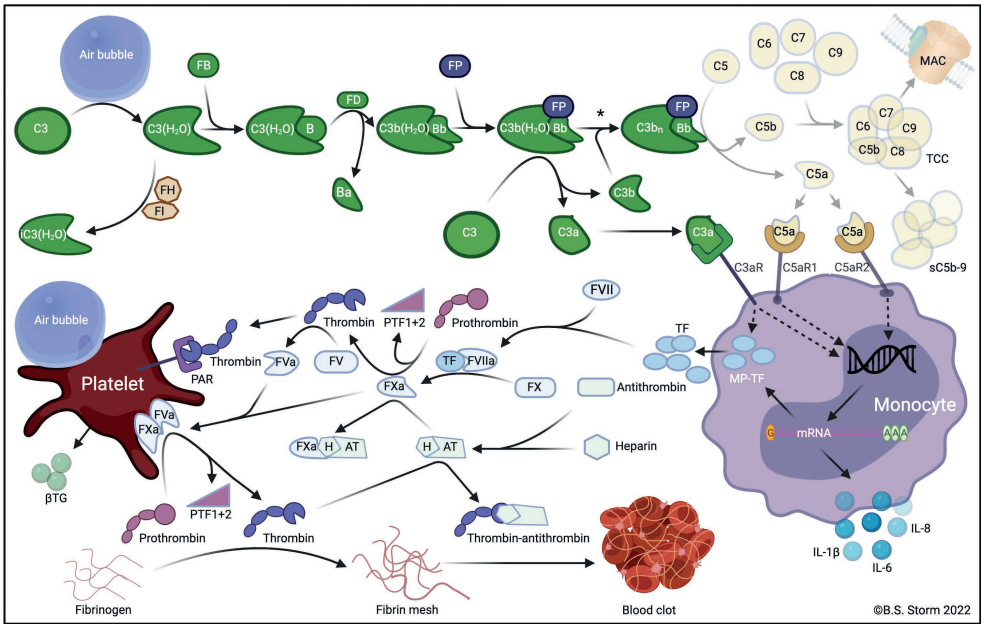

Figure 19 Air activates the alternative pathway and triggers a C3-dependent thromboinflammation. In plasma, C3 undergoes spontaneous slow rate hydrolysis of the internal thioester, forming C3(H_2O). The C3-hydrolysis is accelerated by contact with foreign surfaces, e.g., an air embolus. C3(H_2O) is inactivated to iC3(H_2O) by factor I (FI) in the presence of a cofactor, e.g., factor H (FH). C3(H_2O) may bind FB (FB) to form C3(H_2O)B. Catalyzed by factor D (FD), the small Ba-fragment is cleaved from C3(H_2O)B forming the fluid-phase C3 convertase, C3(H_2O)Bb. The C3 convertase is stabilized by properdin (FP) binding, forming C3b(H_2O)BbP. The stabilized C3 convertase cleaves additional C3 molecules to C3b and C3a or forms the C5 convertase, C3b_nBbP, by binding to one or more C3b fragments deposited on foreign surfaces. The C5 convertase cleaves C5 into C5a and C5b. C5b combines with C6, C7, C8, and C9 to form the terminal complement complex (TCC), C5b-9. TCC may anchor into cell or bacterial membranes and form a pore termed the membrane attack complex (MAC), causing cell lysis. Alternatively, TCC may form a soluble form (sC5b-9) in plasma. The C3a and C5a-anaphylatoxins bind C3a- and C5a-receptors (C3aR and C5aR1 or 2, respectively) on various cells, including monocytes and granulocytes. The activation of anaphylatoxin-receptors C3aR and C5aR1 on monocytes stimulates de novo synthesis and surface expression of tissue factor (TF), extracellular release of microparticles expressing TF (MP-TF), and expression of various inflammatory cytokines, including IL-1β, IL-6, and IL-8. TF binds to coagulation factor VIIa (FVIIa), subsequently activating FX to FXa. FXa catalyzes the cleavage of prothrombin to PTF1+2 and thrombin. Thrombin catalyzes the cleavage of FV to FVa. FVa and FXa combine to form the prothrombinase complex on the surface of activated platelets. The prothrombinase complex cleaves prothrombin into prothrombin fragment 1+2 (PTF1+2) and thrombin. Platelets may be activated by several mechanisms, including thrombin binding to protease-activated receptors 1 and 4 (PAR) and possibly by direct contact with air emboli. Activated platelets release β-thromboglobulin (βTG) and many other mediators. Thrombin cleaves fibrinogen to fibrin, which crosslinks and forms a fibrin mesh leading to the formation of a blood clot. The affinity of antithrombin to thrombin is enhanced by binding heparin to form the heparin-antithrombin complex (HAT), which binds to and inactivates FXa. *The asterisk (*) indicated between the two C3-convertases (upper right) indicates that air C3 activated by emboli is less likely to form an active C5 convertase than C3 activated by solid substances where C3b is bound to the surface, and the C5a-C5aR axis thus plays only a minor role in the air-induced C3-driven thromboinflammation. Note: Only key components relevant to our model are included in the figure.* Reprint from Mollnes TE at al. Application of the C3 inhibitor compstatin in a human whole blood model designed for complement research – 20 years of experience and future perspectives. *Seminars in Immunology*. 2022 under CC-BY 4.0 license.

In our *in vivo* study, pigs with air embolism developed pulmonary hypertension, severe hemodynamic instability, and thromboinflammation with C3a and cytokine deposition in the lungs, underpinning the important role of the lungs in the pathophysiology. This aligns with the clinical picture seen in patients with air embolism, where bronchospasm, pulmonary edema, and hampered gas exchange are important pathophysiological findings [24] We propose a mechanism similar to the C3 driven thromboinflammation observed *in vitro* played an important role in the lung pathophysiology *in vivo*. We sought to elaborate on this by inhibiting C3. However, porcine complement inhibition is limited by the availability of effective complement inhibitors [101], and no C3 inhibitor is commercially available on a large scale. Thus, our study should be regarded as a hypothesis-generating study, and our *in vitro* findings should be tested *in vivo* in a large animal model if and when porcine complement inhibitors become readily available.

Our studies have provided important new insight into air embolism, underscoring the pivotal role of C3-driven thromboinflammation. If our findings translate into human medicine, they may have implications for future therapeutic interventions in patients with air embolism. We suggest that C3 inhibition might attenuate the inflammatory response, not only in patients with air embolism but possibly also in patients with diving-related decompression sickness. Short-term C3 blocking in patients without bacterial infections, for example, using pegcetacoplan [100,174] should be both feasible and safe. C5 blocking, used in many complement-mediated conditions, would have minimal effect in patients with air embolism. Although complement inhibition alone significantly attenuates the coagulation cascade, it will not inhibit platelet activation. Thus, to achieve optimal inhibition of thromboinflammation, complement inhibition should be combined with antiplatelet and possibly anticoagulation therapy. However, as neither complement inhibition nor combined complement inhibition, antiplatelet therapy, and

anticoagulation has not been tested in large animals models of air embolism or patients, these suggestions are speculative only.

8 Conclusion

Avoiding ambient air in test tubes during *in vitro* whole blood experiments reduces "background" complement activation, coagulation, and cytokine release. Thus, *in vitro* immunological experiments should be conducted in air-free environments. Antifoam should be avoided when studying air emboli, and plasma is unsuitable for studying thromboinflammation in detail.

Ambient air and emboli activated C3 and triggered a C3-driven thromboinflammation where the terminal complement pathway played only a minor role *in vitro* in human whole blood. The thromboinflammation was substantially reduced by C3 but not C5 inhibition. The platelets were activated through a complement-independent mechanism.

Air embolism triggered thromboinflammation with increased C3a and cytokines in the lungs *in vivo* in pigs.

Future studies should examine if C3 inhibition, possibly combined with antiplatelet and anticoagulation therapy, is a useful treatment for patients with air embolism-induced thromboinflammation.

9 References

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Journal of Immunological Methods 487 (2020) 112876

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Avoiding ambient air in test tubes during incubations of human whole-blood minimizes complement background activation

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ARTICLE INFO

Keywords:
Whole-blood model
Complement
Ambient air
In vitro experiments
Preanalytical error

ABSTRACT

Background: In vitro, the complement system can be studied in test tubes incubated with anticoagulated human whole-blood. Background activation of complement may mask inflammatory signals. Air bubbles are known to activate complement. We examined if removing ambient air from test tubes before incubation reduced background complement activation.

Methods: Blood from twelve donors was anticoagulated with the thrombin inhibitor lepirudin and incubated with either no air, ambient air or air bubbles in polypropylene tubes at 37 °C for 180 min on a roller mixer. After incubation, EDTA was added, plasma isolated and analyzed for seven complement activation products using ELISA. Results are presented as means with 95% confidence intervals.

Results: Blood incubated without air had significantly lower complement activation compared to blood incubated with ambient air; C4d 273 (192–364) vs. 379 (263–494) ng/mL ($p = 0.002$), C4bc 8.2 (4.1–13) vs. 12 (3.2–21) CAU/mL ($p = 0.01$), C3a 1351 (873–1838) vs. 2944 (2315–3572) ng/mL ($p = 0.0005$), C3bc 31 (17–46) vs. 68 (52–84) CAU/mL ($p = 0.002$), C3bBbP 134 (97–171) vs. 427 (358–506) CAU/mL ($p < 0.0001$), C5a 3.5 (1.9–5 0.2) vs. 15 (1.8–27)) ng/mL ($p = 0.003$), TCC 4.6 (2.8–6.3) vs. 9.9 (7.3–12) CAU/mL ($p = 0.006$). At the end of the experiment blood incubated with air bubbles had a higher complement activation than blood incubated with ambient air with an average 26 fold increase (range 1.6–59) from baseline of all activation products; C4d 551 (337–766) ng/mL, C4bc 21 (5.0–36) CAU/mL, C3a 3983 (3518–4448) ng/mL, C4bc 103 (86–121) CAU/mL, C3bBbP 626 (543–708) CAU/mL, C5a 10 (2.8–18) ng/mL and TCC 10 (6.0–14) CAU/mL.

Conclusion: Avoiding air in test tubes during whole-blood experiments reduced background complement activation substantially and represents an important improvement to the lepirudin whole-blood model. This could also apply to other in vitro models.

1. Introduction

In 2002 we published a unique whole-blood model to study the role of complement in the inflammatory response in a holistic manner (Mollnes et al. 2002). This was made possible by anticoagulating the blood with the thrombin-specific inhibitor lepirudin, which does not interfere with the complement system when collecting blood for in vitro experiments. Undesired background activation of complement and other systems of inflammation in experimental whole-blood models is a common problem during in vitro studies (Lappegård et al. 2004), potentially masking biologically relevant signals in the experiments. In 1980, Track and colleagues discovered that the C3 molecule contained an internal thioester bond. This esther was continuously hydrolyzed at a low degree in the fluid phase and obtained a similar structure to C3b, but C3a was not cleaved off (Tack et al. 1980). This molecule was named iC3b, "C3b-like C3" and C3(H_2O). The C3 hydrolysis concept has recently been reviewed (Fromell et al. 2020) and the role of C3(H_2O) is still under debate. Blood-gas interfaces, such as found on air bubbles in

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https://doi.org/10.1016/j.jim.2020.112876
Received 13 June 2020; Received in revised form 30 September 2020; Accepted 1 October 2020
Available online 06 October 2020

blood, have been shown to activate the complement system in vitro by the thioesther hydrolysis of C3 to C3(H_2O). Interestingly, this hydrolyzation is independent of what type of gas the blood interfaces with, e.g. oxygen and nitrogen have similar effects on the complement system (Ekdahl et al. 1992).

Despite knowledge of this C3 hydrolyzation, it is common laboratory practice to accept the presence of ambient air in test tubes during in vitro experiments. To our knowledge, the effect of ambient air inside test tubes on the complement system during incubations has not previously been examined.

Thus, we studied if eliminating ambient air from test tubes during whole-blood experiments would reduce unwanted background activation of complement. Additionally, we examined if increasing the blood-air interface area by incubation with air bubbles would increase complement activation.

2. Material and methods

Sixty 5 mL polypropylene Nunc 5 mL test tubes (Nalgene, Roskilde, DK) were prefilled with 80 μL lepirudin 2.5 mg/mL (Thermo Fisher Scientific, Roskilde, DK) and preserved at −20 °C. A vacuum of −19 mL air was applied to rethawed tubes using a syringe and a 23 G needle (Becton Dickinson, Franklin Lakes, NJ). Blood from twelve healthy human donors (five woman and seven men) aged between 40- and 50-years was drawn using a BD Vacutainer Eclipse blood collection needle (Becton Dickinson) into three lepirudin filled Nunc test tubes per donor, giving a final blood lepirudin concentration of 50 μg/mL. Blood from each donor was subsequently transferred to a 50 mL polypropylene conical Falcon tube (Corning, Tamaulipas, Mexico) preheated to 37 °C. The blood samples "Baseline", "Ambient air" and "Air bubbles" were prepared in the following way: On a block heater set to 37 °C, 858 μL blood and 142 μL PBS with Ca^{2+} and Mg^{2+} was transferred to three polypropylene 1.8 mL Nunc tubes. To the baseline sample 14.6 μL EDTA was added immediately to block further complement activation, and the tube kept on ice. The "No air" sample was prepared as follows: In a 5 mL Nunc tube 3 mL blood and 497 μL PBS with Ca^{2+} and Mg^{2+} was mixed and from here transferred to a 1.8 mL Nunc tube filling the tube and the tube lid completely. The tube was then carefully capped, ensuring no air was present inside (Fig. 1A). In the "Baseline" and "Ambient air" samples, approximately 3 mL ambient air was left between blood and tube cap (Fig. 1B). To the "Air bubbles" sample, ambient air was bubbled through the blood using a syringe and needle, resulting in a mixture of air bubbles and blood filling the tube completely (Fig. 1C).

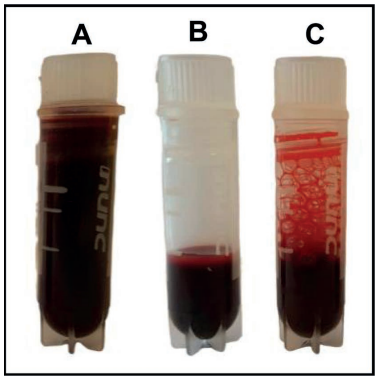

Fig. 1. Blood was incubated in 1.8 mL polypropylene tubes. A. Completely filled with blood, leaving no air inside the tube (the "No air" samples). B. Partly filled with blood leaving ambient air between blood sample and tube cap (the "Baseline" and "Ambient air" samples). C. Partly filled with blood followed by air bubbling, creating a blood-bubble mixture (the "Air bubbles" samples).

All tubes were capped and incubated on a Rock'n'Roller tube roller mixer (Labinco, Breda, NL) at 37 °C for 180 min. After incubation, 1 mL blood was transferred from the "No Air" tube to a new 1.8 mL Nunc tube. To this tube and the two other incubated tubes, 14.6 μL EDTA was added for a final concentration of 10 mM to block further complement activation. The samples were centrifuged at 3000*g* for 20 min at 4 °C, plasma transferred to polypropylene matrix tubes and frozen at −80 °C and later thawed for analysis of C3a, C3bc, C5a and TCC. Additionally, C4d, C4bc and C3bBbP was also analyzed in plasma from six of the donors.

Commercial ELISA kits were used for C4d (C4d fragment, Quidel Corp., Athens, OH), C3a (MicroVue C3a Plus EIA, Quidel) and C5a (C5a Human ELISA kit, HyCult Biotech, Uden, The Netherlands). In-house developed ELISA assays was performed as previously described (Bergseth et al. 2013) and were used for analysis of C4bc, C3bBbP, C3bc and TCC. ELISA plates were read using a Tecan Infinite M200 plate scanner (Tecan Group, Männedorf, Switzerland) and the Magellan 7.1 SP1 software (Tecan Group).

2.1. Statistics

Results were extracted and fold change between groups were calculated using Microsoft Excel for Mac ver. 16.16.9 (Microsoft Inc., Redmond, CA). Statistical analysis and charting of results were done using Prism for Mac ver. 8.4.2 (Graphpad Software, La Jolla, CA).

Missing baseline readouts due to lack of sample material were substituted with a random number between the lowest and the highest baseline result in the series. All results were stated as means with 95% confidence interval. As results did not follow a Gaussian distribution, data were log-transformed, and groups compared using the repeated measures ANOVA with Geisser-Greenhouse correction. Significances were calculated using the Fischer's LSD test. $p < 0.05$ was considered significant.

3. Results

After 180 min of incubation, all complement activation products were increased in all samples, except for C4d in blood incubated without air (Table 1 and Fig. 2). The average level of complement activation products were significantly lower in blood incubated without ambient air in the tubes compared to blood incubated with ambient air in tubes: C4d 273 (192–364) vs. 379 (263–494) ng/mL ($p = 0.002$), C4bc 8.2 (4.1–13) vs. 12 (3.2–21) CAU/mL ($p = 0.01$), C3a 1351 (873–1838) vs. 2944 (2315–3572) ng/mL ($p = 0.0005$), C3bc 31 (17–46) vs. 68 (52–84) CAU/mL (p = 0.002), C3bBbP 134 (97–171) vs. 427 (358–506) CAU/mL ($p < 0.0001$), C5a 3.5 (1.9–5.2) vs. 15

Table 1
The increase in seven complement activation products in lepirudin anticoagulated blood incubated with either no air, ambient air or air bubbles for 180 min.

Complement activation product[a]	Fold increase from baseline		
	No air	Ambient air	Air bubbles
C4d	0.8	1.1	1.6
C4bc	2.0	3.0	5.0
C3a	19	42	57
C3bc	7.4	16	24
C3bBbP	13	40	59
C5a	1.7	7.2	5.0
TCC	12	26	27

[a] C4d, C4bc and C3bBbP $n = 6$. C3a, C5a and TCC $n = 12$.

