Atrial fibrillation and hypercoagulability

Atrial fibrillation and hypercoagulability: a two-way street with many side-roads

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Cover design by Roberta Russo
Editing and graphic layout by Carlo Dodaro

Printed by UNIVERSAL BOOK S.r.l. - Contrada Cutura, 236
87036 Rende (Cosenza), Italy

ISBN 978-909-036-162-8

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Dissertation

To obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus,
Prof. Dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on and by

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Thursday 20 October 2022 at 13:00 hours
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The research described in this thesis was supported by a grant of the Dutch Heart Foundation (DHF CVON2014-09)

Financial support by the Dutch Heart Foundation and Maastricht University for the publication of this thesis is gratefully acknowledged.

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Chapter 1

General introduction

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General introduction

In 1827, by the use of the then recently invented stethoscope, it was Robert Adams who reported irregular pulses associated with mitral stenosis. More than 70 years later, after the Dutch physiologist Willem Einthoven improved the electrocardiograph, the first electrocardiogram of atrial fibrillation (AF) was recorded [1].

AF is the most common form of sustained cardiac tachyarrhythmia with an estimated prevalence of approximately 3% in individuals of European ancestry [2]. AF is characterized by high-frequency, irregular activation of the atria, leading to hemodynamic impairment. It is associated with increased morbidity and mortality, reduced life expectancy and quality of life, and thereby forms a significant burden on the worldwide health care systems [2, 3].

Advancing age is the predominant risk factor for AF. Other established risk factors for developing AF include smoking, alcohol intake, hypertension, obesity, diabetes, myocardial infarction and heart failure (HF) [4, 5]. Within the coming decades, the global AF prevalence is expected to rise up to 7%, not only due to ageing of the general population and the presence of AF-predisposing factors, but also because of the intensified search for undiagnosed AF [4, 6].

Natural time course of AF

AF often starts with short, self-terminating episodes. However, the progressive nature of the arrhythmia is often apparent in the gradual prolongation of the AF episodes until they become persistent [7]. Any change of the atria that contributes to the initiation or maintenance of AF is referred to as "atrial remodeling" and contributes to the so-called "AF-substrate" [8].

Studies on experimental animal models in the 1990's demonstrated that atrial tachycardia leads to electrical remodeling. Rapid atrial pacing in dogs decreased the atrial effective refractory period (AERP) [9]. In goats, atrial pacing at every instant that sinus rhythm (SR) occurred, led to an even stronger decrease of the AERP and a loss of rate adaptation of the refractory period. This was accompanied by a progressive increase in AF susceptibility and duration [10]. The process of electrical remodeling develops within the first days, but it is reversible upon SR restoration, since it is mainly due to AF-related alterations in cardiac ion channels [11, 12].

Just as in most of AF patients, AF episodes become longer over time in animal models of AF [7]. A much slower remodeling process, structural remodeling, has been associated to this gradual increase in AF stability. These alterations take place over a time course of several weeks to many months after the onset of AF [7, 13].

The most important forms of structural remodeling that contribute to the progression of AF are atrial fibrosis, atrial dilation and atrial myocyte hypertrophy [9, 14-16]. Structural remodeling processes are thought to contribute to the complexity of fibrillatory conduction by increasing the number of simultaneously propagating wave fronts [17]. A high AF complexity and abnormalities in conduction are recognized as the main determinants of AF stability [18].

Fibrosis is characterized by an overall increase in fibrous tissue. Three different subtypes of myocardial fibrosis have been described by Weber et al.: replacement, perimysial and endomysial fibrosis [19]. Replacement fibrosis is a reparative response to cell death that leads to scar tissue formation. Perimysial fibrosis is an increase in fibrous tissue that surrounds bundles of myocytes, while endomysial fibrosis reflects increased transverse separation of individual myocytes within muscle bundles. Mainly endomysial and perimysial fibrosis have been shown to lead to impaired transverse conduction between myocytes [20, 21].

Theoretically, myocyte hypertrophy is expected to increase the conduction velocity, which has indeed been shown in a mathematical model of ventricular hypertrophy [22]. However, in a model of canine atrial epicardial preparations, that also mimicked the re-distribution of gap junctions upon cell growth, Spach et al. showed that myocyte hypertrophy led to increased transverse propagation delays and altered anisotropic conduction [23]. Such discontinuous conduction can favor reentry in small circuits [23, 24]. Also, in many cases myocyte hypertrophy is associated with atrial enlargement and the increased surface area provides more space for re-entrant circuits that may further stabilize AF [25].

Previously, our group demonstrated the presence of atrial endomysial fibrosis and myocyte hypertrophy or atrial dilation in the goat model of AF, which resulted in increased complexity of fibrillatory conduction [16, 25, 26].

AF-related risk factors and comorbidities

AF is associated to increased cardiovascular mortality due to sudden death, HF and stroke. The risk of developing thromboembolic stroke increases by five-fold after patients have developed AF [5, 27]. As the etiology of AF is multifactorial, the pre-sence of associated comorbidities, which are often also risk factors for stroke, makes it difficult to assess the real contribution of AF by itself on the coagulation status.

In fact, it is still not fully clarified if "lone AF" (AF in the absence of apparent comorbidities) is sufficient to cause a pro-thrombotic state, or if the presence of other underlying comorbidities and risk factors, is needed. In a study on young very-low-

risk patients with paroxysmal AF, no hypercoagulable state was observed. Nevertheless, these patients were found to have increased levels of Factor (F) IXa-anti-thrombin complexes suggesting the presence of a pre-thrombotic state [28]. Because of this finding, the authors suggested that a second pro-coagulant hit may be needed to provoke a systemic pro-thrombotic response during AF. Clinical studies in AF patients have shown that the stroke risk often increases after many years, when there is advanced age and development of other comorbidities, such as HF [29-31].

Crosstalk between AF and coagulation

The thrombogenic tendency in AF has been related to three main pathophysiological mechanisms that together fulfill the so-called Virchow's triad [1]. The first mechanism is blood stasis which in AF is mainly caused by failure of atrial systole, but also by progressive atrial dilation. The second mechanism includes anatomical and structural changes of the endocardium, where areas of endothelial denudation and damage may predispose to thrombotic aggregation. Finally, the third mechanism of the Virchow's triad refers to alterations in blood constituents which confers a hypercoagulable state. Hypercoagulability in AF patients is often reflected by increased systemic platelet activation, elevated concentrations of pro-thrombotic indices (e.g. prothrombin fragments 1+2, thrombin-antithrombin complex) and altered fibrinolytic activity [32, 33].

Recently, hypercoagulability has been described to play a role in the progression of AF [34]. In fact, activated coagulation factors, such as thrombin and FXa, are able to modulate physiological and pathological processes, such as inflammation and fibrosis, which may contribute to cardiac remodeling [35, 36]. These extravascular (non-hemostatic) functions impact different cell types (e.g. endothelial cells and cardiac fibroblasts) via activation of protease activated receptors (PARs) [37]. The PAR family consists of four isoforms (PAR-1 to -4). Activated coagulation proteases, like thrombin and FXa, cleave PARs at the N-terminus and generate a newly exposed N-tethered ligand that self-activates the receptor [38].

Previously, our group has demonstrated that inhibition of FXa and consequently thrombin, may reduce pathological processes mediated by PAR activation during AF [34]. Inhibition of thrombin via the direct oral anticoagulant (DOAC) dabigatran downregulated the expression of pro-fibrotic markers induced by thrombin on cardiac fibroblasts (CF). Moreover, it was found that the effect of thrombin on this cell type was mainly mediated by PAR-1 activation. In the same study, *in vivo* inhibition of FXa attenuated AF-induced atrial endomysial fibrosis and reduced AF complexity in goats after four weeks of AF [34].

In this context, we speculate on the presence of a crosstalk between AF and hypercoagulability of which a causal relation is difficult to establish. We hypothesize that the AF-associated hypercoagulable state contributes to atrial remodeling and AF substrate development through PAR-activation. Furthermore, we hypothesize that the AF-related coagulation state can be modulated by the coexistence of AF with its underlying risk factors, or by the presence of AF-related comorbidities alone, and thereby accelerating the development of an AF substrate (Figure 1).

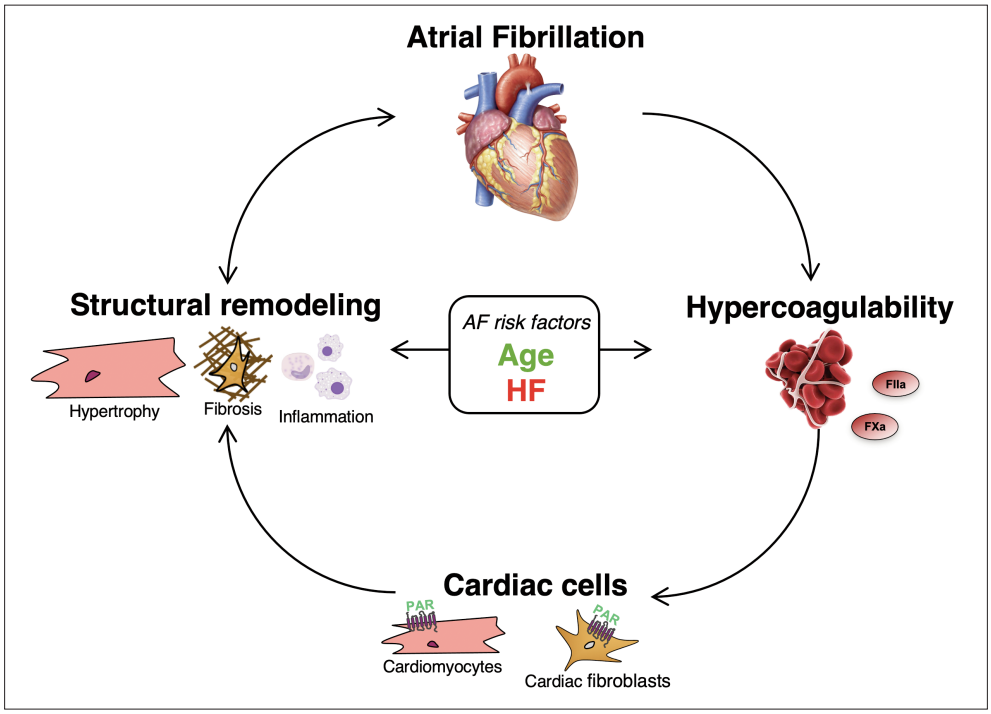

Figure 1. Schematic representation of the general hypothesis of this thesis.
AF is associated to a hypercoagulable state. Activated coagulation factors (e.g. thrombin and FXa) can trigger (atrial) activation of PARs which are expressed on cardiac cells. This leads to structural remodeling processes, such as recruitment of inflammatory cells in the myocardium, fibrosis and myocyte hypertrophy which eventually increase AF complexity and contribute to AF stabilization. The presence of the AF risk factors, age and HF, may modulate the AF-related coagulation state and thereby alter AF substrate development.

Outline of the thesis

The general aim of this thesis was to evaluate how AF progression is linked to hypercoagulability by means of activated blood coagulation (through thrombin and FXa) and consequent PAR-activation. Furthermore, we investigated the effect of the AF risk factors, age and HF, on AF substrate development and coagulation activity.

In **chapter 2** we provide a review of the hemostatic and non-hemostatic functions of the TF:FVIIa complex in the heart and vessels, together with a brief overview of the most important pleiotropic effects of thrombin and FXa.

Previously, thrombin and FXa have been implicated in promotion of cardiac remodeling. In **chapter 3** we investigate the direct effect of thrombin and FXa on the activation of primary cardiac fibroblasts (CF). Furthermore, we characterize the role of PAR activation in the pro-inflammatory cellular signaling mediated by FXa in this cell type.

The goat model of AF was used to answer our research questions *in vivo*. To investigate the main changes in the coagulation system related to AF, we report the customization of the Calibrated Automated Thrombography assay for goat plasma (**chapter 4**). This assay provides a global view of the goat coagulation profile by assessing thrombin generation in plasma.

The aim of **chapter 5** is to test whether inhibition of coagulation during AF attenuates the progression of this arrhythmia. This chapter evaluates the effect of long-term FXa-inhibition by rivaroxaban during four months of AF. Specifically, we test whether rivaroxaban affects fibrotic, hypertrophic and inflammatory signaling and modulates AF substrate development in the goat model of AF.

As age is the most important risk factor for AF, as well as for stroke in patients with AF, we hypothesize that age and AF synergistically interact to produce a prothrombotic state and favor AF substrate development. **Chapter 6** describes the relative contribution of age in the goat model of AF.

Finally, in **chapter 7** we assess the effect of HF, as an important risk factor for AF, on AF substrate development and thrombin potential. We hypothesize that HF induces a substrate that is favorable for the onset and progression of AF, potentially through alterations in coagulation activity.

Overall, the aim of this thesis is to provide a better understanding of the mechanistic link between AF, AF risk factors and coagulation, which could help to characterize disease processes that take place during the early onset of AF.

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Chapter 2

Tissue factor (:Factor VIIa) in the heart and vasculature: more than an envelope

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Thrombosis research, 2018. 168: p.130-13.

Abstract

Blood coagulation comprises a complex cellular and molecular mechanism that maintains vascular integrity, protects against bleeding (hemostasis) and responds to injury. However, several elements of the coagulation system, including several coagulation factors and platelets, are also involved in other physiological and pathological processes.

Tissue factor (TF) is a cell surface glycoprotein expressed in a vast variety of cell types and essential for hemostasis. Upon exposure of the TF-rich subendothelium to the blood stream, Factor VII (FVII) can bind to TF. TF subsequently facilitates the activation of FVII into activated FVII (FVIIa) thereby initiating the extrinsic coagulation pathway followed by the activation of FX and thrombin formation. Besides its hemostatic role in the vasculature, the TF:FVIIa pathway is active in many other compartments and organs where it can take part and mediate different physiological and pathological processes.

The so-called non-hemostatic functions of TF:VIIa play a role in diverse processes such as inflammation, atherosclerosis and vascular and cardiac remodeling. This narrative review aims to reassess the most important and recent findings regarding the complex signaling pathways initiated by the TF:FVIIa complex, with an emphasis on the heart and blood vessels. Understanding how the mechanisms of TF:FVIIa signaling contribute to both physiological and pathological processes, is one of the keys to the development of new treatment strategies in cardiovascular disease.

Keywords: blood coagulation, fibrosis, heart, inflammation, Tissue factor.

Introduction

Blood coagulation comprises a complex cellular and molecular mechanism that maintains vascular integrity, protects against bleeding (hemostasis) and responds to injury. Interactions between immunity and blood coagulation occur at various levels during physiologic as well as pathologic conditions ("thrombo-inflammation"): while inflammation is able to upregulate TF expression (increasing the capacity of initiating coagulation) and decrease the activity of the natural anticoagulant and fibrinolysis pathways, coagulation in turn, can modulate the inflammatory responses [1].

The plasmatic coagulation system starts with the exposure of TF-expressing subendothelial cells to the blood stream and the assembly of a complex between functionally active TF and its ligand FVII(a). The TF:FVIIa complex catalyzes the activation of FX into FXa and FIX into FIXa. Moreover recent evidence suggests direct activation of FVIII when TF:VIIa in complex with FXa [2]. The net effect of any of these reactions is the subsequent generation of thrombin, which yields fibrin out of fibrinogen. At the site of injury (wound bed, inflamed tissue) this mechanism leads to the formation of a clot, which involves a rapid cascade including thrombin driven platelet activation, assembly of platelet surface mediated catalytic complexes and fibrin formation.

In addition to these hemostatic functions several coagulation proteases and platelets are involved in other physiological and pathological processes such as inflammation, remodeling and fibrosis, so-called non-hemostatic functions [3]. The broad biological relevance of these functions becomes more tangible based on consistent data from in vitro and in vivo models, suggesting a number of pleiotropic and extravascular effects of coagulation proteases, on the cardiovascular system. An interesting example is the roles that FXa and thrombin play in triggering pro-fibrotic and pro-inflammatory responses in the heart thus in the progression of some cardiac pathologies. One example is atrial fibrillation (AF), the most common arrhythmia, where increased activation of the coagulation system (also known as AF-associated hypercoagulable state) could contribute to arrhythmia [4].

Since the potential non-hemostatic contributions of platelets, FXa and in particular thrombin, were recently reviewed [5], we focus on the currently somewhat underexposed role of the coagulation trigger, the TF:FVIIa complex in the vasculature with a focus on the heart and blood vessels.

Structure and conformations of Tissue Factor

Tissue factor is a 47-kDa cell-surface glycoprotein that belongs to the cytokine receptor superfamily. In this form, TF is expressed as full-length protein (flTF, from

now on TF). However, it can also be expressed as alternatively spliced TF (asTF) variant, which instead of the transmembrane and cytoplasmic domains, contains a hydrophilic carboxyl-terminal that makes it soluble [6]. TF is also subjected to post-translational modifications which can affect its activity. Specifically, Krudysz-Amblo and colleagues showed that the highly expressed placental TF is N-linked glycosylated in asparagines 11, 124 and 137 and contains high fucosylated and sialylated sugars. Moreover, the unique placental TF glycosylation not only differs from the one of the recombinant counterpart but is most likely responsible for its 5-fold higher activity when compared to the recombinant form. When deglycosylated, the activities of both placental and recombinant TF become comparable [7].

TF is an essential glycoprotein and TF null mice are not viable, dying in utero due to bleeding and with signs of abnormal vascular morphology [8-10]. A minimum level of TF is required for survival, such as achieved in "low TF" transgenic mice that express approximately 1% human TF relative to wild-type levels. Low levels of TF are compatible with development and hemostasis in mice [11]. To date, no congenital TF deficiency or defect have been described in humans, supporting the observation that TF gene deletion is not viable in mice.

Constitutively expressed TF is localized at the surface of vascular smooth muscle cells (VSMCs), pericytes and fibroblasts, and triggers blood clotting upon contact with blood, serving a role as "hemostatic envelope" [12]. In addition, it can also be present on extracellular vesicles (EVs), small membrane vesicles released by cells into the bloodstream mainly to mediate cell-cell communication. They can be derived from apoptotic cells as well as from activated cells (e.g. by platelets, monocytes and endothelial cells) and contribute to hemostasis, as reviewed elsewhere [13].

Many proteases of the coagulation system potentially cleave FVII to FVIIa in a purified system, but the rate of the zymogen-to-enzyme transition increases when FVII interacts with TF [14]. This phenomenon is explained by the unique amino acid sequence of the active site of FVIIa that retains it in a zymogen-like conformation until it binds TF [15]. The vast majority of non-EV derived TF exists in a cryptic configuration, characterized by a decreased pro-coagulant phenotype compared to its non-cryptic (or de-encrypted) configuration, independent of FVII(a) binding [16]. Evidence for the 2 different forms of TF came from a study by Le et al. showing that, in a carcinoma cell line expressing TF upon stimulation with FVIIa, the time needed to achieve maximum pro-coagulant activity was substantially shorter than the time needed to achieve saturated binding of FVIIa to TF, respectively 1 min and 30-60 minutes. At maximum TF pro-coagulant activity, only 10-15 % of the available TF was bound to FVIIa, leaving a high fraction of TF not cooperating in coagulation. Moreover, this study established that the prolonged incubation with FVIIa generated two different classes of TF:FVIIa complexes of which one was classified as

non-functional because of its lack of activity towards FX [17]. However, more conformational studies are needed to confirm these results.

Some authors suggest that most TF that resides in its cryptic form contributes to other cellular processes beyond hemostasis. Ahamed and colleagues showed that a unique monoclonal antibody specific for the cryptic conformation of TF (10H10 AB) was able to inhibit its binding with the G protein-coupled protease-activated receptor 2 (PAR-2) and block the TF:FVIIa cellular signaling in both human umbilical vein endothelial cells (HUVECs) and human HaCaT keratinocytes. Furthermore, it is possible that the activation of PAR-2 by the non-cryptic versus cryptic TF may mediate two distinct cellular responses by inducing the coupling of the receptor with different G proteins [18]. However, some authors raised skepticism regarding specificity of the 10H10 antibody for the cryptic form of TF [19].

The mechanisms controlling decryption of TF remain to be established, but evidence shows that membrane-bound lipids, such as phosphatidylserine, are involved in decryption of TF.

According to what is known so far, the association of TF with phospholipids appears to be required for its procoagulant activity and the presence of phosphatidylcholine in the phospholipid mixture is able to accelerate it. The mechanism by which this happens is unclear. A possible explanation which however remains controversial and only partially clarifies this phenomenon, could be that increased concentration of phospholipids at the outer leaflet of the plasma membrane could enhance the FX binding to the membrane which can lead to an increased interaction with the TF:FVIIa complex. Another plausible model (much more difficult to prove) could be that the direct interaction of PS with TF or TF:FVIIa complex could result in a change of TF quaternary structure that would facilitate its binding with FX [19]. Recently Wang and colleagues showed that sphingomyelin, the major phospholipid in the outer leaflet, may also play a role in regulating TF decryption. They reported that hydrolyzation of macrophages sphingomyelin increased TF activity and TF^{+} EVs release [20].

Additionally it is suggested that that protein disulfide isomerase (PDI), an oxidoreductase protein released by injured cells and platelets, also aids in decryption, as extensively reviewed elsewhere [21, 22].

Cellular signaling of Tissue Factor

Under physiologic conditions, TF:FVIIa signaling contributes to restoration of damaged tissue by regulating inflammation, angiogenesis and tissue remodeling.

The first studies demonstrating TF:FVIIa to trigger intracellular signaling showed cytosolic Ca^{2+} oscillations upon FVIIa binding to TF in different types of

TF-expressing cells (Figure 1A) [23]. This effect appeared to be independent of the presence of the TF cytoplasmic domain and variable among different cell types transfected to overexpress TF or constitutively expressing the receptor [24]. Further studies reported that FVIIa binding to cells transfected with human TF, such as BHK cells, and to a lesser extent fibroblasts and keratinocytes, resulted in the activation of multiple signaling pathways such as the transient activation of P44/42 MAPK which also appeared to be independent of TF cytoplasmic domain [25]. TF:FVIIa downstream signaling affects the expression of many genes, including vascular endothelial growth factor (VEGF), early growth response protein 1 (ERG-1), urokinase receptor (uPAR), and interleukin-8 (IL-8), which are also potentially relevant in pathologies related to TF overexpression such as tumor angiogenesis, cancer and inflammation [26-29]. The relevance of the TF cytoplasmic domain for TF:FVIIa signaling remains incompletely understood; mutations in this domain were not associated with embryonic lethality or impaired hemostasis [30]. A functional role for the TF cytoplasmic domain was first reported in a melanoma metastasis model, where mutations of the TF intracellular domain reduced TF-mediated metastasis but not coagulation activity (Figure 1B) [31]. Later, a study on hematogenous metastasis confirmed these findings and showed that phosphorylation of the cytoplasmic tail at the sites Serine 253 and Serine 258 was required to trigger intracellular signaling associated with the progression of metastasis [32]. In cancer and kidney cells, this phosphorylation was attributed mainly, but not only, to protein kinase C (PKC) [33].

Another relevant mechanism of engagement of intracellular signaling pathways takes place upon activation of protease activated receptors (PARs)(discussed in part 2), which can occur either by activation through the TF:FVIIa complex, or via other proteases like thrombin and FXa (Figure 1C) [3].

Despite the relevant findings that *in vitro* studies have brought to our attention, it is currently still unknown to what extend these different TF:FVIIa directed pathways are operational and relevant *in vivo*.

Crosstalk between TF-FVIIa pathway and inflammation

Inflammation induces TF expression

The TF:FVIIa pathway and inflammatory system interact with each other. TF gene expression seems to be principally mediated by intracellular transduction pathways normally activated by pro-inflammatory stimuli. Lipopolysaccharide (LPS), cytokines and the interaction of cell surface adhesion molecules with components of the extracellular matrix, increase tyrosine phosphorylation of intracellular proteins

in ECs and macrophages, and result in the recruitment of mitogen-activated protein kinases (MAPK) and IκB Kinase (IKK). The activation of these pathways is fundamental for the transcription of the TF gene as demonstrated by the abrogation of TF expression in response to the selective MAPK inhibition in monocytes [34]. Moreover, IKK transduction signaling culminates in the degradation of the inhibitor for nuclear factor-κB (NF-κB), the main nuclear transcription factor involved in TF-induced inflammation in ECs and monocytes. The inflammatory mediators that regulate TF gene expression include several cytokines that stimulate its expression in monocytes and EC, including tumor necrosis factor-α (TNFα), IL-1α, IL-2, IL-6 and IL-12 [35, 36]. Other cytokines with pro-coagulant properties studied *in vitro* include IL-1β, IL-8, interferon-γ (IFN-γ) and monocyte chemoattractant protein 1 (MCP-1) [37]. Recently, IL-33 was shown to induce TF mRNA and protein expression, and increase TF cell surface activity in cultured ECs [38].

Furthermore, TF expression is expressed in monocytes and gets greatly upregulated after activation. Monocyte adhesion and transmigration is mediated by monocyte/macrophage surface adhesion molecules such as VLA-4, Mac-1, which regulate contact with extracellular matrix proteins and the subsequent cell activation and interaction with other inflammatory cells [39]. One of the mechanisms underlying this process involves the MAP kinase proteins that phosphorylate the transcription factor NF-kB which migrates to the nucleus and enhances TF gene expression [34].

Hypoxia and the resulting failure by cells to meet metabolic requirements can also trigger inflammation [40]. Many studies have shown that hypoxic conditions increase TF expression in podocytes, monocytes/macrophages and SMCs and consequently promote fibrin deposition [41, 42]. An example that hypoxia contributes to the progression of atherosclerosis was provided by Marsch et al. showing that reoxygenated as compared to hypoxic atherosclerotic plaque more efficiently cleared apoptotic cells leading to smaller necrotic cores [43]. It is tempting to speculate about the contribution of TF in this process, giving the intertwined relationship TF has with both inflammation and hypoxia. We will further elaborate on this in the atherosclerosis paragraph.

Coagulation-dependent inflammatory signaling

Coagulation can also modulate the inflammatory system by different mechanisms and a strong correlation between activation of the coagulation cascade and an increase in pro-inflammatory cytokines levels in blood has been reported [1]. Coagulation proteases like thrombin are able to stimulate the EC and monocytic production of important inflammatory mediators, such as IL-6 and IL-8 [44, 45]. Furthermore, the direct binding of TF splicing variants to β1 integrins on ECs induced

the expression of adhesion molecules (CAMs) in ECs thus promoting monocyte adhesion to and transmigration of endothelial cells [46].

One of the established mechanisms by which coagulation influences inflammation is through activation of PARs by coagulation proteases [47]. PARs are a subfamily of G-proteins coupled receptors (GPCRs) involved in numerous physiological and pathological processes [3, 48]. PARs are characterized by a single polypeptide chain with a central core domain made of seven transmembrane α-helices connected by three intracellular and extracellular loops. PARs get proteolytically cleaved at the n-terminus which generates a newly exposed ligand that self-activates the receptor [49]. To date, the PAR family comprises four different isoforms (PAR-1 to -4) variously expressed by different cell types including ECs, mononuclear cells, platelets, fibroblasts, and SMCs. The expression pattern of PARs is evident in the vasculature, including the heart [50]. Thrombin is the main activator of PAR-1, -3 and -4, FXa mainly activates PAR-1 and PAR-2 and possibly PAR-3 as reported by Stavenuiter et al. [51, 52]. Other proteases are also able to activate the various PARs: e.g. trypsin and TF:FVIIa (or in complex with FXa) can activate PAR-2, activated protein C (APC) cleaves PAR-1. In addition, transactivation of PAR-2 by PAR-1 can occur when the TF:FVIIa, in complex with FXa, activates PAR-1 (Figure 2).

The mechanism by which the TF:VIIa complex can activate PAR-2, on the same cell membrane, requires TF to bind and activate FVII into FVIIa which in turn cleaves the extracellular domain of the PAR receptor. Similarly, PAR-1 is cleaved and activated by FXa when the coagulation factor is in complex with TF:VIIa [53].

Interestingly, the activation of PAR-1 and PAR-2 represents the most important mechanism driving coagulation-dependent inflammatory responses. In this concept, TF together with FVIIa and the nascent products FXa and thrombin, may play a pivotal role [54]. Although the APC-mediated cleavage of PAR-1 has been reported to have a protective effect on the endothelial barrier, thrombin-mediated PAR-1 activation mostly results in the initiation of proinflammatory events and enhancement of permeability in ECs [55]. PAR-2 mediated signaling has been shown to be responsible for the enhancement of monocytic pro-inflammatory functions, resulting in the production of reactive oxygen species and pro-inflammatory cytokines [54-56].

Moreover, Pawlinski et al. used a murine model of endotoxemia to show that a combination of thrombin inhibition and PAR-2 deficiency, reduces both inflammation and mortality which was also observed in mice expressing low levels of TF [57]. Active PAR-2 is also able to mediate a self-perpetuating positive feedback on TF:FVIIa signaling involving phosphorylation of the cytoplasmic tail of TF by PKC activation, thereby amplifying or continuing the inflammatory response induced by the TF pathway (Figure 1B) [58]. In addition, the TF cytoplasmic domain seems to be capable of self-regulating its expression in a TLR4 erk1/2 dependent and PAR-2

independent manner. This became evident by a study in which isolated macrophages from LPS-induced endotoxemic mice that carried a TF cytoplasmic domain deletion ($TF^{\Delta CT}$), showed upregulated TF expression and procoagulant activity via TLR4 erk1/2 signaling independent of PAR-2 activation [59].