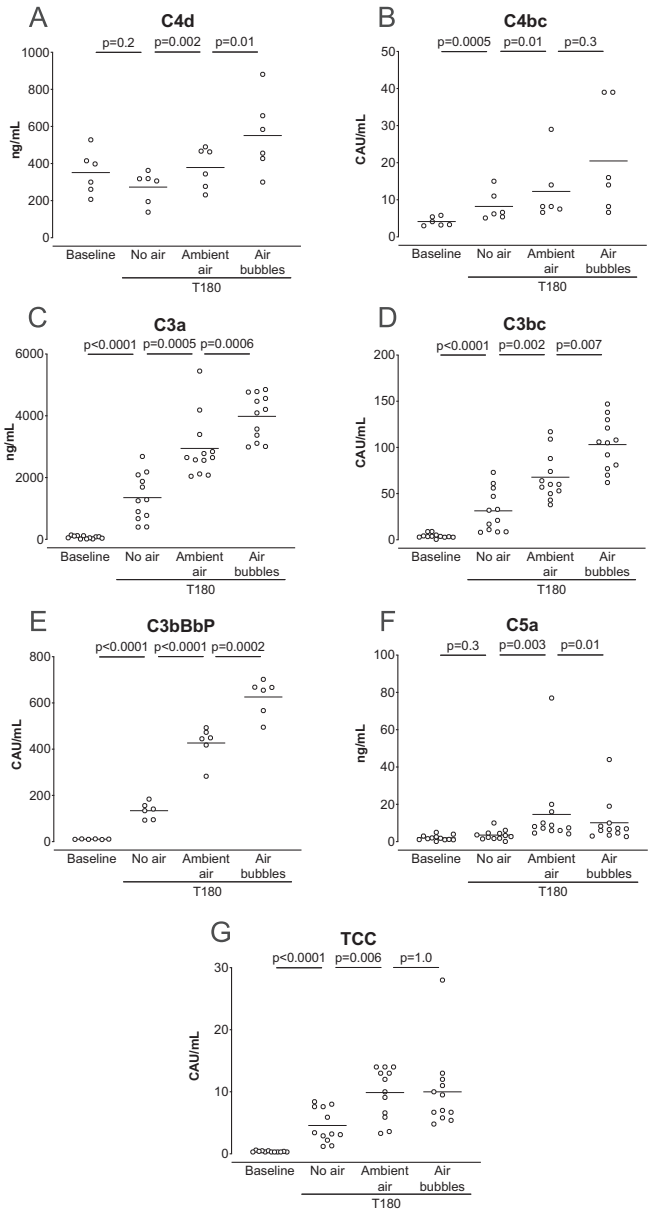

Fig. 2. Lepirudin anticoagulated human whole-blood from 12 donors incubated at 37 °C on a roller mixer in tubes with either no air, ambient air, or a mixture of air bubbles and blood. After 180 min the blood was analyzed for C4d (A), C4bc (B), C3a (C), C3bc (D), C3bBbP (E), C5a (F) and TCC (G). C4d, C3bc and C3bBbP were analyzed in six donors only. Individual readouts are shown as dots with horizontal line at mean. *p*-values were calculated on log-transformed data using repeated measures ANOVA with Geisser-Greenhouse correction and Fischer's LSD test.

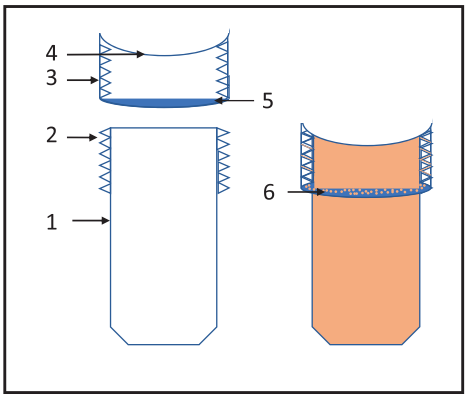

Fig. 3. Suggested design for a new test tube in non-reactive polypropylene plastic material (1) with threading on outside (2 and 3) and convex inside of the cap (4), ensuring air-tight closure of tube. Felt cap insert (5) for absorbing excess blood (red) displaced when capping tube (6). The tube can be produced in various sizes and diameters as needed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(1.8–27)) ng/mL ($p = 0.003$), TCC 4.6 (2.8–6.3) vs. 9.9 (7.3–12) CAU/mL ($p = 0.006$) (Fig. 2). Readouts from the individual donors are shown in supplement 1.

Seven of the 66 baseline readouts and one readout from the "No air" incubations were below lower detection limit and substituted with a random number between zero and the lowest detection limit. Due to limited amount of plasma, eight baseline readouts were substituted with a random number between the lowest and the highest baseline value in the series.

Blood incubated with air bubbles showed an average 26 fold increase from baseline (range 1.6–59) of all complement activation products. For C4d, C4bc, C3a, C3bc and C3bBbP the increase was higher than for blood incubated with ambient air only. For C5a and TCC there was no difference between incubation with bubbles or ambient air only (Table 1 and Fig. 2).

4. Discussion and conclusion

We have shown that removing ambient air from test tubes minimized background complement activation in incubated human whole-blood. We regard this as a substantial improvement of the original whole-blood model (Mollnes et al. 2002), as the very low background activation increases the sensitivity of the model, thus enabling us to decipher the complex inflammatory interplay in even greater detail. Notably, the effect of removing ambient air from the tubes was most pronounced for the activation of C3 and less pronounced for the activation of the C5-9 pathway. This is consistent with the previous findings that the alternative pathway and C3 is the main activating pathway at the surfaces between air and plasma (Ekdahl et al. 1992).

In line with this, we found that blood incubated with air bubbles had a higher complement C3 activation than blood incubated with ambient air only. However, the increases in C3 activation products observed in samples incubated with ambient air amounted to approximately 70% of the levels observed in samples incubated with air bubbles (Fig. 2). This difference in activation could be explained by a larger activating blood-air interface on the bubbles. However, we cannot exclude that other contributing factors, such as mechanical interaction of bubbles with the blood during roller mixer incubation could have contributed to the activation.

Our findings are highly relevant for most in vitro experiments using the whole-blood model, since unwanted background activation of complement by ambient air, which is normally present in experimental setups, may mask subtle but relevant biological findings. Hence, we suggest that in vitro blood experiments should preferably be conducted in completely air-free test tubes and the blood's exposure to ambient air should be minimized.

In order to work in the whole-blood model described, we collected blood in custom prepared lepirudin filled vacuum tubes. As a possible alternative, hirudin filled tubes (S-Monovette 1.6 mL Hirudin, Sarstedt, Nümbrecht, Germany) are commercially available.

It is possible that removal of ambient air from in vitro serum, plasma and whole-blood models also could reduce background activation when studying other biological systems, such as coagulation, cytokines and cell activation. Thus, we recommend that the effect of avoiding ambient air in other in vitro models on biological systems is elucidated further.

For clinical diagnostic purposes we suggest neither the use of our experimental whole-blood model nor the avoidance of ambient air in tubes for these purposes.

A major practical obstacle towards air-free sample handling is the lack of suitable test tubes. To our knowledge, no ordinary test tubes on the market are designed for this purpose. At present, avoiding air during sample handling is difficult, unhygienic due to difficulties filling tubes and tube caps completely, and potentially hazardous due to unavoidable blood spills during handling. Thus, new test tubes, as suggested in Fig. 3, needs to be designed to address this problem.

In conclusion, avoiding ambient air in test tubes during human whole-blood in vitro experiments reduce background complement activation substantially.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2020.112876.

Funding

This study was funded by a research grant from Northern Norway Regional Health Authority (Helse Nord RHF), The Norwegian Council on Cardiovascular Disease and The Odd Fellow Foundation.

Declaration of Competing Interest

None of the authors declare any conflicts of interest.

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Paper II Air bubbles activate complement and trigger C3-dependent hemostasis and cytokine release ex vivo in human whole blood.

Air Bubbles Activate Complement and Trigger Hemostasis and C3-Dependent Cytokine Release Ex Vivo in Human Whole Blood

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Venous air embolism, which may complicate medical and surgical procedures, activates complement and triggers thromboinflammation. In lepirudin-anticoagulated human whole blood, we examined the effect of air bubbles on complement and its role in thromboinflammation. Whole blood from 16 donors was incubated with air bubbles without or with inhibitors of C3, C5, C5aR1, or CD14. Complement activation, hemostasis, and cytokine release were measured using ELISA and quantitative PCR. Compared with no air, incubating blood with air bubbles increased, on average, C3a 6.5-fold, C3bc 6-fold, C3bBbP 3.7-fold, C5a 4.6-fold, terminal complement complex sC5b9 3.6-fold, prothrombin fragments 1+2 (PTF1+2) 25-fold, tissue factor mRNA (TF-mRNA) 26-fold, microparticle tissue factor 6.1-fold, β-thromboglobulin 26-fold (all $p < 0.05$), and 25 cytokines 11-fold (range, 1.5–78-fold; all $p < 0.0001$). C3 inhibition attenuated complement and reduced PTF1+2 2-fold, TF-mRNA 5.4-fold, microparticle tissue factor 2-fold, and the 25 cytokines 2.7-fold (range, 1.4–4.9-fold; all $p < 0.05$). C5 inhibition reduced PTF1+2 2-fold and TF-mRNA 12-fold (all $p < 0.05$). C5 or CD14 inhibition alone reduced three cytokines, including IL-1β ($p = 0.02$ and $p = 0.03$). Combined C3 and CD14 inhibition reduced all cytokines 3.9-fold (range, 1.3–9.5-fold; $p < 0.003$) and was most pronounced for IL-1β (3.2- versus 6.4-fold), IL-6 (2.5- versus 9.3-fold), IL-8 (4.9- versus 8.6-fold), and IFN-γ (5- versus 9.5-fold). Antifoam activated complement and was avoided. PTF1+2 was generated in whole blood but not in plasma. In summary, air bubbles activated complement and triggered a C3-driven thromboinflammation. C3 inhibition reduced all mediators, whereas C5 inhibition reduced only TF-mRNA. Combined C5 and CD14 inhibition reduced IL-1β release. These data have implications for future mechanistic studies and possible pharmacological interventions in patients with air embolism. ***The Journal of Immunology*****, 2021, 207: 2828–2840.**

Venous air embolism may complicate many surgical and medical procedures, including vascular access, open or laparoscopic procedures, biopsies, interventional radiological procedures, and extracorporeal blood circulations such as dialysis, plasmapheresis, or heart and lung machine circulation (1, 2). Air emboli can cause acute hemodynamic collapse and trigger life-threatening inflammatory lung edema (3, 4), with no established specific anti-inflammatory treatment.

The central C3 molecule in the complement system contains an internal thioester bond making C3b able to bind covalently to solid surfaces after cleavage of C3 (5, 6). Low-grade spontaneous hydrolysis of this thioester bond occurs in the fluid phase, changing C3 to C3(H_2O), also termed "iC3" or "C3b-like," where C3a is not cleaved off (7–9). This molecule binds factor B, which is cleaved by factor D, and the initial alternative pathway C3 convertase, C3(H_2O)Bb, is generated and cleaves C3 into C3a and C3b. In vitro, in antifoam-treated human serum and heparin-anticoagulated whole blood, it has been shown that bubbles of ambient air, nitrogen, and oxygen equally triggered the change in C3 configuration to iC3 (10). Furthermore, bubbles of nitrogen, helium, argon, and ambient air equally activated platelets (11). Together, these studies indicate that the gas–plasma interface per se rather than a specific gas composition initiates the complement activation. Whether the C3 activation could trigger further downstream activation, including activation of C5, was not investigated in these studies.

In vivo animal studies have shown that air emboli can trigger inflammation (12, 13), activate platelets, and cause thrombocytopenia

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Received for publication March 31, 2021. Accepted for publication September 20, 2021.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.2100308

This work was suppported by research grants from the Northern Norway Regional Health Authority and the Independent Order of Odd Fellows.

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The online version of this article contains supplemental material.

Abbreviations used in this article: bFGF, basic fibroblast growth factor; BTG, β-thromboglobulin; CAU, complement arbitrary unit; 95% CI, 95% confidence interval; C3NeF, C3 nephritic factor; COVID-19, coronavirus disease 2019; EIA, enzyme immunoassay; MP-TF, microparticle tissue factor; pCO_2, partial pressure of carbon dioxide; PDGF-BB, platelet-derived growth factor-BB; pO_2, partial pressure of oxygen; PTF1+2, prothrombin fragments 1+2; RT, room temperature; TCC, terminal complement complex sC5b9; TF-mRNA, tissue factor mRNA;

and coagulopathy (14). An intimate interaction exists between the complement, hemostatic, and inflammation systems, and activation of one system may trigger the others (15–18). The role of the alternative complement pathway in these interactions has been extensively reviewed (19). C3 and C5 may be pivotal for this interaction, because C3a and C5a can bind to their receptors, C3aR, C5aR1, and C5aR2, and activate many cells, including platelets, leukocytes, and endothelial cells (17, 20, 21). In the present ex vivo study, we used lepirudin-anticoagulated human whole blood without ambient air in the test tubes (22, 23) to study the effect of air embolism, mimicked by air bubbles, on the complement, hemostatic, and cytokine systems. In this paper, we use the term "coagulation" when discussing the coagulation system and the term "hemostasis" when discussing the joint effect of platelet activation, tissue factor, and coagulation.

Lepirudin (recombinant hirudin) is a highly specific thrombin inhibitor that blocks coagulation at the last step before clotting, leaving other cascade systems and inflammatory networks uninhibited and open for mutual interaction (23). In contrast, most other anticoagulants, such as EDTA, citrate, and heparin, interfere with several plasma cascades and cell functions, and they are best avoided when studying the inflammatory responses. Thus, lepirudin anticoagulated whole blood represents the most physiologically relevant ex vivo human model available, to our knowledge.

Antifoam is often used to reduce foam formation in experiments with air bubbles. However, little attention has been paid, first, to whether antifoam may have adverse effects on the inflammatory readouts and, second, whether antifoam is needed for performing such experiments. Thus, we designed experiments to answer these questions.

The primary aim of this study was to investigate the extent to which complement activation contributes to the thromboinflammatory reaction induced by air bubbles. Our three secondary aims were to examine the effect of air bubbles on physiologic parameters, including partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), and pH; the effect of antifoam reagents on the complement and coagulation systems; and the effects of air bubbles on complement and coagulation in whole blood compared with plasma.

Materials and Methods

A series of experiments were conducted (summarized in Figure 1) using the whole blood model (23) with modifications as previously described (22). The study was approved by the Regional Committee for Medicine and Health Research Ethics.

Whole-blood experiments

All disposables and solutions were endotoxin-free. Blood from 16 healthy human donors (7 males and 9 females, aged 30–60 y) was collected using the BD Vacutainer Eclipse blood collection system (Becton Dickinson) into Nunc polypropylene tubes (Nunc, Roskilde, Denmark) prefilled with lepirudin (Refludan; Hoechst, Frankfurt am Main, Germany) to a final concentration of 50 μg/ml blood.

After drawing, each donor's blood was pooled in 50 ml polypropylene conical Falcon tubes (Corning, Tamaulipas, Mexico) preheated to 37°C. On a block heater set to 37°C, we added 858 μl blood from each donor to sets of 1.8-ml Nunc polypropylene tubes prefilled with specific inhibitors of the

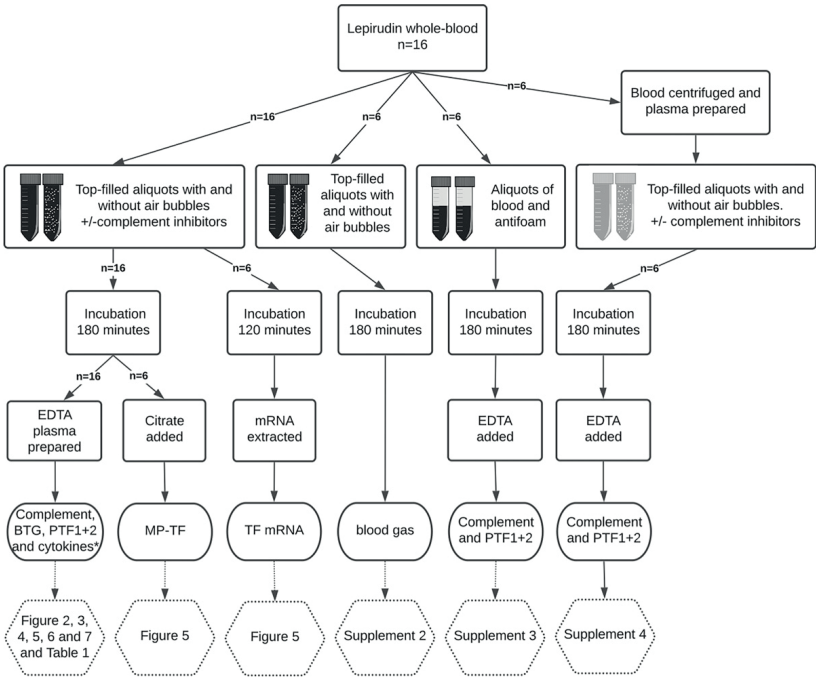

FIGURE 1. Study design. Lepirudin blood and plasma from 16 human donors were incubated in top-filled tubes with and without air bubbles and selected complement and inflammation inhibitors or with antifoam in half-filled tubes. Details of incubations, readouts, and references to figures are stated in the flowchart. *Number of samples included in analyses varies as described in *Materials Methods*.

complement and the TLR systems diluted in sterile PBS with $CaCl_2$ and $MgCl_2$ (PBS) (Sigma-Aldrich, St. Louis, MO) to a final volume of 142 μl as follows: the C3 inhibitor peptide Cp40 (produced at the University of Pennsylvania as previously described [24]) to a final concentration of 20 μM, the recombinant humanized anti-C5 mAb eculizumab (Soliris; Alexion Pharmaceuticals GmbH, Zürich, Switzerland) to a final concentration of 100 μg/ml, the C5aR1 blocking cyclic peptide PMX53 (produced at the University of Queensland, St. Lucia, Australia, as previously described [25]) to a final concentration of 10 μM, the anti-human CD14 recombinant IgG2/4 Ab r18D11 (produced at Nordland Hospital, Bodø, Norway, as previously described [26]) to a final concentration of 15 μg/ml, a combination of Cp40 and r18D11 to a final concentration of 20 μM and 15 μg/ml, respectively, or tubes containing only PBS. Additionally, 3003 μl of blood from each donor was mixed with 497 μl PBS in 5 ml Nunc tubes and from there transferred to 1.8-ml tubes, filling the tubes and the lids completely (as shown in Figure 1A of [22]). To ensure that adequate concentrations of eculizumab and PMX53 were used in our experiments, we titrated these inhibitors; because the C5 concentration in serum and plasma is relatively constant during ex vivo experiments, we tested the eculizumab effect in serum using the Wieslab Complement System Alternative Pathway Wielisa assay (Svar Life Science, Malmö, Sweden) (Supplemental Fig. 1A). In contrast, C5aR expression on the cell surfaces can vary considerably during an experiment. Thus, we tested the effect of PMX53 in whole blood incubated with air using β-thromboglobulin (BTG), microparticle tissue factor (MP-TF), and prothrombin fragments 1+2 (PTF1+2) as readouts (Supplemental Fig. 1B–D).

Except for the "baseline" and "no air" samples, we bubbled ∼2.5 ml of ambient air through the blood using a 21-gauge sterile needle and 20-ml syringe, resulting in a mixture of air bubbles and blood filling the tubes (as shown in Figure 1C of [22]). All tubes except baseline were incubated on a Rock'n'Roller tube roller mixer (Labinco, Breda, the Netherlands) at 37°C for 180 min. To the baseline tubes, we added 14.6 μl EDTA to a final concentration of 10 mM and kept the tube on ice. After incubation, we transferred 1 ml blood from the no-air tubes to new 1.8-ml Nunc tubes and added EDTA to all incubated samples to a final concentration of 10 mM. We centrifuged the samples at 3000 *g* for 20 min at 4°C, transferred the plasma to 1.0-ml polypropylene Matrix 2D storage tubes (Thermo Fisher Scientific, Waltham, MA), and stored the samples at −80°C for later analysis. Immediately before incubation and after incubation, we transferred 225 μl blood from six of the donors to new tubes, added 25 μl citrate (Vacuette; Greiner Bio-One GmbH, Frickenhausen, Germany), centrifuged the samples at 1,500 *g* for 15 min at room temperature (RT), isolated and centrifuged the supernatant at 13,000 *g* for 2 min, and stored plasma at −80°C until analysis of MP-TF. Additionally, we prepared and incubated blood from six donors (three males and three females) as described above. After 120 min of incubation, 650 μl of blood was transferred to 5-ml Matrix polypropylene tubes (Thermo Fisher Scientific) containing 1.8 ml of PAXgene RNA stabilization solution (BD Biosciences, San Jose, CA). The tubes were carefully tilted 10 times, kept at RT for 2 h, and stored at −80°C for later analysis. Also, blood from six donors was prepared and incubated as described above with either no air in tubes, ambient air bubbles, or ambient air bubbles and either the mouse anti-human factor D mAb (clone 166-32; kindly provided by Genentech, South San Francisco, CA) in a final concentration of 50 μg/ml or Cp40 in a final concentration of 20 μM (three donors).

Blood gas experiments

We obtained blood from six donors, and for each donor, we prepared one baseline tube, three tubes with no air, and three tubes with air bubbles as described above. We drew 1 ml blood from the baseline tube on a safePICO heparinized syringe (Radiometer, Copenhagen, Denmark) and analyzed the blood on an epoc MC55A blood analyzer (Siemens Healthcare GmbH, Eschborn, Germany), and incubated the no-air and air tubes at 37°C on a Rock'n'Roller tube roller mixer. After 60, 120, and 180 min of incubation, we removed a set of samples with and without air from the incubator, drew 1 ml of blood on a safePICO syringe, and analyzed the samples on the epoc MC55A.

Antifoam experiments

In pilot experiments, we found the required amount of the pure silicone polymer antifoam A (Merck KGaA, Darmstadt, Germany) to be at least 1 μl/5 ml blood and antifoam B (10% polydimethylsiloxane in aqueous solution; Merck) to be at least 2.5 μl/5 ml blood for a full antifoaming effect during continuous bubbling of air through the blood. To examine for possible adverse effects of the antifoam reagents on the complement and coagulation systems, we collected blood from six donors, as described above. From each donor, we transferred 1 ml blood to three 1.8-ml Nunc tubes. We added either 2 μl antifoam A, 6 μl antifoam B, or nothing to these aliquots and incubated the samples for 180 min at 37°C on a Rock'n'Roller tube roller mixer. After incubation, we added EDTA, isolated plasma, and stored the samples for subsequent analysis as described above.

Plasma experiments

We collected blood from six donors in Nunc polypropylene tubes prefilled with lepirudin to a final concentration of 50 μg/ml blood as described above. We centrifuged the tubes at 3000 *g* for 20 min at RT and transferred the supernatant to 2 ml Biopur polypropylene Eppendorf tubes (Eppendorf, Hamburg, Germany). We centrifuged these tubes at 10,000 *g* for 30 min at RT and transferred the cell-free plasma to 50 ml polypropylene conical Falcon tubes placed on a block heater at 37°C. We transferred aliquots of 358 μl plasma to 1.8-ml Nunc tubes prefilled with 142 μl sterile PBS with $MgCl_2$ and $CaCl_2$ and aliquots of 2506 μl plasma to 5-ml Nunc tubes prefilled with 994 μl PBS. From there, we transferred the diluted plasma to 1.8-ml Nunc tubes, filling the tubes and the tube lids completely. From three donors, we transferred 358-μl aliquots to 1.8-ml Nunc tubes containing 40 μM Cp40 or 200 μg/ml eculizumab in PBS to a final volume of 142 μl, yielding equipotent concentrations of inhibitors in blood and plasma experiments (assuming a plasma volume of 50%). After collecting baseline samples, we added air bubbles to all tubes, except no-air tubes, as described under *Whole blood experiments* above, and incubated the tubes for 180 min at 37°C on a Rock'n'Roller, added EDTA to a final concentration of 20 mM, transferred the plasma to 1-ml Matrix storage tubes, and stored these at −80°C for later analysis.

Complement and hemostasis analyses

In EDTA plasma from whole blood and plasma incubations, we analyzed C3bc, C3bBbP, and the terminal complement complex sC5b9 (TCC) by ELISAs developed at our laboratory, described in detail previously (23, 27, 28), using pooled human sera from healthy donors activated with zymosan (10 mg/ml) and heat-aggregated human IgG (1 mg/ml) as a reference standard set to 1000 complement arbitrary units (CAU/ml). We analyzed C4d using the MicroVue C4d fragment enzyme immunoassay (EIA) kit (Quidel Corporation, Athens, OH), C4a using the MicroVue C4a fragment EIA kit (Quidel), C3a using the MicroVue C3a Plus EIA kit (Quidel), C5a using a kit from Hycult Biotech (Uden, the Netherlands), PTF1+2 using the Enzygnost F1+2 kit (Siemens Healthcare, Marburg, Germany), and BTG using the Human CXCL7/NAP-2 DuoSet ELISA kit (R&D Systems, Abingdon, UK). We analyzed MP-TF in citrated plasma using the Zymuphen MP-TF kit (Hyphen Biomed, Neuville de Oise, France). We conducted all analyses as per the manufacturers' instructions. Due to slight changes in the protocol during the study period, C4d from 9, C3bBbP from 10 and BTG from 15 whole-blood incubations were analyzed.

Cytokine analysis

In EDTA plasma from 13 whole-blood incubations, we analyzed the following cytokines using the Bio-Plex human 27-plex kit and the Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA): IL-1β, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, MCP-1, MIP-1α, MIP-1β, eotaxin-1, IP-10, basic fibroblast growth factor (bFGF)6, G-CSF, GM-CSF, IFN-γ, platelet-derived growth factor-BB (PDGF-BB), RANTES, TNF, and vascular endothelial growth factor.

Gene expression analysis

All equipment used was RNase- and DNase-free. From thawed, PAXgene-treated whole blood, two aliquots of 1.25 ml were transferred to 2-ml Eppendorf tubes, and the tubes were centrifuged at 5000 *g* for 10 min. The supernatant was carefully poured from each tube, and the pellets were resuspended in 0.2 ml PBS without calcium or magnesium. From the resuspended cell lysate, we isolated total RNA using the MagNA Pure 96 Instrument and MagNA Pure 96 Cellular Large Volume Kit (Roche Diagnostics GmbH, Mannheim, Germany) as per the manufacturer's instructions. We analyzed the RNA concentrations using a Thermo Scientific NanoDrop spectrophotometer (Life Technologies, Carlsbad, CA) and RNA integrity number using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). The mean RNA integrity number was 8.0. We used the TaqMan RNA-to-Ct 1-Step Kit (Thermo Fisher Scientific) for gene expression studies as per the manufacturer's instructions. We used 20 ng RNA in a total reaction volume of 20 μl. Cycling conditions were set according to the kit insert, and qPCRs were run in triplicates in MicroAmp Fast 96-Well Reaction Plates on the QuantStudio 6 Real-time PCR System instrument (Thermo Fisher Scientific) using predeveloped TaqMan gene expressions assays for the candidate gene tissue factor (TF, Hs01076029_m1, F3; Thermo Fisher Scientific) and the human β_2-microglobulin (B2M, 4333766F; Thermo Fisher Scientific) as an endogenous control. The relative tissue factor mRNA (TF-mRNA) levels were quantified using the comparative ΔΔCt method. All results are given as fold changes (relative quantification) compared with blood incubated without air.

Statistical methods

Data were organized and fold change was calculated in Excel for Mac 16.37 (Microsoft Corporation, Redmond, WA). Prism 8.4.2 for Mac (GraphPad Software, La Jolla, CA) was used for statistical analysis and graphing, except for cytokine imputations and statistics. These were calculated in STATA for Windows version 16 (StataCorp LLC, College Station, TX). Before statistical analysis, we logarithmically transformed all complement activation product readouts and PTF1+2, BTG, and cytokine readouts to fit a Gaussian distribution. To avoid missing values due to results missing at random, we excluded two donors from the complement and PTF1+2 datasets by listwise deletion; one sample incubated with Cp40 was lost, and one donor had an extreme C3bc value outlier. The complete datasets were analyzed using a repeated-measures ANOVA with Greenhouse-Geisser correction to account for the within-subject correlations. The effect of anti-factor D and Cp40 on C3bc and C3bBbP was analyzed using a mixed model due to the incomplete data. Due to corrupted measurements of cytokines in three incubations with air bubbles and eculizumab, three with air and PMX53, and four with air bubbles and combined r18D11 and Cp40, we applied multiple imputations by chained equations under the missing at random assumption. We analyzed the data using a linear mixed-effects model with random intercept. The imputation of missing cytokine values was performed to correct the bias in estimates otherwise emerging from the high between-subject compared with within-subject variability in the data. Blood gas readouts from each sample time point were compared using multiple *t* tests. All *p* values were corrected for multiple testing by the Benjamini-Hochberg method for controlling the false discovery rate. We considered a p value <0.05 significant for all analyses. Data are presented as means with 95% confidence intervals (95% CI).

Results

Whole-blood experiments

Complement activation. C4d at baseline was 483 ng/ml (95% CI, 292 to 678 ng/ml) (Figure 2A). After incubation, C4d was 538 ng/ml (95% CI, 386 to 690 ng/ml), in blood incubated without air compared with 628 ng/ml (95% CI, 518 to 737 ng/ml), in blood incubated with air bubbles ($p = 0.09$). C3a at baseline was 80 ng/ml (95% CI, 43 to 117 ng/ml) (Figure 2B). After incubation, C3a was 709 ng/ml (95% CI, 593 to 825 ng/ml), in blood incubated without air compared with 4632 ng/ml (95% CI, 4031 to 5233 ng/ml), in blood incubated with air bubbles ($p < 0.0001$). C3bc at baseline was 3.8 CAU/ml (95% CI, 2.8 to 4.8 CAU/ml) (Figure 2C). After incubation, C3bc was 16 CAU/ml (95% CI, 11 to 20 CAU/ml), in blood incubated without air compared with 88 CAU/ml (95% CI, 68 to 108 CAU/ml), in blood incubated with air bubbles ($p < 0.0001$). C3bBbP at baseline was 21 CAU/ml (95% CI, 8.3 to 33 CAU/ml) (Figure 2D). After incubation, C3bBbP was 146 CAU/ml (95% CI, 97 to 195 CAU/ml), in blood incubated without air compared with 544 CAU/ml (95% CI, 303 to 785 CAU/ml), in blood incubated with air bubbles ($p < 0.0001$).

C5a at baseline was 0.9 ng/ml (95% CI, 0.1 to 1.6 ng/ml) (Figure 2E). After incubation, C5a was 2.8 ng/ml (95% CI, 1.4 to 4.2 ng/ml), in blood incubated without air compared with 13 ng/ml (95% CI, 8.0 to 18 ng/ml) in blood incubated with air bubbles ($p < 0.0001$). TCC at baseline was 1.0 CAU/ml (95% CI, 0.5 to 1.5 CAU/ml) (Figure 2F). After incubation TCC was 2.6 CAU/ml (95% CI, 1.5 to 3.8 CAU/ml), in blood incubated without air compared with 9.3 CAU/ml (95% CI, 6.9 to 12 CAU/ml), in blood incubated with air bubbles ($p < 0.0001$).

Complement inhibitors and correlations: early (C3) versus late (TCC) activation. In blood incubated with air bubbles and the C3 inhibitor Cp40, C3a, C3bc, C3bBbP, C5a, and TCC were reduced to baseline values ($p < 0.0001$) (Figure 2B–F). In incubations with air bubbles and the C5 inhibitor eculizumab, C5a and TCC were reduced to baseline values ($p < 0.0001$) (Figure 2E and F). In blood incubated with air bubbles, C4d was not inhibited by eculizumab, but C4d was increased by Cp40 ($p = 0.007$) (Figure 2A). As expected, the C5aR1 antagonist PMX53 did not reduce the complement activation products (Figure 2A–F).

In blood incubated without air, an increase in alternative and terminal pathway activation was found, with a strong correlation between C3bc and TCC ($r = 0.65$; $p = 0.01$) (Figure 3A) and a moderate correlation between C3bBbP and TCC ($r = 0.56$; $p = 0.03$) (Figure 3B). In blood incubated with air bubbles, the relative increase in alternative pathway activation was substantially higher than the terminal pathway, and no correlations were seen between C3bc and TCC ($r = 0.10$; $p = 0.7$) (Figure 3C) or C3bBbP and TCC ($r = 0.02$; $p = 0.9$) (Figure 3D).

Complement alternative pathway activation: anti-factor D versus Cp40. To precisely delineate the role of the alternative pathway compared with the classical and lectin pathways in air bubble–induced C3 activation, C3BbP and C3bc formation was evaluated in the absence versus presence of a specific anti-human factor D Ab or the C3 inhibitor Cp40. After incubation of blood with air bubbles and no inhibitor, C3bBbP levels were 811 CAU/ml (95% CI, 501 to 1122 CAU/ml), compared with 59 CAU/ml (95% CI, 41 to 76 CAU/ml), in incubations with anti-factor D ($p < 0.0001$) and 14 CAU/ml (95% CI, 3.2 to 25 CAU/ml), in incubations with Cp40 ($p = 0.0005$) (Figure 4A). In contrast, after incubation of blood with air bubbles and no inhibitor, C3bc was only increased to 197 CAU/ml (95% CI, 126 to 269 CAU/ml), underscoring the importance of the alternative pathway in air bubble–induced C3 activation (Fig 4B). Compared with incubations without inhibitor, C3bc levels were reduced to 44 CAU/ml (95% CI, 12 to 77 CAU/ml), in incubations with anti-factor D ($p = 0.002$) and nearly abolished with a reduction to 4.4 CAU/ml (95% CI, 0 to 11 CAU/ml), in incubations with Cp40 ($p = 0.007$). This observation indicates a minor but genuine contribution of the classical pathway and the lectin pathway to air bubble–induced C3 activation, which was also reflected as a slight accumulation of C4d in the presence of Cp40 (Figure 2A).

Hemostasis and effect of complement inhibition. TF-mRNA comparative ΔΔCt (relative quantification) increased 26-fold in blood incubated with air bubbles compared with blood incubated without air ($p = 0.04$) (Figure 5A). The three complement inhibitors substantially reduced TF-mRNA virtually to baseline: Cp40 by 5.4-fold ($p = 0.04$), eculizumab by 12-fold ($p = 0.04$), and PMX53 by 7.6-fold ($p = 0.04$).

MP-TF at baseline was 3.2 pg/ml (95% CI, 2.0 to 4.4 pg/ml) (Figure 5B). After incubation without air, MP-TF was 3.4 pg/ml (95% CI, 2.3 to 4.5 pg/ml), compared with 14 pg/ml (95% CI, 9.6 to 19 pg/ml), in incubations with air bubbles ($p = 0.008$). MP-TF was reduced by Cp40 to 7.2 pg/ml (95% CI, 4.0 to 10 pg/ml) ($p = 0.02$) but was not reduced by eculizumab or PMX53.

PTF1+2 at baseline was 336 pmol/l (95% CI, 54 to 618 pmol/l) (Figure 5C). After incubation without air, PTF1+2 was 2071 pmol/l (95% CI, 761 to 3381 pmol/l), compared with 51,166 pmol/l (95% CI, 31,468 to 70,863 pmol/l), in incubations with air bubbles ($p < 0.0001$). The three complement inhibitors substantially reduced PTF1+2: Cp40 to 22,222 pmol/l (95% CI, 13,974 to 30,471 pmol/l) ($p = 0.002$), eculizumab to 24,950 pmol/l (95% CI, 17,582 to 32,318 pmol/l) ($p = 0.02$), and PMX53 to 28,499 pmol/l (95% CI, 17,043 to 39,956 pmol/l) ($p = 0.02$).

BTG at baseline was 117 ng/ml (95% CI, 69 to 166 ng/ml) (Figure 5D). After incubation without air, BTG was 121 ng/ml (95% CI, 91 to 151 ng/ml), compared with 1489 ng/ml (95% CI, 1222 to 1756 ng/ml), in blood incubated with air bubbles ($p < 0.0001$). PMX53 reduced BTG to 1297 ng/ml (95% CI, 1080 to 1513 ng/ml) ($p = 0.02$), whereas Cp40 or eculizumab did not reduce BTG significantly.

Cytokine release and effect of complement inhibition. Whole blood incubated with air bubbles induced, on average, an 11-fold increase (range, 1.5–78) in 25 of 27 cytokines ($p < 0.0001$)

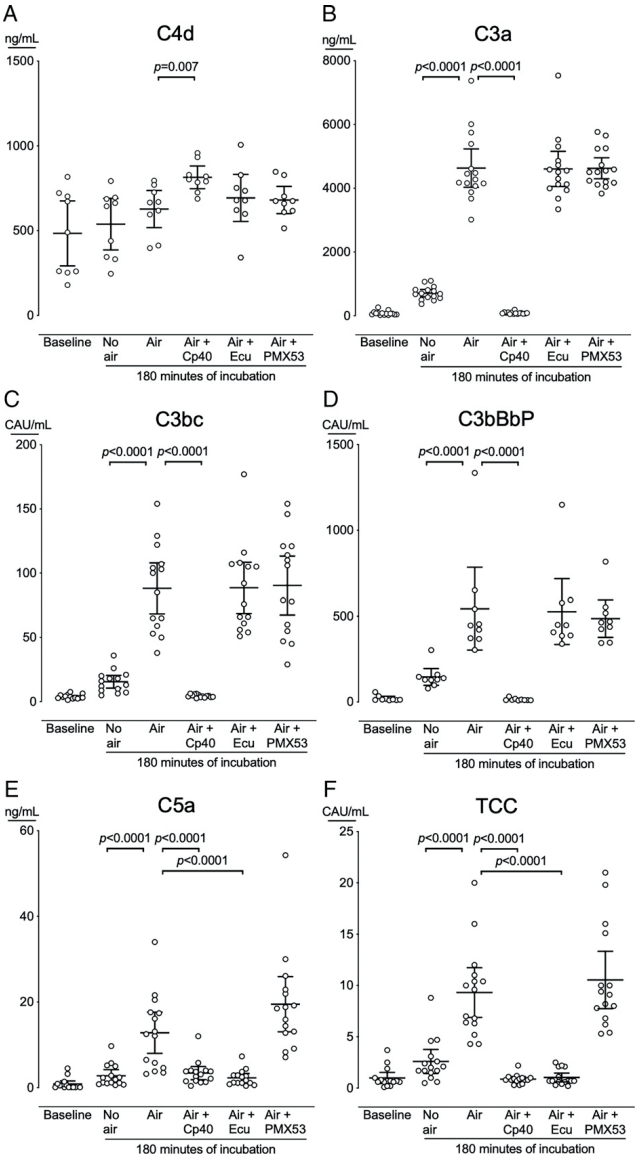

FIGURE 2. Complement activation in blood incubated without or with air bubbles. Human whole blood from 16 donors was incubated for 180 min in either completely blood-filled tubes (no air); with air bubbles in the tubes (air); or air bubbles and the C3 inhibitor Cp40, the C5 inhibitor eculizumab (Ecu), or the C5a receptor 1 inhibitor PMX53. The blood was analyzed for C4d (**A**), C3a (**B**), C3bc (**C**), C3bBbP (**D**), C5a (**E**), and TCC (**F**). Horizontal lines represent means. Error bars are 95% confidence intervals. Only significant p values ($p < 0.05$) are shown.