TF-FVIIa role on vascular remodeling and atherosclerosis

Atherosclerosis is a chronic, progressive inflammatory disease of the vessel wall characterized by the formation of plaques that narrow the lumen. Unstable atherosclerotic plaques have a highly pro-thrombotic content and are prone to rupture, triggering atherothrombosis, the cause of acute coronary syndrome and non-embolic stroke. Under physiological conditions, TF expression is hardly expressed in the intima and media of the vessel wall. However, during atherosclerosis, TF is increasingly and widely expressed throughout the plaque [60]. Importantly, most pro-coagulant activity inside plaques is considered to be TF mediated and dependent on monocytes [61].

Migration of VSMCs, one of the morphological features of atherosclerosis, is an important determinant of plaque stability because of their remodeling activities that stabilize the fibrous cap [62]. The cytosolic domain of TF contributes in FVIIa dependent migration of VSMCs. Active site inhibited FVIIa (ASIS), which has higher affinity to TF than wt FVIIa, increased the migration of wt aortic SMCs [63, 64]. However, in $TF^{\Delta ct/\Delta ct}$ aortic SMCs (SMCs with a TF cytosolic domain deletion) this effect of ASIS was abolished, suggesting a FVIIa dependent inhibition of VSMC migration that relies on signaling cascades downstream of the cytosolic domain of TF [29, 63, 64]. Marutsuka et al. further tried to elucidate this mechanism and were able to show that migration relied on the communication of TF:FVIIa with PAR-2 [65]. More proof of this TF:FVIIa:PAR-2 axis came a few years later when in an angiogenesis model, TF:FVIIa mediated migration of ECs was shown to be dependent on both the cytosolic domain of TF and PAR-2, of which the activity of the latter was tightly regulated by the TF cytosolic domain [66]. TF mediated increased motility of VSMCs is effectuated by TF induced signaling through the Wnt/β-catenin pathway, with Rho GTPases as key mediators. Additionally, more confirmatory data on the signaling molecules involved in TF:FVIIa:PAR2 mediated migration of cells, came some years later from a knock-down study of β-catenin and PI3K (upstream of β-catenin) inhibition, which both where able to attenuate PAR-2 mediated metastasis of breast cancer cells. Although this study used immortal MCF7 epithelial cells, these and other findings suggest a common pathway in VSMCs [67].

Additional circumstantial evidence of a role of TF in vascular remodeling came from a study showing that microbiota increased glycosylation of TF in the small in-

testines, which led to PAR-1 mediated TF signaling that ultimately initiated the Ang-1-Tie-2 axis, known to promote remodeling of blood vessels [68-70].

Cells that are in a hypoxic environment produce various proteins (e.g. VEGF, HIF-1alpha and EGR-1) to induce angiogenesis as a self-sustaining mechanism to improve oxygen supply [71, 72]. When the atherosclerotic plaque starts to grow towards the lumen, angiogenesis is needed to maintain nutrition and oxygenation of the expanding plaque contents. This process is associated with increased expression of cellular TF in the plaque, comparable to upregulated TF expression in tumor cell models to induce angiogenesis and resolve hypoxia. In tumor cells this process involves the activation of integrins that subsequently signal through PI3K and MAPK-akt pathway to upregulate HIF-1α and VEGF [73]. Whether this, in parallel, creates a TF mediated hypercoagulable state by inducing the upregulation of decrypted TF, is not totally understood. However, when considering that activated endothelial cells release PDI during inflammation, it is arguable that in the tumor environment, but also in atherosclerotic plaques, the upregulated form of TF gets decrypted by PDI and creates a hypercoagulable state as seen in both microenvironments of the pathologies. Indeed, in atherosclerotic plaque material TF pro-coagulant activity is evident, depending on the state of lesion development [74]. Already in 1988 Wilcox and colleagues reported high TF mRNA expression in several regions of human atherosclerotic plaques and an extensive TF protein localization in the extracellular matrix of their necrotic cores. The majority of the TF-mRNA and protein positive cells were macrophages and monocytes [60]. In addition, many coagulation factors were detected in advanced atherosclerotic plaques suggesting the presence of coagulation activity within the lesion. This hypothesis was confirmed when also fibrin and fibrin cleavage products were found in the advanced lesions [61]. Borissoff et al, analyzed the relationship between atherosclerosis and the procoagulant phenotype of the plaques. Interestingly while the presence of both pro and anti-coagulant proteins was detected in both early and old plaques, the activity of some of them such as TF, FXa, and thrombin were significantly more pronounced in early atherosclerotic lesions than in stable advanced atherosclerotic lesions. These findings reconfirmed that thrombin generation is an important and active process during atherogenesis. Furthermore, the enhanced pro-coagulant phenotype of the early lesions suggested that the activation of the coagulation proteases may be essential for the initial burst towards atherosclerosis progression, triggering cellular migration, adhesion, angiogenesis and inflammation. In this context the role of TF as trigger for the activation of coagulation appears once again to be crucial [74].

TF:FVIIa pathway in the heart

Localization and structural function of cardiac TF

TF provides hemostatic protection from mechanical injury to the blood vessels, particularly in organs rich in TF expression, including the lungs, brain, heart, testis, uterus, and placenta [75]. In the human adult heart, TF content is highest in the left ventricle, intermediate in the left atrium and right ventricle and lowest in the right atrium likely due to the differences in mechanical stress and risk of damage these compartments are subjected to [76]. Pawlinski et al. demonstrated a correlation between low cardiac myocyte TF expression and increase of cardiac hemostatic defects. In this study, low-TF mice show hemosiderosis, indicative of local hemorrhage, and fibrosis in their hearts under both physiological and pathological conditions [77]. Similar results were obtained in mice with low FVII expression but not with low-FVIII and FIX levels, suggesting a specific role of the TF:FVIIa pathway in cardiac "hemostasis" [78]. Human and murine cardiomyocytes constitutively express TF during early and late stages of their development, also suggesting a morphogenic role in cardiogenesis [75, 79]. Interestingly, immunohistochemical studies on adult cardiomyocytes showed TF to localize to the transverse parts of the intercalated discs together with cytoskeletal and adhesion proteins [80]. This peculiar intracellular distribution may additionally reflect a non-hemostatic role of TF in the maintenance of the structure and function of the cardiac muscle. The transverse components of the intercalated discs are rich in adhesion molecules that interact with cytoskeleton proteins, with which TF may establish a connection through its cytoplasmic domain. A possible analogue is that both in endothelial and cancer cells, the cytoplasmic domain of the receptor can bind a specific actin binding protein, ABP-280, and support cellular adhesion and migration through induction of the phosphorylation of focal adhesion kinase [81]. Luther and colleagues demonstrated that the content of TF in the left ventricle of hypertensive people with ventricular hypertrophy, a condition characterized by a decreased number of intercalated discs per muscle mass, was significantly reduced in comparison to the content in the left ventricle of healthy patients. Moreover, they showed that necrotic lesions and/or dilatation of the cardiac muscle of people dying of septic shock, correlated with a significant decrease in TF protein concentration compared to the non-septic myocardium [76]. These observations suggest a loss in myocardial TF in association with pathological changes in the heart, either as cause or consequence. Another role of TF in the heart is pointed out by Boltzen et al. showing a positive correlation between TF expression and protection from programmed cell death. Murine cardiomyocytes expressing high levels of both asTF and flTF, present reduced phosphatidylserine exposure and

upregulation of anti-apoptotic proteins upon TNF-α stimulation leading to a reduction of the caspase-cascade activation and cardiomyocyte death [82].

TF:VIIa in the heart

Besides its protective functions, cardiac TF is also implicated in a wide array of pathological processes such as inflammation and fibrosis. In a rabbit coronary ligation model, the use of monoclonal antibodies against TF reduced infarct size and inflammation in the heart after myocardial ischemia-reperfusion (I/R) injury. In parallel, inhibition of the TF-thrombin pathway, led to a decrease in cytokine expression associated with leukocyte infiltration in the heart [83]. Similarly, in a murine I/R injury model, the inactivation of the TF:FVIIa complex reduced myocardial I/R injury most likely through a reduction of inflammation driven by the NF-kB pathway [84]. Taken together, these results suggest that, also in the heart, the activation of the TF pathway is able to trigger inflammatory responses. Among the several molecular mechanisms by which this process can take place, one in particular seems to require the activation of cardiac PAR-2 which has been shown to trigger pro-inflammatory signaling in cultured cardiomyocytes [85].

The PAR-2-mediated inflammatory responses in the myocardium may result from its activation on different cell types. PAR-2 signaling is important not only for leukocyte adhesion and rolling but also triggers the expression of pro-inflammatory mediators in ECs and cardiomyocytes [86]. Antoniak et al. demonstrated that PAR-2 deficiency in mice can decrease inflammation following I/R injury, and since TF:FVIIa complex is one of the triggers of PAR-2 activation, its specific binding to the receptor might be the crucial mechanism driving this pathological process [87, 88].

Interestingly, in the same rabbit model of I/R injury, both TF mRNA levels and pro-coagulant activity were increased in the regions of the left ventricle exposed to ischemia (at risk regions) in comparison to non-at-risk regions as well as to the left ventricle of the sham-operated animal group. These findings suggest that hypoxic conditions in the heart can enhance not only TF expression but also its pro-coagulant activity. Additional findings supporting this hypothesis come from *in vitro* studies on different types of cancer cells which demonstrated that hypoxia is responsible for the upregulation of TF pathway signaling, enhancement of TF pro-coagulant activity and downregulation of TF pathway inhibitor (TFPI) expression [89,90]. In this context, the effects of hypoxic conditions on the signaling and pro-coagulant activity of the TF pathway, could be relevant for understanding the link with certain cardiac pathologies. Atrial fibrillation for instance, the most common form of sustained arrhythmia, is a progressive age-related disease also characterized by a hypercoagulable state and an increased risk of thromboembolic events. Little is known about the

molecular mechanisms by which the coagulation system becomes activated during this pathological condition. In acute AF, a mismatch between oxygen demand and supply in the atria and an upregulation of the HIF pathway in the right atrial appendages takes place [91,92]. Moreover, both TF plasma levels and protein expression in atrial endothelia of AF patients are increased in comparison to the healthy control group, pointing to a possible correlation between hypoxia and AF-related hypercoagulable state [93, 94]. Further studies are needed to assess whether the hypoxic environment in the heart during AF is sufficient to trigger activation of the TF-pathway, and if that is the case, to understand the underlying molecular mechanisms. In addition, local coagulation protease activity may trigger and/or aggravate AF, although the specific contribution of TF in that process needs to be established [4].

The TF:FVIIa pathway is also implicated in the onset of cardiac fibrosis, possibly unrelated to the impaired hemostasis, also characteristic for the low TF mice. Low-TF mice develop cardiac fibrosis in an age and sex dependent manner. Females present less pronounced cardiac remodeling and collagen deposition in comparison to males that additionally develop fibrosis earlier in life [95]. This phenomenon seems to be dependent on the effect that gender has on uPA expression in this model. In tissues, uPA converts plasminogen into plasmin which degrades matrix components and activate matrix metalloproteinases (MMPs) thus affecting cardiac remodeling.

Others demonstrated that female sex hormones can reduce uPA expression in different cell types, an effect opposed by thrombin [95, 96]. In female low-TF mice, uPA expression is lower compared to the males suggesting that in this scenario, thrombin production might be not sufficient to counteract the uPA-lowering effects of estrogen and that TF and/or thrombin may control cardiac uPA expression, thus influencing cardiac remodeling in a gender dependent manner [95].

Therapeutic strategies

It is nowadays clear that the biological functions of the TF:FVIIa pathway are not confined to blood coagulation and maintenance of the hemostatic balance in the vasculature; it is active in many other compartments and organs where it can take part in and mediate many different processes. Obviously, it remains difficult to dissect the contributions of TF:FVIIa from those of the downstream proteases generated upon initiation of the coagulation cascade, including FXa and thrombin.

It is challenging to identify effective therapeutic strategies able to target its pathological activities while preserving its protective functions. The direct blockage of PARs or the complete inhibition of the TF pathway will increase the risk of bleeding,

hence selective modulation of TF:FVIIa signaling is needed [97]. So far, different anti-TF interventions have been tested in experimental studies with promising antithrombotic efficacy, but also with anti-inflammatory and anti-fibrotic potential. An example is active site inactivated FVIIa (ASIS) that competes with FVII for TF bin-ding, forming ASIS:TF which is unable to initiate coagulation. ASIS' affinity for TF remains lower than FVIIa affinity therefore even when high concentrations of the protein are administered, some active TF:FVIIa complexes can still form and activate the coagulation system. This peculiarity would have made ASIS an interesting antithrombotic drug with relatively few anti-hemostatic side effects as determined in studies on rabbit models of arterial thrombosis in which the drug was administrated topically or by infusion into the carotid artery [98, 99]. However, the trial that followed these promising preclinical studies failed to prove its safety pointing out an increased mortality and risk of major bleeding in the group of patients receiving the highest dose of the drug [100]. Among the different compounds tested as selective inhibitors of the TF:FVIIa pathway, recombinant TFPI (rTFPI) is the most broadly studied drug. TFPI, mainly synthetized by vascular ECs, is the natural endogenous TF:FVIIa complex inhibitor which binds to the active site of factor Xa and subsequently inhibits TF:VIIa activity forming the quaternary complex TF-factor VIIa/TFPI-factor Xa [101]. rTFPI inhibits atherosclerotic plaque thrombogenicity and subsequent platelet and fibrinogen deposition *in vitro*. Moreover, its local gene transfer and overexpression at a site of balloon injury in porcine carotid arteries, prevented platelet-dependent thrombus formation without detectable derangements in hemostasis [102]. Some pathological circuits driven by the TF pathway can also be targeted by novel and more sophisticated approaches such as at specific post-transcriptional modifications of the TF gene. Serine/arginine-rich (SR) proteins and the respective SR proteins kinases are important splicing factors which orchestrate the alternative splicing of genes including TF. In human ECs, SRF1, a particular SR protein, inhibits the expression of both TF isoforms under inflammatory conditions suggesting that pro-inflammatory signals may also affect TF expression at a post-transcriptional level [103]. In this context, components of the alternative TF splicing machinery may be considered potentially attractive therapeutic targets. Recently, non-coding microRNAs have also been discovered to modulate TF expression not only in cancer cells but also in ECs. An example is miRNA9b, the most upregulated miRNA in patients with angina-related pro-thrombotic state, which targets TF mRNA and downregulates its expression. MicroRNA-19b may have anti-thrombo-tic properties in patients with unstable angina by targeting TF [104]. However, cynomolgus monkeys were recently subjected to TF monoclonal antibody treatment and the authors observed hemorrhagic lesions in various organs [105].

Thus, how tempting anti-TF therapy may sound, it will be very challenging to cope with the enormous bleeding potential anti-TF therapy has. Targeting the TF:FVIIa complex will only be a promising therapeutic value if implemented properly, preserving its reparative and protective functions. However, currently we are on the verge of elucidating TF:VIIa signaling and before properly tuning its function into therapeutic value, both its protective and reparative function as well as its disruptive role should be unraveled.

Acknowledgements

The authors gratefully acknowledge the Dutch Heart Foundation (CVON 2014-09, Reappraisal of Atrial Fibrillation: Interaction between HyperCoagulability, Electrical Remodeling, and Vascular Destabilization in the Progression of Atrial Fibrillation - RACE V) for financial support. We appreciate the critical reading and input on the revised manuscript by dr. Nigel Mackman, McAllister Heart Institute, University of North Carolina, Chapell Hill, North Carolina, USA.

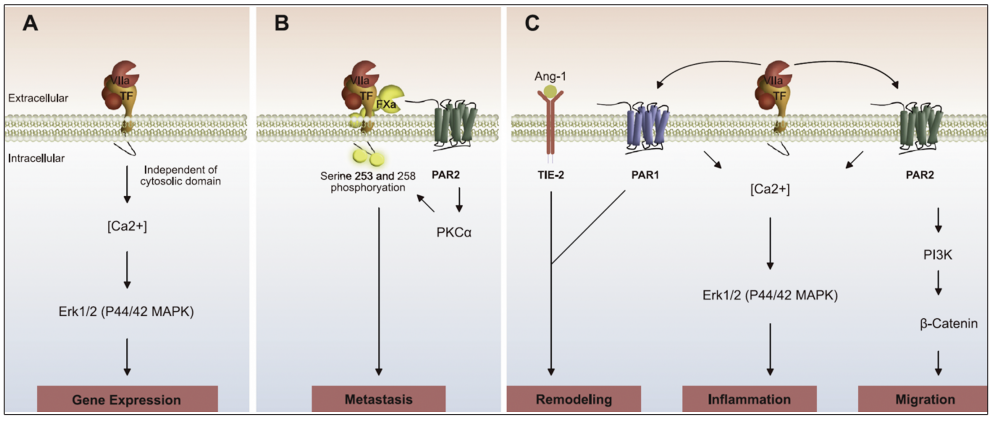

Figure 1. TF:VIIa-mediated intracellular signaling.
A. TF:FVIIa triggers intracellular calcium oscillation independently of the TF cytoplasmic domain. Upon this signaling, the transient activation of P44/42 MAPK takes place and leads to VEGF, ERG-1, uPAR gene expression. B. A functional role for the TF cytoplasmic domain was described in a melanoma metastasis model. Phosphorylation of its tail at sites Serine 253 and 258 by PKC triggers the progression of metastasis. C. Additionally, TF:VIIa engages intracellular signaling through activation of PAR-1,2 receptors. This leads to remodeling of the vessel wall and cellular migration respectively (C).

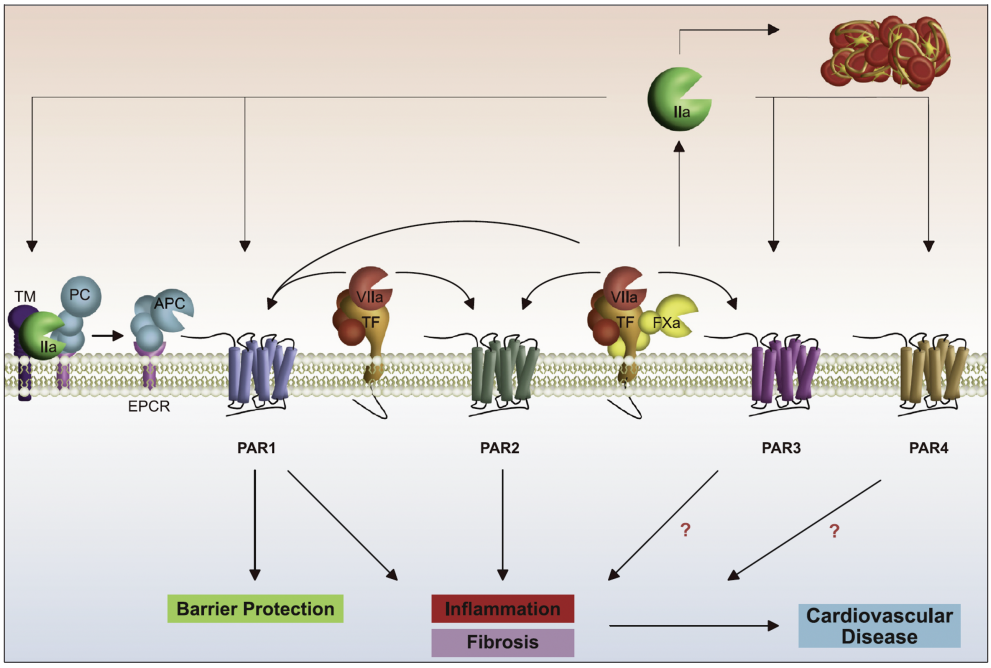

Figure 2. The role of coagulation proteases in cardiovascular disease.
After the initiation of the coagulation cascade, various serine proteases of the coagulation system get activated and ultimately aid in the formation of a stable blood clot. Alternatively, these serine proteases can activate protease activated receptors (PAR) that trigger intracellular signaling contributing to the development of various cardiovascular diseases, including atherosclerosis and atrial fibrillation. The role of PAR3 and PAR4 in cardiovascular disease is still speculative and has to be elucidated.

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Chapter 3

Coagulation Factor Xa induces pro-inflammatory responses in cardiac fibroblasts via activation of Protease-Activated Receptor-1

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Cells, 2021. 10(11): p. 2958.

Abstract

Background

Coagulation factor (F) Xa induces proinflammatory responses through activation of protease-activated receptors (PARs). However, the effect of FXa on cardiac fibroblasts (CFs) and the contribution of PARs in FXa-induced cellular signalling in CF has not been fully characterised.

Methods

To answer these questions, human and rat CFs were incubated with FXa (or TRAP-14, PAR-1 agonist). Gene expression of pro-fibrotic and proinflammatory markers was determined by qRT-PCR after 4 and 24 h. Gene silencing of *F2R* (PAR-1) and *F2RL1* (PAR-2) was achieved using siRNA. MCP-1 protein levels were measured by ELISA of FXa-conditioned media at 24 h. Cell proliferation was assessed after 24 h of incubation with FXa ± SCH79797 (PAR-1 antagonist).

Results

In rat CFs, FXa induced upregulation of *Ccl2* (MCP-1, > 30-fold at 4 h in atrial and ventricular CF) and *Il6* (IL-6, ± 7-fold at 4 h in ventricular CF). Increased MCP-1 protein levels were detected in FXa-conditioned media at 24 h. In human CF, FXa upregulated the gene expression of *CCL2* (> 3-fold) and *IL6* (> 4-fold) at 4 h. Silencing of *F2R* (PAR-1 gene), but not *F2RL1* (PAR-2 gene), downregulated this effect. Selective activation of PAR-1 by TRAP-14 increased *CCL2* and *IL6* gene expression, this was prevented by *F2R* (PAR-1 gene) knockdown. Moreover, SCH79797 decreased FXa-induced proliferation after 24 h.

Conclusions

In conclusion, our study shows that FXa induces overexpression of proinflammatory genes in human CFs via PAR-1, which was found to be the most abundant PARs isoform in this cell type.

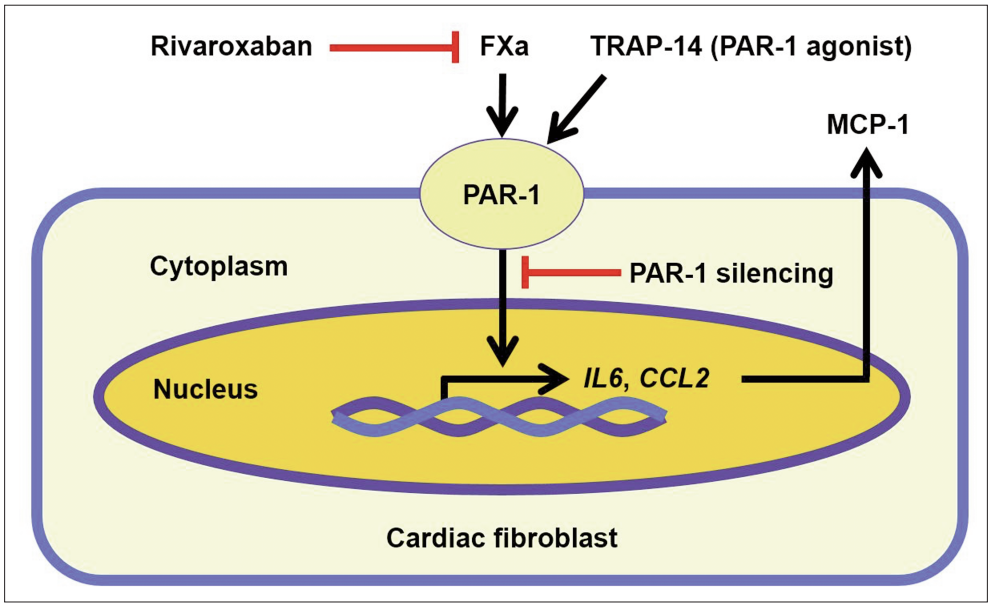

Figure A. Graphical abstract.

Keywords: coagulation FXa, cardiac fibroblasts, inflammation, PARs.

Introduction

Cardiac fibroblasts (CFs) are essential to maintain homeostasis of the heart [1]. Upon different types of cardiac injury (e.g., myocardial infarction, hypertensive heart disease), quiescent CFs acquire a more proliferative, migratory, and secretory phenotype, and can differentiate into myofibroblasts that express contractile proteins, such as alpha-smooth muscle actin (α-SMA), and increase collagen deposition [2-4].

Moreover, CFs have been reported to play an important role in the inflammatory processes that occur within the heart upon acute and chronic injury [5]. In the acute phase after myocardial infarction (MI), CFs respond to proinflammatory stimuli (e.g., reactive oxygen species (ROS), interleukin (IL)-1, and tumour necrosis factor alpha (TNF-α)) in the ischemic microenvironment by altering their gene expression profile and acting as inflammatory supporter cells [6]. In this phase, CFs produce large amounts of cytokines, such as IL-6, IL-1, and chemokines, such as IL-8 and monocyte chemoattractant protein (MCP)-1, supporting and modulating the recruitment of leukocytes that clear the tissue of dead cells and matrix debris [5, 6].

Recently, coagulation factors, such as thrombin and factor (F) Xa, have been implicated in the activation of CFs and promotion of cardiac remodelling [7, 8]. These non-haemostatic effects are mediated by activation of a family of G protein-coupled receptors, the protease-activated receptors (PARs) [9]. The PAR family comprises four different isoforms (PAR-1 to -4) variously expressed by different cell types including platelets, endothelial cells, mononuclear cells, and CFs. Serine proteases, such as thrombin and FXa, proteolytically cleave PARs at their N-terminus, thereby generating a new tethered ligand, which self-activates the receptor [10].

PAR-1 activation by thrombin promotes the expression of pro-fibrotic markers, such as α-SMA and transforming growth factor beta 1 (TGF-β1), but also of proinflammatory markers, such as IL-6 and MCP-1, in adult rat CFs [7].

Similarly, FXa has been reported to be a strong in vitro inducer of proinflammatory responses acting mainly via activation of PAR-2 [11]. In fact, PAR-2 activation has often been associated with pathological processes characterized by an increased inflammatory state (e.g., atherosclerosis) [12]. Recently, however, Friebel and colleagues reported that reduced expression of PAR-2 in endomyocardial biopsies of patients with heart failure with preserved ejection fraction (HFpEF) aggravated lymphocyte and macrophage infiltration and myocardial fibrosis [12, 13]. Moreover, Shinozawa et al. showed that FXa upregulated the expression of the proinflammatory MCP-1 in human umbilical vein endothelial cells and that this effect was markedly inhibited by a selective PAR-1 inhibitor [14].

These apparently contrasting findings suggest that PAR-1 activation may also play a role in mediating FXa proinflammatory activity. Moreover, the specific contribution of PAR-1 and PAR-2 activation on FXa signalling on CF has not been fully characterised yet.

Therefore, the aim of this study was to investigate the effect of FXa on adult rat and human CFs and clarify the role of PAR-mediated proinflammatory signalling in these cells.

Materials and methods

Isolation and culture of adult rat CF

Adult surplus rats (Lewis or Wistar strains, aged between 5 and 52 weeks) were euthanized according to the guidelines of Animals Act 1986 (Scientific Procedures).

The hearts were excised, and atrial and ventricular tissue was separately minced into small pieces and digested with 2 mg/mL of Worthington Collagenase Type II (Merck KGaA, Darmstadt, Germany) as previously described [15, 16]. Both atrial and ventricular CFs were isolated using differential plating and cultured until 80% confluency in Dulbecco's Modified Eagle's medium (DMEM, no. 22320, Gibco, Invitrogen, Breda, the Netherlands) supplemented with 10% foetal bovine serum (FBS, Gibco) and gentamicin (50 μg/mL, Gibco). Cells were harvested, frozen and stored in liquid nitrogen, and used from passage 1-3.

Experiments were performed with the approval of the Animal Ethical Committee of Maastricht University (DEC-2007-116, July 31, 2007) and conform to the national legislation for the protection of animals used for scientific purposes.

Isolation and culture of human atrial CFs

All atrial biopsies were obtained from patients undergoing different cardiac surgery procedures, after informed patient consent and approval of the Institutional Ethics Committee of the Maastricht University (the Netherlands, Ref. 162012), or the local ethical committee of the Leeds Teaching Hospitals NHS Trust (United Kingdom, Ref. 01/040). CFs were isolated as described above and cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco), gentamicin (50 μg/mL, Gibco), 1% Insulin-Transferrin-Selenium-Sodium Pyruvate (ITS-A, Gibco), and basic fibroblast growth factor (1 ng/mL, Gibco). Cells were harvested, frozen and stored in liquid nitrogen, and used from passage 1-4.

Stimulation of CFs with thrombin and FXa

Rat atrial or ventricular CFs were seeded at 10,000 cells/cm^2, cultured for 24 h, and serum-starved for 24 h prior exposure to bovine thrombin (8 pM, Synapse, Maastricht, the Netherlands) or bovine FXa (100 nM, Synapse).

Similarly, human atrial CFs were seeded at 10,000 cells/cm^2, cultured for 24 h, and serum-starved for 24 h, and subsequently exposed to purified human FXa (100 nM, Hyphen, Biomed, Neuville-sur-Oise, France) or PAR-1 agonist (TRAP-14, 100 μM, Bachem, Bubendorf, Switzerland). The selective FXa Inhibitor, rivaroxaban (400 ng/mL, Bayer, Leverkusen, Germany), was incubated with FXa for 10 min before addition to the cells. Effects on gene expression were determined at 4 and 24 h.

MCP-1 assay

Conditioned media of rat CFs incubated with FXa for 24 h, were collected and stored at -80 °C for analysis. MCP-1 DuoSet ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN., USA).

Proliferation assay

Human CFs (200 cells/cm^2) were seeded in 96-well plates, cultured for 24 h, and serum-starved for 24 h. Subsequently, cells were incubated with human FXa (100 nM) with or without PAR-1 antagonist (1 μM, SCH79797, Tocris, Bioscience, Bristol, UK). All the stimuli were prepared in the presence of BrdU (10 μM) and 0.1% FBS. After 24 h of incubation with the stimuli, Cell Proliferation ELISA, BrdU (Roche, Basel, Switzerland) was performed according to the manufacturer's instructions.

Gene silencing

Adult human CFs were transfected with 10 nM *F2R* (PAR-1) or *F2RL1* (PAR-2), specific Silencer Select Pre-Designed siRNA (PAR-1: SASI-Hs01-00240436, PAR-2: SASI-Hs02-00339249, Merck) or MISSION SiRNA Universal Negative Control No. 1 siRNA (SCI001, Merck), using Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA) in OptiMEM (Gibco), as per the manufacturer's instructions. Medium was replaced with DMEM supplemented with 0.1% FBS 24 h later. Cells were used for experimentation 48 h after transfection.

Gene expression analysis

Total RNA was collected using the Micro-Elute Total RNA kit (Omega-Bio-Tek, Norcross, GA, USA) and reverse transcribed with Iscript into cDNA (Biorad,

Hercules, CA, USA) according to the manufacturer's protocol. Gene expression levels were measured with a CFX96 Touch Real-Time PCR detection system (BioRad) and SYBR Green Supermix technology (BioRad). Expression levels were normalized for Cyclophilin-A and calculated using the comparative threshold cycle method (ΔCt). The gene expression values were multiplied by 1000 to enhance readability. The sequences of the specific primers used are provided in Table S1 and S2.

Statistical analysis

All data in the figures are expressed as median bars, with *n* representing the number of cell isolations from different rats or patients, unless stated otherwise. Rat RT-qPCR analyses involving CTRL and either thrombin or FXa as the stimulus, and human RT-qPCR on *F2R* (PAR-1) and *F2RL1* (PAR-2), were statistically tested using the non-parametric Wilcoxon signed rank test. MCP-1 ELISA results were statistically analysed using the non-parametric Friedman test for paired samples and Dunn's multiple comparison test. Human RT-qPCR including CTRL with FXa (or TRAP-14) and either siPAR-1 or siPAR-2 or rivaroxaban were tested for significance using the non-parametric Friedman test and Dunn's multiple comparison test. The proliferation assay was statistically tested using a repeated-measures one-way ANOVA and Šidák's post-hoc test. *p*-values < 0.05 were considered to be statistically significant. PRISM (version 9.0.0, GraphPad) was used to compute all statistics.

Results

Thrombin and FXa induce upregulation of pro-fibrotic and proinflammatory genes in adult rat CFs

Thrombin induced upregulation of Acta2 mRNA at 4 and 24 h in atrial CFs (1.6-fold, 1.9-fold, respectively), while in ventricular CFs, this upregulation was significant only at 24 h (3.1-fold, Figure 1A). Exposure of ventricular CFs to FXa upregulated Acta2 mRNA at both 4 and 24 h (1.6-fold and 2.7-fold, respectively, Figure 1B). No effect of FXa was observed on Acta2 expression in atrial cells. Thrombin and FXa also upregulated the expression of Tgfb1 (TGF-β1), another well-known pro-fibrotic marker, in both atrial and ventricular cells at 4 h (Thrombin: 2.2-fold and 1.9-fold, respectively, Figure 1C, FXa: 2.1-fold and 1.9-fold, respectively, Figure 1D).

Four-hour incubation of atrial or ventricular CFs with thrombin increased the gene expression of *Ccl2* (MCP-1) by 8.5-fold and 6.6-fold, respectively (Figure 1E).

This induction was still significantly increased in atrial cells at 24 h (2.1-fold), although it was not as strong as at the earlier time point. In ventricular CF, *Ccl2* expression returned to baseline levels after 24 h. Interestingly, incubation of atrial and ventricular CF with FXa triggered fast and strong upregulation of *Ccl2* mRNA levels. After 4 h of incubation, *Ccl2* expression was increased by 34.2-fold in atrial and 32.4-fold in ventricular CFs. At 24 h, FXa was still able to induce overexpression of *Ccl2* by 16.1-fold in atrial and 10.4-fold-in ventricular CFs (Figure 1F). Furthermore, the strong induction of *Ccl2* expression mediated by FXa was reflected by the 20.6-(atrial) and 36.0-fold (ventricular) upregulation of MCP-1 protein levels in the conditioned media of CFs exposed to FXa for 24 h. Thrombin also increased secretion of MCP-1 although not as strongly (5.6-fold in both atrial and ventricular CFs, Figure 2). Moreover, thrombin upregulated the expression of another proinflammatory gene, *Il6* (IL-6), in ventricular CFs at 4 h (2.7-fold, Figure 1G). In atrial cells, the expression of *Il6* was not affected. These cells also showed a significantly higher baseline expression compared to ventricular CFs. *Il6* expression was also upregulated by FXa in ventricular cells at both time points (6.9-fold at 4 h and 3-fold at 24 h, Figure 1H). Atrial CF *Il6* mRNA levels showed a trend towards an increase at 4 h of FXa ($p = 0.06$).

Finally, gene expression analysis revealed that adult rat CFs expressed PAR-1- and PAR-2-encoding genes, *F2r* and *F2rl1*, respectively (Figure 3), while mRNA levels of *F2rl2* and *F2rl3* (expressing the PAR-3 and PAR-4 proteins, respectively) were not detectable. Furthermore, rat CFs showed a much higher expression level of *F2r* compared to *F2rl1* mRNA, suggesting that PAR-1 is the most abundantly expressed PARs isoform in these cells. Exposure of atrial and ventricular cells to thrombin or FXa for 4 h significantly upregulated *F2r* gene expression (atrial:1.7-fold and 1.7-fold, respectively, ventricular: 1.7-fold and 1.9-fold, respectively, Figure 3A,B). Similarly, thrombin and FXa upregulated the gene expression of *F2rl1* in ventricular cells at 4 h (2.9-fold and 2.8-fold, respectively, Figure 3C,D), while no significant difference was found in atrial CFs.

Silencing of PAR-1 attenuates FXa-mediated upregulation of CCL2 and IL6 in primary human atrial CFs

The effect of FXa on the upregulation of *CCL2* and *IL6* gene expression was confirmed in primary human atrial CFs (3.4-fold and 4.6-fold, respectively, Figure 4A,B). Knockdown of the genes *F2R* or *F2RL1* (encoding PAR-1 and PAR-2 proteins) produced an 85% and 61% reduction of their relative expression, respectively (Figure 4C,D). Interestingly, silencing of *F2R* significantly reduced the effect of FXa on IL6 mRNA expression, while *F2RL1* knockdown did not significantly attenuate this effect (Figure 4A,B). Similarly, the FXa-induced increase in *CCL2* expression

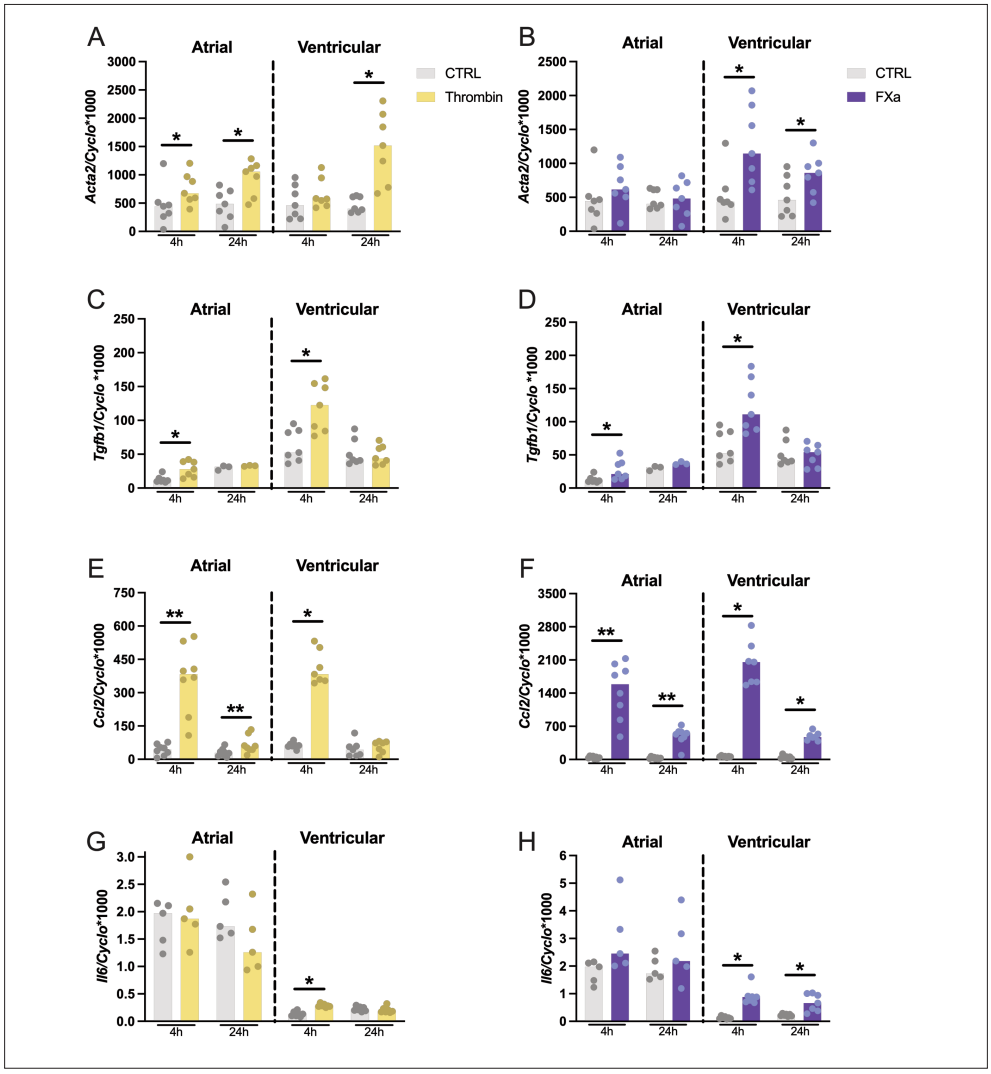

Figure 1. Effect of thrombin (A, C, E, G) and FXa (B, D, F, H) on mRNA expression of pro-fibrotic and proinflammatory genes. Rat atrial (n = 3-8) and ventricular (n = 7) CF were stimulated with 8 pM of thrombin or 100 nM of FXa for 4 and 24 h. Gene expression of *Acta2* (A, B), *Tgfb1* (C, D), *Ccl2* (E, F), and *Il6* (G, H) was measured by RT-qPCR. Data were normalized to the housekeeping gene Cyclophilin-A. Results are expressed as median bars, with dots indicating separate CF isolations. Statistical analysis was performed using the non-parametric Wilcoxon test for paired samples ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

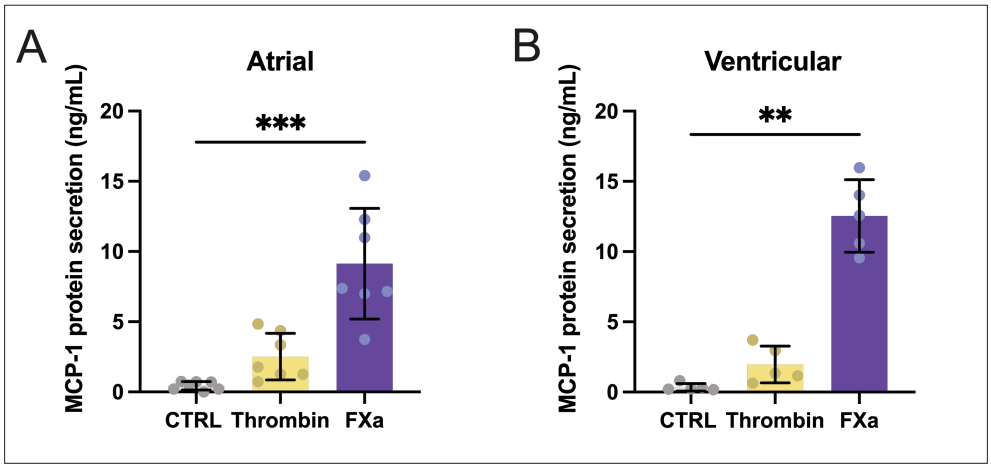

Figure 2. Effect of thrombin and FXa on MCP-1 protein secretion at 24 h in atrial CFs, $n = 7$ (A), and ventricular CFs, $n = 5$ (B). Statistical analysis was performed using the non-parametric Friedman test for paired samples (atrial: overall $p < 0.0001$; ventricular: overall $p < 0.0001$) and Dunn's multiple comparison test ($^{**}p < 0.01$, $^{***}p < 0.001$). Results are expressed as means ± SD.

was lower upon *F2R* silencing, although this effect failed to reach statistical significance. Silencing of *F2RL1* seemed to downregulate the FXa-mediated *CCL2* increase but not as strongly as *F2R* knockdown (Figure 4A,B).

Furthermore, inhibition of FXa by its selective inhibitor rivaroxaban completely prevented the upregulation of *CCL2* and *IL6* (Figure 4E,F).

PAR-1 agonist induces upregulation of CCL2 and IL6 in human CFs

PAR-1 activation by TRAP-14, a selective PAR-1 agonist, significantly upregulated the gene expression of *CCL2* (5.1-fold) and *IL6* (15.9-fold) mRNAs in human atrial CFs at 4 h, respectively (Figure 5A,B). As expected, PAR-1 silencing fully prevented this effect while PAR-2 knockdown did not.

PAR-1 antagonist downregulates the effect of FXa on human CF proliferation

Exposure of human CFs to FXa for 24 h stimulated cell proliferation compared to control-treated cells (1.5-fold). This proliferative effect of FXa was reduced by a selective PAR-1 antagonist (SCH79797), although this reduction failed to reach statistical significance (Figure 6).

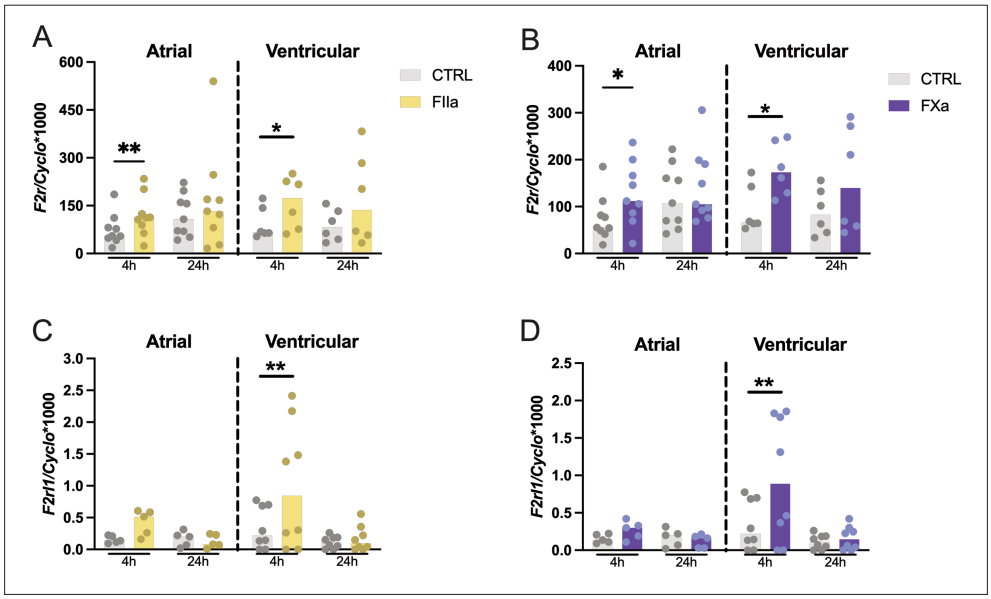

Figure 3. Effect of thrombin and FXa on mRNA expression of *F2r* (PAR-1 gene) (A, B) and *F2rl1* (PAR-2 gene) (C, D). Rat atrial (n = 5-9) and ventricular (n = 6-8) CFs were stimulated with 100 nM of FXa for 4 and 24 h. Gene expression of *F2r* and *F2rl1* was measured by RT-qPCR. Data were normalized to the housekeeping gene Cyclophilin-A. Results are expressed as median bars, with dots indicating individual rat CF isolations. Statistical analysis was performed using the non-parametric Wilcoxon test for paired samples ($^{*}p < 0.05$, $^{**}p < 0.01$).

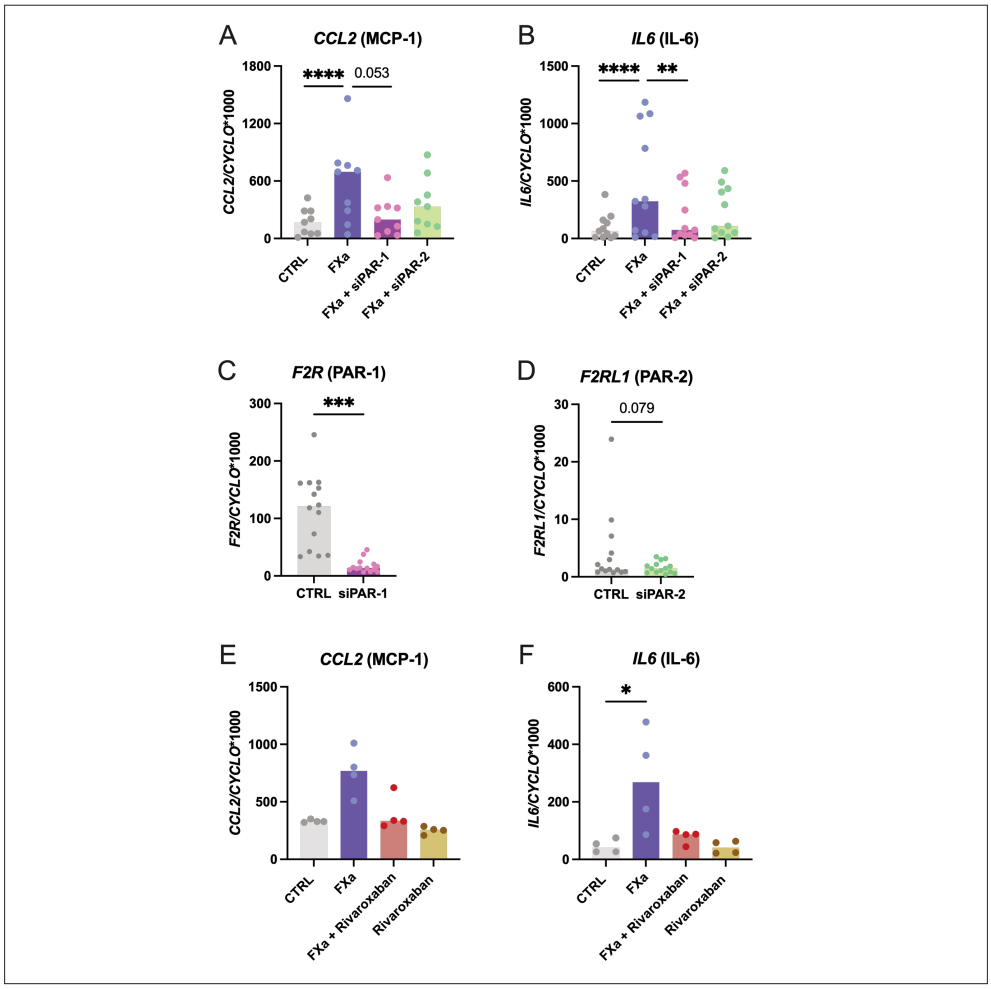

Figure 4. Effect of PARs silencing on FXa-mediated *CCL2* and *IL6* expression in human CFs (A, B; n = 8-9). Before exposure to FXa (100 nM), human atrial CFs were transfected with 10 μM of either scrambled *F2R* (PAR-1) or *F2RL1* (PAR-2) specific siRNA and incubated for 48 h. Gene expression was measured by RT-qPCR after 4 h of incubation with FXa. Data were normalized to the housekeeping gene Cyclophilin-A. Statistical analysis was performed using the non-parametric Friedman test for paired samples (*CCL2*: overall $p < 0.001$; *IL6*: overall $p < 0.001$) and Dunn's multiple comparison test ($^{**}p < 0.01$, $^{****}p < 0.0001$). Effect of silencing on *F2R* (PAR-1) and *F2Rl1* (PAR-2) gene expression (C, D; n = 14). Data were analysed using the non-parametric Wilcoxon test for paired samples ($^{***}p < 0.001$). Effect of FXa inhibition by rivaroxaban on *CCL2* and *IL6* expression in human CFs (E, F; n = 4). Statistical analysis was performed using the non-parametric Friedman test for paired samples (*CCL2:* overall $p < 0.001$*; IL6:* overall $p < 0.001$) and Dunn's multiple comparison test ($^{*}p < 0.05$). Results are expressed as median bars, with dots indicating individual human CF isolations.

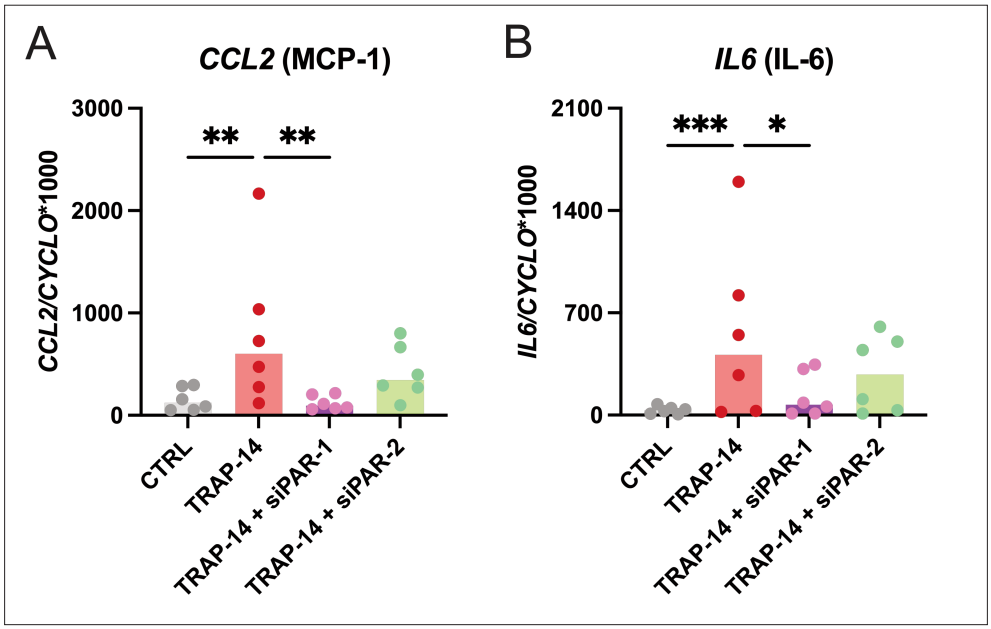

Figure 5. Effect of PARs silencing on *CCL2* and *IL6* expression (A, B; $n = 6$). Human atrial CFs were transfected with 10 μM of either scrambled PAR-1 or PAR-2 specific siRNA before exposure to TRAP-14 (100 μM). Gene expression was measured by RT-qPCR after 4 h of incubation. Data were normalized to the housekeeping gene Cyclophilin-A. Results are expressed as median bars, with dots indicating individual human CF isolations. Statistical analysis was performed using the non-parametric Friedman test (*CCL2*: overall $p < 0.0001$; *IL6*: overall $p < 0.0001$) test for paired samples and Dunn's multiple comparison test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

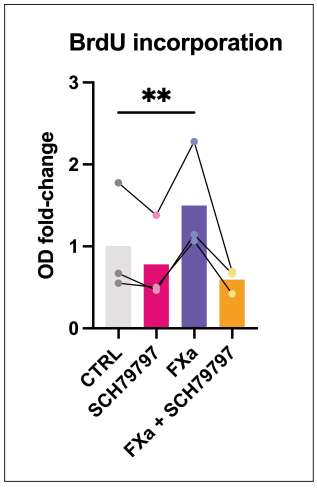

Figure 6. Proliferation assay on human atrial CFs ($n = 3$). Cells were incubated with 100 nM FXa with and without SCH79797 (1 μM). Proliferation was measured 24 h after exposure to the stimuli via BrdU colorimetric assay. Results are expressed as fold-change relative to CTRL (mean bars). Statistical analysis was performed using the repeated measures one-way ANOVA ($p < 0.01$) and Šidák's multiple comparison test ($^{**}p < 0.01$). Lines depict paired data points.

Discussion

This study shows that FXa upregulates the gene expression of two key regulators of inflammatory processes, *CCL2* and *IL6*, in primary adult human atrial CFs. Surprisingly, the FXa pro-inflammatory effect was mainly mediated by PAR-1 activation, which appears to be the most abundant isoform expressed in CFs. PAR-1 agonist also increases *CCL2* and *IL6* gene expression, confirming that PAR-1 activation plays a pivotal role in inflammatory processes mediated by CFs.

Moreover, our data show that the coagulation factors thrombin and FXa lead to increased expression of well-known pro-fibrotic genes in primary adult rat CFs, supporting the possible link between (pathological) activation of the coagulation system and cardiac remodelling.

Coagulation factors induce pro-fibrotic and proinflammatory gene expression changes in rat CFs

In this study we made use of primary adult rat CFs to explore the effect of thrombin and FXa on CF activation. In line with what is reported in the literature [7], thrombin induced overexpression of the *Acta2* and *Tgfb*1 genes in atrial CFs already after 4 h. In a similar fashion, FXa upregulated the expression of these pro-fibrotic markers mainly in ventricular CFs. This suggests that both thrombin and FXa may play a role in the activation of CFs within the process of cardiac remodelling.

PARs are the main mediators of cellular signalling elicited by coagulation factors including thrombin and FXa [9]. To elucidate their role in this process, we tested whether PAR activation would also lead to upregulation of their mRNA levels. Interestingly, both thrombin and FXa were able to upregulate the expression of the rat *F2r* and *F2rl1* genes, encoding PAR-1 and PAR-2, respectively, in CFs. These results are in line with what has previously been described by our group and suggest the existence of a positive feedback loop on PAR expression upon their activation [7].

Most importantly, our study confirmed the proinflammatory effect of FXa and provided additional insight on its cellular signalling on CFs. As described by several in vitro and in vivo studies, we found that exposure of rat CFs to FXa resulted in a strong and rapid upregulation of *Ccl2* (in both atrial and ventricular CFs) and *IL6* (in ventricular CFs) mRNA levels [11,12]. Moreover, *Ccl2* overexpression was accompanied by increased MCP-1 protein levels, detected in the conditioned media of CFs exposed to FXa for 24 h.

Coagulation factors elicit inflammatory signalling through PAR-1 in human CFs

FXa-mediated proinflammatory changes were confirmed in primary adult human atrial CFs, where the expression of *CCL2* and *IL6* increased upon stimulation with FXa for 4 h.

To investigate the mechanism by which FXa leads to inflammatory responses in human CFs, *F2R* (PAR-1) and *F2RL1* (PAR-2) were silenced. Surprisingly, *F2RL1* silencing did not significantly alter the FXa-mediated upregulation of *CCL2* and *IL6*. In contrast, our experiments revealed that *F2R* silencing did prevent the upregulation of *CCL2* and *IL6* by FXa. These results suggest that FXa elicits its proinflammatory role mainly via PAR-1 activation. Moreover, in line with what was reported by Snead and colleagues, at baseline, human (and rat) CFs expressed much higher *F2R* than *F2RL1* mRNAs, indicating that PAR-1 might be the predominant isoform in these cells [17].

Our findings seem to disagree with the literature about the role of PAR-2 as the main mediator of FXa proinflammatory activity [9, 18]. For example, Bukowska and colleagues reported that incubation of human atrial tissue slices with FXa increased mRNA expression of inflammatory molecules, such as intercellular adhesion molecules and IL-8. These effects were mediated by PAR-2 activation and downregulated by the direct FXa inhibitor, rivaroxaban [19]. A possible explanation to the seemingly contrasting data between our study and previous reports is the fact that the cardiac tissue slices contained all cardiac cell types, while the specific effect of FXa on CFs was not characterized. Recently, the same research group showed that exposure of A549 cells (model of type two alveolar epithelial cells) to FXa upregulates the gene expression of inflammatory molecules, such as MCP-1. Interestingly, in these cells, the effect of FXa on proinflammatory markers was prevented by PAR-1 inhibition [20]. These data support our findings and suggest that FXa proinflammatory signalling via PAR-1 may also play a relevant role in other organs and cell types.