(Table I), including the classical pro- and anti-inflammatory cytokines (Figure 6A–F) and chemokines (Figure 7A–F). Because cytokines are frequently induced both by complement and by the TLRs, the inhibition of the TLR coreceptor CD14 by anti-CD14 Ab r18D11 was included. Cp40 reduced all the 25 cytokines, on average, 2.7-fold (range, 1.4–4.9) ($p < 0.05$) (Table I). Cp40 and r18D11 combined reduced all the 25 cytokines 3.9-fold (range, 1.3–9.5) ($p < 0.003$). r18D11 alone reduced only IL-1β (Figure 6B), IL-6 (Figure 6C), and bFGF, on average, 6-fold ($p < 0.04$) (Table I). Inhibition at the level of C5 with eculizumab reduced only IL-1β (Figure 6B), IL-8 (Figure 7A), and MCP-1 (Figure 7B), on average, 1.7-fold

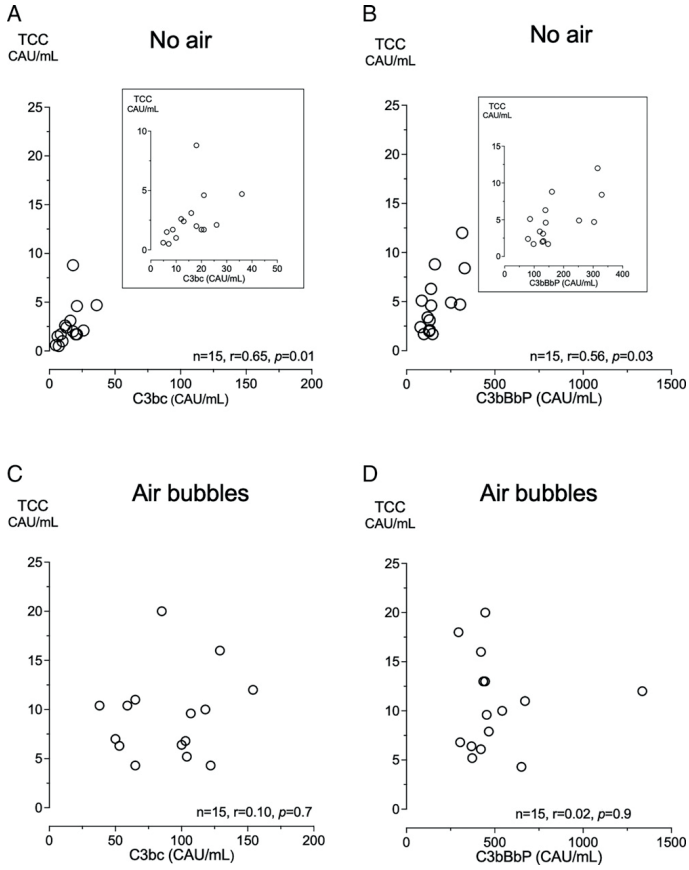

FIGURE 3. Correlation between early (C3bc and C3bBbP) and late (TCC) complement activation. After 180 min of incubation of human whole blood, there was a strong Spearman correlation between C3bc and TCC and a moderate correlation between C3bBbP and TCC in samples incubated without air (**A** and **B**), but there was no correlation in samples incubated with air bubbles (**C** and **D**).

($p < 0.03$), and inhibition of C5aR1 with PMX53 reduced only IL-8 (Figure 7A) and MCP-1 (Figure 7B), on average, 1.9-fold ($p < 0.006$) (Table I).

Blood gas and chemistry. In blood incubated with air bubbles compared with blood incubated without air, pO_2, pCO_2, pH, lactate, ionized Ca^{2+}, and HCO_3^- did not differ at the start of the incubation (Supplemental Fig. 2A–F). Throughout the incubation period, pO_2 (Supplemental Fig. 2A), lactate (Supplemental Fig. 2B), and pH (Supplemental Fig. 2C) were higher and pCO_2 (Supplemental Fig. 2D) was lower in blood incubated with air bubbles than in blood incubated without air ($p < 0.0001$, $p < 0.03$, $p < 0.05$, and $p < 0.003$, respectively). At the end of the 180-min incubation, ionized Ca^{2+} (Supplemental Fig. 2E) and HCO_3^- (Supplemental Fig. 2F) were lower in blood incubated with air bubbles ($p = 0.03$ and $p = 0.01$, respectively).

Effects of antifoam on complement and coagulation. Possible adverse effects of antifoam were examined in blood incubated with antifoam A or B. Antifoam B substantially increased C3bc, TCC, and PTF1+2 ($p < 0.0001$, $p < 0.0001$, and $p = 0.0002$, respectively) compared with blood incubated without antifoam (Supplemental Fig. 3A–C). Antifoam A did not affect these readouts. In our experimental setup, we avoided foam formation by incubating tubes with blood and air bubbles on a roller mixer rather than continuously bubbling air through the blood. This eliminated the need for the addition of antifoam agents that could induce adverse effects in the blood.

Plasma experiments

C4d did not differ between plasma incubated without air or with air bubbles (Supplemental Fig. 4A). C3bc was 35 CAU/ml (95% CI, 17 to 54 CAU/ml) in plasma incubated without air compared with 157 CAU/ml (95% CI, 120 to 193 CAU/ml) in plasma incubated with air bubbles ($p = 0.004$) (Supplemental Fig. 4B). C3bBbP was 296 CAU/ml (95% CI, 183 to 408 CAU/ml) in plasma incubated without air compared with 1219 CAU/ml (95% CI, 783 to 1654 CAU/ml) in plasma incubated with air bubbles ($p = 0.004$) (Supplemental Fig. 4C). TCC was 5.3 CAU/ml (95% CI, 0 to 12 CAU/ml) in plasma incubated without air compared with 18 CAU/ml (95% CI, 2.0 to 34 CAU/ml) in plasma incubated with air bubbles, but the difference was nonsignificant

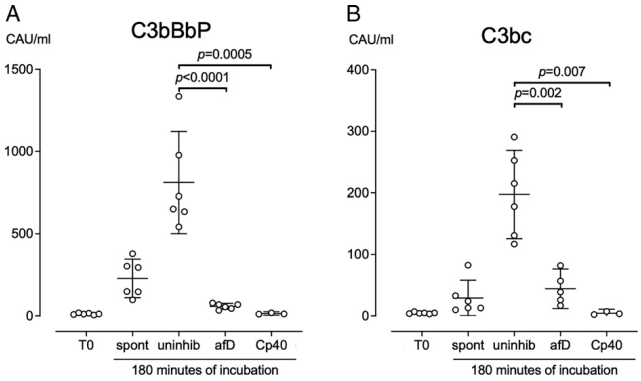

FIGURE 4. The specific role of the alternative pathway. Lepirudin-anticoagulated human whole blood from six donors (Cp40 was incubated in blood from three donors) was incubated for 180 min on a roller mixed at 37°C with no air in tubes (spont), with ambient air bubbles (uninhib), or with ambient air bubbles and either anti-factor D (afD) to a final concentration of 50 μg/ml or the C3 blocking peptide Cp40 to a final concentration 20 μM. Plasma was analyzed for C3bc (**A**) and C3bBbP (**B**) using ELISA. Horizontal lines represent means. Error bars are 95% confidence intervals.

(p = 0.09) (Supplemental Fig. 4D). Notably, in contrast to whole blood, PTF1+2 did not differ between incubations with air bubbles and without air (Supplemental Fig. 4E). As expected, the C3 inhibitor Cp40 abolished the C3bc and C3bBbP formation, and both Cp40 and eculizumab abolished the TCC formation (Supplemental Fig. 4B–D).

Discussion

In the present study, we used air bubbles incubated in a whole-blood model of venous air emboli. We showed how air bubbles induced substantial thromboinflammation, including activation of complement, coagulation, and platelets, and the release of 25 cytokines, including ILs, chemokines, and growth factors. This inflammatory response was mainly C3 driven, and C3 inhibition substantially reduced hemostasis, measured by TF-mRNA, MP-TF, and PTF1+2 and all 25 cytokines.

In contrast, during air bubbling, C5 activation was less pronounced and showed no correlation with C3 activation. C5 inhibition reduced TF-mRNA and PTF1+2 to the same extent as C3 inhibition and marginally inhibited platelet BTG release, which was the only marker that C3 did not inhibit. Interestingly, C5 inhibition reduced only three cytokines, IL-1β, IL-8, and MCP1, and the effect of C5 inhibition was mainly C5aR1 mediated. Surprisingly, inhibiting only CD14, an important cofactor for the TLRs typically inducing cytokines in blood incubated with bacteria (29, 30), reduced only IL-1β, IL-6, and bFGF. In contrast, C3 inhibition reduced all 25 cytokines, and combined C3 and CD14 inhibition further potentiated the reduction. To our knowledge, this study is the first human whole-blood study elucidating the role of complement in air bubble–induced hemostasis and cytokine release, revealing a crucial role for C3 in thromboinflammation. The alternative pathway was the primary driver of this C3 activation because blocking of factor D and C3 equally reduced the levels of C3bc and C3bBbP.

Oxygen, nitrogen, and air bubbles have previously been shown in vitro in serum and blood to change the C3 configuration to iC3, also termed "C3b-like" or "C3(H_2O)" (10), indicating that the complement activation depends on the gas–plasma interface on the surface of the air bubbles per se more than on the composition of gas inside the bubbles. This is in accordance with the hypothesis put forward by Atkinson and Farries that complement can discriminate between a "foreign surface" and a "self-surface" through the binding of the alternative pathway regulatory proteins, factors B and H (31); C3 binds the activating factor B with a higher affinity than the inhibiting factor H on a foreign surface. Thus, it is reasonable to suggest that the blood–gas surface interaction acts at a foreign surface and triggers activation.

A pivotal step in the complement activation by air bubbles is the hydrolysis and conformational change of the C3 molecule to C3(H_2O), as described several decades ago (10, 32). In line with these studies, we found that air bubbles triggered a strong generation of the C3 convertase C3bBbP, suggesting a similar activation mechanism. Notably, in blood incubated with air bubbles, there was no correlation between C3 activation (C3bBbP or C3bc) and C5 activation (TCC). We have introduced the term "dissociation" of complement to indicate the different activation potency of the convertases, with a highly efficient C3 convertase and a C5 convertase with low or no activity. This rarely seen but well-known phenomenon is discussed below.

Complement activation frequently occurs on particles or solid surfaces such as microbes or damaged endothelium, and typically an efficient C5 convertase is generated. The potent mediator C5a induces a strong inflammatory response through its receptor C5aR1 and, to a lesser extent, through C5aR2 (33). An example of this is described in a recent study of patients with coronavirus disease 2019 (COVID-19) (34), in whom complement activation products from the classical (C4d), the alternative (C3bBbP), the common (C3bc), and the terminal pathway (C5a and sC5b-9) were measured. In that study, patients with COVID-19 had a typical "sepsis-like" complement system activation, with a significant and strong correlation between all activation products. Under such conditions, inhibition of C5, C5a, or C5aR1 dampens the inflammatory response generated from the terminal pathway, and, although approved for use only for a couple of rare diseases (35), an inhibitor of C5 cleavage is the only complement inhibitor in routine clinical use. Interestingly, C3 inhibition in patients with COVID-19 afforded broader therapeutic control by attenuating both C3a and sC5b-9 generation and preventing factor B consumption, which is associated with a more robust decline of neutrophil counts, attenuated neutrophil extracellular trap release, faster serum lactate dehydrogenase decline, and more prominent lymphocyte recovery (36).

In contrast to this "whole complement system activation," which involves the two central components, C3 in the early phase and C5 in the terminal phase, we have previously described a "dissociation" phenomenon between C3 and C5 activation in patients with C3 nephritic factors (C3NeFs) (37). NeFs are autoantibodies binding to and stabilizing the complement convertases. In patients with C3NeF, the C3 convertase continuously activates C3, whereas the C5 convertase is inefficiently formed with minimal or no terminal pathway activation. The "dissociation" phenomenon has been confirmed by others (38), and the NeFs are now classified as C4NeF, C3NeF, and C5NeF.

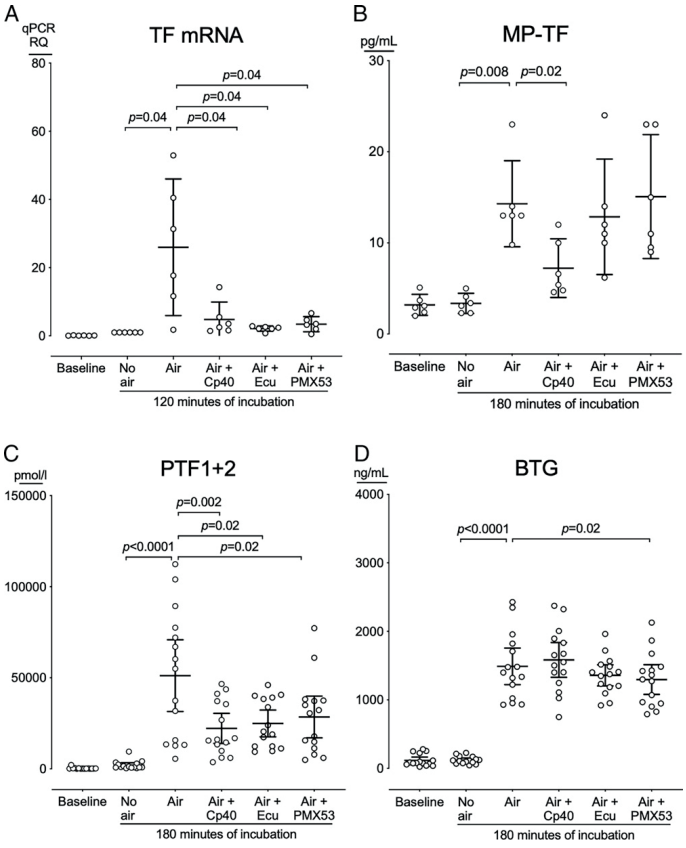

FIGURE 5. Hemostasis activation in blood incubated without or with air bubbles. Human whole blood from six donors was incubated with either no air; air bubbles (air); or air bubbles and Cp40, eculizumab, or PMX53. TF-mRNA was analyzed after 120 min (**A**), MP-TF (**B**), PTF1 + 2 (**C**), and BTG (**D**) were analyzed after 180 min. Horizontal lines represent means. Error bars are 95% confidence intervals. Only significant p values ($p < 0.05$) are shown.

In the present study on air bubbles, the inflammatory response was mainly dependent on C3 and could hardly be attenuated by C5 inhibition. A few of the hemostatic readouts, including TF and PTF1+2, were dependent on C5a but equally well abolished by C3 inhibition, suggesting that air bubble–induced inflammatory responses should be inhibited at the level of C3. The mechanisms need to be further investigated, but the main limitation for further investigation of the role of C3 is the lack of a highly specific C3aR antagonist, because those currently available are not selective and have both agonistic and antagonistic effects (39). The differences observed in the effect of the C3 inhibitor Cp40 and C5 inhibitor eculizumab or the C5aR1 blocker PMX53 on the readouts TF-mRNA, MP-TF, BTG, and PTF1+2 could not be explained by insufficient amounts of inhibitor. On the basis of our experience using these inhibitors for more than a decade, we used all inhibitors well in excess of the required concentration. Furthermore, in pilot experiments in our model system, we increased the inhibitor concentration 10-fold with no additional inhibitory effect observed on MP-TF, BTG, or PTF1+2.

Bubbles triggered a statistically nonsignificant increase in C4d, in line with our previous findings (22). C3 inhibition further amplified the C4d increase. This indicates that air bubbles activated not only the alternative but also the classical or lectin pathways. The exact mechanisms for these findings have not been elucidated before, to our knowledge. However, we suggest some theoretical explanations. Bubbles might have activated the C4 directly by hydrolysis, such as the C3 activation (10), because both the C4 and C3 molecules contain internal thioester bonds, and activated C4 not covalently bound to a surface will rapidly be inactivated by hydrolyzation (40). Additionally, the C4 could also have been indirectly activated, for example, by cross-talk with activated platelets (20). Finally, C3 blocking could have led to an increased C4 activation with the release of C4d after C4b deposition to amino and hydroxyl groups normally occupied by activated C3, similar to complement deposition on bacteria, as we have observed (41). Whether a similar mechanism exists on the gas–blood interface is speculative and has not been documented but cannot be excluded.