Moreover, our data show that, in human atrial CFs, rivaroxaban downregulated the effect of FXa on *IL6* and *CCL2* expression. These results indicate that FXa is the actual mediator of these proinflammatory changes and exclude the possible contribution of thrombin (of which small traces may be present in FXa preparation) in the upregulation of these markers.

Furthermore, in line with what was previously described by Guo et al. in neonatal rat CFs, we showed that FXa enhanced the proliferation of human CFs and that this effect seemed to be attenuated by PAR-1 antagonist [21].

Finally, to further elucidate the role of PAR-1 signalling, CFs were incubated with a selective PAR-1 agonist that significantly upregulated the gene expression of *CCL2* and *IL6* after 4 h. As expected, the upregulation of these genes was fully prevented by PAR-1 knockdown but not by PAR-2 knockdown. Interestingly, upon PAR-2 silencing, PAR-1 agonist was not able to induce an upregulation as strong as PAR-1 agonist alone, suggesting that PAR-2 might still play a role in the gene expression of these markers possibly via its transactivation by PAR-1 [22].

Taken together, these data confirm that PAR-1 activation triggers early upregulation of proinflammatory genes in human CFs and therefore plays a pivotal role in inflammatory responses mediated by CFs.

Study limitations

In the present study, primary adult human atrial CFs were used to investigate the signalling receptor involved in the proinflammatory effect of FXa found in rat CFs. As extensively reported in the literature, prolonged in vitro culturing of CFs promotes their activation and differentiation into myofibroblasts. To preserve the CF phenotype, our experiments were performed on cells at a low passage number (the highest passage number was 4). However, this, in combination with the low proliferation rate and the relatively small number of donors, limited the number of experiments (and conditions) we could perform. Nevertheless, we were able to carry out key experiments and answer our primary research questions.

Finally, we reported the effect of FXa on the upregulation of only two proinflammatory genes, *CCL2* and *IL6*. Despite the fact that these markers are two well-known regulators of proinflammatory processes, further analysis on other regulators and targets can be helpful to clarify the mechanism of action of FXa signalling on CFs. Moreover, co-culture experiments and migration assays on CFs in combination with immune cells can be performed to further characterize the proinflammatory character of FXa and study the functionality of inflammatory changes in CFs.

Conclusions

This study demonstrates that FXa induces proinflammatory responses in human atrial CFs by upregulating the expression of two well-known regulators of inflammatory processes, *CCL2* and *IL6*. Moreover, our data show that FXa elicits cellular signalling mainly via activation of PAR-1, which was found to be the most abundantly expressed PARs isoform in primary adult human (and rat) CFs.

Supplementary materials

The following are available online at www.mdpi.com/xxx/s1, Table S1: Gene-specific Rattus norvegicus primer sequences used for quantitative real-time PCR, Table S2: Gene-specific Homo sapiens primer sequences used for quantitative real-time PCR.

Author contributions

Conceptualization, E.D., B.S., S.V., H.M.H.S., N.A.T., H.t.C., U.S., F.A.v.N., performed experiments and produced data E.D., C.M., C.J.T., data analyses, E.D., B.S., C.M., F.A.v.N., writing-original draft preparation, E.D., B.S., writing-review and editing, E.D., B.S., A.v.H., S.V., H.M.H.S., N.A.T., H.t.C., U.S., F.A.v.N. All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by grants of the Netherlands Heart Foundation (CVON2014-09, RACE V Reappraisal of Atrial Fibrillation: Interaction between hyperCoagulability, Electrical remodeling, and Vascular Destabilisation in the Progression of AF) and the European Union (ITN Network Personalize AF: Personalized Therapies for Atrial Fibrillation: a translational network, grant number 860974, CATCH ME: Characterizing Atrial fibrillation by Translating its Causes into Health Modifiers in the Elderly, grant number 633196, MAESTRIA: Machine Learning Artificial Intelligence Early Detection Stroke Atrial Fibrillation, grant number 965286, REPAIR: Restoring cardiac mechanical function by polymeric artificial muscular tissue, grant number 952166).

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of the Maastricht University (the Netherlands, Ref. 162012), or the local ethical committee of the Leeds Teaching Hospitals NHS Trust (United Kingdom, Ref. 01/040).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

The data presented in this study are available from the corresponding author upon request.

Acknowledgments

We are grateful to Karen Porter (University of Leeds) for provision of human atrial fibroblasts.

Conflicts of interest

The authors declare no conflict of interest.

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Chapter 4

Thrombin generation by Calibrated Automated Thrombography in goat plasma: optimization of an assay

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Research and Practice in Thrombosis and Haemostasis, 2021. 5(8): p. e12620.

Abstract

Background

The goat model of atrial fibrillation (AF) allows investigation of the effect of AF on coagulation. However, assays for goat plasma are not available from commercial sources. Calibrated Automated Thrombography (CAT) provides a global view of the coagulation profile by assessing *in vitro* thrombin generation (TG). We describe the customization of the CAT assay in goat platelet-poor plasma (PPP) and in FXa-inhibitor-anticoagulated PPP.

Methods

TG was initiated in the presence of phospholipids (PL) and either i) PPP Reagent, Reagent Low or Reagent High, ii) goat brain protein extraction (GBP) or iii) Russell's viper venom-factor X activator (RVV-X). Contact activation was assessed by adding corn trypsin inhibitor (CTI). Different concentrations of prothrombin complex concentrate (PCC) were used to determine the sensitivity of both the GBP and RVV-X method. To obtain FXa-inhibitor anticoagulated plasma, rivaroxaban was added to plasma.

Results

TG settings with human reagents were not suitable for goat plasma. TG triggered with GBP increased peak height and ETP values. Similarly, the RVV-X method produced comparable TG curves and was more sensitive to PCC titration. Finally, both methods were able to detect the decrease in clotting potential induced by FXa-inhibition.

Conclusions

This is the first study that reports the customization of the CAT assay for goats. The GBP and RVV-X methods were comparable in triggering TG in goat plasma. The RVV-X method seemed to better discriminate changes in TG curves due to increases in clotting potential as well as to FXa-inhibition by rivaroxaban in goat plasma.

Introduction

Atrial fibrillation (AF) is one of the most common forms of sustained arrhythmias in clinical practice [1]. AF is associated with an increased risk of stroke and other thromboembolic events as a consequence of abnormal changes in blood flow, the vessel wall and most importantly, the hemostatic system [2]. People with chronic AF exhibit a hypercoagulable state that is characterized by increased plasma levels of pro-thrombotic markers (e.g. prothrombin fragment 1.2, thrombin-antithrombin complexes and fibrin turnover) [3]. Moreover, young paroxysmal AF patients with low risk for stroke display increased factor (F) IXa-AT plasma levels as a reflection of a prethrombotic state induced by 'lone AF', (i.e. AF without underlying structural heart disease) [4].

However, little is known about the molecular mechanism by which the coagulation system becomes activated during AF, nor on the effects of activated coagulation factors on cardiac remodeling and AF progression [5].

To unravel these critical aspects, our group uses an experimental goat model in which AF is induced by atrial burst pacing, leading to a progressive increase in AF episode duration until AF does not terminate spontaneously anymore (persistent AF) [6]. This model allows the investigation of changes in the heart (e.g. electrical and structural) and blood that occur within days to months of AF [7, 8]. Ongoing research by our group focusses on the mechanisms behind the inhibitory effects of anticoagulation on atrial structural remodeling, as previously shown for nadroparin in the AF goat model [5].

In order to monitor AF-induced alterations of the coagulation system and to investigate the pathways involved in the AF-related hypercoagulable state, suitable coagulation assays for goat plasma are needed, but these are not available from commercial sources.

Calibrated Automated Thrombography (CAT) is a well-known functional assay to assess plasma thrombin generation upon *in vitro* activation of the coagulation system [9, 10]. It relies on the ability of thrombin to cleave a low affinity fluorogenic substrate (Z-Gly-Gly-Arg-AMC), thereby allowing continuous recording of thrombin's enzymatic activity [10]. Moreover, the thrombin generation curve reflects the contribution of either the procoagulant or the anticoagulant pathways, providing a global view on the overall coagulation profile of the sample [11, 12]. For these characteristics, the CAT assay was selected as primary hemostatic tool to monitor anticoagulant therapy and to assess hypercoagulability in the goat model of AF [unpublished data].

However, the most common reagents used to trigger thrombin generation in the CAT assay are human-derived and/or optimized for human plasma. Therefore, these may not be able to trigger reliable thrombin generation curves in goat plasma.

Moreover, commercially available goat-derived reagents for the CAT assay are lacking. To overcome species-specific differences and reliably measure thrombin generation in goat plasma, modifications of the assay are needed.

This paper describes the customization and optimization of the CAT assay for the assessment of thrombin generation in goat platelet-poor plasma (PPP). Furthermore, it reports the optimal test conditions for the evaluation of the clotting potential in FXa-inhibitor-anticoagulated goat plasma.

Materials and methods

Animals

Blood samples and brain tissues were collected from untreated female Dutch milk goats with no atrial fibrillation. Average body weight was 68.5 ± 11.6 kg and average age was 2.8 ± 1.1 years.

Animal procedures were conducted in accordance with national and institutional guidelines for the use of laboratory animals and were approved by the local ethics committees for animal experiments of Maastricht University (DEC2014-025).

Blood collection and plasma preparation

Blood was collected from the jugular vein using BD Precision Glide needles and BD Vacutainers (3.2%(w/v) citrate). The first 10 mL of venous blood was discarded. Goat PPP was obtained following two centrifugation steps at room temperature: the first at 2,000 x g for 5 minutes (min) and the second at 11,000 x g for 10 min, according to our in-house protocol[13]. Goat normal pooled plasma (GNP) was obtained by pooling together the PPP obtained from 12 goats. Plasma aliquots were stored at -80 ˚C until use.

Human normal pooled plasma (HNP) was prepared in-house by pooling plasma from at least 80 healthy volunteers not using any medication as described previously [13].

Goat brain protein extraction

Goat brain specimens were freeze-dried. Freeze-dried tissues were brought to room temperature (RT) and grinded. The powder was dissolved in a solution of N-Octyl-B-D-Glucopyranoside (50 nM, Sigma-Aldrich) and vortexed for 30 min. The preparation was then centrifuged at maximum speed (13,000 x g) for 10 min at RT. The supernatant was transferred into a new microcentrifuge tube and centrifuged a second time as described above.

Different dilutions of goat brain protein extraction (GBP) were obtained by diluting the GBP stock solution with Hepes NaCl (HN) buffer (Sigma-Aldrich). A protein assay (Bio-Rad) was used to quantify the total protein content in 50-times diluted GBP preparation which was found to be equal to 16.6 mg/mL.

Thrombin generation

Thrombin generation was measured by means of the CAT method [13]. Fluorescence was measured in an Ascent Reader (Thermolabsystems OY, Helsinki, Finland) equipped with a 390/460 nm filter set, and thrombin generation curves were calculated using Thrombinoscope software (Thrombinoscope B.V., Maastricht, The Netherlands). Correction for inner filter effects and substrate consumption was performed by calibrating the results from each thrombin generation analysis against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator (Thrombin Calibrator, Thrombinoscope B.V., Maastricht, The Netherlands).

Unless stated otherwise, measurements were conducted in triplicate on 80 mL of goat or human PPP in a total volume of 120 mL (20 mL fluorogenic substrate, Calcium chloride [FluCa] and 20 mL trigger reagent).

Thrombin generation was initiated by adding either i) PPP Reagent, Reagent Low or Reagent High, containing human recombinant TF (Stago), ii) GBP or iii) Russell's viper venom-factor X activator (RVV-X, ITK Diagnostics BV) in the presence of phospholipids (PL, Avanti Polar Lipids). Measurements performed in human plasma with PPP Reagent Low, PPP Reagent or PPP Reagent High, were comparable to reactions performed with in-house reagents using 1, 5 or 20 pM as final TF concentration, respectively.

Inhibition of the intrinsic pathway was achieved by adding corn trypsin inhibitor (CTI, Haematologic Technologies) to the reaction mixture. Different concentrations of prothrombin complex concentrate (PCC, Sanquin Plasma Products B.V), were used to increase the TG in goat plasma. To obtain FXa-inhibition, anticoagulated plasma rivaroxaban (Bayer AG) was added to goat plasma prior the assay.

Results

PPP Reagent Low-induced thrombin generation in goat plasma

TF is one of the most common triggers used to initiate thrombin generation in the CAT assay. Depending on the concentration of TF, the sensitivity of the assay can be adjusted to a specific coagulation (and/or anticoagulant) pathway.

Since the CAT triggered by low levels of TF has been shown to activate both intrinsic and extrinsic pathways, this TF concentration was chosen to study the thrombin generation in goat plasma.

Initially, due to a lack of goat-derived reagents, thrombin generation was initiated with in-house reagents containing human TF. However, the thrombin generation curve obtained in goat plasma showed substantially lower ETP (308.2 ± 23.8 nM • min) and Peak height (44 ± 4.9 nM) values than in human plasma (ETP: 1361.7 ± 45.7 nM • min, Peak: 159.2 ± 5.9 nM) and as such, was unsuitable for further reliable measurements (Figure 1).

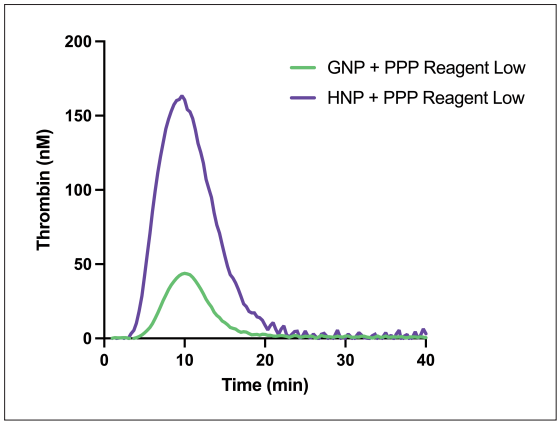

Figure 1. Calibrated Automated Thrombography (CAT) in human and goat normal pooled plasma.
Thrombin generation was triggered by adding PPP Reagent Low to human (HNP, blue curve) and goat (GNP, green curve) plasma (average curves of n=3).

Effect of increasing phospholipids concentration and plasma dilution on thrombin generation

To raise the amount of generated thrombin in goat plasma, we increased the concentration of PL in the reaction mixture. As shown in Figures 2A and 2B, PL concentrations above 4 µM produced an increase of both Peak height and ETP of the TG curves. The highest increase in both parameters (Peak: 217.3 ± 18.9 nM and ETP: 921.1 ± 64.7 nM • min) was reached at 30 µM of PL, while Lag time was not affected.

At low TF concentrations, high PL concentrations tend to enhance the contribution of contact activation. Under these circumstances, thrombin generation is highly dependent on the activation of the intrinsic coagulation pathway (Figure 2C) [14].

To optimize the thrombin generation curve and develop a CAT equally sensitive to pro- and anti-coagulant forces, measurements were performed in diluted plasma. As reported by Tchaikovski et al., reactions carried out in diluted mouse plasma helped to overcome the activity of natural coagulation inhibitors (e.g. TF pathway inhibitor and antithrombin) and to increase the Peak height and ETP of the thrombin generation curves [15]. Figure 2D shows that in goat plasma, the largest increase in Peak height was achieved at a plasma dilution of 1:2 (156.6 ± 3.2 nM, 4.5-fold compared to non-diluted plasma). At this dilution the Lag time shortened by 1.6-fold. At higher plasma dilutions, Peak heights dose-dependently decreased while ETP values remained stable (Figures 2D and 2E).

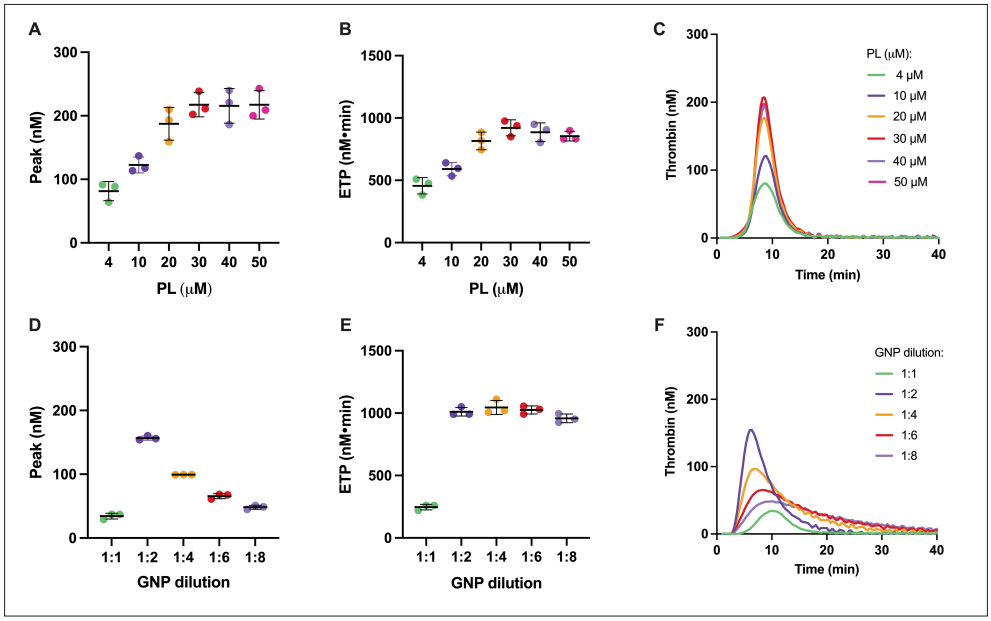

Figure 2. Effect of PL titration and plasma dilution on thrombin generation in goat plasma.
Thrombin generation was measured in the presence of PPP Reagent Low and 4, 10, 20, 30, 40 and 50 µM of PL (A-C). Effect of PL titration on peak height (A), ETP (B) and TG average curves (C). Thrombin generation was measured in diluted GNP plasma (1:1, 1:2, 1:4, 1:6, 1:8) in the presence of PPP Reagent Low (D-F). Effect of plasma dilutions on Peak height (D), ETP (E) and TG average curves (F). Results are expressed as mean ± SD, n=3.

As reported by Tchaikovski et al., plasma dilutions are expected to reduce the contribution of the natural coagulation inhibitors in mouse plasma [15]. In goat plasma, this effect was not tested. However, to exclude this possibility and to increase the TG parameters without affecting the sensitivity of the assay to the anti-coagulant pathways, we decided to explore alternative strategies.

Goat brain protein extraction-induced thrombin generation

As one of the most highly vascularized organs of the body, the brain contains large amounts of TF, which can be extracted and used to initiate coagulation *in vitro*.

Figures 3A-C show thrombin generation initiated in goat plasma with 4 µM of PL and different dilutions of goat brain protein extraction (GBP) used as a source of TF. To obtain an estimate of the concentration of goat TF, the TG curves were compared to the curves obtained in human plasma in the presence of 4 µM of PL and increasing concentrations of human TF.

The results show that thrombin formation initiated with 400-, 200- and 50-times diluted GBP was reproducible (Supplementary table 1) and comparable to thrombin generated in human plasma triggered with PPP Reagent Low, PPP Reagent or PPP Reagent high, respectively.

To rule out contact activation, the goat intrinsic pathway was inhibited by CTI. Figure 3D indicates a slight decrease in thrombin generation when CTI was added to the reaction mix, suggesting that GBP may lead to activation of the goat contact system.

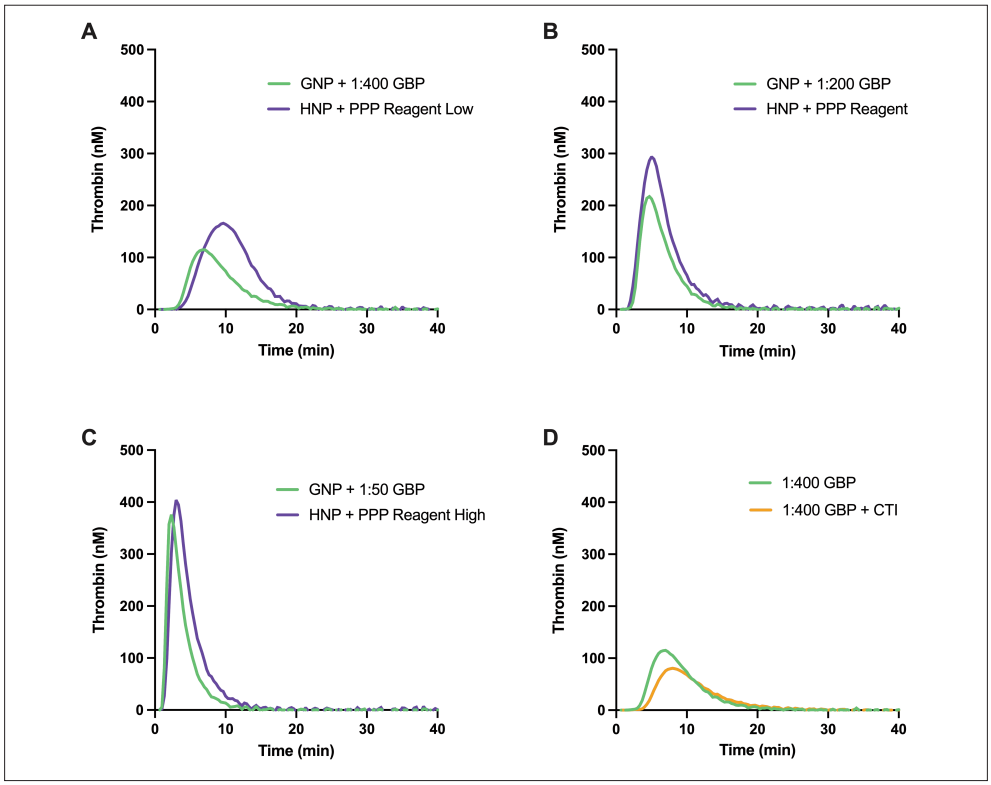

Figure 3. Thrombin generation induced by goat brain protein extraction (GBP).
Thrombin generation was initiated with in-house reagents PPP Reagent Low, PPP Reagent, or PPP Reagent High in HNP plasma (blue curve) and with 400-, 200- and 50-times diluted GBP in GNP plasma (green curve) (A-C). Effect of CTI on thrombin generation induced by GBP (D). All curves are average curves of n=3.

Improving assay sensitivity: GBP vs RVV-X-induced thrombin generation

Once the optimal conditions for a reliable thrombin generation curve were established, the assay sensitivity to small increases in the goat clotting potential was tested.

To enhance the thrombin formation in GNP, increasing concentrations of PCC were added to the reaction mixture. Thrombin generation was initiated with diluted GBP. Subsequently, the Russell's viper venom-factor X activator (RVV-X), a known exogenous activator of coagulation that cleaves and activates FX into FXa, was used as alternative trigger (Supplementary table 2) [16].

As illustrated in Figures 4A-B, both methods were able to detect the increments of generated thrombin induced by PCC titration. Comparable Peak height values were reached with both methods at different PCC concentrations (Figure 4C). However, the RVV-X method resulted in a higher relative Peak increase as compared to the GBP method (Peak % increase at 0.75 U/mL of PCC, 201% and 145%, respectively, Figure 4D). This indicates that the RVV-X method may better discriminate between TG curves that reflects small changes of the goat clotting potential, as compared to the GBP method.

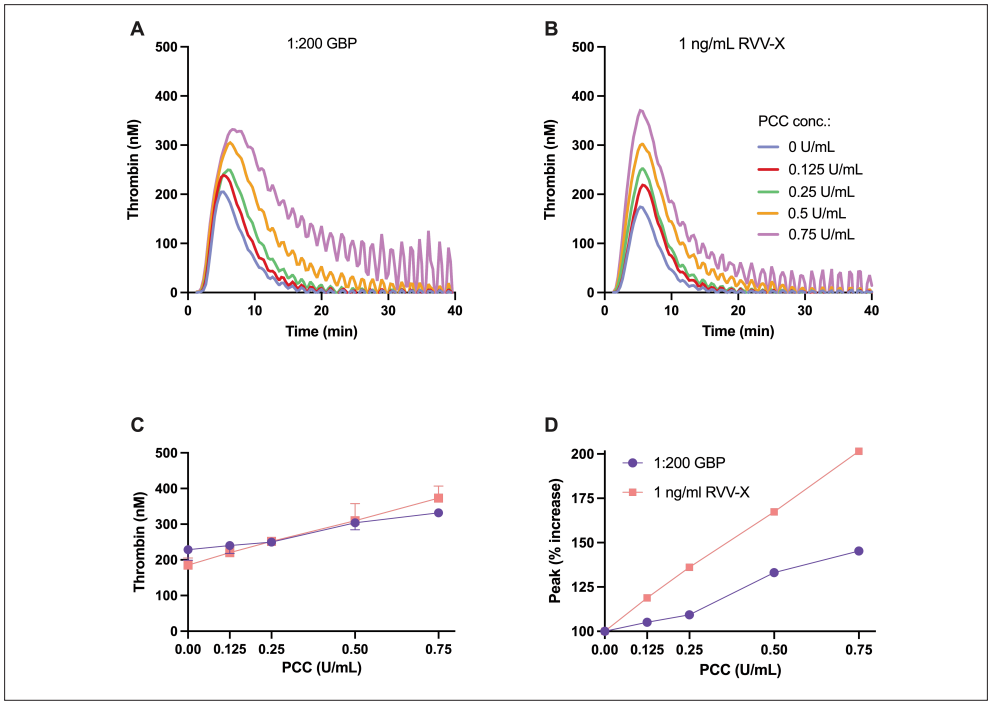

Figure 4. Effect of PCC titration on thrombin generation.
Thrombin generation was initiated with 200-times diluted GBP and 4 µM of PL or 1 ng/mL of RVV-X and 4 µM of PL in GNP plasma. All curves are average curves. The legend in panel B also applies to panel A (A-B). Effect of PCC titration on Peak height values obtained with GBP (blue) and RVV-X (orange) (C). Percentage of increase of peak height values relative to 0 U/mL of PCC All curves are average curves. The legend in panel D also applies to panel C (D). Results are expressed as mean ± SD.

Thrombin generation in anticoagulated goat plasma

In a previous study of our group [unpublished data], goats with AF received oral rivaroxaban treatment (3 mg/kg twice daily). In order to establish the optimal assay conditions to measure the thrombin generation in anticoagulated goat plasma, rivaroxaban was added to the GNP prior to the measurement. To assess which of the two methods made the assay more sensitive to rivaroxaban titration, thrombin generation was measured in the presence of either GBP or RVV-X (Figures 5A-B). Both methods appeared to detect the decreases in Peak height values caused by rivaroxaban (Figure 5C). However, the RVV-X method seemed to better discriminate the TG curves at rivaroxaban concentrations ranging between 10 and 200 ng/mL, as indicated by the smaller Peak percentage decrease compared to the one obtained by the GBP method (Peak percentage decrease at 50 ng/mL rivaroxaban, 67% and 85%, respectively, Figure 5D).

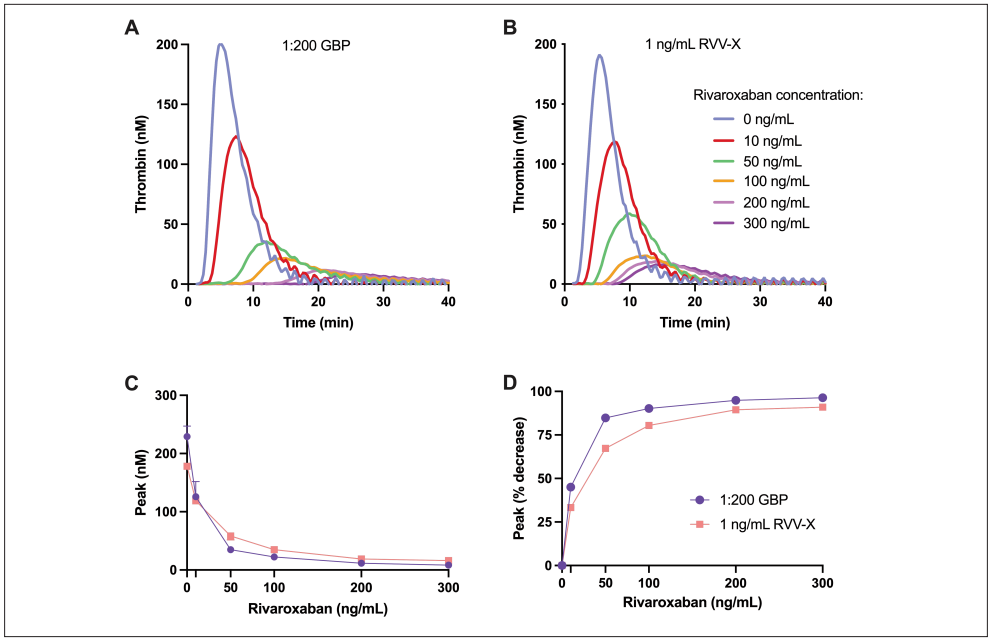

Figure 5. Effect of rivaroxaban titration on thrombin generation.
Thrombin generation was initiated with 200-times diluted GBP and 4 µM of PL or 1 ng/mL of RVV-X and 4 µM of PL in GNP plasma. All curves are average curves. The legend in panel B also applies to panel A (A-B). Effect of rivaroxaban titration on peak height values obtained with GBP (blue) and RVV-X (orange) (C). Percentage of decrease of peak height values relative to 0 ng/mL of rivaroxaban. All curves are average curves. The legend in panel D also applies to panel C (D). Results are expressed as mean ± SD.