Air bubbles triggered a vigorous complement-mediated cytokine response. Cp40 effectively attenuated the response for all cytokines, and the combination of Cp40 and r18D11 further reduced the response slightly. In contrast, single inhibition with eculizumab reduced only IL-1β, IL-8, and MCP1, implying that the modest

Table I. Cytokine response to incubation without or with air bubbles or with air and inhibitors of either C3, C3 and CD14 combined, C5, or C5a receptor 1

Cytokine	No Air Versus Air Bubbles		Air Bubbles Versus Air Bubbles and Cp40		Air Bubbles Versus Air Bubbles and Cp40/r18D11		Air Bubbles Versus Air Bubbles and Eculizumab		Air Bubbles versus Air Bubbles and PMX53	
	Fold Increase	FDR Adjusted *p* Value[a]	Fold Decrease	FDR Adjusted *p* Value[a]	Fold Decrease	FDR Adjusted *p* Value[a]	Fold Decrease	FDR Adjusted *p* Value[a]	Fold Decrease	FDR Adjusted *p* Value[a]
PDGF-BB	78	***<0.0001***	2.9	***<0.0001***	5.6	***<0.0001***	1.1	0.5	1	0.7
IL-8	29	***<0.0001***	4.9	***<0.0001***	8.6	***<0.0001***	2	***0.001***	2	***0.002***
IFN-γ	20	***<0.0001***	5	***0.0001***	9.5	***<0.0001***	1.4	0.2	1.2	0.4
RANTES	20	***<0.0001***	1.8	***<0.0001***	2.1	***0.0001***	1.2	0.2	1.2	0.3
IL-1ra	14	***<0.0001***	4.2	***<0.0001***	3.5	***<0.0001***	1.3	*0.3*	1.1	0.5
G-CSF	14	***<0.0001***	3.3	***0.0005***	3.4	***0.0004***	1	0.7	0.9	0.7
VEGF	11	***<0.0001***	4.2	***<0.0001***	4.4	***<0.0001***	1.2	0.4	1.1	0.4
MCP-1	11	***<0.0001***	3.9	***<0.0001***	2.4	***<0.0001***	2	***0.005***	1.8	***0.005***
MIP-1α	9.6	***<0.0001***	4.3	***<0.0001***	5.2	***0.0001***	1.1	0.4	1	0.6
IL-1β[b]	8	***<0.0001***	3.2	***<0.0001***	6.4	***<0.0001***	1.5	***0.02***	1.3	0.2
IL-17α	7.2	***<0.0001***	2.1	***<0.0001***	2.8	***<0.0001***	1.1	0.4	1.1	0.3
IL-15	5.8	***<0.0001***	2.1	***0.0004***	3.6	***<0.0001***	1	0.8	1.1	0.5
IL-10	5.2	***<0.0001***	2.7	***<0.0001***	4	***<0.0001***	1.3	0.2	1.3	0.2
IL-6[b]	5	***<0.0001***	2.5	***0.0004***	9.3	***<0.0001***	1.2	0.2	1.2	0.2
GM-CSF	4.9	***<0.0001***	1.5	***0.04***	4.4	***0.0001***	0.9	0.8	1	0.7
IL-9	4.7	***<0.0001***	1.4	***0.02***	1.3	***0.002***	1	0.5	1	0.4
IL-5[c]	4.3	***<0.0001***	2.9	***0.0001***	3	***0.0001***	1	0.7	1	0.8
bFGF[b]	4.1	***<0.0001***	1.5	***0.0008***	2.4	***<0.0001***	1.1	0.3	1.1	0.4
IL-2	4	***<0.0001***	1.9	***0.0001***	3.4	***0.0001***	1.1	0.3	1.2	0.3
MIP-1β	3.8	***<0.0001***	2	***0.002***	3	***0.0009***	0.8	0.8	0.7	*0.4*
IL-4	3.5	***<0.0001***	2.2	***0.0005***	1.9	***0.0002***	1.1	0.4	1.1	0.5
TNF	3.4	***<0.0001***	2.4	***0.0008***	3	***<0.0001***	1.3	0.3	1.3	0.3
IL-7	2.3	***<0.0001***	1.7	***0.003***	1.6	***0.0002***	1.2	0.1	1	0.3
IL-13	1.8	***<0.0001***	1.5	***0.0003***	1.5	***0.0002***	1.1	0.3	1	0.4
Eotaxin	1.5	***<0.0001***	1.7	***<0.0001***	1.7	***<0.0001***	1.1	0.2	1.1	0.4

[a]Log-transformed data. Linear mixed-effects model with random intercept. Multiple comparisons corrected using the FDR method with $q = 0.05$. Discoveries (FDR adjusted $p < 0.05$) are shown in italic and bold type.
[b]Compared with incubations with air bubbles only, incubations with air bubbles and r18D11 reduced IL-1β 6.4-fold ($p = 0.03^a$), IL-6 9.3-fold ($p = 0.02^a$), and bFGF 2.4-fold ($p = 0.05^a$).
[c]Due to low bead number, only nine incubations were analyzed for IL-5.
FDR, false discovery rate; IL-1ra, IL-1 receptor antagonist; VEGF, vascular endothelial growth factor.

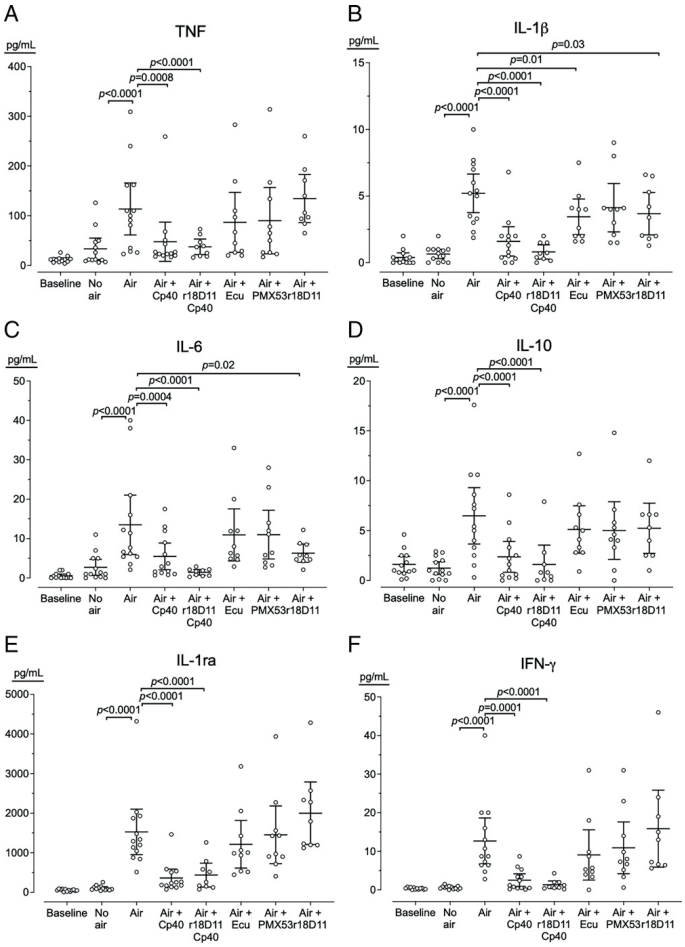

FIGURE 6. Pro- and anti-inflammatory cytokine released in blood incubated without or with air bubbles. Human whole blood from 13 donors was incubated 180 min with either no air; air bubbles (air); or air bubbles and Cp40, Cp40 and r18D11 (aCD14), eculizumab (Ecu), PMX53, or r18D11. The blood was analyzed for the cytokines TNF (**A**), IL-1β (**B**), IL-6 (**C**), IL-10 (**D**), IL-1 receptor antagonist (IL-1ra) (**E**), and IFN-γ (**F**). Horizontal lines represent means. Error bars are 95% confidence intervals. Only significant p values ($p < 0.05$) are shown.

activation taking place through the C5 convertase was still sufficient to induce these key cytokines, of which IL-8 previously has been shown to be mainly dependent on complement activation (42). Single inhibition with r18D11 reduced only IL-1β, IL-6, and bFGF. IL-1β is a major product following the formation of the inflammasomes, such as NLRP3 (43). Despite these interesting effects of C5 inhibition, collectively, our findings suggest that air bubbles activated inflammatory cells primarily through a C3-dependent mechanism.

Air bubbles activated the hemostasis with a substantial increase in the coagulation split product PTF1+2; the platelet-specific BTG; the cytokines RANTES and PDGF-BB, which are both released from activated platelets (44); and tissue factor, which can be released in microparticles from various cells, including monocytes (45). In contrast, in blood incubated without air, all readouts remained at baseline, showing that contact activation in the plastic tubes was not a major contributor to the hemostasis. In blood incubated with air bubbles, Cp40, eculizumab, and PMX53 abrogated TF-mRNA and equally reduced PTF1+2. The effects of eculizumab on air-induced TF-mRNA and PTF1+2 align with our observations in studies of *Escherichia. coli* in human whole blood (46). Cp40, but not eculizumab or PMX53, partly reduced MP-TF, RANTES, and PDGF-BB, suggesting both a C3-driven and a non–complement-driven TF release of presynthesized encrypted or intracellular TF in MP-TF from various blood cells, including monocytes (47). In contrast, de novo TF production, measured as an increase in TF-mRNA, was completely C5aR1 dependent. The BTG release from platelets was not reduced substantially by either Cp40, eculizumab, or PMX53, suggesting that the platelet activation was mainly non–complement dependent. The coagulation measured as PTF1+2 was activated both through a C5/C5aR1-dependent mechanism, possibly TF upregulation on the surface of monocytes, and through a

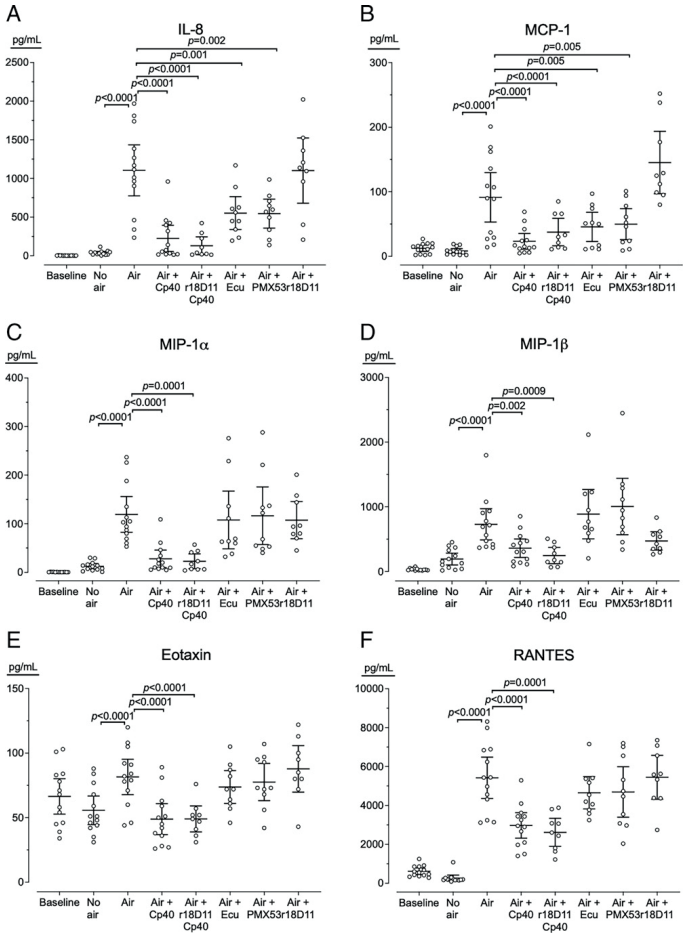

FIGURE 7. Chemokines released in blood incubated without or with air bubbles. Human whole blood from 13 donors was incubated 180 min with either no air; air bubbles (air); or air bubbles and Cp40, Cp40 and r18D11 (aCD14), eculizumab (Ecu), PMX53, or r18D11. The blood was analyzed for the chemokines IL-8 (**A**) MCP-1 (**B**), MIP-1α (**C**), MIP-1β (**D**), eotaxin (**E**), and RANTES (**F**). Horizontal lines represent means. Error bars are 95% confidence intervals. Only significant p values ($p < 0.05$) are shown.

complement-independent mechanism. PTF1+2 remained at baseline levels in plasma experiments, showing that the air-induced coagulation was highly dependent on blood cells. Both monocytes and platelets can activate coagulation (48), and we speculate that these cells played a pivotal role in the air-induced coagulation. However, our study was not designed to pinpoint the exact mechanisms for the hemostasis, and further studies are needed to elaborate on this in detail.

Importantly, our findings consistently support the use of C3 inhibition to reduce air-induced coagulation.

The results presented in this paper are based on the use of lepirudin anticoagulated whole blood without antifoam or ambient air in the tubes as an experimental system, which we suggest should be used to study thromboinflammation (23). In contrast to serum- and plasma-based models, whole blood contains both plasma proteins and all cell populations including platelets and leukocytes, as well as platelets, enabling the study of cell-released inflammatory mediators and the cross-talk between the whole inflammatory network. Importantly, we could not observe any activation of coagulation as measured by PTF1+2 upon incubation with air bubbles in plasma, underscoring the importance of experimenting in whole blood when studying this readout.

Antifoam is often used when studying air bubbles in vitro. We tested two different antifoams and found that antifoam B showed substantial adverse effects by activating complement and coagulation by increasing C3bc, C3bBbP, and PFT1+2. Thus, we designed a model system where incubation of air bubbles rather than continuously bubbling air through blood eliminated the need for antifoam. Heparin is known to interfere with complement, coagulation, and inflammation (23). Also, we have previously shown that ambient air in tubes triggers complement activation (22). Antifoam, heparin, and ambient air were avoided in our experimental model, minimizing undesired "background" activation, enabling us to study the complement, inflammation, and coagulation cascades with a higher signal-to-noise ratio than would otherwise have been possible.

Our study's main limitation is that it was performed ex vivo, and caution should be observed in translating the data to the in vivo situation. Although whole blood is the closest available model to a physiological condition, it needs to be anticoagulated, whereby thrombin is inhibited. Furthermore, the epithelial cells with their glycocalyx are missing, and this precludes investigation of their interactions with the blood cascade systems.

If our ex vivo findings translate to in vivo conditions, we suggest that clinicians should maintain a strong focus on the detection of air emboli, such as by using echocardiography as described in a pig model (49), during high-risk procedures such as thoracic surgery, hysteroscopy, or extracorporeal blood circulation. Our findings suggest that C3 inhibition might dampen the thromboinflammatory response and could be a future approach for treatment. The recent clinical approval of the first compstatin-based C3 therapeutic, pegcetacoplan (Empaveli; Apellis Pharmaceuticals, Waltham, MA) (50, 51), opens up opportunities for applying therapeutic C3 modulation to a broad spectrum of thromboinflammatory indications in which C3 activation evokes undesirable effects, including surgery-associated venous air embolism.

In conclusion, air bubbles in lepirudin-anticoagulated human whole blood activated complement, predominantly through the alternative pathway, and subsequently triggered thromboinflammation. C3 inhibition attenuated the complement activation, hemostasis, and cytokine release, whereas C5 inhibition had only minor effects.

Acknowledgments

Paul Van Buren, Sr., proofread the manuscript.

Disclosures

T.M.W. is an inventor of patents pertaining to complement inhibitors for inflammatory disease. He has acted as a consultant to companies that are commercially developing complement inhibitors and has received an honorarium from Alexion Pharmaceuticals for participation in industry conferences and meetings. He holds no stocks, shares, or other financial interests in any of these companies. J.D.L. is the founder of Amyndas Pharmaceuticals, which develops complement inhibitors for therapeutic purposes; is inventor of a broad patent portfolio that describes the therapeutic use of complement inhibitors, some of which are developed by Amyndas Pharmaceuticals, inventor of the compstatin technology licensed to Apellis Pharmaceuticals [i.e., 4(1MeW)7W/POT-4/APL-1 and pegylated derivatives such as APL-2/pegcetacoplan and APL-9]; and has received consulting fees from Achillion, Baxter, LipimetiX, Ra Pharma, Sanofi, and Viropharma. T.E.M. is a member of the scientific advisory board of Ra Pharma/UCB. The other authors have no financial conflicts of interest.

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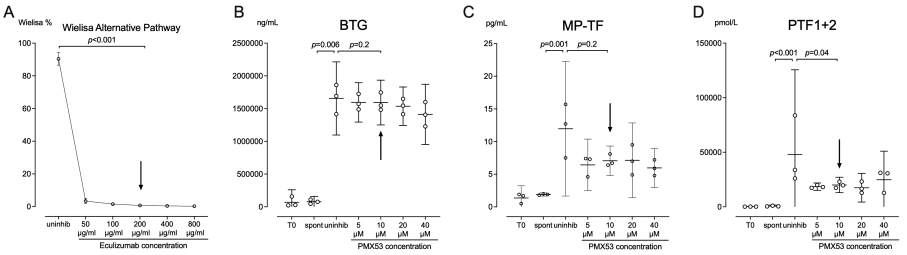
Supplemental figure S1

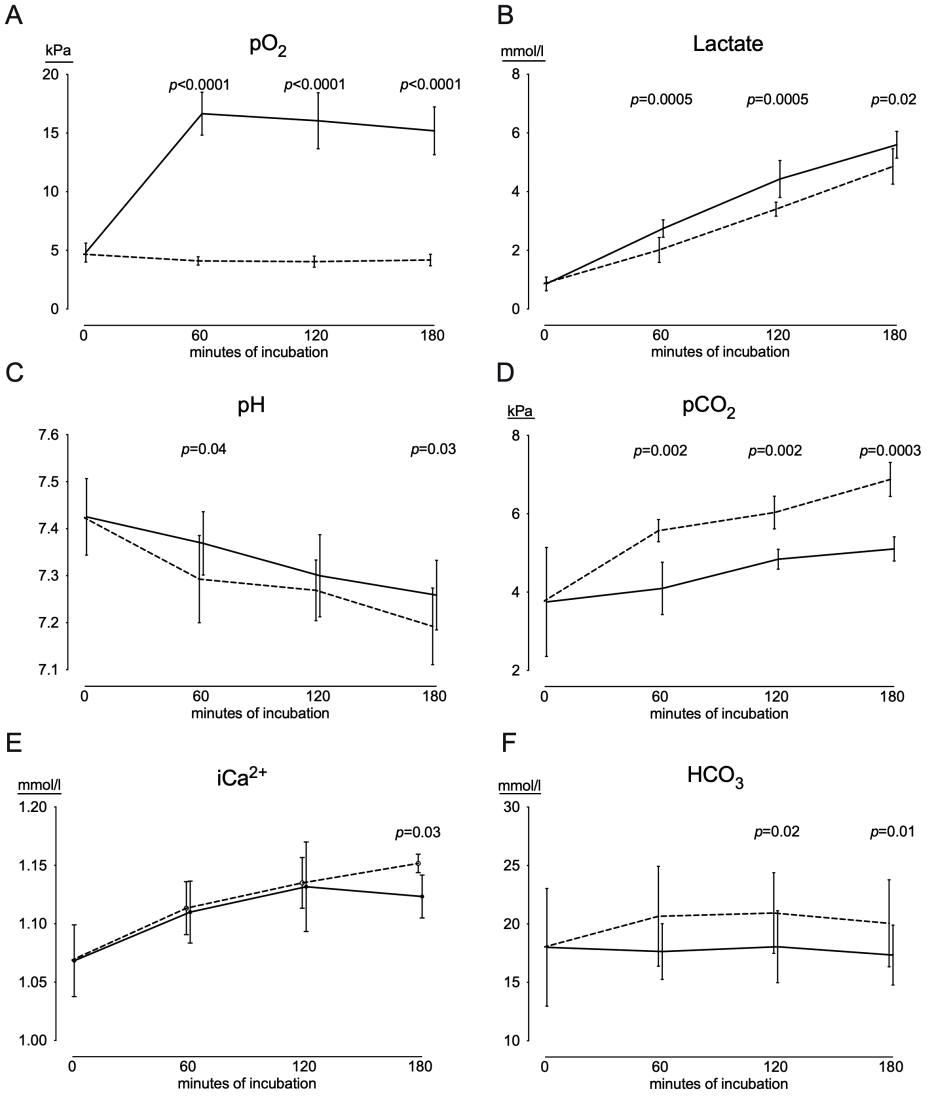
Eculizumab titration in alternative pathway Wielisa and PMX53 titration in whole blood



Panel A: Normal human serum was incubated without or with Eculizumab in five concentrations for five minutes at 37°C and analyzed using a Wieselab Complement system alternative pathway Wielisa (showing complement activity between 0% and 100%). Eculizumab effectively reduced the AP activity in all tested concentrations. *Panel B-D:* Lepirudin-anticoagulated human whole blood from three donors was incubated without *(spont)* or with air bubbles *(uninhib)* or with air bubbles and PMX53 in four concentrations for 180 minutes on a roller mixer at 37°C. Plasma was isolated, inactivated by EDTA, and analyzed using ELISA for BTG *(panel A)*, MP-TF *(panel B),* and PTF1-2 *(panel C)*. Incubation with air significantly increased all readouts. Incubation with PMX53 did not reduce the BTG in any tested concentration, non-significantly reduced MP-TF, and significantly reduced PTF1+2 equally in all tested concentrations. Arrow marks the concentration used in the *in vitro* whole blood air bubble study. Arrow marks the equivalent concentration used in the *in vitro* whole blood air bubble study. Graphs show means with 95% CI. *p* values were calculated using ratio paired t-test.

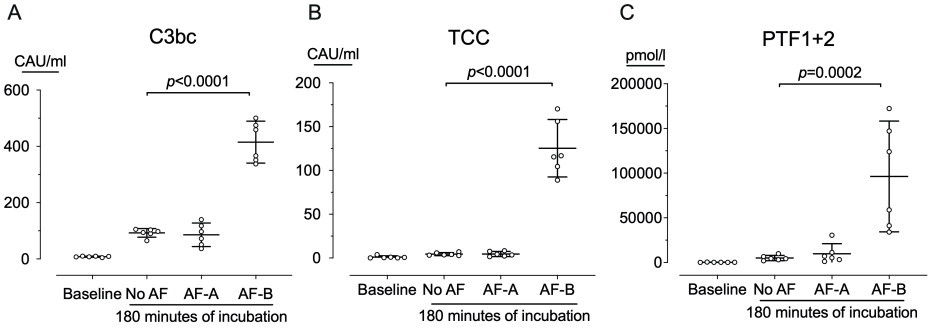
Supplemental figure S2

Blood gas and blood chemistry



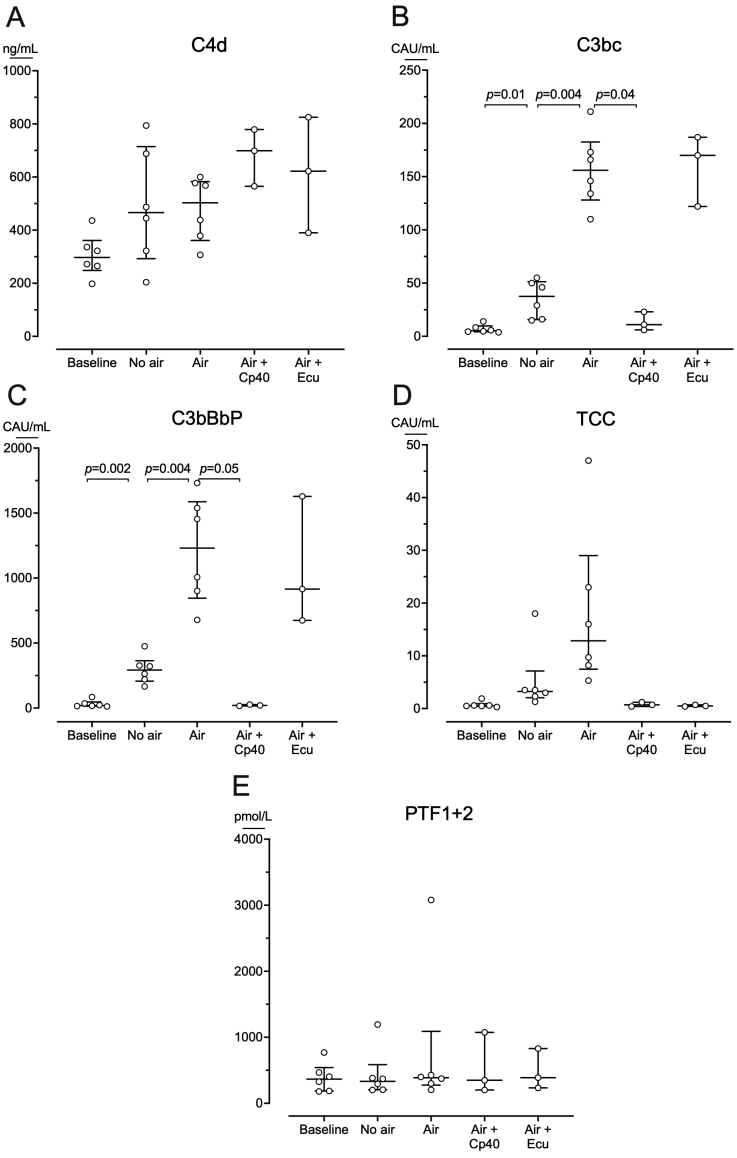
Lepirudin-anticoagulated human whole-blood from six donors was incubated for 180 minutes at 37°C on a roller mixer either in tubes without air (dotted lines) or with air bubbles added to the tubes (solid lines). pO2 (panel A), lactate (panel B), pH (panel C), pCO_2 (panel D), iCa^{2+} (panel E) and HCO_3 (panel F) sampled after 0, 60, 120, and 180 minutes of incubation. Graphs show means with 95%CI. *p*-values were calculated by multiple t-tests. Only significant *p*-values ($p<0.05$) are shown.

Antifoam incubations in whole blood



Whole blood from six donors was incubated 180 minutes on 37°C on a roller mixer in 1 mL aliquots with ambient air present in the tubes and with either no additives (*No AF*), 2 μL Antifoam A (*AF-A*), or 6 μL Antifoam B (*AF-B*). Graphs show means with 95%CI. *p*-values were calculated using RM-ANOVA with Geisser-Greenhouse correction and Fischer's exact t-test on log-transformed data. Only significant *p*-values ($p<0.05$) are shown.

Plasma air bubble incubations Supplemental figure S4



Fresh plasma from six donors was incubated for 180 minutes with either no air (n=6), air bubbles (*Air*; n=6), air bubbles and Cp40 (*Air+Cp40*; n=3) or air bubbles and eculizumab (*Air+Ecu*; n=3) and analyzed for C4d (*A*), C3bc (*B*), C3bBbP (*C*), TCC (*D*), and PTF1+2 (*E*). Graphs show means with 95% CI. *p*-values were calculated using a mixed-effects model. Only significant *p*-values ($p<0.05$) are shown.

Paper III Venous Air Emboli Activates Complement C3, and Trigger Inflammation and Coagulation in vivo in a Porcine Model.

frontiers in Immunology

ORIGINAL RESEARCH
published: 15 March 2022
doi: 10.3389/fimmu.2022.839632

Venous Air Embolism Activates Complement C3 Without Corresponding C5 Activation and Trigger Thromboinflammation in Pigs

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Edited by:
Mihály Józsi,
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Reviewed by:
Janos Szebeni,
Semmelweis University, Hungary
Markus Bosmann,
Boston University, United States

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Specialty section:
This article was submitted to Inflammation, a section of the journal Frontiers in Immunology

Received: *20 December 2021*
Accepted: *21 February 2022*
Published: *15 March 2022*

Citation:
Storm BS, Ludviksen JK, Christiansen D, Fure H, Pettersen K, Landsem A, Nilsen BA, Dybwik K, Braaten T, Nielsen EW and Mollnes TE (2022) Venous Air Embolism Activates Complement C3 Without Corresponding C5 Activation and Trigger Thromboinflammation in Pigs. Front. Immunol. 13:839632. doi: 10.3389/fimmu.2022.839632

Introduction: Air embolism may complicate invasive medical procedures. Bubbles trigger complement C3-mediated cytokine release, coagulation, and platelet activation *in vitro* in human whole blood. Since these findings have not been verified *in vivo*, we aimed to examine the effects of air embolism in pigs on thromboinflammation.

Methods: Forty-five landrace pigs, average 17 kg (range 8.5-30), underwent intravenous air infusion for 300 or 360 minutes (n=29) or served as sham (n=14). Fourteen pigs were excluded due to e.g. infections or persistent foramen ovale. Blood was analyzed for white blood cells (WBC), complement activation (C3a and terminal C5b-9 complement complex [TCC]), cytokines, and hemostatic parameters including thrombin-antithrombin (TAT) using immunoassays and rotational thromboelastometry (ROTEM). Lung tissue was analyzed for complement and cytokines using qPCR and immunoassays. Results are presented as medians with interquartile range.

Results: In 24 pigs receiving air infusion, WBC increased from 17×10^9/L (10-24) to 28 (16-42) ($p<0.001$). C3a increased from 21 ng/mL (15-46) to 67 (39-84) ($p<0.001$), whereas TCC increased only modestly ($p=0.02$). TAT increased from 35 µg/mL (28-42) to 51 (38-89) ($p=0.002$). ROTEM changed during first 120 minutes: Clotting time decreased from 613 seconds (531-677) to 538 (399-620) ($p=0.006$), clot formation time decreased from 161 seconds (122-195) to 124 (83-162) ($p=0.02$) and α-angle increased from 62 degrees (57-68) to 68 (62-74) ($p=0.02$). In lungs from pigs receiving air compared to sham animals, C3a was 34 ng/mL (14-50) versus 4.1 (2.4-5.7) ($p<0.001$), whereas TCC was 0.3 CAU/mL (0.2-0.3) versus 0.2 (0.1-0.2) ($p=0.02$). Lung cytokines in pigs receiving air compared to sham animals were: IL-1β 302 pg/mL (190-437) versus 107 (66-120), IL-6 644 pg/mL (358-1094) versus

25 (23-30), IL-8 203 pg/mL (81-377) versus 21 (20-35), and TNF 113 pg/mL (96-147) versus 16 (13-22) (all p<0.001). Cytokine mRNA in lung tissue from pigs receiving air compared to sham animals increased 12-fold for IL-1β, 121-fold for IL-6, and 17-fold for IL-8 (all p<0.001).

Conclusion: Venous air embolism in pigs activated C3 without a corresponding C5 activation and triggered thromboinflammation, consistent with a C3-dependent mechanism. C3-inhibition might represent a therapeutic approach to attenuate this response.

Keywords: air embolism, porcine model, complement, inflammation, cytokines, coagulation, thromboinflammation

INTRODUCTION

Venous air embolism, a condition where air inadvertently enters the bloodstream, may complicate many medical and surgical interventions (1–3), or bubbles may form in the bloodstream during rapid decompression when diving (4). Animal studies and human case reports have shown that air embolism triggers acute pulmonary hypertension and hamper pulmonary gas exchange (5–7). Air may also transverse the pulmonary circulation, enter the arterial circulation, disrupt blood circulation, and cause organ ischemia and damage, such as acute myocardial infarction or stroke (6, 8, 9). Patient case reports have described prolonged severe inflammation after air embolism (6, 10), and one case report described a suspected link between perioperative air embolism and disseminated intravascular coagulation, albeit with an unknown pathophysiological mechanism (11). *In vitro* experiments in human serum and heparinized blood have shown that air activates C3 to C3(H_20), also termed iC3 (12–14), and *in vitro* studies in lepirudin anticoagulated human whole blood have shown that air emboli trigger a complement C3-driven thromboinflammation (15), which is attenuated by C3 inhibition (15). *Ex vivo* rat studies have shown how air emboli trigger a pulmonary inflammation involving both monocytes and granulocytes and complement (16), recent *in vivo* studies of divers suffering from decompression sickness have shown how microbubbles trigger an acute inflammation (17), and human *in vivo* and *in vitro* studies of bubble-oxygenators has shown that bubbles activate C3 in fully heparinized blood (12). Heparin anticoagulation, however, precludes detailed studies into thromboinflammation and is known to interact with complement in a dosage-dependent manner (18, 19). Despite the numerous *in vitro*, *ex vivo*, and *in vivo* studies on air emboli, the role of the complement system in the air-induced thromboinflammation has to our knowledge, not previously been examined in detail *in vivo* in minimally anticoagulated larger animals.

Measures for the prevention, detection, and immediate treatment of air embolism are well described (3). However, these measures only address the immediate emergency treatment and do not address the management of air-induced thromboinflammation. Based on our recent *in vitro* human whole blood study (15), we hypothesize that C3 activation plays a key role and that C3 inhibition can attenuate the detrimental thromboinflammatory effects of air embolism. Unfortunately, no C3 inhibitor shown to work in swine is available for therapeutic use in porcine studies. Thus, this hypothesis remains untested.

The aim of this exploratory study was to examine the effects of venous air embolism on the complement system, the cytokine network, and coagulation *in vivo* in a porcine model.

Abbreviations: CAU, complement activation units; CFT, clot formation time; IL, interleukin; IQR, interquartile range; NATEM, non-activated thromboelastometry test; NeF, nephritic factor; ROTEM, rotational thromboelastometry; TAT, thrombin-antithrombin complex; TCC, terminal complement complex; TNF, tumor necrosis factor.

MATERIALS AND METHODS

We developed a novel porcine model of venous air embolism based on previous animal studies by Durant, Oppenheimer, and Vik (5, 20, 21). The protocol was approved by The Norwegian Animal Research Authority (FOTS ID9466) and performed per the Norwegian Laboratory Animal Regulations and EU directive 2010/64/EU.

Anesthesia, Instrumentation, and Monitoring

We retrieved 45 Norwegian domestic landrace pigs weighing on average 17 kg (range 8.5-30 kg) from two local farms. Before retrieval, the animals were selected to undergo venous air infusion or serve as sham animals. The animals were anesthetized with azaperone 4 mg, ketamine 500 mg, and atropine 0.5 mg intramuscularly. An iv. cannula was placed in an ear vein, and anesthesia was maintained with a continuous infusion of morphine 2 mg/kg/h, midazolam 0.15 mg/kg/h, and pentobarbital 4 mg/kg/h. We endotracheally intubated the pigs with a Portex ID 6 mm endotracheal tube (Smiths Medical International Ltd, Kent, United Kingdom) and ventilated them with a tidal volume of 10-15 mL/kg, a respiratory rate of 20/minute, an inspired oxygen fraction of 21%, and a positive end-expiratory pressure of 0 cmH_2O using a Datex-Ohmeda Engström Carestation intensive care ventilator (GE Healthcare, Madison, WI).

We placed a pediatric 9T esophageal echo probe (General Electric, Horten, Norway) in the esophagus and connected it to a Vivid 7 pro echo doppler machine (General Electric). Using

sterile cut-down technique, we inserted a 4 Fr. 8 cm Leadercath arterial catheter (Vygon Ltd., Swindon, UK) in the right carotid artery, an 8 Fr. Avanti+ Vascular Sheath Introducer (Cordis, Santa Clara, CA), and a 7.5 Fr. Swan-Ganz CCOmbo pulmonary artery catheter (Edwards Lifesciences Corporation, Irvine, CA) in the right external jugular vein, a 4 Fr. 8 cm PiCCO thermodilution catheter (Pulsion/Getinge, Gothenburg, Sweden) in the femoral artery, and a suprapubic catheter with a temperature sensor in the bladder. The pulmonary artery catheter, sheath introducer, and arterial cannula were connected to a TruWave x3 T001660A Pressure Monitoring Set (Edwards Lifesciences Corporation), and the PiCCO catheter was connected to a PV8215 PiCCO Monitoring Kit (Pulsion). Both systems were connected to a pressurized 500 mL Ringer's acetate bag (Fresenius Kabi, Oslo, Norway) with 1250 IU Heparin (LEO Pharma AS, Oslo, Norway) added to a final concentration of 2.5 IU/mL. A continuous infusion of 3 mL/h of heparinized Ringer's acetate was delivered through each of the four pressure lines, i.e., 30 IU Heparin pr hour was administered to the animals. If flushing of the catheters was needed, normal saline was used. The catheters were not flushed with heparinized Ringer. After instrumentation, the animals were stabilized for thirty minutes before starting the air infusion or sham observation.

We recorded continuous invasive arterial, pulmonary, and venous blood pressures, ECG, plethysmographic arterial saturation (SpO_2), end-tidal expired CO_2 ($EtCO_2$), and hourly intermittent cardiac output by thermodilution using an Intellivue MP70 monitor (Philips Healthcare, Cambridge, CA) as per manufacture's instructions. Additionally, hourly intermittent and continuous cardiac output was measured using the Pulsion Pulsed index Continuous Cardiac Output PiCCO2 monitor (Pulsion) as per manufactures instructions. Before the experiments, we performed echocardiography, and throughout the experiments, cardiac function and buildup of air and thrombi in the pulmonary artery and systemic egress of air were monitored using continuous echocardiography.

Exclusion Criteria

Animals with infections, either observed upon retrieval or diagnosed at the post-mortem lung autopsy or defined as a white blood cell count or complement C3a level three standard derivations above mean baseline values, were excluded (**Figure 1**). Animals with persistent foramen ovale, detected by preoperative echocardiography with an agitated saline injection or by post-mortem autopsy, and animals with iatrogenic complications, such as ventricular fibrillation upon inflation of the pulmonary artery catheter or vessel injury due to cannulation, were excluded from the study. Also, one animal in the sham group was excluded due to severe volvulus, which became evident during the observation period, and one due to an unintended infusion of air through an unpurged intravenous line.

Air Infusion

After instrumentation and baseline blood sampling, we administered a continuous air infusion of 3-7 mL/kg/hour by syringe driver through an ear vein. The infusion rate was either maintained or increased with 1-2 mL/kg every sixty minutes for 300 minutes (21 pigs) or 360 minutes (3 pigs) or until the animals died (**Figure 2A**). The air infusion protocol was based on previous studies (5, 20, 21) and titrated to cause severe hemodynamic instability, but not systemic egress of air embolism or the death of the animals. Initially, a 360-minute

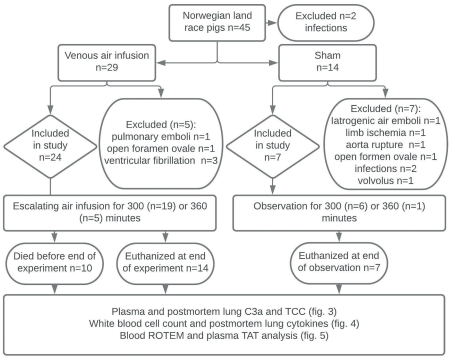

FIGURE 1 | Experimental flowchart. Forty-five Norwegian landrace pigs from two farms were selected by convenience sampling to undergo 300 or 360 minutes of titrated air infusion or serve as sham animals. Animals with infections, pulmonary emboli during animal preparation, persistent foramen ovale, ventricular fibrillation, iatrogenic venous air embolism, limb ischemia, or iatrogenic aortic rupture were excluded. Ten pigs receiving air infusion died due to the air infusion after a median infusion time of 243 minutes. Animals alive at the end of the experiments were euthanized. Blood was sampled at regular intervals throughout the experiments, and lung tissue was sampled post-mortem. Blood and lung samples were analyzed as detailed in the figure.