Discussion

In this study we reported different methods for the customization and optimization of the CAT assay in goat PPP. Furthermore, we identified the optimal assay conditions to evaluate thrombin generation in anticoagulated goat plasma.

The goat model is a well-established and extensively researched model for the pathogenesis of AF [6]. Because AF can be maintained in the goat for several months, this animal model has also become an interesting tool to investigate the long-term effects of AF on the coagulation system. Moreover, it has been used to elucidate the effect of FXa-inhibition on atrial structural remodeling and AF progression [5]. For these reasons, it has become crucial to develop hemostatic assays suitable for goat plasma.

The CAT assay is able to reliably assess changes of the clotting potential and monitor anticoagulant therapy [17]. Additionally, unlike other techniques (e.g. ELISAs), it does not require many species-specific reagents, which makes this test easier to be performed in goat plasma. The biggest advantage of the CAT is to provide a complete view on the coagulation profile of the sample by evaluating the contribution of both pro- and anti-coagulant pathways [10].

Depending on the type and concentration of the trigger, the sensitivity of the CAT can be adjusted to a specific (pro- or anti-coagulant) pathway. In this study we aimed to establish the optimal assay conditions to equally evaluate the contribution of extrinsic and intrinsic pathways of the goat coagulation system.

Very little is known about the goat hemostatic system as well as about differences and/or homologies between the goat and the human coagulation factors. Studies performed in the 70's, reported that overall coagulation values were similar between the two species. However, it was measured that the goat plasma had a shorter activated thromboplastin time and a longer thrombin time as compared to human plasma [18, 19]. In our study, we observed that the standard settings developed to measure thrombin generation in human plasma did not yield reliable thrombin generation curves in goat plasma. This may be explained by the fact that the human TF in PPP Reagents may not have a high affinity for the goat FVIIa. In fact, goat and human TF share 68.4% of identity, while goat and human FVII only 66.1% [20].

In order to increase the amount of generated thrombin, we explored different strategies. In line with previous studies on human and murine plasma, performing the assay with increased concentrations of PL or with diluted plasma seemed to enhance goat thrombin generation [15, 21]. However, increasing PL concentrations may make the assay highly dependent on the intrinsic pathway, while plasma dilution may decrease the assay sensitivity to anticoagulation pathways [14].

To keep the CAT assay equally sensitive to all coagulation processes, we initiated thrombin generation with GBP as source of TF.

Interestingly, GBP led to a reliable increase in Peak height and ETP values. Our data revealed that this assay modification provided a reproducible method to perform the CAT assay in goat plasma. However, it also showed a few limitations. Firstly, this method can only be carried out if the source of goat TF is available (e.g. goat brain or other TF-rich goat organs). Secondly, we were not able to purify nor to assess the exact concentration of goat TF in the whole protein extraction. This means that GBP preparations may contain other membrane proteins (e.g. thrombomodulin) and lipids which may affect the assay sensitivity. Such observation is also supported by the fact GBP seems to also trigger the goat contact activation.

To overcome these limitations, we explored another assay modification. Thrombin generation was initiated with RVV-X. This metalloprotease cleaves FX into FXa and activates the common coagulation pathway [16]. Despite the fact that this is a non-standardized method and does not reproduce a common mechanism of activation of the coagulation system, the RVV-X method yielded good thrombin generation curves. Furthermore, this method seemed to better discriminate small increases of clotting potential as compared to the GBP method.

Finally, we established the most sensitive settings to measure thrombin generation in FXa-inhibitor-anticoagulated goat plasma. To do so, we compared the curves obtained with the GBP and the RVV-X method in the presence of rivaroxaban. Our findings showed that both methods were able to detect the decrease in clotting potential produced by FXa inhibition. However, the RVV-X method better discriminated the decrease in Peak height induced by rivaroxaban.

Conclusions

To the best of our knowledge, this is the first study that reports the customization and optimization of the CAT assay for a large animal model. Two distinct methods, GBP and RVV-X, were established and found to be comparably able to trigger thrombin generation in goat plasma. Ultimately, the RVV-X method seemed to better discriminate changes in TG curves induced by small increases in clotting potential as well as by FXa-inhibition by rivaroxaban in goat plasma.

Contributions

Elisa D'Alessandro and Billy Scaf were responsible for the execution of the study and all the experimental work. Rene van Oerle and Arne van Hunnik provided

guidance on technical aspects and animal handling. Frans A. van Nieuwenhoven, Sander Verheule, Ulrich Schotten, Hugo ten Cate and Henri M.H. Spronk were involved in the supervision and coordination of the study.

Conflict of interest

HtC received consultancy fees from Bayer, Pfizer, Leo, Portola/Alexion and received research grants from Bayer, unrelated to the presentwork. HtC is a consultant for Alveron and shareholder of CoagulationProfile, a spin-off company of Maastricht University. US received consultancy fees or honoraria from Università della Svizzera Italiana (USI, Switzerland), Roche Diagnostics (Switzerland), EP Solutions Inc. (Switzerland), Johnson & Johnson Medical Limited (United Kingdom), Bayer Healthcare (Germany). US received research grants from Roche and EP Solutions. Bayer Healthcare (Germany) supported pharmacokinetic study of this investigation. US is co-founder and shareholder of YourRhythmics BV, a spin-off company of the University Maastricht.

Funding

This work was supported by grants of the Netherlands Heart Foundation (CVON2014-09, RACE V Reappraisal of Atrial Fibrillation: Interaction between hyperCoagulability, Electrical remodeling, and Vascular Destabilisation in the Progression of AF) and the European Union (ITN Network Personalize AF: Personalized Therapies for Atrial Fibrillation: a translational network, grant number 860974; CATCH ME: Characterizing Atrial fibrillation by Translating its Causes into Health Modifiers in the Elderly, grant number 633196; MAESTRIA: Machine Learning Artificial Intelligence Early Detection Stroke Atrial Fibrillation, grant number 965286; REPAIR: Restoring cardiac mechanical function by polymeric artificial muscular tissue, grant number 952166).

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Supplementals

Supplemental Table 1. Intra and inter-assay variability for different TG test parameters measured in goat plasma with the GBP method (n=3).

	1:400 GBP				1:200 GBP			
	Mean	SD	Inter-assay CV (%)	Mean intra-assay CV (%)	Mean	SD	Inter-assay CV (%)	Mean intra-assay CV (%)
Lag time (min)	3.5	0.1	1.6	6.9	2.4	0.1	3.7	2.7
ETP (nM*min)	843.1	81.5	9.7	6.1	1084.4	58.3	5.4	3.1
Peak (nM)	115.7	7.6	6.6	10.6	219.6	4.8	2.2	3.7
Velocity index (nM/min)	34.7	2.3	6.5	10.6	94.7	6.8	7.1	8.9

Supplemental Table 2. Intra and inter-assay variability for different TG test parameters measured in goat plasma with the RVV-X method (n=3).

	1 ng/mL RVV-X			
	Mean	SD	Inter-assay CV (%)	Average intra-assay CV (%)
Lag time (min)	1.7	0.1	3.2	7.2
ETP (nM*min)	858.7	77.5	9	4.5
Peak (nM)	229.4	18.5	8.1	4.7
Velocity index (nM/min)	113.2	11.8	10.4	7.3

CHAPTER 5

Rivaroxaban prevents atrial myocyte hypertrophy in a goat model of persistent atrial fibrillation

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*Contributed equally to the writing of the manuscript.

In preparation for publication.

Abstract

Background

Atrial fibrillation (AF) is associated with an increased risk of stroke and an activation of the coagulation system. Little is known about the effect of hypercoagulability on AF development and progression. Direct oral anticoagulants (DOACs) may be protective against cardiac structural remodeling processes. In this study, we investigated the effects of rivaroxaban treatment on electrophysiological characteristics, structural remodeling and profibrotic and inflammatory signaling in the atria of goats with persistent AF.

Methods

AF was maintained by burst pacing for 16 weeks. Three groups of goats were tested: CTRL AF (control AF, n=10), RIVA AF (daily rivaroxaban treatment during AF, n=11), and SHAM (no AF, n=10). Rivaroxaban plasma levels, as well as plasma thrombin generation, were carefully monitored. Flecainide cardioversion attempts were performed to assess AF stability. A final experiment was performed to measure hemodynamics and AF complexity, as well as to collect tissue for histological and gene expression analyses.

Results

All groups were comparable in age and body weight. Thrombin generation was strongly inhibited in RIVA AF (249 ± 42nM at baseline vs. 69 ± 33nM after 16 weeks of AF, $p<0.001$). Atrial histological analysis revealed myocyte hypertrophy in CTRL AF (13.5μm [95%CI: 12.9, 14.0]) compared to SHAM (12.5μm [95%CI: 12.0, 13.0], $p<0.05$), which was fully prevented by rivaroxaban (12.2μm [95%CI: 11.7, 12.7], $p<0.01$). The degree of endomysial fibrosis was similar between the groups. Hemodynamics and AF stability were not altered by rivaroxaban treatment. AF complexity was comparable overall, except for LA fractionation index which was higher in RIVA AF (2.6 ± 0.9) compared to CTRL AF (1.8 ± 0.4, $p=0.04$). Left atrial expression of the pro-inflammatory gene CCL2 was downregulated in RIVA AF compared to CTRL AF ($p=0.01$).

Conclusion

Prolonged oral treatment with rivaroxaban, provided a sustained reduction of systemic clotting potential, prevented cardiomyocyte hypertrophy and caused anti-inflammatory signaling.

Introduction

Atrial fibrillation (AF), the most common chronic cardiac arrhythmia, is prevalent in approximately 3% of the adult population and accounts for a 5-fold risk increase in thromboembolic stroke [1-5]. Patients with AF present a hypercoagulable state, which is characterized by increased platelet activation (e.g. β-thromboglobulin), elevated concentrations of pro-thrombotic indices (e.g. prothrombin fragments 1+2, thrombin-antithrombin complex) and altered fibrinolytic activity (e.g. increased amounts of D-dimer, tissue-plasminogen activator and t-PA inhibitor-1 [6-10].

In recent years more and more pleiotropic effects of coagulation factors have been documented. For example, activated coagulation factors, such as thrombin and Factor Xa (FXa), may contribute to a substrate for AF [11] through their pro-inflammatory and profibrotic effects mediated by protease activated receptors (PARs), their most relevant biological receptors [12]. The PAR family consists of four isoforms (PAR1 to -4), which are expressed on a variety of cell types (e.g. fibroblasts and myocytes) and in various organs, including the heart [13]. Activation of PARs by coagulation factors and other proteases contributes to pro-inflammatory responses in different cell types, as well as cardiomyocyte hypertrophy [11, 13-15].

Structural remodeling processes, such as fibrosis and myocyte hypertrophy [16, 17], impair propagation of the electrical excitation wave between cardiomyocytes. F [18, 19]. Experimental studies have shown that interstitial fibrosis causes localized conduction delays and heterogeneities [19, 20]. Moreover, an increase in cardiomyocyte size may also contribute to reduced conduction velocity due to decreased end-to-end coupling between hypertrophic myocytes [17]. Such abnormalities contribute to unidirectional conduction block and electrical dyssynchrony, thereby enhancing AF propensity.

Previously, we demonstrated that the inhibition of FXa (and thrombin), by the low molecular weight heparin (LMWH) nadroparin, reduced AF-induced endomysial fibrosis in the atria of goats after 4 weeks of pacing-induced AF [11]. These results suggest that inhibition of FXa may reduce the pathological mechanisms mediated by the activation of PARs. Direct oral anticoagulants (DOACs), which are more predictable and need less laboratory monitoring compared to LMWH [21], selectively inhibit the activity of FXa or thrombin. Therefore, DOACs (e.g. rivaroxaban, dabigatran etc.) might be more efficient in targeting pathological processes that lead to cardiac remodeling.

In this study, we tested the hypothesis that long-term FXa-inhibition by rivaroxaban in goats with AF can (1) attenuate profibrotic, prohypertrophic and inflammatory signaling, (2) modulate atrial structural remodeling processes and (3) delay the development of an electrophysiological substrate for AF.

Methods

All animal experiments were conducted in accordance with European and national guidelines for the use of laboratory animals and were approved by the Maastricht University ethics committees for animal experiments (DEC2014-025).

Rivaroxaban pharmacokinetics in goats

An extensive pharmacokinetic (PK) study was performed to determine the appropriate administration route and dosage level to reach stable rivaroxaban plasma concentrations in goats.

Three different dosing routes were tested: first, a single intravenous injection of 0.5mg/kg; second, an oral dosage of 2mg/kg; and third, a single subcutaneous injection of 1mg/kg (Table 1 and Figure 1A).

The first assessment showed that the oral dosing route (2mg/kg) was feasible and reached plasma levels that were just within the therapeutic range. Subsequently, a 4mg/kg oral dosage was tested; this resulted in higher plasma concentrations that were at the lower end of the therapeutic range (Figure 1B).

Ultimately, a twice daily oral dosage of 3mg/kg was tested for three days with additional blood samples taken up to 36h after the last application. This dosing regimen resulted in a plasma concentration that remained in the therapeutic range for more than 12h after each administration. Hence, an administration of twice per day resulted in plasma concentrations within the therapeutic range for >24h (Figure 1C), and was used for this study.

Table 1. Pharmacokinetics of rivaroxaban for different dosing routes in goats.

Route	Oral		Subcutaneous		Intravenous	
Dose	2mg/kg		1mg/kg		0.5mg/kg (bolus)	
Tested goats	n=4		n=4		n=4	
	gMean	gSD	gMean	gSD	gMean	gSD
AUC (0-24h) [mg*h/L]	0.50	1.23	0.09	1.54	0.79	1.39
C_{max} [mg/L]	0.03	1.12	0.01	1.34	0.67	1.11
Half-life (h)	14.60	1.46	57.10	1.23	1.43	1.60

The geometric mean (gMean) and geometric standard deviation (gSD) given for n=4 goats per dosing route. AUC = area under the curve / C_{max} = rivaroxaban peak concentration.

Main study - General

The main study consisted of three experimental groups: AF only (CTRL AF, n=10), AF + rivaroxaban (RIVA AF, n=11), and sham (SHAM, n=10). In the CTRL AF group, AF was maintained for 16 weeks by burst pacing, as described below. In the RIVA AF group, 16 weeks of AF was combined with rivaroxaban treatment, which was initiated one week prior to AF induction. The sham-operated group (SHAM) underwent the same operation procedures as the other groups, but AF was never induced.

Electrode implantation for AF induction

Induction of anesthesia was performed by intravenous (IV) injection of thiopenthal (20mg/kg, Rotexmedica). Anesthesia was maintained with sufentanyl (6ug/kg/h IV, Hameln Pharma) and propofol (5 - 20mg/kg/h IV, Fresenius Kabi).

For the implantation of a triangular custom-made patch of 10 electrodes, a left-sided thoracotomy through the 4th intercostal space was performed. From there, the patch was sutured to the pericardium overlying the left atrial free wall. Additionally, two-electrode custom made patches were sutured to the pericardium overlying the left ventricle, as well as on the right ventricle. The wires of the electrodes were combined in one silicone tube that was tunneled under the skin and exteriorized at the back of the goat. This was done to allow pacing and electrogram recording with an external custom-made electrophysiology system.

In both AF groups, in addition to the custom-made electrodes, a commercial bipolar epicardial lead (CapSure Epi, Medtronic) was implanted on the pericardium of the left atrial free wall and connected to a subcutaneously implanted pacemaker (Itrel, Medtronic). After the electrode implantation procedure, goats were allowed to recover for 2 to 3 weeks.

Atrial fibrillation induction

The custom-made electrophysiology setup was used for AF induction and continuous monitoring of atrial electrical activity, as previously described [22]. In summary, AF was induced by burst pacing (50Hz, ≤ 10mA) once sinus rhythm (SR) was detected for 1 second. Goats remained connected up to 14 days, until AF episodes lasted > 24h. Subsequently, the implanted pacemaker was used to maintain AF (50Hz, 4 times threshold) until the end of the experiment at 16 weeks of AF (first 5 weeks of AF: 1 burst per minute, next 5 weeks: 1 burst per 5 minutes and from then on: 1 burst per 10 minutes).

Blood sampling and rivaroxaban monitoring

Blood was sampled at up to 8 different time points for monitoring of the rivaroxaban plasma levels throughout the experimental period in the RIVA AF group (Figure 1D). Blood samples for the thrombin generation analysis were taken at time points pre-Riva (RIVA AF), pre-AF (CTRL AF and RIVA AF) and at the moment of the final experiment (SHAM; ±8 weeks after the implantation, CTRL AF and RIVA AF; ±18 weeks after implantation). Blood was drawn from the jugular vein using a needle and holder (PrecisionGlide, BD) and collected in 3.2% (w/v) citrate vacutainers (BD). Subsequently, the blood was processed into platelet poor plasma (PPP) and stored at -80°C as described previously [23].

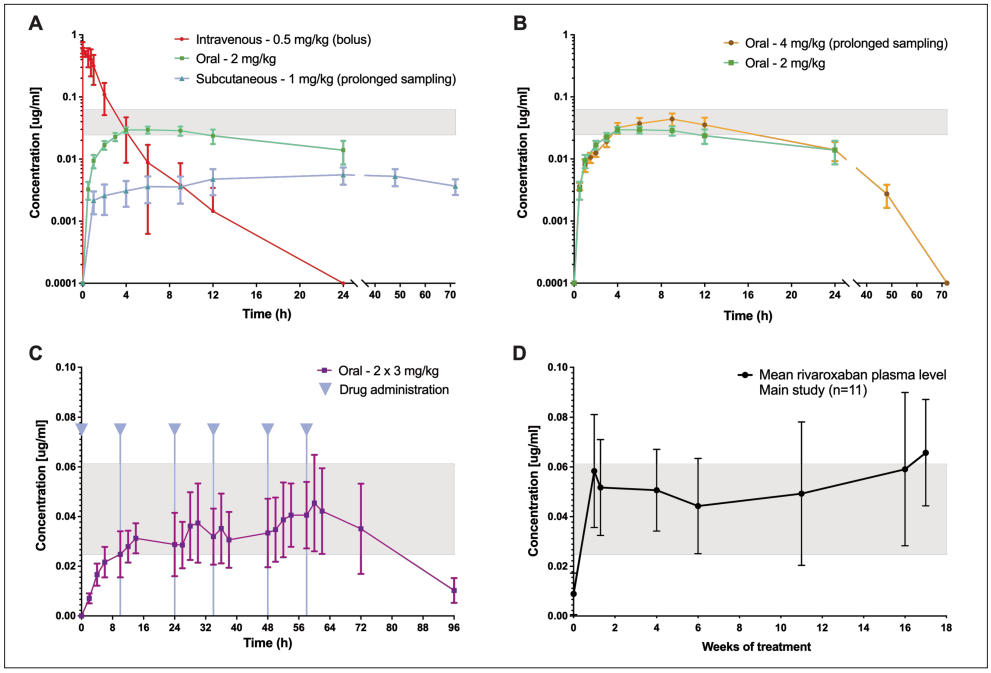

Figure 1. Grey boxes indicate the therapeutic window. A: Pharmacokinetics (PK) of three different rivaroxaban dosing routes (base 10 logarithmic y-axis, n=4 goats per route). B: PK of a double dosage of the previously tested oral route (base 10 logarithmic y-axis, n=4 goats). C: PK of the final twice daily oral dosage (prolonged administrations, n=3 goats), D: Rivaroxaban plasma levels of the RIVA AF group throughout the experimental period (n=11 goats).

Thrombin generation assay

Thrombin generation (TG) was measured in goat citrated platelet poor plasma by means of the calibrated automated thrombography method (Thrombinoscope

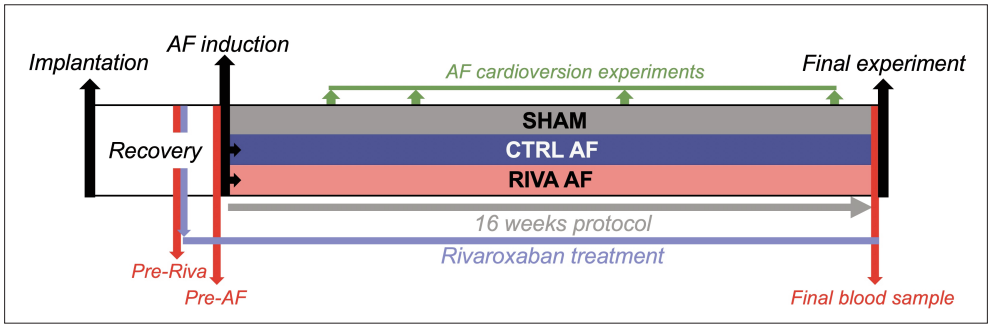

Figure 2. Time line of the main study. The red arrows indicate the different blood sampling moments, the green arrows indicate the (maximum) 4 cardioversion attempts, the black arrows indicate crucial experimental procedures and the blue arrow indicates the initiation of rivaroxaban treatment.

B.V.). The analysis was performed to assess the coagulation state of the animals at different time points throughout the experiment (Table 3). This analysis provides a thrombin generation curve that reflects the ability of the plasma specimen to generate thrombin, also known as clotting potential. The typical thrombin generation curve is characterized by different parameters such as lag time (initiation phase), ETP (endogenous thrombin potential), peak height (highest thrombin concentration) and start tail (time needed for generated thrombin to be fully inactivated) [24].

As commercial reagents, including human recombinant tissue factor (TF), did not yield proper TG curves (data not shown), TG in goat plasma was triggered with goat brain protein extraction (GBP) as source of TF and procoagulant phospholipids (D'Alessandro et al., 2021). The final concentration of GBP used was defined as the one able to produce a thrombin generation curve comparable to that obtained in normal pooled human plasma using PPP Reagent LOW (low TF trigger). Correction for inner filter effects and substrate consumption was performed by calibrating the results from each thrombin generation analysis against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator (Thrombin Calibrator, Thrombinoscope B.V.). Fluorescence was measured in an Ascent Reader (Thermolabsystems OY) equipped with a 390/460nm filter set, and thrombin generation curves were calculated using Thrombinoscope software (Thrombinoscope B.V.).

Cardioversion attempts

To assess the stability of AF, pharmacological cardioversion attempts were performed at 3, 5, 10 and 15 weeks of AF. A cardioversion attempt consisted of 90 minutes of flecainide administration (IV 6mg/kg/h, Tambocor, Mylan) or until SR occurred. Experimental endpoints were AF termination, doubling of the QRS-width

and ventricular arrhythmia. Cardioversion was considered successful if AF terminated during drug infusion or during a 2-hour wash-out period. If two consecutive attempts did not terminate AF, the subsequent cardioversion attempts were not performed.

During the cardioversion experiments, atrial and ventricular electrograms were continuously monitored and recorded. The difference in AF cycle length between the start of flecainide infusion (baseline) and during the last minute before cardioversion (peak, if cardioversion occurred), or at 90min flecainide were determined.

Final experiment (i, ii, iii)

Induction of anesthesia was performed as described above (see 'electrode implantation for AF induction'), including administration of the muscle relaxant rocuronium (0.3mg/kg/h, Fresenius Kabi). A standard limb leads ECG was connected for continuous monitoring.

i. *Hemodynamics*

A pressure-tip catheter (Sentron Europe BV) was inserted into the carotid artery to measure aortic and left ventricular pressures. In the CTRL AF and RIVA AF goats, pressures were measured during AF and SHAM animals were measured during SR.

IDEEQ software (Maastricht University) was used to determine left-ventricular end-diastolic and end-systolic pressure, dP/dt_{max} and dP/dt_{min}. Additionally, mean aortic pressures were obtained from the aortic pressure signals. A Swan Ganz catheter (7.5FR / 110cm, Edward Lifesciences) was inserted into the jugular vein and advanced to the pulmonary artery to determine cardiac output by thermodilution (10mL cold saline) using a Sat-2 Oximeter (Baxter). The mean of 5 consecutive measurements was taken for analysis.

ii. *Atrial contact mapping for assessment AF complexity*

A left-sided thoracotomy was performed to expose the heart for direct atrial contact mapping. Subsequently, two custom-made, round, high-density mapping electrodes (249 electrodes, interelectrode distance = 2.4mm) were placed on the free wall of the left and right atria. Local unipolar electrograms were recorded at a sampling rate of 1.039kHz, bandwidth of 0.56 - 408Hz and a 16-bit A/D resolution. AF recordings of 60 seconds were used for analysis.

To determine AF complexity, AF-electrogram files were analyzed offline using custom-made analysis software (MATLAB 8.1, MathWorks). The previously described algorithm [25] is based on a probabilistic approach that allows the identifica-

tion of local activation times. AF cycle length (AFCL) was determined based on activation time intervals. The conduction velocity (CV) was calculated for each activation by fitting a plane for the activation times of eight spatiotemporal neighboring activations. Waves were defined by clusters of activation times that were connected in space and time by an apparent CV of ≥ 20cm/s. A wave was classified as peripheral if the starting point occurred at the edge of the electrode and breakthrough if the starting point occurred within the mapping array. Waves of < 3 electrodes were considered to be noise and eliminated.

iii. *Tissue collection*

Tissues were harvested after the atrial mapping procedure, following a stabilization period of 30 minutes during which the heart was left untouched. First, the complete heart was removed and weighed and samples were taken from various cardiac regions by two experimenters. Simultaneously, other organs (lungs, left and right kidney, liver and brain) were weighed and sampled by other experimenters. All organ samples were snap frozen in liquid nitrogen and stored at -80°C. Finally, tibial length was measured from the left hind leg.

Analysis of structural remodeling

Cryosections (7μm thickness) were cut transmurally from left and right atrial free wall samples and stained with wheat germ agglutinin (WGA, ThermoFisher) to visualize cell membranes. A detailed protocol for the staining procedure was described previously [26].

Images of the stained sections were acquired at 400X magnification with a Leica DM4B microscope and an MC170 HD camera (Leica). Images of the epi- and endocardial regions were collected, with at least 5 images per region for each atrium per goat. The analysis was performed using the automated analysis software "JavaCyte" [26]. The images were analyzed for endomysial fibrosis (inter-myocyte distance) and myocyte hypertrophy. To manually confirm the accuracy of this analysis, thresholded images were examined and compared to the original images.

Gene expression analysis

Total RNA was extracted using the Direct-zol RNA Miniprep Plus Kit (Zymo) and reverse transcribed with Iscript (BioRad) into cDNA. Gene expression levels were measured with a CFX96 Touch Real-Time PCR detection system (BioRad) and SYBR Green Supermix technology (BioRad). Expression levels were normalized for the housekeeping gene *HPRT* (Hypoxanthine phosphoribosyltransferase 1) and calculated using the comparative threshold cycle method (ΔCt).

SHAM group gene expression was taken as reference and, therefore, CTRL AF and RIVA AF expression levels are shown as fold changes compared to SHAM. The sequences of the specific primers used are provided in Supplemental Table 1.

Statistical analysis

All data are expressed as mean ± standard deviation (SD), unless stated otherwise. Normality was tested by the Shapiro-Wilk test with an alpha of 0.05. In normally distributed data, experimental group differences were tested for significance using a one-way ANOVA with Tukey's post-hoc test for multiple comparisons. In non-normally distributed data, a Kruskall-Wallis test and Dunn's post-hoc test for multiple comparisons were used. A Student's t-test was performed if only two groups were compared, or the Mann-Whitney test in case of non-normality. A paired samples t-test, and a Friedman test for non-normally distributed data, were used in situations where repeated measures were taken. To deal with missing values between time points, a mixed model analysis (MMA) for repeated measures was fitted to the data using subject (goats) as random factor and time point as fixed factor. For the assessment of structural remodeling, a MMA was performed with goat and picture number as random variables and treatment, atrium and cardiac wall as fixed factors. The cardioversion experiment outcome was tested for significance using Fisher's exact test on a 2x2 contingency table.

P-values <0.05 were considered to be significant. Software programs SPSS (Version 26, IBM) and PRISM (version 9.0.0, GraphPad) were used to compute all statistics.

Results

Physical characteristics

In total, 31 female Dutch milk goats were used. The three study groups were comparable in terms of age, body weight and height (assessed by measuring tibia length). Sixteen weeks of atrial fibrillation, with and without rivaroxaban treatment, did not affect heart, lung, kidney and liver weights (Table 2).

Plasma rivaroxaban levels

In goat plasma, a prothrombin time (PT) prolongation of 20-50% was reached with plasma rivaroxaban concentrations between 0.025 and 0.061ug/mL (Bayer AG, personal communication, December 2015). Plasma rivaroxaban levels were monitored at 8 time points throughout the treatment period. At all time points RI-

Table 2. Physical characteristics of the three experimental groups.

	SHAM n=10	**CTRL AF** n=10	**RIVA AF** n=11	**ANOVA**
	Mean ± SD	Mean ± SD	Mean ± SD	*P*-value
Age (months)	36.9 ± 17.2	39.9 ± 16.4	33.4 ± 13.7	0.64
Body weight (kg)	62.8 ± 11.8	66.5 ± 11.3	68.5 ± 11.6	0.54
Tibia length (cm)	25.5 ± 1.7	25.2 ± 2.9	24.1 ± 1.3	0.30
Organ weights (g)				
Heart	320.1 ± 61.3	326.9 ± 72.2	309.4 ± 63.3	0.83
Lung	482.9 ± 76.0	465.2 ± 61.2	489.8 ± 72.9	0.72
Left kidney	77.8 ± 12.3	83.6 ± 15.8	75.1 ± 13.0	0.37
Right kidney	79.6 ± 12.6	83.6 ± 16.9	74.2 ± 13.5	0.35
Liver	903.7 ± 190.2	856.1 ± 176.0	873.2 ± 202.2	0.85
Ratios				
Heart weight / tibia length	12.5 ± 2.2	13.2 ± 3.5	12.8 ± 2.3	0.29
Heart weight / body weight	4.9 ± 0.6	4.9 ± 0.6	4.5 ± 0.5	0.27

Group comparisons were done by one-way ANOVA. SHAM n=10, CTRL AF n=10, RIVA AF n=11 goats.

VA AF goats showed plasma rivaroxaban levels that were above the lower limit of the therapeutic range (0.025μg/mL, Figure 1D).

Thrombin generation analysis

Rivaroxaban treatment resulted in a clear inhibition of clotting potential in RIVA AF from the moment prior to AF induction until 16 weeks of AF. The SHAM and CTRL AF thrombin generation parameters were comparable at the final time point. AF did not increase clotting potential in CTRL AF compared to SHAM. On the contrary, a slight decrease between the pre-AF and final time point was observed (Table 3).

Hemodynamics

Hemodynamics after 16 weeks of AF were comparable in CTRL AF and RIVA AF. Moreover, despite the difference in heart rhythm between SHAM (SR) and both AF groups, the cardiac output, left ventricular diastolic pressure and aortic pressure, did not differ between groups.

Table 3. Thrombin generation assay parameters per group and different time points.

	Pre-Riva	**Pre-AF**	**Final**	
	Mean ± SD	Mean ± SD	Mean ± SD	*P*-value
Lag time (min)				
SHAM	-	-	5.2 ± 1.0	
CTRL AF	-	3.9 ± 1.1	4.7 ± 1.2*	<0.001†^
RIVA AF	4.2 ± 0.4	10.1 ± 2.3**	12.1 ± 3.4**	
ETP (nM•min)				
SHAM	-	-	942.3 ± 448.3	
CTRL AF	-	841.9 ± 175.2	829.2 ± 121.4	<0.001†^
RIVA AF	1022.4 ± 258.9	466.7 ± 77.2**	454.9 ± 183.3**	
Peak height (nM)				
SHAM	-	-	193.5 ± 64.6	
CTRL AF	-	196.2 ± 45.5	179.6 ± 25.7*	<0.001†^
RIVA AF	248.9 ± 41.7	78.4 ± 15.4**	68.7 ± 33.0**	
Start tail (min)				
SHAM	-	-	22.4 ± 3.3	
CTRL AF	-	20.2 ± 1.6	20.9 ± 1.7*	<0.001†^
RIVA AF	20.5 ± 1.7	29.1 ± 3.5**	33.1 ± 5.5**	

Group comparisons for the final time point were done by Kruskall-Wallis test and Dunn's post-hoc test for multiple comparisons (SHAM n=10, CTRL AF n=10, RIVA AF n=11 goats): Lag time †= RIVA AF vs. SHAM ($p<0.001$) and ^= vs. CTRL AF ($p<0.001$), ETP †= RIVA AF vs. SHAM ($p<0.001$) and ^= vs. CTRL AF ($p=0.002$), Peak height †= RIVA AF vs. SHAM ($p<0.001$) and ^= vs. CTRL AF ($p=0.001$), Start tail †= RIVA AF vs. SHAM ($p=0.003$) and ^= vs. CTRL AF ($p<0.001$). *Paired samples t-tests CTRL AF; Pre-AF vs. Final (n=9): Lag time $p=0.01$, ETP $p=0.91$ Peak height $p=0.04$, Start tail $p=0.02$. **Friedman test RIVA AF; Pre-Riva vs. Pre-AF and vs. Final (n=11): Lag time $p<0.001$, ETP $p<0.001$, Peak height $p<0.001$, Start tail $p<0.001$.

The right atrial pressure was significantly higher in CTRL AF and RIVA AF compared to SHAM. In addition, an AF-related decrease in left ventricular systolic pressure, LV maximal positive dP/dT (a measure of LV contractility) and LV maximal negative dP/dT (a measure of LV relaxation) was only found to be significant between RIVA AF and SHAM. Moreover, a trend was found for a difference in heart rate between these two groups ($p=0.07$, Table 4).

Efficacy of pharmacological cardioversion

AF stability was not significantly altered by rivaroxaban treatment. At 3 weeks of AF, cardioversion attempts resulted in 1 out of 5 (20%) successful cardioversions for

Table 4. Hemodynamics and cardiac pressures per experimental group.

	SHAM n=10	**CTRL AF** n=9	**RIVA AF** n=9	*P*-value
	Mean ± SD	Mean ± SD	Mean ± SD	
Cardiac output (L/min)	4.2 ± 1.3	3.6 ± 0.7	3.2 ± 0.9	0.10
Left ventricular pressures (LVP; mmHg)				
Diastolic	9.2 ± 4.7	9.3 ± 5.0	10.5 ± 2.6	0.44
Systolic	109.0 ± 15.3	93.4 ± 18.2	87.4 ± 11.0	0.01^
dP/dT-max	1817 ± 507	1272 ± 409	1290 ± 567	0.02^
dP/dT-min	-2155 ± 597	-1666 ± 371	-1588 ± 475	0.10
Rate (bpm)	75 ± 20	84 ± 14	103 ± 31	0.08
Right atrial pressure (RAP; mmHg)	3.6 ± 1.7	8.1 ± 3.4	7.5 ± 3.0	0.004††^
Aortic pressure (mmHg)	98.7 ± 12.9	88.4 ± 16.6	84.8 ± 15.6	0.13

Cardiac output, right atrial pressure and aortic pressure group comparisons were done by one-way ANOVA and Tukey's post-hoc test for multiple comparisons. RAP; ††= SHAM vs. CTRL AF (p=0.006); ^= SHAM vs. RIVA AF (p=0.02). Left ventricular pressures group comparisons were done by Kruskall-Wallis test and Dunn's post-hoc test for multiple comparisons. LVP; ^= SHAM vs. RIVA AF: systolic p=0.03, dP/dT_{max} p=0.03. SHAM n=10, CTRL AF n=9, RIVA AF n=9 goats.

CTRL AF and in 4 out of 9 (44.4%) successful cardioversions for RIVA AF (p>0.05). At 5 weeks of AF, 2 out of 7 (28.6%) CTRL AF goats and 1 out of 8 (12.5%) RIVA AF goats successfully cardioverted as a result of flecainide infusion (p>0.05). At 10 weeks of AF, one CTRL AF goat still cardioverted, but none did in the RIVA AF group. Missing values, as compared to other experimental parameters in which CTRL AF had n=10 and RIVA AF had n=11, were due to exclusion of some experiments because of technical considerations.

Rivaroxaban treatment did not interfere with the ability of flecainide to increase the AFCL. In fact, the AFCL increase at the 3-week flecainide cardioversion experiment was stronger in RIVA AF (62.6 ± 8.4ms increase) compared to CTRL AF (30.4 ± 12.6ms increase, p<0.001). At the 5 week experiment, AFCL increased to a similar extent in both AF groups.

AF complexity during the final experiment

AF complexity and conduction patterns in the atrial free walls were assessed by epicardial mapping. A more complex AF pattern consists of a higher number of simultaneously propagating wavefronts, leading to a more dissociated activation pat-

tern. The fractionation index of the left atrium was significantly higher in RIVA AF as compared to CTRL AF. Rivaroxaban treatment did not affect any other AF complexity parameter in LA or RA (Table 5).

Table 5. AF complexity parameters determined in the left atrium and the right atrium.

Left atrium	**CTRL AF** Mean ± SD	**RIVA AF** Mean ± SD	*P*-value
Waves (n/cycle)	10.7 ± 2.2	10.9 ± 3.9	0.85
Break throughs (n/cycle)	5.0 ± 1.3	5.4 ± 2.5	0.68
Conduction velocity (cm/sec)	63.0 ± 4.9	66.0 ± 6.9	0.29
AF cycle length (ms)	124.1 ± 14.2	128.4 ± 12.8	0.29
Max. dissociation (ms)	25.9 ± 4.7	26.8 ± 6.0	0.70
Fractionation index	1.8 ± 0.4	2.6 ± 0.9	0.04
Right atrium			
Waves (n/cycle)	5.9 ± 2.3	6.8 ± 2.9	0.45
Break throughs (n/cycle)	2.4 ± 1.2	3.1 ± 1.7	0.34
Conduction velocity (cm/sec)	67.9 ± 11.6	65.8 ± 6.6	0.63
AF cycle length (ms)	104.7 ± 10.9	115.6 ± 13.2	0.06
Max. dissociation (ms)	17.9 ± 3.7	19.6 ± 6.1	0.47
Fractionation index	1.5 ± 0.4	1.6 ± 0.5	0.54

Left atrial AF cycle length data was analyzed using Mann-Whitney test. All other group comparisons were done using Student's t-test. CTRL AF n=10, RIVA AF n=10 goats.

Analysis of structural remodeling

The overall degree of endomysial fibrosis was comparable in the three study groups. Further subdivision for the epi- and endocardial layer of each atrium revealed no differences in endomysial fibrosis (Figure 3). Atrial mycocyte size was significantly increased in CTRL AF compared to SHAM (p=0.01). This AF-related atrial myocyte hypertrophy was fully prevented in RIVA AF (p=0.001, Figure 4). Further subdivision confirmed this finding in the left atrial (LA) epicardium (p=0.01), LA endocardium (p=0.02) and right atrial endocardium (p=0.003, Figure 7).

Gene expression analysis

A total of 14 genes were selected as target for qPCR analysis of the RA and LA. These target genes were chosen on the base of their documented role in either structural or vascular remodeling, coagulation or inflammation (Supplemental Table 1).

In accordance with the increased myocyte size found in our histological analysis, RT-qPCR analysis revealed that 16 weeks of AF induced upregulation of two well-

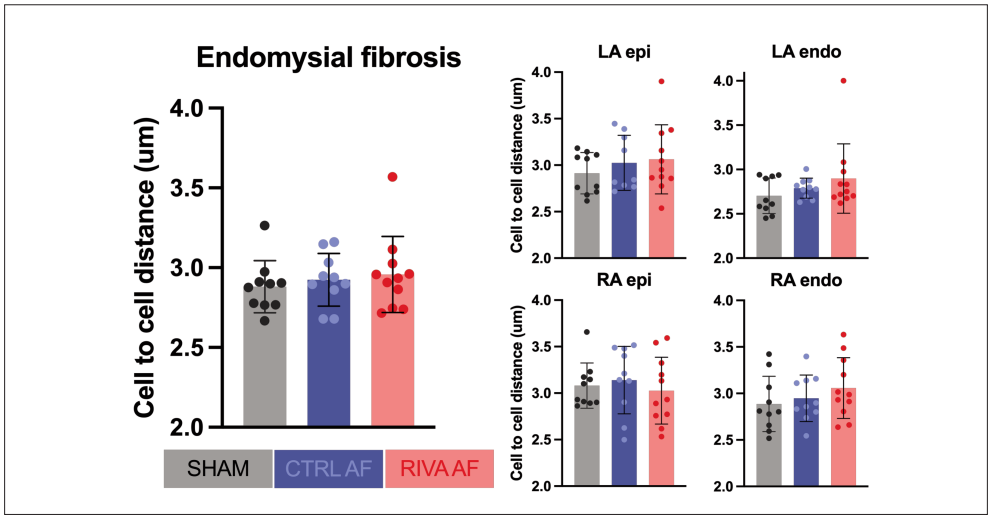

Figure 3. Endomysial fibrosis, quantified as cell-to-cell (C2C) distance between neighboring myocytes, specified per atrium and cardiac wall (epi- and endocardium). MMA estimated overall means per group; SHAM: 2.81μm (95%CI: 2.65, 2.97), CTRL AF: 2.84μm (95%CI: 2.68, 3.00), RIVA AF: 2.90μm (95%CI: 2.75, 3.06). SHAM n=10, CTRL AF n=10, RIVA AF n=11 goats.

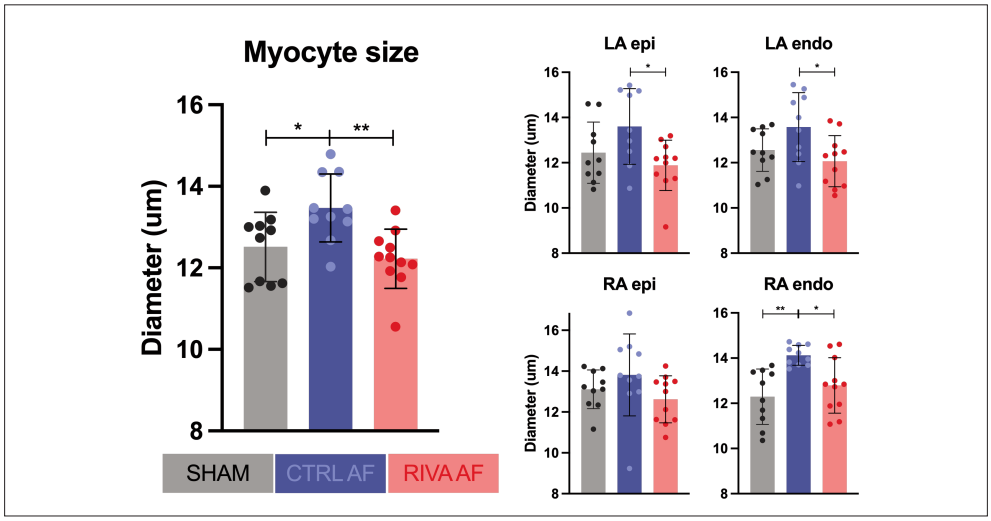

Figure 4. Myocyte hypertrophy specified per atrium and cardiac wall. MMA estimated overall means per group; SHAM: 12.51μm (95%CI: 11.99, 13.03), CTRL AF: 13.47μm (95%CI: 12.94, 13.99), RIVA AF: 12.23μm (95%CI: 11.73, 12.73). Significant pairwise comparisons: SHAM vs. CTRL AF (p=0.01), CTRL AF vs. RIVA AF (p=0.001). Significance of pairwise comparisons per atrium and cardiac wall is indicated in the graph. SHAM n=10, CTRL AF n=10, RIVA AF n=11 goats.

known pro-hypertrophic markers, *NPPA* and *NPPB*, in both the LA (Table 6) and the RA (Table 7). The upregulation of *NPPA* and *NPPB* was not prevented by rivaroxaban, suggesting that FXa inhibition may not be sufficient to fully prevent pro-hypertrophic signaling.

Interestingly, LA expression of the pro-fibrotic markers *ACTA2* and *COL1A1* was upregulated after 16 weeks of AF in CTRL AF without an additional rivaroxaban effect. This suggests the presence of pro-fibrotic molecular changes in the goat LA during AF.

Our analysis also shed light on the expression of a key pro-inflammatory mediator, *CCL2*, and on the potential anti-inflammatory properties of rivaroxaban. We found that, although *CCL2* was not upregulated by AF, rivaroxaban treatment significantly downregulated its expression in the LA. A similar expression pattern was found in the RA, although not significant (p=0.13).

Finally, we looked at the expression of PAR1-4 encoding genes (*F2R*, *F2RL1*, *F2RL2*, and *F2RL3*). Our data revealed that goat LA and RA expressed PAR1, -2 and -3 mRNAs, while PAR4 mRNA was not detected. In the LA, *PAR3* gene expression was downregulated by AF. In contrast, RA *PAR3* did not show significant changes but instead *PAR1* and *PAR2* were both significantly downregulated by AF.

Ultimately, *CAV1*, a gene encoding for an intracellular protein involved in PARs-internalization, was found to be significantly upregulated in both atria after 16 weeks of AF. This effect was not altered by rivaroxaban treatment.

Table 6. RT-PCR gene expression levels of the left atrium, expressed as fold change relative to SHAM.

	SHAM			**CTRL AF**			**RIVA AF**					
Left atrium	Ref.	SD	n	Fold change	SD	n	Fold change	SD	n	*P*-value	**SHAM vs. CTRL AF**	**CTRL AF vs. RIVA AF**
ACTA2	1	0.47	9	1.83	0.88	10	2.10	1.10	10	0.04	0.05	0.66
ANGPT2	1	0.81	10	0.90	0.36	10	0.81	0.39	10	0.78	-	-
CAV1	1	0.31	10	1.61	0.44	10	1.57	0.50	11	0.007	0.004	0.71
CCL2	1	0.70	10	0.85	0.45	10	0.30	0.23	10	0.006	0.72	0.01
COL1A1	1	0.70	10	3.00	2.03	10	2.60	1.13	10	0.002	0.002	1.00
F2R	1	0.44	10	0.98	0.44	10	1.19	0.45	11	0.67	-	-
F2RL1	1	1.19	9	1.57	1.33	10	0.88	0.58	10	0.24	-	-
F2RL2	1	0.79	10	0.44	0.53	10	0.72	0.42	10	0.05	0.02	0.05
F3	1	0.92	10	0.48	0.31	9	0.57	0.70	10	0.50	-	-
IL6	1	0.82	10	0.66	0.47	9	0.51	0.60	10	0.13	-	-
NPPA	1	0.48	9	4.32	2.52	10	4.85	2.44	11	0.002	0.005	0.61
NPPB	1	1.08	9	22.64	16.97	10	27.91	20.88	11	<0.001	0.001	0.71
VEGFA	1	0.40	10	1.02	0.41	10	1.15	0.47	11	0.71	-	-
VWF	1	0.80	9	1.75	1.11	10	2.34	1.71	11	0.10	-	-

Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test for the left atrium.

Table 7. RT-PCR gene expression levels of the right atrium, expressed as fold change relative to SHAM.

	SHAM			CTRL AF			RIVA AF					
Right atrium	Ref.	SD	n	Fold change	SD	n	Fold change	SD	n	*P*-value	**SHAM vs. CTRL AF**	**CTRL AF vs. RIVA AF**
ACTA2	1	0.66	10	1.51	1.51	10	2.24	1.23	11	0.02	0.20	0.12
ANGPT2	1	0.68	10	0.96	0.42	10	1.33	0.83	11	0.39	-	-
CAV1	1	0.46	10	2.12	0.67	10	1.80	0.78	11	0.005	0.001	0.32
CCL2	1	0.75	10	0.78	0.36	10	0.46	0.30	11	0.08	-	-
COL1A1	1	0.53	10	0.75	0.96	10	1.05	1.27	11	0.20	-	-
F2R	1	0.48	10	0.52	0.24	10	0.89	0.44	11	0.04	0.01	0.10
F2RL1	1	0.68	10	0.07	0.08	9	0.56	0.70	11	0.01	0.002	0.08
F2RL2	1	0.31	9	1.17	1.10	9	1.12	1.34	10	0.70	-	-
F3	1	1.34	10	0.72	0.55	10	0.88	1.42	11	0.39	-	-
IL6	1	0.53	10	0.75	0.96	10	1.05	1.27	11	0.20	-	-
NPPA	1	0.61	10	2.77	1.34	10	3.12	1.86	11	0.001	0.002	0.83
NPPB	1	1.10	9	8.19	5.74	10	9.03	9.36	11	0.001	<0.001	0.60
VEGFA	1	0.71	10	1.04	0.32	10	1.80	1.49	11	0.18	-	-
VWF	1	0.85	10	1.64	0.78	10	2.63	2.09	11	0.07	-	-

Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test for the right atrium.

Discussion

Our study shows that FXa-inhibition, via rivaroxaban treatment, decreases the clotting potential and prevents AF-related atrial myocyte hypertrophy in the goat model of persistent AF. Moreover, rivaroxaban appears to inhibit atrial inflammatory signaling. AF stabilization over time and atrial endomysial fibrosis, as well as the overall degree of atrial electrophysiological changes due to AF, were not altered by rivaroxaban treatment.

In the goat model of AF, electrical remodeling (shortening in atrial refractoriness) takes place during the first couple of days after AF onset, while structural remodeling processes in the subsequent weeks to months are associated with further AF stabilization [22, 27]. Several studies demonstrated that the success rate of pharmacological cardioversion declines with increasing time in AF [28, 29]. In this study, AF stability was assessed by the efficacy of flecainide to cardiovert AF. Similar to the previous studies we found a decline in the success rate of flecainide cardioversions, but this progressive stabilization of AF was not affected by rivaroxaban treatment.

Our histological findings are in line with in vivo studies that have found a protective effect of rivaroxaban on hypertrophy in non-AF small animal models. Delbeck et al. reported that rivaroxaban treatment prevented the development of right-ventricular hypertrophy in a rat model of pulmonary hypertension [30]. Recently, Guo et al. showed that rivaroxaban resulted in a reduction of cardiac hypertrophy in a

mouse model of cardiac pressure-overload after transverse aortic constriction (TAC) [31]. The same study also reported reduced expression of the hypertrophic markers *NPPA* and *NPPB* in TAC-rivaroxaban mice compared to TAC-only mice.

Similarly, in our study, the AF-induced hypertrophic growth of atrial myocytes is accompanied by a clear upregulation of *NPPA* and *NPPB* in both left and right atrium. However, while FXa-inhibition prevented the increase in myocyte size due to AF, it did not prevent the upregulation of these two hypertrophic markers. This may be explained by the fact that, in addition to FXa signaling, other mechanisms may contribute to myocyte hypertrophy in the goat model of AF. The increased (right) atrial pressure that we observed during AF in our study, may lead to atrial stretch, which potentially contributes to the upregulation of *NPPA* and *NPPB* via other signaling pathways. In this scenario, rivaroxaban, which did not alter right atrial pressures, is not sufficient to prevent upregulation of *NPPA* and *NPPB*.

In this study, we focused on atrial myocyte hypertrophy and fibrosis as components of atrial structural remodeling. Both have been related to electrophysiological changes contributing to complex fibrillatory conduction patterns [11, 17, 32, 33].

Our finding that myocyte diameter was homogeneously increased throughout the atrial wall after an extensive period of persistent AF (CTRL AF) is consistent with our previous reports [19, 27, 32]. However, the prevention of myocyte hypertrophy in the RIVA AF group did not affect AF complexity as shown by the fact that none of the AF complexity markers was lower in RIVA AF compared to CTRL AF. A possible explanation for this could be the relatively minor atrial myocyte size difference (± 10%) that was found between RIVA AF and CTRL AF goats.

Moreover, prevention of myocyte hypertrophy did not result in a significant difference in heart weight to body weight, or heart weight to tibia length ratio, compared to SHAM and RIVA AF. This could be due to the minor atrial myocyte size differences between groups, but also to the fact that atria and ventricles were not weighed separately.

The second structural remodeling component we focused on is fibrosis, an increase in fibrous tissue for which three different subtypes were described by Weber et al.: perimysial, endomysial and replacement fibrosis. Perimysial fibrosis is an increase fibrous tissue that surrounds bundles of myocytes, while endomysial fibrosis reflect an increased separation of individual myocytes within muscle bundles. Replacement fibrosis is a reparative response to cell death leading to scar tissue [34].

In a previous study, we extensively described endomysial fibrosis in the goat model of AF, which preferentially occurred in the outer millimeter of the atrial wall and resulted in significant conduction disturbances during AF [33]. For this reason, endomysial fibrosis was used as the main read-out for fibrosis in the present study. It was quantified as inter-myocyte distances, as described by Winters et al. [26]. In the

present study, endomysial fibrosis was not increased after 16 weeks of AF maintenance. No effect of AF was found on endomysial fibrosis in any of the subdivided regions (LA and RA, endo- and epicardial layer). It should be noted, however, that for practical reasons histological analysis in the present study was performed in frozen tissue, while, in the previous study, plastic-embedded specimens were studied. This difference may have led to a lower sensitivity in detecting endomysial fibrosis.

Other studies have demonstrated that rivaroxaban attenuates processes that lead to cardiac fibrosis [31, 32, 35]. Kondo et al. showed that rivaroxaban administration decreased atrial interstitial fibrosis in a murine model of pressure overload. Moreover, rivaroxaban significantly attenuated atrial fibrosis in rats with isoproterenol-induced AF [36, 37]. However, in our model, no additional effect was found for rivaroxaban treatment during AF, compared to sinus rhythm (SHAM) or AF without treatment (CTRL AF). The absence of a difference in endomysial fibrosis between CTRL AF and RIVA AF in this study was reflected by a comparable degree of AF complexity between the two groups.

Atrial gene expression analysis, however, revealed an upregulation of the pro-fibrotic genes *COL1A1* and *ACTA2* in the LA of the CTRL AF group compared to SHAM. This upregulation was not affected by rivaroxaban treatment. Despite the fact that no relevant differences in endomysial fibrosis were found, these results suggest that the molecular processes leading to fibrosis may be activated in the goat LA during AF.

Additionally, the current study may offer insights into the potential anti-inflammatory effects of rivaroxaban. The gene *CCL2* encodes for the monocyte chemoattractant protein-1 (MCP1), one of the key regulators of pro-inflammatory processes. The SHAM and CTRL AF groups showed similar expression levels of *CCL2*. This suggests that AF alone did not upregulate the expression of this gene in our model. Nevertheless, rivaroxaban significantly downregulated *CCL2* expression in the LA and showed a similar trend in the RA. These results indicate a link between plasma FXa inhibition and reduced pro-inflammatory signaling in the heart. This observation is in line with other studies which reported downregulation of pro-inflammatory gene expression in the heart upon plasma FXa inhibition in mice [35, 36, 38]. Nevertheless, expression analysis of our second pro-inflammatory marker, *IL6*, did not show the same pattern.

Changes in *PARs* expression have been found in several pathological conditions. *PAR1* and *PAR2* were more frequently expressed in the heart of patients with ischemic and idiopatic dilated heart failure and *PAR2* was also upregulated in the blood of patients with atherosclerosis [39, 40].

We found measurable expression of *PAR1-3* mRNAs in goat atria. Our analysis showed that, in the LA of the CTRL AF group, *PAR3* expression is significantly

downregulated, while in the RA, *PAR1* and *PAR2* expression were decreased compared to SHAM.

These findings, however, are not in line with other studies that report on the relationship between changes in PARs expression and cardiac hyperthrophy. Antoniak et al. demonstrated that cardiac-specific *PAR2* overexpression in mice lead to larger hearts and altered expression of pro-hypertrophic genes [39]. Additionally, Pawlinski et al. reported hypertrophic effects related to *PAR1* overexpression and that PAR1-deficiency in mice resulted in a significant reduction of LV dilation and impairment of LV function after myocardial infarction [41]. Species differences in the regulation of PAR expression may underly these discrepancies.

On the other hand, Friebel et al. recently reported contrasting findings [42]. They discovered that, in endomyocardial biopsies of patients with heart failure with preserved ejection fraction, a relationship existed between low *PAR2* expression and increased infiltration of inflammatory cells, as well as myocardial fibrosis. Interestingly, in our study, *CAV1*, a gene encoding an intracellular protein involved in PARs internalization, was upregulated in both LA and RA. This suggests that PARs internalization and/or recycling might be enhanced during AF.

Study limitations

In our study, 4 months of AF did not increase the coagulation potential. On the contrary, we observed a slight decrease in peak height of the thrombin generation curve. These results seem to be in contrast with clinical studies that describe a hypercoagulable state in patients with AF. However, our coagulation assessment is only based on TG analysis, which might not be the most sensitive method to pick up changes of coagulation in goat plasma. For this reason, additional assays could give a better insight on the goat haemostatic profile during AF. An alternative explanation can be that AF alone and/or AF duration might not be sufficient to trigger a procoagulant response in goats.

Initially, the first time point for the pharmacological cardioversion experiment was at 5 weeks of AF. After the first unsuccessful flecainide experiments, this was set to the earlier time point of 3 weeks AF. Together with some technical complications, especially in the CTRL AF group, this limited the final group size. Such discrepancies reduced the statistical power to detect potential differences in early AF stabilization.

Also, this study reports on the effects of AF on systemic coagulation activity. No additional information is known about the plasma levels and/or the activity of specific coagulation factors (e.g. FXa) in the tissues. Therefore, besides describing the overall clotting potential of these animals, we did not further study the contribution

of specific pro-coagulant or anti-coagulant pathways, particularly because of known species differences in coagulation pathways that hamper the usage of commercially available coagulation assays.

Conclusion

In an established goat model of AF, prolonged oral treatment with a direct FXa inhibitor, rivaroxaban, provided a sustained reduction of systemic clotting potential, resulting in diminished cardiomyocyte hypertrophy, a key element in the pathogenesis of AF. These data further corroborate the non-canonical molecular interplay between coagulation proteases (i.e. FXa) and the heart. The consequences for patients on long term treatment with FXa inhibiting direct oral anticoagulants merit further investigation.

Translational perspective

This article describes, for the first time in a large animal model, the experimental evidence that show the protective effect of FXa-inhibition by rivaroxaban on atrial structural remodeling induced by 4 months of atrial fibrillation. Rivaroxaban treatment decreases the clotting potential and prevents AF-related myocyte hypertrophy in the goat model of persistent AF. These data show that systemic FXa-inhibition during atrial fibrillation, may locally protect the heart from atrial structural changes which play a crucial role in the pathogenesis of AF. Confirmation of these findings in human studies, may help understanding and preventing the mechanisms that lead to AF progression.