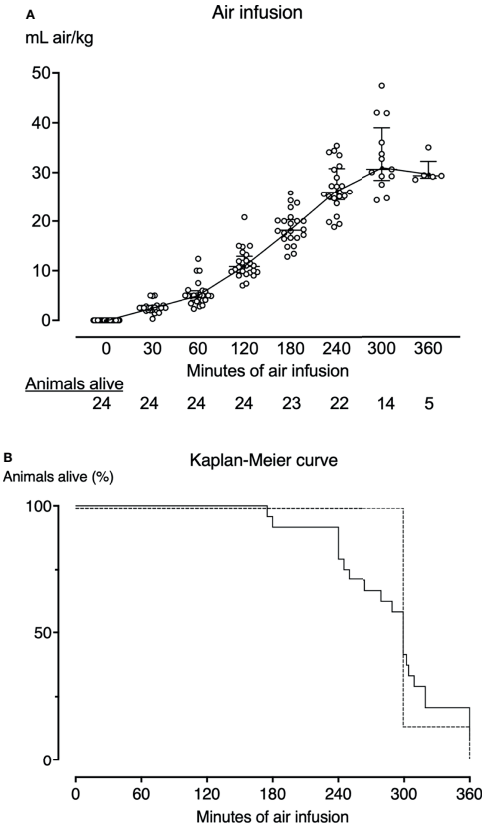

FIGURE 2 | Air infusion protocol and survival curves. **(A)** Twenty-four pigs received intravenous air infusion based on body weight. The infusion rate was increased hourly. Each point represents one pig. The horizontal lines indicate the median infusion rate. Error bars span the interquartile range. Animals are only included in the graph until death. Pigs alive at each sample point are listed below the graph. **(B)** Kaplan-Meier survival curve of the animals that received air infusion (solid line) and sham animals (dotted line). Pigs that received air infusions died after a median of 300 minutes (IQR 249-320). All sham animals lived until the end of the observation period of either 300 (n=6) or 360 minutes (n=1).

observation time was chosen to allow adequate time for the synthesis of cytokines. To reduce the preterm mortality of animals, this was reduced to 300 minutes after the initial experiments. We adjusted the respiratory tidal volume, respiratory rate, and inspired oxygen fraction throughout the experiments to maintain arterial pH 7.34-7.40 and SpO_2 above 90%. We infused 2-3 mL/kg/h of Ringer's acetate to compensate for insensible fluid losses. If mean arterial pressure dropped below 55 mmHg, we administered repeated boluses of 100 mL Ringer's acetate and infused noradrenaline (Abcur, Helsingborg, Sweden) in the range 0.01 to 0.8 μg/kg/minute. Animals still alive at the end of the experiment were euthanized by intravenous injection of 30-50 mmol potassium chloride (B. Braun, Melsungen, Germany).

Blood and Tissue Sampling and Analysis

We sampled arterial blood from the carotid artery at baseline (just before the air infusion was started at zero minutes), after 30, 60, 180, 240, and 300 minutes of air infusion, and at the end of the experiments. Approximately 100 mL of blood was sampled

from the animals throughout the experiments. The blood was sampled using a Vacutainer closed vacuum system, Vacuette EDTA tubes and 3.2% sodium-citrate tubes, and serum tubes with gel (all Vacuette, Greiner Bio-One GmbH Frickenhausen, Germany), and a safePICO heparinized blood gas syringe (Radiometer, Copenhagen, Denmark). To avoid unintended contamination of the samples with heparin from the line flush solution, 5 mL of blood was aspirated into a sterile syringe immediately before sampling. Additionally, after drawing blood into the heparinized blood gas syringe, 2 mL of blood was drawn and discarded before subsequent sampling. Blood drawn on heparinized syringes was analyzed immediately after drawing for lactate, pH, pO_2, and pCO_2 on the ABL80 Flex blood gas analyzer (Radiometer, Copenhagen, Denmark). A set of EDTA tubes were stored at room temperature for up to 8 hours and analyzed for white blood count using the ADVIA 2120i (Siemens Healthcare GmbH, Erlangen, Germany) or IDEXX ProCyte Dx (IDEXX Laboratories, Westbrook, ME). A set of EDTA tubes were immediately centrifuged at 4°C at 1500 g for 15 min and plasma isolated and frozen at -80°C for later analysis of complement and cytokines, as detailed below. PAXgene tubes were carefully tilted ten times, left at room temperature for a minimum of two hours, frozen at -20°C overnight, and then at -80°C until RNA extraction and cytokine mRNA analysis.

Post-mortem, we opened the thorax and sampled tissue from the lower lobe of the left lung. These samples were snap-frozen on dry ice in NUNC tubes (Thermo Scientific, Roskilde, Denmark) with no additive for further homogenization and analysis.

Homogenization of Lung Tissue

For cytokine mRNA analysis, approximately 20 mg of tissue was transferred to gentleMACS M-tubes (Miltenyi Biotec, Bergisch Gladbach, Germany), and 800 µL Trizol reagent (Thermo Fisher Scientific, MA) was added to the samples. The samples were homogenized using program 7 on the Dispomix homogenizer (Miltenyi Biotec). After homogenization, the samples were left at RT for 5 minutes, centrifuged at 1,400 *g* for two seconds, and transferred to 1.5 mL Eppendorf PCR Clean Safe-Lock Tubes (Eppendorf, Enfield, CT) and stored at -80°C for later mRNA isolation and PCR analysis.

For complement analysis, approximately 100 mg of tissue was transferred to gentleMACS M-tubes, and a mixture of 10 µL Protease Inhibitor Cocktail Set I (Merck KGAA, Darmstadt, Germany) and 1 mL CytoBuster Protein Extraction Reagent (Millipore Sigma, Burlington, MA) was added to the samples, and the samples were homogenized using program 7 on the Dispomix homogenizer (Miltenyi Biotec). After homogenization, the samples were incubated for 5 minutes on ice, centrifuged for 20 minutes at 2,500 *g* at 4°C, and the supernatant was transferred to 1 mL Matrix tubes (Thermo Fisher Scientific) and stored at -80°C for later analysis. Likely, receptor-bound C3a detached during the lysis process, making it available for subsequent ELISA detection.

Analysis of Complement in Plasma and Lung Tissue

We measured complement C3a using ELISA with porcine-specific C3a monoclonal antibodies as previously described in detail (22). The antibody binds to a neoepitope exposed when C3a is cleaved off C3, and the assay only detects free C3a in the fluid phase. We measured TCC using ELISA with the anti-human-C9 neoepitope antibody clone aE11 produced in-house as capture antibody and a porcine cross-reacting anti-human C6, Quidel (San Diego, CA) as detection antibody as described in detail (23, 24). We have previously documented that the aE11 cross-reacts with porcine TCC (23).

Analysis of Cytokines in Plasma

We analyzed EDTA plasma for the following cytokines using immunoassays: Tumor necrosis factor (TNF) and interleukin (IL)-6 using the Porcine TNF and IL-6 Quantikine sandwich ELISA kit (R&D Systems Inc.) with optical density measured by Infinite M200 Pro microplate reader (Tecan Trading AG, Switzerland); IL-1β, IL-6, and IL-8 using a porcine MILLIPLEX map Kit (Merck, EMD Millipore Corporation, Billerica, MA) and IL-10 using Invitrogen ProcartaPlex Multiplex Porcine Immunoassay (Bender MedSystems GmbH, Vienna, Austria), and the fluorescence intensity analyzed on a Bio-Plex 200 Multiplex Analyzer (Bio-Rad Laboratories). All analyses were performed in accordance with the manufacturer's instructions.

Analysis of Cytokines in Lung Tissue

We analyzed samples of homogenized lung tissue for IL-6 using the Milliplex map Kit (Merck) and IL-10 using Invitrogen ProcartaPlex Multiplex Immunoassay for Porcine assay (Bender MedSystems GmbH, Vienna, Austria). The fluorescence intensity was analyzed on a Bio-Plex 200 Multiplex Analyzer (Bio-Rad Laboratories). We analyzed the homogenized tissue for TNF, IL-1β, and IL-8 using Quantikine sandwich ELISA kits (R&D Systems Inc.) and measured the optical density using an Infinite M200 Pro microplate reader (Tecan Trading AG). All analyses were performed in accordance with the manufacturer's instructions. Results are given pr. mL homogenate.

Analysis of Cytokine mRNA in Lung Tissue

Paxgene blood, 1.5 mL, was transferred to 2 mL Eppendorf tubes (Eppendorf) and centrifuged at 5,000 *g*, 10 min. The supernatant was carefully poured from each tube, and the pellet was resuspended in 1 mL nuclease-free water. The RNA was re-pelleted by centrifugation at 5,000 *g* for 10 minutes. MagMAX for Stabilized Blood Tubes RNA Isolation Kit (Thermo Fisher Scientific) was used for total RNA isolation in accordance with the manufacturer's instructions. The extracted RNA was eluted in 50 µL of Elution Buffer, quantified using Thermo Scientific Nanodrop 2000 (Thermo Fisher Scientific), and controlled with the Agilent 2100 Bioanalyzer (Agilent Technologies Santa Clara, CA). The mean RNA Integrity Number was 9.6.

RNA was extracted from homogenized lung tissue using TRIzol Reagent and RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) and subsequent DNase treatment (Thermo Fisher Scientific) as described previously (25). RNA was quantified using the NanoDrop 2000 and integrity controlled using the Agilent 2100 BioAnalyzer. The mean RNA integrity number was 8.1.

For gene expression studies, the TaqMan RNA-to-Ct 1-step Kit (Thermo Fisher Scientific) was used. The amount of input RNA was 25 ng in a total volume of 20 µL. Cycling conditions were set according to the kit insert, and qPCR was run in triplicates for each candidate gene in MicroAmp Fast 96-Well Reaction Plates (all reagents from Thermo Fisher Scientific) using QuantStudio 6 Flex Real-Time PCR System instrument (Thermo Fischer Scientific). Predeveloped TaqMan porcine gene expression assays (Thermo Fisher Scientific) for the following candidate genes were used: IL-1β (Ss03393804_m1), IL-8 (Ss03392437_m1), and TNF (Ss03391318_g1). IL-6 (PIG_IL6) was custom-made by Thermo Fisher Scientific. Ribosomal Protein S 18 (Ss03391029_g1) was used as endogenous control and was stably expressed in all samples. The relative quantification (RQ) of cytokine mRNA expression was calculated using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method. All results are presented as fold change (RQ) compared to sham animals.

Analysis of Thrombin-Antithrombin Complex (TAT) in Plasma

We quantified the TAT in EDTA plasma using the human Enzygnost TAT micro (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany), documented to cross-react with porcine TAT (26) in accordance with the manufacturer's instructions. The optical density was measured using an Infinite M200 Pro microplate reader (Tecan Trading AG).

Whole Blood Rotational Thromboelastometry (ROTEM)

We used the ROTEM delta (Tem Innovations GmbH, Munich, Germany) to analyze the kinetic of the clot formation. Citrated blood sampled at 0, 30, 60, and 120 minutes of air infusion were incubated at 37°C for five minutes and analyzed using the non-activated thromboelastometry test (NATEM) according to the manufacturer's instructions. 300 µL citrated blood was added in a disposable cup with 20 µL star-TEM reagents containing $CaCl_2$ (Tem Innovations GmbH). The clot formation was detected by inhibition of the movement of the pin in the cup, and several parameters were measured or calculated. Clotting time (CT, in seconds), clot formation time (CFT, in seconds), α-angle (in degrees), and maximum clot formation (MCF, in millimeters) were analyzed in this study.

Data Analysis and Statistical Methods

All data was collected and organized, and missing values were imputed in Microsoft Excel for Mac version 16.54 (Microsoft Inc., Redmond, CA). In animals that died before 300 minutes of observation, missing data were imputed using the Last-Observation-Carried-Forward method. Changes from baseline were calculated for leukocytes, TAT, and ROTEM readouts, and RQ were calculated for cytokine mRNA qPCR in Microsoft Excel. Statistical analysis and data charting was done in Prism for macOS version 9.3.0 (Graphpad Software, La Jolla, CA). Results from post-mortem analysis of lung cytokines, cytokine mRNA, and complement in pigs receiving air infusion were compared with sham animals using the two-tailed Mann-Whitney U-test. Changes from baseline to time of death in plasma complement, TAT, and leukocytes and changes from baseline to 120 minutes of observation in whole blood ROTEM were analyzed using the Wilcoxon matched-pairs signed-rank test. All analyses were corrected for multiple comparisons using Benjamini and Hochberg's original false discovery rate (FDR) method with an FDR of 1%. A p-value <0.05 was considered significant. Results are presented as medians with interquartile range. Graphs of repeated samples are presented as changes from baseline. As our data did not follow a Gaussian, data are presented as medians with interquartile range. qPCR relative quantifications are presented as geometric means with 95% CI.

Five pigs had one or more plasma TCC results below the lower detection limit, and one pig had lung tissue TCC results below the lower detection limit. These missing values were imputed with a random number between 0.01 and the lower detection limit. Six pigs had lung cytokines results below the lower detection limit but with reliable results on the extrapolated standard curve. These results were included in the analysis. One lung tissue IL-8 analysis failed and yielded no result. This missing value was replaced with the median lung tissue IL-8 from all pigs. One pig had an erroneous ROTEM baseline reading due to a heparin bolus given shortly before the baseline sampling. The baseline ROTEM for this animal was imputed by averaging the ROTEM readings thirty minutes before and thirty minutes after baseline sampling.

RESULTS

Animals Included in the Study

Fourteen of 45 animals were excluded from the study due to infections, open foramen ovale, or perioperative adverse events (**Figure 1**). Twenty-four animals receiving air infusion and seven sham animals were included in the study. Baseline observations sampled after the initial instrumentation but before air infusion did not differ between pigs allocated to air infusion and sham animals (**Table 1**).

Despite careful titration of the air infusion, ten of the 24 animals (42%) receiving air infusion died before the end of the experiments with a clinical picture of acute right heart failure, after a median of 243 minutes (IQR 240-261) (**Figure 2B**). Fourteen of the 24 animals receiving air infusion, and all sham animals lived until the end of the experiments. The last observations were carried forward for the ten animals that died before 300 minutes of observation.

Complement in Plasma and Lung Tissue

In pigs receiving air infusion, baseline median plasma C3a was 21 ng/mL (IQR 15-46). Plasma C3a increased steadily after 120 minutes of air infusion and was 3.2-fold higher (67 ng/mL [IQR 39-84]) at death than at baseline ($p<0.001$) (**Figure 3A**). In sham animals, no C3a increase was observed during the observation period. In pigs receiving air infusion, baseline median plasma TCC was 0.8 CAU/mL (IQR 0.6-1.1). During the experiment, plasma TCC increased to a lesser extent and was 1.25-fold higher (1.0 CAU/mL [IQR 0.7-1.4]) at death than at baseline ($p=0.02$)

TABLE 1 | Characteristics of pigs included in the study at baseline[1].

		Pigs receiving air infusion (n=24) Median (IQR)	Sham animals (n=7) Median (IQR)	p[2]
Weight	kg	17 (10-24)	11 (9.5-22)	0.8
ROTEM				
Clotting time (CT)	seconds	613 (531-677)	519 (453-604)	0.2
Clot formation time (CFT)	seconds	161 (122-195)	133 (126-167)	0.6
α-angle	degrees	57 (62-68)	66 (59-68)	0.6
Maximum clot formation (MCF)	millimeter	64 (59-73)	65 (63-68)	0.6
Biochemistry				
White blood cell count (WBC)	$x10/^{9}L$	17 (10-24)	16 (11-18)	0.6
ELISA				
Thrombin-antithrombin complex (TAT)	µg/mL	35 (28-42)	23 (16-29)	0.2
C3a	ng/mL	21 (15-46)	12 (7.9-20)	0.2
TCC	CAU/mL	0.8 (0.6-1.1)	0.7 (0.3-0.8)	0.4

[1]*Sampled after 30 minutes after instrumentation, before air infusion (T0).* [2]*Wilcoxon signed-rank test. p values corrected for multiple comparisons using Benjamin and Hochberg's FDR method.*

(**Figure 3B**). In sham animals, no TCC increase was observed during the observation period.

In post-mortem lung tissue samples from pigs that received air infusion, median C3a was 34 ng/mL (IQR 14-50) versus 4.1 ng/mL (IQR 2.4-5.7) in sham animals (p<0.001) (**Figure 3C**), and median TCC was 0.3 CAU/mL (IQR 0.2-0.3) in pigs that received air infusion versus 0.2 CAU/mL (IQR 0.1-0.3) in the sham animals (p=0.02) (**Figure 3D**).

White Blood Cell Count

In pigs receiving air infusion, baseline median plasma white blood cell count was $17{\cdot}10^{9}$ cells/L (IQR 10-24). Plasma white blood cell count increased steadily after 120 minutes of air infusion and was 1.6-fold higher ($28{\cdot}10^{9}$ cells/L [IQR 16-42]) at death than at baseline (p<0.001) (**Figure 4A**). In sham animals, only a slight increase in white blood cell count was noted.

Cytokines in Plasma and Lung Tissue

During the experiments, no significant changes in plasma cytokines were observed in pigs receiving air infusion or sham animals. In contrast, in pigs receiving air infusion compared to sham animals, the post-mortem mean lung tissue cytokines mRNA expressions were increased: 12-fold for IL-1β, 121-fold for IL-6, and 17-fold for IL-8 (all p<0.001) (**Figure 4B**). The TNF mRNA expression did not differ between the groups. In line with the mRNA findings, the median lung tissue cytokines measured as protein in pigs receiving air infusion compared to sham animals were: IL-1β 302 pg/mL (IQR 190-437) versus 107 pg/mL (IQR 66-120), IL-6 644 pg/mL (IQR 358-1094) versus 25 pg/mL (IQR 23-30), IL-8 203 pg/mL (IQR 81-377) versus 21 pg/mL (IQR 20-35), and TNF 113 pg/mL (IQR 96-147) versus 16 pg/mL (IQR 13-22) (all p<0.001) (**Figure 4C**). Thus, TNF increased as measured by protein but not by mRNA.