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Supplementals

Supplemental Table 1. Target genes and primer sequences used for RT-PCR analysis.

Gene symbol	Target gene	Forward primer (5'->3')	Reverse primer (5'->3')
ACTA2	Actin alpha 2	GGGCCGTTTTCCCGTCTATC	TCATCACCCACGTAGCTGTC
ANGPT2	Angiopoietin 2	GACGGACGTTGAAGCCCAA	TCCAGGAAGCTGTTCTTATCTTG
CAV1	Caveolin 1	GGGGGCAAATACGTAGACTCA	GGGCTTGTAGATGTTGCCCT
CCL2	C-C motif chemokine 2	CTCGCTCAGCCAGATGCAAT	TGTCAGCCTCTGTATGGGGA
COL1A1	Collagen type I alpha 1 chain	TGTCCTAACGCCAAAGTCCC	TGGTCCGTGGTTGATTCCTG
F2R	Coagulation factor II thrombin receptor	AGAAGGACGTTTGTGGGTCC	AGAAGGACGTTTGTGGGTCC
F2RL1	Coagulation factor II thrombin receptor like 1	GTCGCAGGAGACAAGAGACTG	CTTTCCAGTGAGGACGGAGG
F2RL2	Coagulation factor II thrombin receptor like 2	GCTTCCTGTCAAAGCGGCAT	TTGGTGGTTTCTGTCCAGCC
F2RL3	Coagulation factor II thrombin receptor like 3	GCAGAAGGCGATGACAGCAC	GGAGCTCCAGAGTGTTGCT
F3	Coagulation factor III	GCACTAGCCACGAGAAAGGT	GAGTCACAGACAGGACGACG
IL6	Interleukin 6	CTTCACAAGCGCCTTCAGTC	GCTTGGGGTGGTGTCATTCT
NPPA	Natriuretic peptide A	TTTCAAGAATTTGCTGGACCGTT	ATACTTGTGAGGGCACAGCC
NPPB	Natriuretic peptide B	CTCTTCTTGCACCTGTCGCT	CCAGCAGCTCCTGTAACCCA
VEGFA	Vascular endothelial growth factor A	CCTTCACCATGCCAAGTGGT	GTCTCAATGGGACGGCAGAA
VWF	Von Willebrand factor	TTGATGGGGAGGTGAACGTG	TTCAGGAACACGGAGACAGC
Housekeeping gene			
HPRT1	Hypoxanthine phosphoribosyltransferase 1	CGACTGGCTCGAGATGTGAT	ATCCAACAGGTCGGCAAAGA

CHAPTER 6

Atrial fibrillation and age synergistically increase clotting potential and promote atrial structural remodeling in goats

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In preparation for publication.

Abstract

Introduction

Age is a risk factor for atrial fibrillation (AF) as well as for stroke in patients with AF. The effect of ageing on AF pathogenesis and coagulation activity is not well understood. In this study we evaluated the effect of age on coagulation status, atrial remodeling and AF progression in the goat model of pacing-induced AF.

Methods

Four groups of goats were studied: Young goats in sinus rhythm (n=9), young goats in AF (n=7), old goats in sinus rhythm (n=6), and old goats in AF (n=8). The age difference between the young (<3 years) and old (>8 years) animals was approximately 6 years. All groups were matched for body weight. AF was induced and maintained by burst pacing for 4 weeks. Coagulation activity at baseline and after 4 weeks of AF was measured by thrombin generation assays. Stabilization of AF was monitored during the first day of AF induction. At 2 and 4 weeks of AF, pharmacological cardioversion attempts with vernakalant were performed to assess AF stability. During a final open-chest experiment hemodynamics and AF complexity parameters were measured. Finally, atrial tissue was collected for histology and gene expression analysis.

Results

The thrombin generation parameters peak height ($p=0.02$) and endogenous thrombin potential (ETP; $p=0.02$) demonstrated a significantly enhanced clotting potential after 4 weeks of AF in old, but not in young goats. AF stabilized faster in old goats than in young goats ($p=0.02$) during the first day of AF induction, while AF stability at 2 and 4 weeks of AF maintenance was comparable between the groups. Complexity of AF and hemodynamics were not affected by age alone. AF, however, significantly increased the right atrial pressure in old ($p=0.02$), but not in young goats. AF induced atrial myocyte hypertrophy ($p<0.01$) and left atrial epicardial endomysial fibrosis ($p=0.04$) in old, but not in young goats. This was associated with upregulation of hypertrophic and fibrotic marker genes.

Conclusion

This study shows the synergism between advanced age and AF, leading to enhanced coagulation potential, accelerated AF stabilization and atrial structural remodeling. Complexity of atrial conduction was affected by AF, but not by age.

Introduction

Atrial fibrillation (AF) is the most common cardiac arrhythmia and its prevalence increases with age [1]. Among people below 50 years of age, AF is present in less than 0.5% of the population and this number rises to 10-17% in the age class of ≥80 years [2, 3]. Due to ageing of our population, the number of AF patients in the European Union above 55 years will double in the upcoming decades [4-6]. Since AF is an independent risk factor for stroke, leading to a nearly 5-fold increase in stroke risk, the doubling of AF prevalence is a major concern [1, 7-9]. In addition, increased age is also a strong risk factor for stroke and, therefore, a component of the CHA_2DS_2-VASc score that is used to decide on antithrombotic treatment in patients with AF [9-11].

To date, it is still not fully elucidated whether lone AF (AF without traditional risk factors and underlying structural heart disease, in patients <60 years of age) is sufficient to induce a prothrombotic state and cause thrombotic events. Some studies have demonstrated that lone AF is not a benign condition, but that it causes a prothrombotic state [12, 13]. However, most reports suggest that in lone AF patients there is no increased risk for stroke. In fact, the stroke risk increases only after many years, when there is advanced age and development of other risk factors[8, 14-16]. These findings emphasize the relevance of interaction between risk factors, in particular age and AF, for the development of a hypercoagulable state.

Despite the fact that age is a very important risk factor of AF, the age-related changes in atrial structure and function that increase the propensity to AF, are incompletely understood. For example, some, but not all, studies have demonstrated that age is associated with an increasing degree of atrial fibrosis [17, 18].

We hypothesize that age and AF synergistically interact to produce a pro-thrombotic state and favor AF substrate development. In this study we evaluated the effect of age on coagulation status, atrial remodeling and AF progression, in the goat model of pacing-induced AF.

Methods

The study was conducted in accordance with European and national guidelines for the use of laboratory animals and was approved by the local ethics committee for animal experiments at Maastricht University (AVD1070020173984).

Experimental groups

Female Dutch milk goats were used in this study. Each goat had a history of bearing and high milk production, but at the moment of study inclusion the lactation

was stopped by a dry-off period. Only the goats with a negative test for paratuberculosis, normal ranges of red and white blood counts and normal liver and kidney blood values were included.

The animals were divided in 4 experimental groups: young sinus rhythm (Y-SR: sinus rhythm, n=9), young AF (Y-AF, n=7), old sinus rhythm (O-SR: sinus rhythm, n=6), and old AF (O-AF, n=8). The mean age per experimental group was 2.7, 2.7, 8.8 and 9.2 years, respectively.

The groups were matched for body weight, which were on average 73.9 (Y-AF), 71.4 (Y-SR), 71.2 (O-SR) and 76.3 (O-AF) kilograms. These inclusion criteria led to 4 comparable groups at baseline, comprising 2 different age classes.

All the animals underwent the same initial surgical procedure. In the AF groups, AF was induced and maintained for 4 weeks, whereas in the SR groups the stimulation electrodes were implanted but AF was not induced (Figure 1).

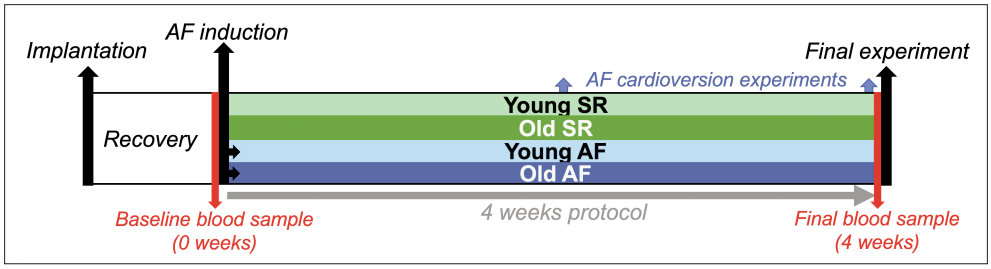

Figure 1. Experimental time line. AF was only induced in Y-AF and O-AF.

Electrode implantation for AF induction

Anesthesia was induced by intravenous (IV) injection of sodium thiopenthal (20 mg/kg, Rotexmedica) and maintained by sufentanyl (6 μg/kg/h IV, Hameln Pharma) and propofol (5-20 mg/kg/h IV, Fresenius Kabi).

For the implantation of a triangular custom-made patch of 10 electrodes, a left-sided thoracotomy through the 4th intercostal space was performed. From there, the patch was sutured on the pericardium overlying the left atrial free wall. In addition, custom-made bipolar-electrode patches were sutured on the pericardium overlying the left ventricle and the right ventricle. The electrode wires, incorporated in a silicone tube, were tunneled under the skin and exteriorized at the neck, allowing pacing and electrogram recording with an external device.

In both AF groups, a bipolar epicardial lead (CapSure Epi, Medtronic) was implanted next to the custom-made electrodes on the pericardium of the left atrial free wall and was connected to a subcutaneously implanted pacemaker (Itrel, Medtronic). All animals were allowed to recover for 2 weeks after surgery.

Atrial fibrillation induction and stabilization

AF was induced and maintained by burst pacing (50 Hz, 1 s, ≤10 mA) once sinus rhythm (SR) was detected for >1 second. Goats remained connected to the stimulation set-up for 5 consecutive days. After that period the implanted pacemaker was used to maintain AF (50 Hz, 4 times threshold, 1 burst per 5 minutes) until sacrifice. AF stabilization was assessed at the first day of AF initiation by calculating the number of AF paroxysms during the first 24 hours.

Blood sampling and thrombin generation assay

Blood was sampled at baseline (just before AF induction) and at the final time point (4 weeks of AF, Figure 1). Blood was drawn from the jugular vein using an 18G needle with holder (PrecisionGlide, BD) and collected in 3.2%(w/v) citrate tubes (Vacutainer, BD). Subsequently, the blood was processed into platelet poor plasma (PPP) and stored at -80˚C as described by Loeffen et al. [19].

The coagulation status was assessed by means of the of the calibrated automated thrombography method (Stago). The lag time (initiation phase), ETP (endogenous thrombin potential) and peak height (highest thrombin concentration) were derived from the thrombin generation (TG) curve [20].

As described previously (D'Alessandro et al. 2021 / chapter 3) TG in goat plasma was triggered with goat brain protein extraction (GBP) as source of tissue factor (TF) and procoagulant phospholipids. The final concentration of GBP was defined as the concentration that was able to produce a thrombin generation curve comparable to the concentration obtained in normal pooled human plasma using PPP Reagent LOW (low TF trigger). Correction for inner filter effects and substrate consumption was performed by calibrating the results from each thrombin generation analysis against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator (Thrombin Calibrator, Stago). Fluorescence was measured in an Ascent Reader (Thermolabsystems OY) equipped with a 390/460 nm filter set, and TG curves were calculated using Thrombinoscope software (Stago).

Cardioversion experiment

Pharmacological cardioversion attempts were performed at 2 and 4 weeks of AF, to assess AF stability. A cardioversion attempt consisted of maximally 60 minutes of vernakalant (Merck, Darmstadt, Germany) administration (IV, the first 30 minutes 3.7 mg/kg and the next 30 minutes 4.5 mg/kg) and a 30-minute wash-out period, or until SR occurred [21]. During the cardioversion experiment, atrial and ventricular electrograms were continuously monitored and recorded. Cardioversion was consi-

dered successful if AF terminated during the drug infusion or during the wash-out period. If AF did not terminate at the 2-week time point, AF was considered to be permanent and the attempt at 4 weeks of AF was not executed.

Final experiment

Anesthesia was induced and maintained as described above (see the section 'electrode implantation for AF induction'), with the additional administration of the muscle relaxant rocuronium (0.3 mg/kg/h, Fresenius Kabi). A standard limb lead ECG was connected for continuous monitoring.

A. *Hemodynamics*

Pressure tip catheters (7F, Sentron Europe BV) were placed in the left carotid artery and jugular vein to measure aortic and left-ventricular (LV) and right ventricular (RV) pressures, respectively. In SR goats, cardiac pressures were measured during SR, while in AF goats pressures were measured during AF.

IDEEQ software (Instrument Development Engineering and Evaluation, Maastricht University, Maastricht, The Netherlands) was used to determine end-diastolic and end-systolic pressure and maximum positive and negative rate of pressure change (dP/dt). In addition, mean aortic pressures were obtained from the aortic pressure signals. A Swan Ganz catheter (7.5 FR / 110 cm, Edward Lifesciences) was used to determine cardiac output by thermodilution (10 mL cold saline) using a Sat-2 Oximeter (Baxter). The cardiac output was calculated as an average of 5 consecutive measurements.

B. *Atrial contact mapping for assessment of AF complexity*

Once the hemodynamic measurements were completed, a left-sided thoracotomy was performed to expose the heart for direct atrial contact mapping. Two custom-made high-density mapping electrodes (249 electrodes, interelectrode distance 2.4 mm) were placed on the free walls of the left and right atrium. Local unipolar electrograms were recorded at a sample rate of 1.039 kHz, bandwidth of 0.56-408 Hz and a 16-bit A/D resolution.

To determine AF complexity, the unipolar electrograms were analyzed offline using a custom-made analysis software developed in Matlab (The MathWorks, Inc., Natick, Massachusetts, USA) [22]. The algorithm identifies local activation times based on a probabilistic approach. AF cycle length (AFCL) was determined using the local activation times. The waves were defined by clusters of activation times that are connected in space and time by an apparent conduction velocity (CV) of $\geq$ 20 cm/s. CV was calculated for each activation within the wave by fitting the activation

times of the neighboring activations with a plane. A wave was classified as peripheral if the starting point occurred at the edge of the electrode, and as a breakthrough if the starting point occurred within the mapping array. Waves of < 3 electrodes were excluded from the analysis.

C. *Tissue collection*

After the atrial mapping procedure, a stabilization period of 30 minutes followed during which the heart was left untouched. Subsequently, the complete heart was removed and weighed. Tissue samples were taken from various cardiac regions, including left and right atrial free wall. Lungs, left and right kidney, liver and brain were weighed and left tibial length was measured.

Histological analysis

Cryosections (7 μm thickness) were cut transmurally from left and right atrial free wall samples and stained with wheat germ agglutinin (WGA, ThermoFisher) to visualize extracellular matrix [23]. Images of the stained sections were acquired at 400X magnification with a Leica DM4B microscope and an MC170 HD camera (Leica microsystems, Amsterdam, the Netherlands). Images of the epi- and endocardial regions were collected, with at least 5 images per region for each atrium per goat. The analysis was performed using the automated analysis software "JavaCyte", as described by Winters et al. [23]. The images were analyzed for endomysial fibrosis (inter-myocyte distance) and myocyte hypertrophy.

Gene expression analysis

Total RNA was extracted using the Direct-zol RNA Miniprep Plus Kit from (Zymo research, Irvine, USA) and reverse transcribed with Iscript (BioRad, Veenendaal, the Netherlands) into cDNA. Gene expression levels were measured with a CFX96 Touch Real-Time PCR detection system (BioRad) and SYBR Green Supermix technology (BioRad). Expression levels were normalized for the housekeeping gene HPRT (Hypoxanthine phosphoribosyltransferase 1) and calculated using the comparative threshold cycle method (ΔCt).

Statistical analysis

All data are expressed as mean ± standard deviation (SD), unless stated otherwise. Normality was tested by the Shapiro-Wilk test with an alpha <0.05. In normally distributed data, experimental group differences (e.g. gene expression) were tested for significance using a one-way ANOVA with Šidák's post-hoc test for multiple

comparisons. In non-normally distributed data (e.g. gene expression and physical characteristics), a Kruskall-Wallis test with Dunn's post-hoc test for multiple comparisons were used. A Student's t-test was performed if only 2 groups were compared, or the Mann-Whitney test was used in case of non-normal distribution (AF stabilization). A paired samples t-test, and Wilcoxon test for non-normally distributed data, were used in situations where repeated measures were taken (TG data). For the analysis of AF complexity, a two-way ANOVA with factors Age (young vs. old) and AF (acute vs. 4 weeks) and Tukey's post-hoc test for multiple comparisons was performed. For the assessment of structural remodeling, a mixed model analysis was used with goat and picture as random variables and treatment, atrium and cardiac wall as fixed factors. The cardioversion experiment outcome was tested for significance using Fisher's exact test on a 2x2 contingency table.

P-values <0.05 were considered to be significant. Software programs SPSS (Version 26, IBM) and PRISM (version 9.0.0, GraphPad) were used to perform the statistical analyses.

Results

Physical characteristics

In line with the design of this study, age significantly differed between the young and old groups of goats, while body weight and tibial length were comparable (Table 1).

Organ weights such as heart, lung, left and right kidney showed minor differences between groups, although not between relevant study group comparisons. The heart weight to tibial length ratio was greater in O-AF as compared to Y-AF (p=0.03, Table 1).

Hemodynamics

Hemodynamic assessment revealed no age-related differences in cardiac pressures between the groups with the same heart rhythm (Y-SR vs. O-SR and Y-AF vs. O-AF, Table 4). However, AF had significant impact on hemodynamic characteristics within the two age classes. In the young goats, AF decreased the LV systolic pressure (p=0.01), maximal slope of LV pressure curve ($dP/dt_{max,}$ p=0.01), RV systolic pressure (p=0.02), RV dP/dt_{max} (p=0.01) and aortic pressure (p=0.01). In the old goats, AF decreased LV systolic pressure (p=0.03), and increased right atrial (RA) pressure (p=0.02). Interestingly, no significant differences in cardiac output were found between groups, although a tendency was found for AF to negatively affect cardiac output in the young groups (Table 2).

Table 1. Physical characteristics of the four experimental groups.

	Y-SR	**O-SR**	**Y-AF**	**O-AF**	**Kruskal-Wallis**	**Dunn's post-hoc**			
	n=9	n=6	n=7	n=8	*P*-value	**Y-SR vs. O-SR**	**Y-SR vs. Y-AF**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
Age (years)	2.7 ± 0.5	8.8 ± 0.8	2.7 ± 0.4	9.2 ± 0.6	<0.0001	0.03	>0.99	>0.99	<0.01
Body weight (kg)	71.4 ± 8.8	71.2 ± 7.0	73.9 ± 5.2	76.3 ± 11.7	0.65	-	-	-	-
Tibia length (cm)	23.0 ± 1.2	23.5 ± 1.6	24.3 ± 0.8	24.1 ± 1.1	0.24	-	-	-	-
Organ weights (g)									
Heart	329.7 ± 50.1	364.0 ± 34.3	356.4 ± 29.4	432.7 ± 76.9	0.01	>0.99	>0.99	0.41	0.13
Lung	475.3 ± 92.5	593.9 ± 52.0	544.1 ± 53.3	645.2 ± 135.9	0.01	0.07	>0.99	>0.99	0.49
Left kidney	89.2 ± 18.4	105.4 ± 9.6	91.4 ± 9.4	110.7 ± 20.2	0.03	0.48	>0.99	>0.99	0.10
Right kidney	85.4 ± 18.9	102.3 ± 10.9	90.3 ± 10.2	108.8 ± 20.2	0.04	0.30	>0.99	>0.99	0.25
Liver	1041.0 ± 213.2	960.3 ± 109.4	980.0 ± 114.4	1080.0 ± 250.3	0.87	-	-	-	-
Normalized weight									
Heart weight / tibial length	14.1 ± 2.0	15.5 ± 1.2	14.7 ± 1.2	17.9 ± 2.7	<0.01	>0.99	>0.99	0.32	0.03
Heart weight / body weight	4.6 ± 0.6	5.1 ± 0.5	4.8 ± 0.6	5.8 ± 1.1	0.06	0.53	>0.99	>0.99	0.60

Group comparisons were done by Kruskal-Wallis test and Dunn's post-hoc test for multiple comparisons. Values are mean ± SD.

Table 2. Hemodynamics and cardiac pressures of the four experimental groups.

	Y-SR	**O-SR**	**Y-AF**	**O-AF**	**Kruskal-Wallis**	**Dunn's post-hoc**			
	n=9	n=6	n=7	n=8	*P*-value	**Y-SR vs. O-SR**	**Y-SR vs. Y-AF**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
Left ventricular pressures (mmHg)									
Diastolic	10.6 ± 5.5	10.6 ± 4.9	8.0 ± 4.9	8.8 ± 4.2	0.57	-	-	-	-
Systolic	121.6 ± 16.3	113.6 ± 16.9	85.2 ± 17.8	75.3 ± 19.3	<0.0001	>0.99	0.01	0.03	>0.99
dP/dtmin	-2182 ± 533	-1774 ± 480	-1680 ± 530	-1279 ± 541	0.03	0.85	0.42	0.54	0.84
dP/dtmax	2038 ± 221	1730 ± 430	1318 ± 419	1179 ± 406	<0.001	0.89	0.01	0.21	>0.99
Right ventricular pressures (mm/Hg)									
Diastolic	10.1 ± 4.2	7.1 ± 5.1	8.1 ± 2.9	11.4 ± 4.6	0.28	-	-	-	-
Systolic	39.0 ± 7.2	36.8 ± 11.2	26.6 ± 6.0	31.6 ± 7.6	0.04	>0.99	0.02	>0.99	0.89
dP/dtmin	-430 ± 142	-409 ± 111	-295 ± 40	-308 ± 92.8	0.04	>0.99	0.12	0.22	>0.99
dP/dtmax	704 ± 152	565 ± 165	408 ± 159	380 ± 107	<0.01	0.70	0.01	0.31	>0.99
Right atrial pressure (mmHg)	8.2 ± 3.4	6.4 ± 1.9	8.3 ± 2.5	11.4 ± 3.1	0.04	0.86	>0.99	0.02	0.24
Aortic pressure (mmHg)	103.0 ± 10.3	92.0 ± 24.9	76.7 ± 14.1	68.3 ± 20.9	<0.001	>0.99	0.01	0.18	>0.99
Cardiac output (L/min)	4.9 ± 0.8	4.3 ± 1.2	3.3 ± 1.3	3.9 ± 1.6	0.14	>0.99	0.08	>0.99	>0.99

Group comparisons were done by Kruskal-Wallis test and Dunn's post-hoc test for multiple comparisons. Values are mean ± SD.

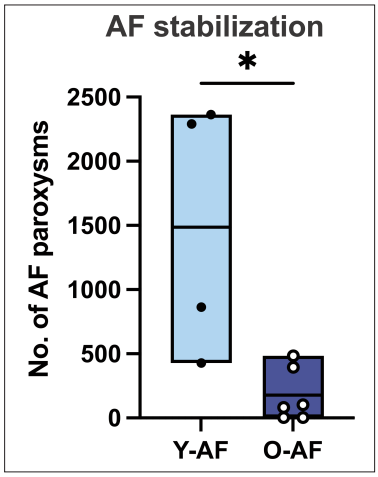

Figure 2. Early AF stabilization. The number of AF paroxysms during the first 24 hours of AF induction in Y-AF (n=4) vs. O-AF (n=6, p=0.02), plotted by box and whiskers (min to max).

AF stabilization

Stabilization of AF was assessed by the number of AF paroxysms during the first 24 hours and occurred faster in O-AF (179±374 paroxysms) compared to Y-AF (1487±614 paroxysms, p=0.02) goats (Figure 2).

Cardioversion experiments

At 2 and 4 weeks, AF stability was assessed by cardioversion attempts using the antiarrhythmic drug vernakalant. Cardioversion efficacy of vernakalant was not found to be significantly different between the young and old AF group. In Y-AF, 6 out of 7 (86%) goats cardioverted at 2 weeks of AF, whereas 5 out of 8 (63%) goats cardioverted in O-AF (p=0.57). At the 4 weeks experiment, 3 goats in each AF group still cardioverted. Overall, this resulted in a 43% cardioversion success rate in Y-AF and 37% in O-AF (p>0.99) after 4 weeks of AF.

The average time to cardioversion did not differ between the groups. At 2 weeks of AF, it was 42.6 ± 13.2 min in Y-AF and 55.0 ± 6.7 min in O-AF (p=0.13). At 4 weeks AF it was 50.0 ± 25.1 min in Y-AF and 63.7 ± 10.2 min in O-AF (p=0.80).

AF complexity

In the two-way ANOVA analysis including all animals, AF resulted in an increase of the number of waves (p<0.01) and breakthroughs (p<0.01) per AF cycle. This difference was accompanied by slower conduction (p<0.01) and increased maximal dissociation (p=0.03) in the left atrium (LA). In the right atrium (RA), we found that AF tended to lead to decreased conduction velocity (p=0.07) and a significant reduction of AFCL (p<0.001, Table 3).

However, post hoc tests between specific groups showed that in the young groups (Y-SR vs. Y-AF) there was no difference in complexity between acutely induced AF and 4 weeks of AF. Also, age did not affect complexity of conduction in acutely induced AF (Y-SR vs. O-SR), or after 4 weeks of AF (Y-AF vs O-AF, Table 3). Nevertheless, in old but not in young goats, 4 weeks of AF led to slowing of conduction during AF in the LA (p=0.04) and shortening of the AFCL in the RA (p=0.02, Table 3).

Table 3. AF complexity parameters of both atria in the four experimental groups.

					Two-way ANOVA			Post-hoc			
	Y-SR	**Y-AF**	**O-SR**	**O-AF**	**Age**	**AF**	**Interaction**	**Y-SR vs. Y-AF**	**Y-SR vs. O-SR**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
						P-value					
Left atrium	n=7	n=7	n=6	n=8							
Waves (n/cycle)	7.23 ± 1.83	10.14 ± 2.22	7.7 ± 3.34	11.2 ± 3.02	-	<0.01	-	-	-	0.10	-
Break throughs (n/cycle)	2.91 ± 1.04	5.06 ± 1.57	3.50 ± 2.05	5.29 ± 2.07	-	<0.01	-	-	-	-	-
Conduction velocity (m/sec)	0.77 ± 0.14	0.69 ± 0.07	0.87 ± 0.18	0.68 ± 0.07	-	<0.01	-	-	-	0.04	-
AF cycle length (ms)	136.7 ± 14.8	129.70 ± 16.5	141.7 ± 23.1	130.3 ± 25.4	-	-	-	-	-	-	-
Max. dissociation (ms)	23.09 ± 4.42	27.87 ± 5.76	22.35 ± 8.98	30.16 ± 8.02	-	0.03	-	-	-	-	-
Fractionation index	1.11 ± 0.42	1.73 ± 0.77	1.32 ± 0.62	1.70 ± 0.76	-	0.06	-	-	-	-	-
Right atrium	n=7	n=7	n=6	n=7							
Waves (n/cycle)	7.27 ± 1.99	6.76 ± 2.35	7.27 ± 2.23	8.33 ± 2.80	-	-	-	-	-	-	-
Break throughs (n/cycle)	2.79 ± 0.82	3.01 ± 1.39	3.35 ± 1.63	3.83 ± 1.49	-	-	-	-	-	-	-
Conduction velocity (m/sec)	0.77 ± 0.13	0.71 ± 0.09	0.75 ± 0.12	0.66 ± 0.05	-	0.07	-	-	-	-	-
AF cycle length (ms)	129.8 ± 13.8	105.3 ± 13.0	146.6 ± 25.6	111.2 ± 24.7	-	<0.001	-	-	-	0.02	-
Max. dissociation (ms)	22.50 ± 2.60	20.24 ± 6.00	23.93 ± 5.27	24.06 ± 7.69	-	-	-	-	-	-	-
Fractionation index	1.10 ± 0.32	1.16 ± 0.32	1.45 ± 0.48	1.37 ± 0.90	-	-	-	-	-	-	-

All values are mean ± SD. Group comparisons were done by two-way ANOVA, with factors Age (young vs. old) and AF (acute vs. 4 weeks) and Tukey's post-hoc test for multiple comparisons.

Thrombin generation assay

The clotting potential at both baseline and the final time point did not significantly differ between the experimental groups (Table 4). However, 4 weeks of AF induced a significant increase in clotting potential in old goats (O-AF), as shown by peak height and ETP values (both p=0.02, Figure 3 and Table 5). Interestingly, comparable findings were not observed in young goats (Y-AF). The results indicate that 4 weeks of AF provoke an increase in pro-thrombotic tendency only in aged animals.