Coagulation

In pigs that received air infusion, baseline median clotting time was 613 seconds (IQR 531-677) (**Figure 5A**), baseline median clot formation time was 161 seconds (IQR 122-195) (**Figure 5B**), and baseline median α-angle was 62 degrees (IQR 57-68) (**Figure 5C**). Already after 30 minutes of air infusion, clotting time and clot formation time had decreased and α-angle increased, and after 120 minutes of air infusion, both clotting time and clot formation time were significantly reduced compared to baseline, to 538 seconds (IQR 399-620) and 124 seconds (IQR 83-162) (p=0.006 and p=0.02, respectively). The α-angle significantly increased compared to baseline, to 68 degrees (IQR 62-74) (p=0.02). In contrast, in sham animals, neither clotting time, clot formation time, or α-angle changed significantly during the observation period (**Figures 5A–C**). The maximum clot formation did not change significantly during the experiments.

In pigs that received air infusion, baseline median TAT was 35 µg/mL (IQR 28-42). TAT increased after 180 minutes of air infusion and continued to increase throughout the experiments (**Figure 5D**). At the end of the experiments, median TAT was 1.5-fold higher (51 µg/mL [IQR 38-89]) than at baseline (p=0.002). In contrast, TAT remained at baseline in sham animals throughout the observation period.

DISCUSSION

In this study, we have shown *in vivo* in a porcine model how venous air embolism triggered a thromboinflammation with activation of the complement system, leukocytosis, release of proinflammatory cytokines, and activation of coagulation. Notably, air embolism triggered a relatively selective and robust C3 activation as measured by C3a, without a corresponding C5 activation, as measured by TCC.

The complement system is an integral part of the innate immune system, protecting the body against invading pathogens and endogenous cellular damage. The system consists of several plasma peptides, which bind to antibodies, lectins, or foreign surfaces, resulting in classical, lectin, or alternative pathway activation, respectively (27). It has previously been shown that air activates the complement system mainly through the alternative pathway (14, 15) by hydrolysis of C3 to generate C3 (H_2O), also termed C3b-like or iC3 (14). This initial alternative C3 activation generates the C3(H_2O)B, where factor B is cleaved by factor D to form Ba, which is released, and Bb, which together

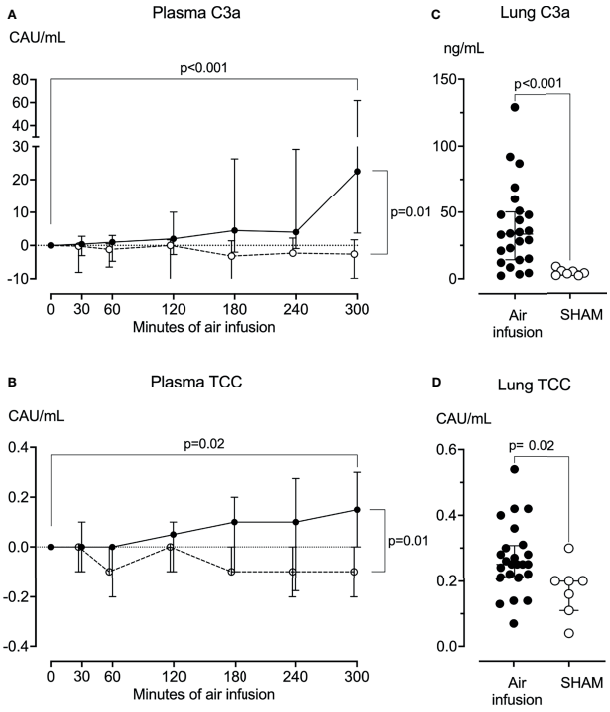

FIGURE 3 | Complement activation products in plasma and lung tissue. **(A)** Plasma C3a was measured at regular intervals throughout the experiments. C3a increased significantly from baseline in animals receiving air infusion (solid line) but not in sham animals (dotted line). **(B)** Plasma TCC measured at regular intervals throughout the experiments increased significantly from baseline in animals receiving air infusion (solid line) compared to the sham animals (dotted line). **(C)** In lung tissue sampled post-mortem, C3a was significantly higher in animals receiving air infusion than in sham animals. **(D)** In lung tissue sampled post-mortem, TCC was slightly but statistically significantly higher in animals receiving air infusion than sham animals. Dots in panels **(A, B)** and horizontal lines in panels **(C, D)** represent medians. Error bars span the interquartile range. Note Y-axis is split in panel **(A)** The two-tailed Mann-Whitney test was used between groups, and the Wilcoxon signed ranks test for within-group comparisons. P-values in panels **(A, B)** are corrected for multiple comparisons using Benjamin and Hochberg's FDR method.

with properdin (P) binds to C3(H_2O) and forms the first alternative C3 convertase, C3(H_2O)BbP. This convertase further activates C3 to be cleaved to C3b and C3a, and the final alternative pathway C3 convertase C3bBbP is formed. Under normal circumstances, C3bBbP forms the C5-convertase ($C3b_2BbP$), which catalyzes the formation of the terminal C5b-9 complement complex (TCC). Activated C3- and C5-split products have been shown to interact with thrombocytes and leukocytes (28, 29) and may potentially trigger a *thromboinflammation* involving both leukocytes, platelets, and coagulation. Interestingly, and in line with our previous *in vitro* findings in human whole blood (15), we discovered that air embolism predominantly activated C3 with only a minor C5 activation, contrary to what is otherwise observed in general when complement is activated, for example, by bacteria or damage or pathogen-associated molecular patterns. The C5a-C5aR axis is known to play an important role in complement-mediated thromboinflammation associated with a wide array of diseases (27, 30), and the monoclonal anti-C5 antibody eculizumab has been used clinically for many years to treat complement-driven diseases, such as paroxysmal nocturnal hemoglobinuria and atypical hemorrhagic uremic syndrome (31). C3a receptors have been shown on both platelets and activated astrocytes, neutrophils, and monocytes (32, 33), and we have previously shown how C3 inhibition attenuates air-induced thromboinflammation (15).

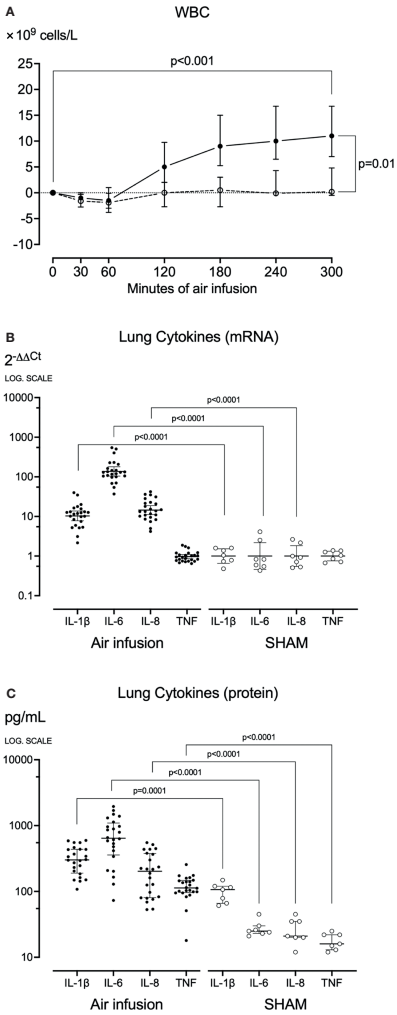

FIGURE 4 | Blood White Cell Count and Lung cytokines. **(A)** White blood cells were counted at regular intervals throughout the experiments. After sixty minutes of air infusion, white blood cells increased significantly from baseline in animals receiving air infusion (solid line) but not in sham animals (dotted line). **(B)** In lung tissue sampled post-mortem cytokine mRNA was analyzed using qPCR, and results were calculated using the $2^{-\Delta\Delta Ct}$ method. **(C)** In lung tissue sampled post-mortem, cytokines were measured using ELISA or multiplex. Animals receiving air infusion are represented as closed circles and solid lines, and sham animals as open circles and dotted lines. Circles in panel **(A)** and horizontal lines in panel **(C)** represent medians, and error bars span the interquartile range. The horizontal lines in panel **(B)** represent the geometric means, and error bars span the 95% CI of the mean.

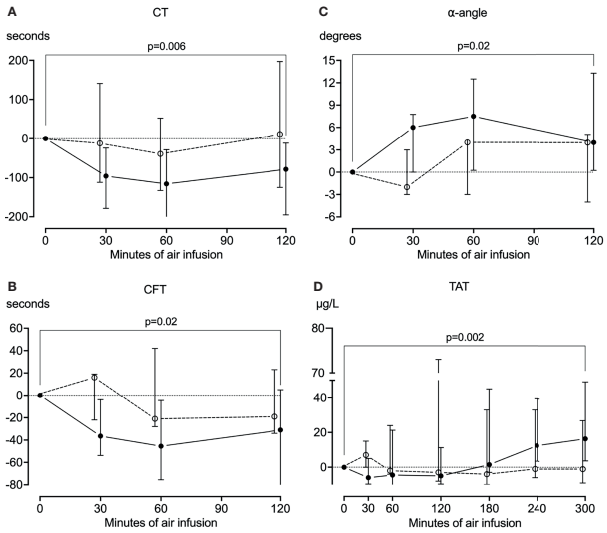

FIGURE 5 | Coagulation. Whole blood was drawn from the animals every half hour for the first two hours of the experiments, and the coagulation was analyzed using rotational thromboelastometry (ROTEM). In animals receiving air infusion, a significant reduction from baseline in the clotting time **(A)** and clot formation time **(B)** and a significant increase from baseline in the α-angle **(C)** was observed. These parameters mainly remained at baseline in sham animals, albeit with a pronounced heterogenicity. **(D)** Plasma was drawn from the animals throughout the experiments and analyzed for thrombin-antithrombin (TAT) using ELISA. TAT increased significantly from baseline in pigs receiving air infusion and remained at baseline in sham animals. Solid line represents pigs receiving air infusion. Dotted lines are sham animals. Circles represent medians, and error bars span the interquartile range.

Recently, a specific C3 inhibitor, pegcetacoplan (Empaveli, Apellis Pharmaceuticals, Waltham, MA), was approved to treat paroxysmal nocturnal hemoglobinuria (34), and further studies should evaluate if C3 inhibitors can be used to dampen thromboinflammation triggered by air embolism.

The aforementioned observed relative selective C3 activation without a corresponding C5 activation is most likely related to the mechanism by which air activates C3. The efficacy of the complement system convertases depends on whether the C3 activation occurs on the solid phase, where C3 binds to surfaces to generate a potent convertase, or in the fluid phase, where a less potent convertase is formed. As mentioned above, air bubbles have been shown to generate a fluid-phase C3 convertase *in vitro* in plasma experiments (14), and it is reasonable to assume that an identical fluid-phase convertase is formed *in vivo* in pigs with air embolism. The formation of a less efficient fluid-phase convertase could explain why the C5 convertase is insufficiently generated, which we documented with limited TCC formation compared to C3 activation. This contrasts the complement activation otherwise seen. For example, in a porcine study of polymicrobial sepsis (35), both C3a and TCC increased substantially, with C3a appearing first followed by TCC (22).

Another interesting imbalance between C3 and C5 convertase potency occurs when autoantibodies, nephritic factors (NeFs) form against the complement convertases, as recently reviewed (36). NeFs may bind to the C3, C4, or C5 convertase (C3NeF, C4Nef, and C5Nef, respectively). NeF binding stabilizes the convertases, resulting in pathological ongoing complement activation. As with air embolism, we have previously shown that some C3NeFs activate C3 without a corresponding C5 activation (37). NeFs cause an autoimmune kidney failure in patients, particularly if the C5 is activated (36).

The imbalance between C3 and C5 activation is important concerning the potential treatment of patients with air embolism. Most complement-mediated diseases involve the C5a or the C5b-9, and treatment involves inhibition of C5 to keep the C3 open for complement-driven bacterial defense. However, in the case of venous air embolism, C3 inhibition would be required to reduce the thromboinflammatory response effectively. It remains to be shown if other pathophysiological conditions are similarly mainly driven by C3 activation with only a modest activation of C5.

In our study, we measured C3a using an ELISA assay with a porcine-specific antibody (22) and TCC using a human assay

shown to work well in swine (23, 24), as no reliable porcine C5a assay is commercially available. This is a reasonable approach, as C5a and TCC (sC5b-9) are released in equimolar concentration in plasma. TCC has a plasma half-life of 50-60 minutes (38) compared to C5a with a half-life of one minute (23), making it a robust marker of terminal pathway activation. Further details on assays for the detection of complement activation products in humans and animals are reviewed in detail (39).

In pigs that received air infusion, but not in sham animals, we found increased levels of proinflammatory cytokines in lung tissue, increased circulating white blood cells, and coagulation system activation. Combined with our human *in vitro* findings (15), we have shed new light on the mechanisms for inflammation, coagulation, and the interplay between complement, inflammation, and coagulation. Our data suggest that complement activation played an important role mainly through the activation of C3. *In vitro*, we showed how selective inhibition of C3 reduced complement activation, cytokine release, and coagulation. Most of the inflammatory mediators were purely mediated by C3-activation, but a few were C5a dependent. The majority of mediators were abolished by C3 inhibition. Thus, there is a theoretical rationale for C3 inhibition as a treatment of air embolism *in vivo*. Unfortunately, as no inhibitor reacting with porcine C3 is currently available largescale for *in vivo* studies, we could not verify these findings in our porcine model.

The accumulation of C3a and increased cytokine concentrations in lung tissue of pigs with air embolism indicates the importance of the lungs in the pathophysiology of venous air embolism and air-induced thromboinflammation. Venous air emboli are transported with the bloodstream to the lung capillaries, where the air is absorbed into the alveoli and exhaled. However, large amounts of air may overwhelm the lung's filtering capacity, occlude the capillaries and obstruct blood flow, trigger a local inflammatory process, or transverse the lungs to the systemic circulation (7, 9, 40). In humans, air bubbles have been shown to activate the alternative complement pathway *in vivo, ex vivo*, and *in vitro* (12, 14, 15). We suggest that lodged air bubbles in the pig's lung capillaries activated C3 by a similar mechanism and subsequently triggered inflammation and coagulation alike in humans. This would explain the increase in lung tissue cytokines and circulating white blood cells observed in pigs receiving air infusion. The pulmonary inflammation may have triggered lung edema. The combination of air bubbles, thrombi, and edema may have further hampered blood flow and gas exchange, resulting in pulmonary hypertension, a drop in end-tidal CO_2, and acute right heart failure, as we and others have found in animals studies and human cases (6, 20, 41, 42).

In our study, air infusion triggered coagulation, measured by ROTEM and TAT. We recently showed *in vitro* in human whole blood that coagulation was activated through a complement-dependent mechanism, where C3 activation played a pivotal role, and through a complement-independent mechanism, which we did not identify in detail (15). We speculate that air embolism activated the coagulation through similar mechanisms *in vivo* in pigs. The ROTEM and TAT results were most likely diminished and underestimated, as we had to administer a heparin infusion of approximately 30 IU/h to avoid coagulation of arterial and venous lines and the buildup of thrombi on the pulmonary artery catheter. Additionally, in animals receiving air infusion, but not in sham animals, we also had to administer intermittent heparin boluses of approximately ten IU due to thrombi in the pulmonary artery catheter.

Despite many similarities between humans and pigs, human immunoassays may not reliably cross-react and work in pigs. In this study, we limited complement readouts to C3a and TCC, and we used only cytokine assays shown to work in pigs as detailed in (43). Many commercial immunological assays, including complement and cytokine assays, are marketed as working in pigs. Over the years, we have thoroughly tested most available complement assays, comparing them to the reliable C3a and TCC assays described above and in (22–24); disappointingly, in such comparisons, most assays failed to detect complement activation reliably. Likewise, we have recently studied porcine-specific cytokine assays, finding that many of these are also unreliable (43).

Our study has some limitations; it was a single-center, non-randomized, and non-blinded exploratory study. The animals were assigned to air infusion or to serve as sham animals at the examiner's discretion. However, we do not suggest that these limitations affected our results.

Despite careful titration of the air infusion, several animals died before the intended 300 minutes of air infusion, with most premature deaths occurring between 240 and 300 minutes of air infusion (**Figure 2**). These premature deaths could have been mitigated by using a less-aggressive air infusion protocol, for example, by not increasing the infusion rate after 180 minutes of air infusion or by terminating the experiments after 180 minutes of air infusion. However, we titrated the air infusion to maximize the effects of air embolism on the thromboinflammation to allow for complement production and time for the synthesis of cytokines. It is possible that reducing the air infusion or shortening the experiments would have reduced complement readouts and yielded lower post-mortem cytokine levels. However, we measured cytokines by mRNA and by protein quantification, enabling us to detect cytokines with high sensitivity. Cytokine mRNA forms rapidly before proteins are synthesized, but, as we observed concerning TNF and IL-1β, may also be downregulated and thus not detected by PCR when proteins are still present and detected by ELISA in the tissue. In our study, pigs that died prematurely were more inflammatory affected than animals surviving the experiment, and it is unlikely that premature death impacted our results negatively.

CONCLUSION

Venous air embolism triggered thromboinflammation *in vivo* in pigs, reflected by increased plasma complement C3 activation, leukocytosis, and coagulation. Furthermore, post-mortem pulmonary tissue homogenates revealed increased C3a and

cytokines IL-1β, IL-6, IL-10, and TNF, and increased synthesis of IL-1β, IL-6, IL-8, and IL-10 mRNA. A corresponding terminal pathway activation did not follow the C3 activation. These findings align with previous *in vitro* human whole blood studies, suggesting that C3-inhibition is relevant for further studies in the treatment of venous air embolism.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors upon request, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Norwegian Animal Research Authority (FOTS ID9466) and performed perthe Norwegian Laboratory Animal Regulations and EU directive2010/64/EU.

AUTHOR CONTRIBUTIONS

BS, TM, and EN conceptualized and designed the study. BS, EN, BN, and KD conducted animal experiments. BS, DC, HF, JL, KP, and AL acquired and analyzed the biological materials. BS and TB conducted the statistical analyses. BS, EN, and TM drafted the manuscript. All authors critically revised the manuscript and approved the final version.

FUNDING

The study was funded by an unrestricted research grant from Northern Norway Regional Health Authority (Helse Nord RHF).

ACKNOWLEDGMENTS

We thank Chief Bioengineer Reidar Johansen, Department of Laboratory Medicine, Nordland Hospital, Gravdal, Norway, for valuable assistance with sample analysis, and Paul van Buren, Jr., and Sr., for proofreading the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix I Literature search on air embolism

To construct Figure 3, I conducted an advanced PubMed search (www.PubMed.gov), using the search term (air[Title] AND (embolism[Title] OR emboli[Title])) AND (case[Text Word] OR case report[Text Word]) AND ("1900"[Date - Completion] : "2019"[Date - Completion]), where completion date was increased in 10 years intervals. I subsequently repeated this search replacing “case report” and “case” with “study” and “experiment”, or with “review.” The search was run on March the 30th 2022, including all results up until this date. The search results were organized, and the graph was constructed in Prism 9 for Mac OS ver 9.3.1 (GraphPad Software, San Diego, CA).

ISBN: 978-82-7589-870-6

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