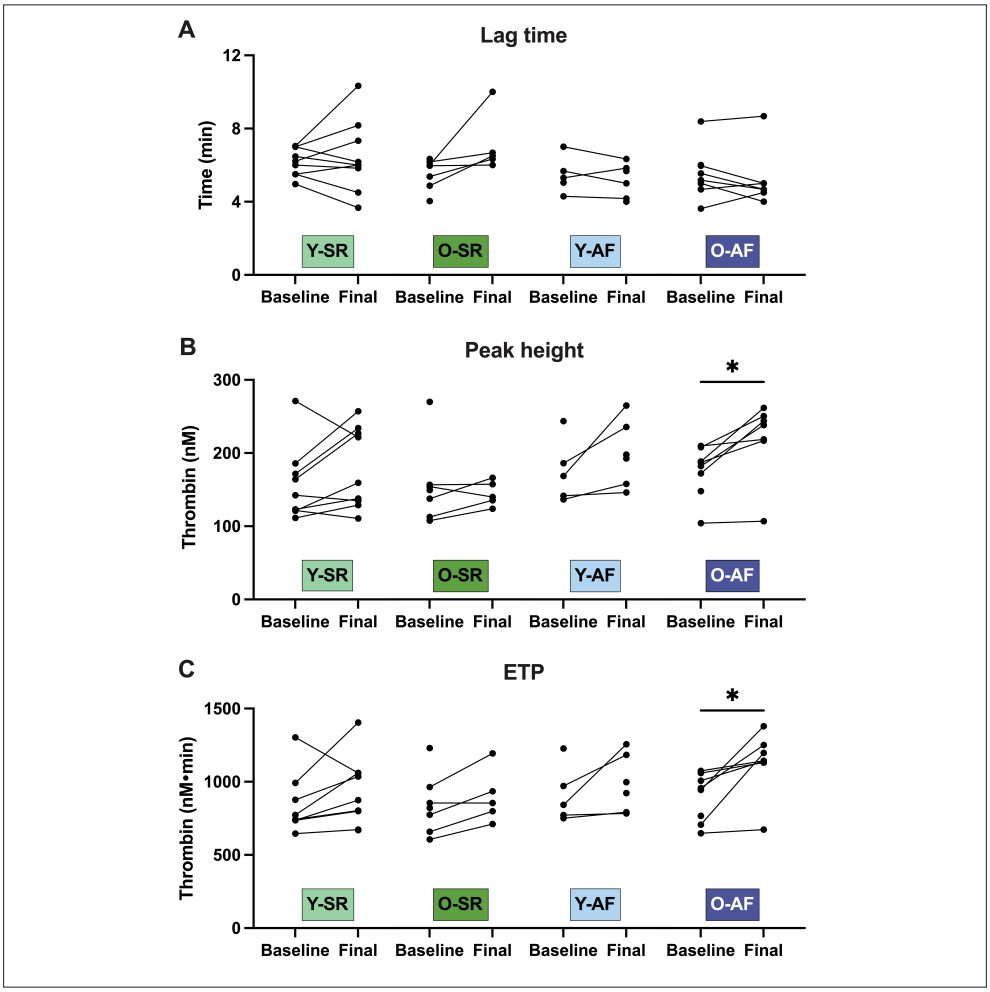

Figure 3. Thrombin generation parameters lag time (A), peak height (B) and ETP (C) indicated per experimental group at baseline vs. final, analyzed by Wilcoxon matched-pairs test. Exact outcome per analysis can be found in Table 5 (within groups) and Table 4 (between groups comparison).

Table 4. Between groups comparison of thrombin generation parameters.

Between groups	**Y-SR**	**Y-AF**	**O-SR**	**O-AF**	**Kruskal-Wallis**	**Y-SR vs. Y-AF**	**Y-SR vs. O-SR**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
Baseline	n=9	n=5	n=7	n=8	*P*-value				
Lag Time (min)	6.2 ± 0.8	5.5 ± 1.0	5.5 ± 0.8	5.5 ± 1.4	0.28	-	-	-	-
Peak (nM)	156.9 ± 50.1	175.3 ± 43.2	155.4 ± 54.2	174.9 ± 34.7	0.37	-	-	-	-
ETP (nM•min)	850.8 ± 210.8 (n=8)	912.5 ± 195.6	843.5 ± 208.0	895.3 ± 165.0	0.82	-	-	-	-
Final	n=9	n=6	n=5	n=7	*P*-value				
Lag Time (min)	6.4 ± 2.0	5.2 ± 0.9	7.1 ± 1.6	5.2 ± 1.6	0.05	-	-	0.06	-
Peak (nM)	179.1 ± 55.3	199.2 ± 45.2	144.5 ± 17.1	219.5 ± 52.2	0.12	-	-	0.09	-
ETP (nM•min)	931.0 ± 234.8	988.8 ± 197.3	898.2 ± 184.1	1131.0 ± 219.9	0.25	-	-	-	-

Between groups analysis was done with Kruskal-Wallis test and Dunn's multiple comparisons test.

Table 5. Within groups comparison of thrombin generation parameters.

Within groups	**Y-SR** Baseline vs. Final (n=9)		*P*-value	**Y-AF** Baseline vs. Final (n=4)		*P*-value
Lag Time (min)	6.2 ± 0.8	6.4 ± 2.0	0.82	5.6 ± 1.2	5.333 ± 0.9	0.38
Peak (nM)	156.9 ± 50.1	179.1 ± 55.3	0.13	158.2 ± 23.3	201.1 ± 58.2	0.13
ETP (nM•min)	850.8 ± 210.8	963.8 ± 227.9	0.11	833.8 ± 98.8	1004.0 ± 251.2	0.13
	O-SR Baseline vs. Final (n=5)		*P*-value	**O-AF** Baseline vs. Final (n=7)		*P*-value
Lag Time (min)	5.7 ± 0.5	7.1 ± 1.6	0.06	5.5 ± 1.5	5.2 ± 5.2	0.38
Peak (nM)	133.7 ± 22.9	144.5 ± 17.1	0.19	178.8 ± 35.6	219.5 ± 52.2	0.02
ETP (nM•min)	770.8 ± 144.7	898.2 ± 184.1	0.06	913.7 ± 169.2	1131.0 ± 219.9	0.02

Paired analysis was done by Wilcoxon matched-pairs signed rank test.

Histology

The presence of structural remodeling was assessed by endomysial fibrosis, which was quantified as the myocyte-to-myocyte distance. Atrial histological analysis overall (mixed model) did not show any significant differences between the groups (Figure 4A, Table 6). Further subdivision for the left and right atrial epicardium and endocardium revealed that endomysial fibrosis was significantly increased in the epicardial layer of the LA in O-AF as compared to O-SR, while no differences were found for the other subdivisions (Figure 4C-F, Table 6).

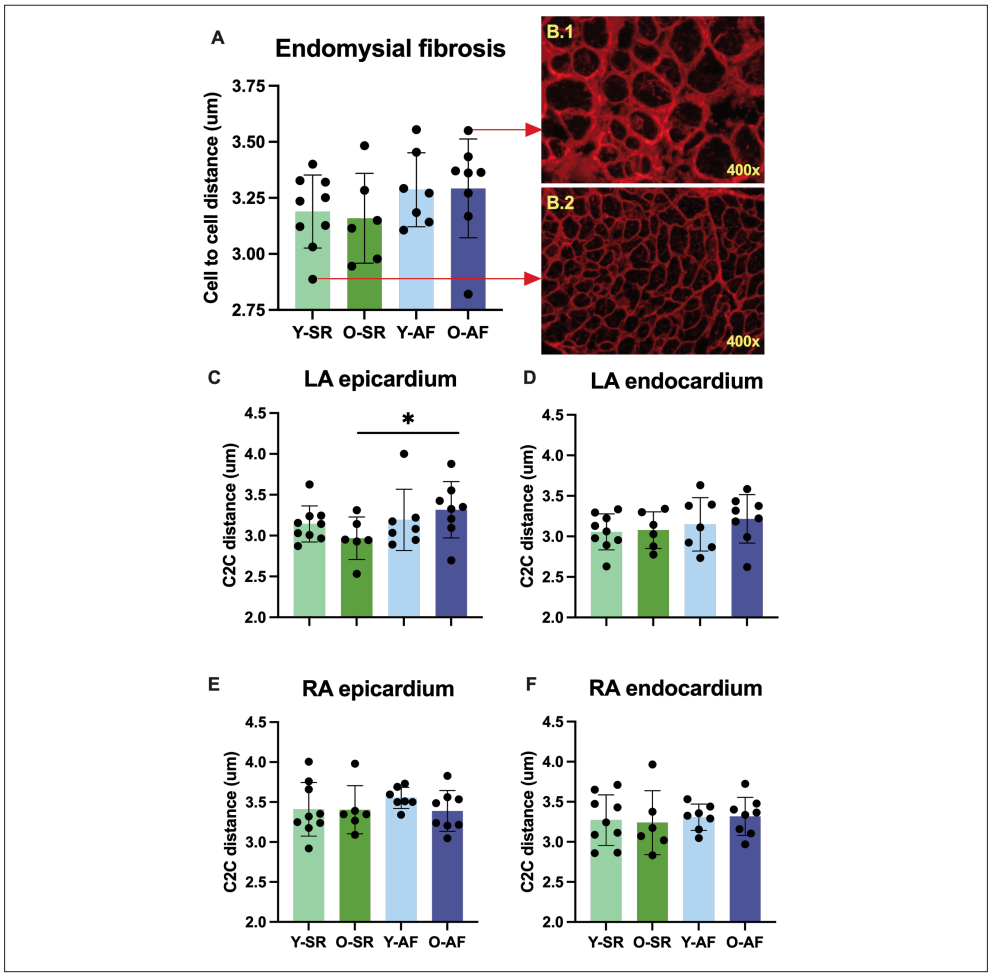

Figure 4. Endomysial fibrosis, indicated per experimental group (A) and for different regions (C-F). Two example images of endomysial fibrosis are shown in B.1 and B.2.

The other structural remodeling parameter, atrial myocyte size, significantly differed between the groups overall (mixed model, Figure 5A, Table 6). The post-hoc analysis revealed that age did not affect myocyte size in sinus rhythm (Y-SR vs. O-SR), nor did AF in young goats (Y-SR vs. Y-AF). However, the synergism of old age and AF led to an increase in atrial myocyte size (O-SR vs. O-AF $p<0.01$) and a trend was found towards larger myocytes in O-AF as compared to Y-AF ($p=0.08$). The effect of increased myocyte size in O-AF compared to O-SR was found in the left atrial (LA) epicardium ($p=0.02$), LA endocardium ($p=0.02$) and RA endocardium ($p<0.01$), with a trend for the RA epicardium ($p=0.06$). The difference between O-AF and Y-AF was found to be significant in the LA epicardium (Figure 5C-F and Table 6).

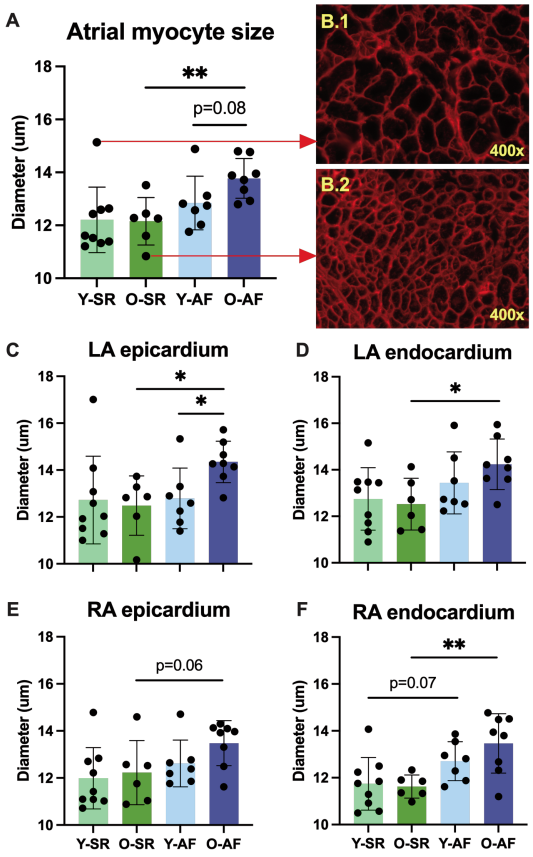

Figure 5. Atrial myocyte size, indicated per experimental group (A) and for different regions (C-F). Two example images are shown in B.1 and B.2.

Table 6. Histological analysis of the structural remodeling parameters endomysial fibrosis (cell-to-cell distance) and myocyte size, evaluated per atrium and cardiac wall in the four experimental groups.

	Y-SR		**O-SR**		**Y-AF**		**O-AF**		**Type III**	**LSD multiple comparisons**			
	n=9		n=6		n=7		n=8		**fixed effects**	**Y-SR vs. Y-AF**	**Y-SR vs. O-SR**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
	Mean (μm)	95% CI	Mean (μm)	95% CI	Mean (μm)	95% CI	Mean (μm)	95% CI	*P*-value				
Cell to cell distance													
LA epicardium	3.01	2.78 - 3.34	2.89	2.57 - 3.20	3.10	2.80 - 3.41	3.23	2.93 - 3.53	0.23	-	-	0.04	-
LA endocardium	3.06	2.87 - 3.24	3.08	2.85 - 3.31	3.15	2.94 - 3.36	3.21	3.02 - 3.41	0.64	-	-	-	-
LA overall	2.99	2.67 - 3.30	2.91	2.59 - 3.24	3.05	2.73 - 3.34	3.15	2.83 - 3.47	0.29	-	-	0.07	-
RA epicardium	3.34	3.12 - 3.58	3.34	3.07 - 3.61	3.51	3.25 - 3.76	3.33	3.08 - 3.58	0.58	-	-	-	-
RA endocardium	3.27	3.07 - 3.47	3.24	3.00 - 3.48	3.31	3.08 - 3.53	3.32	3.11 - 3.53	0.95	-	-	-	-
RA overall	3.33	3.15 - 3.52	3.32	3.10 - 3.54	3.43	3.22 - 3.63	3.34	3.15 - 3.54	0.88	-	-	-	-
Overall (LA & RA)	3.15	2.98 - 3.32	3.12	2.92 - 3.31	3.24	3.06 - 3.42	3.25	3.07 - 3.42	0.45	-	-	-	-
Myocyte size													
LA epicardium	12.72	11.75 - 13.69	12.48	11.30 - 13.67	12.79	11.69 - 13.88	14.35	13.31 - 15.38	0.06	-	-	0.02	0.04
LA endocardium	12.74	11.88 - 13.60	12.53	11.48 - 13.57	13.43	12.46 - 14.41	14.23	13.32 - 15.15	0.05	-	-	0.02	-
LA overall	12.70	11.84 - 13.56	12.47	11.42 - 13.52	13.08	12.11 - 14.05	14.28	13.37 - 15.19	0.04	-	-	0.01	0.07
RA epicardium	12.23	11.24 - 13.22	12.48	11.34 - 13.61	12.80	11.73 - 13.87	13.73	12.70 - 14.76	0.08	-	-	0.06	-
RA endocardium	11.74	11.03 - 12.45	11.62	10.76 - 12.48	12.70	11.90 - 13.50	13.46	12.71 - 14.21	<0.01	0.07	-	<0.01	-
RA overall	11.89	11.18 - 12.60	11.94	11.08 - 12.80	12.68	11.88 - 13.48	13.49	12.74 - 14.24	0.01	-	-	<0.01	-
Overall (LA & RA)	12.22	11.52 - 12.91	12.17	11.32 - 13.02	12.85	12.06 - 13.64	13.79	13.06 - 14.53	0.01	-	-	<0.01	0.08

Mixed model analysis (MMA) estimated marginal means and 95% confidence intervals given per group, per atrium and cardiac wall. Post-hoc multiple comparison testing based on Least Significant Difference (LSD).

Gene expression analysis

Thirteen genes were selected that encode proteins with a documented role in either structural or vascular remodeling, coagulation or inflammation (Supplemental Table 1). The outcome of the gene expression analysis is given in Table 7 (left atrium) and Table 8 (right atrium).

Of the selected targets, the hypertrophic gene, *NPPA* was upregulated in O-AF compared to O-SR in both RA ($p<0.01$) and LA ($p=0.05$), and in the O-AF compared to Y-AF in the RA ($p<0.01$). Similarly, *NPPB* expression was found significantly increased in the O-AF group compared to the age-matched group (O-SR) in both atria (LA: $p=0.01$; RA: $p<0.01$).

Moreover, AF increased expression of the pro-fibrotic *COL1A1* in the LA. This increase was significant between Y-AF and Y-SR ($p=0.03$) and O-AF and O-SR ($p=0.05$). In the RA, the expression of this gene was upregulated in O-AF as compared to O-SR ($p=0.01$). *ACTA2*, the other pro-fibrotic gene assessed, was not affected by AF, nor by age. Finally, RA expression analysis revealed a downregulation of the angiogenic gene *VEGFA* after 4 weeks of AF in old but not in young goats ($p=0.02$).

Discussion

This study shows that age and AF synergistically increase the coagulation potential, and promote atrial structural remodeling by increasing myocyte hypertrophy and endomysial fibrosis. AF stabilization was faster in old goats. As expected, AF increases complexity of atrial conduction. In contrast, age does not affect the response to pharmacological cardioversion, neither AF complexity.

The goat model of ageing

In humans, age is the most important risk factor for AF development [1]. The overall prevalence of AF is 5.5%, but the numbers rise strongly from 0.7% at the age of 55 years, to >17% in people of ³85 years of age [2, 3]. In our model, the age difference between young and old goats was approximately 6 years. The young goats were on average 2.7 years of age and the old goats had an average age of 9 years. In goats, the life span varies per breed and is dependent on many factors. On average, dairy goats can reach 12-15 years of age, but for commercial reasons (loss of fertility and decreasing milk production) the average life span of a Dutch white milk goat in the Netherlands is 3.8 years, which limited the inclusion of even older goats in this study [24] [nieuweoogst.nl].

Table 7. Left atrial gene expression analysis.

	Y-SR		**O-SR**		**Y-AF**		**O-AF**		**ANOVA**	**Post-hoc**			
Left atrium	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	*P*-value	**Y-SR vs. Y-AF**	**Y-SR vs. O-SR**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
ACTA2	1.13 ± 0.52	8	0.95 ± 0.54	3	1.68 ± 0.68	5	1.81 ± 1.34	7	0.27	-	-	-	-
ANGPT2	0.08 ± 0.05	8	0.07 ± 0.01	3	0.10 ± 0.04	5	0.11 ± 0.09	7	0.86	-	-	-	-
CAV1	2.62 ± 0.75	8	2.61 ± 0.8	3	3.40 ± 0.81	5	4.00 ± 0.61	7	<0.01	0.27	>0.99	0.05	0.54
CCL2	45.10 ± 34.87	8	17.08 ± 1.65	3	41.49 ± 10.07	5	36.57 ± 16.03	7	0.38	-	-	-	-
COL1A1	1.45 ± 1.34	8	0.54 ± 0.52	3	10.74 ± 8.26	5	5.73 ± 4.38	7	<0.01	0.03	>0.99	0.05	>0.99
F2R	0.88 ± 0.31	8	0.70 ± 0.42	3	1.11 ± 0.31	5	1.27 ± 0.38	7	0.09	0.71	0.91	0.11	0.90
F2RL2	0.37 ± 0.24	8	0.39 ± 0.15	3	0.55 ± 0.41	5	0.53 ± 0.23	7	0.59	-	-	-	-
F3	0.62 ± 0.45	8	0.22 ± 0.06	3	0.48 ± 0.31	4	0.33 ± 0.13	6	0.24	-	-	-	-
IL6	8.75 ± 9.38	8	2.35 ± 1.66	3	8.37 ± 5.67	5	6.42 ± 4.22	7	0.54	-	-	-	-
NPPA	19.66 ± 10.37	8	14.29 ± 5.1	3	37.45 ± 22.18	5	42.37 ± 15.1	7	0.02	0.18	0.97	0.05	0.97
NPPB	7.70 ± 7.75	8	4.22 ± 3.97	3	34.60 ± 17.14	5	54.67 ± 22.42	7	<0.01	0.13	>0.99	0.01	>0.99
VEGFA	1.47 ± 0.55	8	1.10 ± 0.18	3	1.15 ± 0.21	5	1.15 ± 0.68	7	0.45	-	-	-	-
VWF	0.91 ± 0.59	8	0.93 ± 0.82	3	1.15 ± 0.62	5	1.08 ± 0.37	7	0.87	-	-	-	-

Relative expression data were normalized for the housekeeping gene *HPRT*. Group comparisons were done by one-way ANOVA and Šidák's post-hoc test for multiple comparisons. *ACTA2*, *ANGPT2*, *COL1A1*, *NPPB* and *VEGFA* were non-normally distributed and were analyzed by Kruskal-Wallis test and Dunn's post-hoc test for multiple comparisons. Values are mean ± SD.

Table 8. Right atrial gene expression analysis.

	Y-SR		**O-SR**		**Y-AF**		**O-AF**		**ANOVA**	**Post-hoc**			
Right atrium	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	*P*-value	**Y-SR vs. Y-AF**	**Y-SR vs. O-SR**	**O-SR vs. O-AF**	**Y-AF vs. O-AF**
ACTA2	0.94 ± 0.43	9	0.94 ± 0.49	5	0.82 ± 0.17	7	0.88 ± 0.45	8	0.89	-	-	-	-
ANGPT2	0.06 ± 0.02	9	0.09 ± 0.04	5	0.07 ± 0.03	7	0.09 ± 0.03	8	0.10	-	-	-	-
CAV1	1.96 ± 0.26	8	2.88 ± 1.48	5	3.16 ± 0.74	7	3.23 ± 0.37	8	<0.01	0.02	0.15	0.88	1.00
CCL2	43.24 ± 26.63	9	40.3 ± 22.27	5	44.32 ± 31.30	7	46.24 ± 32.03	8	0.99	-	-	-	-
COL1A1	1.47 ± 1.07	8	0.51 ± 0.25	5	4.68 ± 4.10	7	4.57 ± 3.71	8	<0.01	0.23	0.30	0.01	>0.99
F2R	0.46 ± 0.29	9	0.53 ± 0.21	5	0.56 ± 0.19	7	0.91 ± 0.28	8	0.02	>0.99	>0.99	0.21	0.17
F2RL2	0.21 ± 0.21	9	0.31 ± 0.16	5	0.22 ± 0.15	7	0.47 ± 0.33	8	0.14	-	-	-	-
F3	0.17 ± 0.11	8	0.15 ± 0.06	5	0.19 ± 0.06	7	0.33 ± 0.19	8	0.06	>0.99	>0.99	0.14	0.79
IL6	7.89 ± 8.01	9	4.83 ± 2.78	5	4.09 ± 3.49	7	4.16 ± 2.41	7	0.87	-	-	-	-
NPPA	10.45 ± 6.19	9	13.47 ± 6.00	5	13.46 ± 8.16	7	30.94 ± 10.28	8	<0.001	0.92	0.94	<0.01	<0.01
NPPB	12.09 ± 13.58	8	6.91 ± 5.00	4	20.52 ± 6.69	6	67.76 ± 33.51	8	<0.01	0.67	>0.99	<0.01	0.40
VEGFA	1.30 ± 0.39	9	2.20 ± 0.82	5	1.05 ± 0.38	7	1.10 ± 0.57	8	0.03	>0.99	0.32	0.02	>0.99
VWF	0.53 ± 0.24	9	0.58 ± 0.24	5	0.46 ± 0.15	7	0.66 ± 0.22	8	0.33	-	-	-	-

Relative expression data were normalized for the housekeeping gene *HPRT*. Group comparisons were done by one-way ANOVA and Šidák's post-hoc test for multiple comparisons. *ACTA2*, *ANGPT2*, *COL1A1*, *F2R*, *F2RL2*, *F3*, *IL6*, *NPPB*, *VEGFA* and *VWF* were non-normally distributed and were analyzed by Kruskal-Wallis test and Dunn's post-hoc test for multiple comparisons. Values are mean ± SD.

As in humans, we expected that also in goats the health conditions and diseases acquired during their life span could influence their susceptibility to AF onset or its progression. Therefore, to exclude the contribution of apparent comorbidities, we included only animals without signs of active infectious diseases, with normal blood counts and with normal liver and renal blood parameters.

Age-related (early) progression of AF

AF is a progressive disease, characterized by the transition from paroxysmal to persistent and, ultimately, to permanent AF [25]. This progression also occurs in the goat model of pacing-induced AF, in which AF episode duration increases over time until AF becomes sustained [26]. In this study, we found that early AF stabilization, assessed by the number of AF paroxysms required to maintain AF during the first 24 hours, was significantly lower in old goats than in young goats. These results suggest the presence of a pre-existing substrate for AF in old goats, which increases the AF stabilization rate during the first day after AF onset.

To evaluate AF stability once the arrhythmia has become sustained, we performed cardioversion experiments with vernakalant at 2 and 4 weeks of AF. The cardioversion attempts showed that AF stability did not significantly differ between young and old goats. As expected, the cardioversion success rate declined over time from 86% to 43% in young and from 63% to 37% in old goats between the 2- and 4-week time point. This loss of efficacy of antiarrhythmic drugs with increasing AF duration is in agreement with our previous reports, indicating the presence of ongoing remodeling processes that drive AF stabilization even after AF has become sustained [21, 27].

AF-related atrial remodeling in aged goats

The occurrence of AF depends on a trigger, as initiator, and an appropriate substrate to maintain the arrhythmia [28, 29]. The trigger in our model was SR-triggered burst pacing. AF substrate development is the result of atrial remodeling, for which two main processes have been described: electrical and structural remode-ling [29-31].

Electrical remodeling, taking place during the first days after AF initiation, is characterized by the shortening of atrial refractoriness and action potential duration. Structural remodeling, a much slower process, is considered to be responsible for the progression and stabilization of AF [32, 33].

There are several animal studies that have investigated age-associated structural remodeling in relation to electrical conduction and AF. Spach et al. found age-related slowing of transverse conduction in dog atria, which was due to extensive col-

lagenous septa between groups of myofibers [34, 35]. Koura et al. reported an increase in myocyte width and fat cell infiltration between myofibers, associated with enhanced anisotropy of conduction in old dogs [36]. Also, a comparative study between young and old rats by Hayashi et al. found that AF could only be induced in old rats [37]. The difference in inducibility was associated with a significant amount of atrial interstitial fibrosis that was present in the old rats. These age-dependent changes in structure, associated with conduction disturbances, form an arrhythmogenic substrate that has also been suggested to be relevant for AF vulnerability in humans [29]. However, not all histological studies of human atria have confirmed an association between age and AF [17]. Nevertheless, in a recent large histological study our research group confirmed a significant association of age with fibrosis, although the effect size was limited (cell to cell distance increase / 5 years: LA appendage: 0.20 ± 0.09 μm, p=0.04; RA appendage 0.25 ± 0.15 μm, p=0.04; PhD thesis Joris Winters, unpublished data).

In our study, endomysial fibrosis was investigated as the main read-out parameter for atrial fibrosis, since in the goat model it was associated with significant conduction disturbances during AF [38, 39]. Endomysial fibrosis leads to increased separation of individual myocytes within muscle bundles and it was previously detected in goats with 6 months of AF [38]. In line with these findings, the current study shows that in old goats, 4 weeks of AF led to increased endomysial fibrosis in the epicardium of the LA wall. Possibly because the atrial pressure is higher in the LA compared to the RA, which is therefore more prone to stretch (and stretch-induced fibrosis) [30]. Nevertheless, AF seemed to induce upregulation of the pro-fibrotic gene *COL1A1* in both atria, pointing at the presence of pro-fibrotic changes within the RA and LA. These results agree with our previous study which revealed that adult goats with 16 weeks of AF showed increased atrial expression of *COL1A1* [unpublished data / chapter 4]. These observations indicate that in the goat model, AF may be able to initiate atrial profibrotic signaling already after 4 weeks in old animals, but that the development of endomysial fibrosis may not become fully detectable until a later stage.

Another important aspect of structural remodeling is myocyte size. In our previous study, we showed that 16 weeks of AF induced a significant increase of atrial myocyte size in adult goats. In the present study, we found that 4 weeks of AF caused atrial myocyte hypertrophy in old, but not in young goats. This suggests that in old goats, a shorter AF duration is sufficient to induce detectable hypertrophic growth of myocytes and that advanced age may accelerate the effect of AF on myocyte growth, even in the absence of relevant comorbidities. This assumption is also supported by our finding that in the AF groups, old animals had an increased heart weight (normalized to tibial length) compared to the young animals.

The histological findings on myocyte hypertrophy were supported by qPCR analysis of LA and RA samples, which revealed an upregulation of the hypertrophic marker genes *NPPA* and *NPPB* in the old AF group compared to old sham. In this context, the increased (right) atrial pressure in the old goats with AF may represent one of the potential mechanisms responsible for atrial stretch and therefore for the upregulation of these hypertrophic genes [40, 41].

Moreover, although 4 weeks of AF are expected to increase AF complexity as compared to acutely induced AF, this was only found to be significant between the groups of old animals [39]. The increased myocyte size in the old AF compared to old SR group might explain the difference in LA conduction velocity. In fact, it has been shown that an increase in cardiomyocyte size may contribute to reduced conduction velocity due to decreased end-to-end coupling between hypertrophic myocytes. The resulting conduction disturbances may enhance AF propensity [34, 42].

Synergistic effect of age and AF on coagulation

In healthy individuals, the blood coagulation potential increases with advancing age [43-45]. In the majority of AF patients, the increased risk for thromboembolic events has been linked to a confluence of three factors comprising Virchow's triad of risk factors for thrombosis: aberrant blood flow, vessel wall abnormalities and alterations in blood constituents (e.g. coagulation factors) [46]. Elderly people show a subtle increase in plasma levels of pro-coagulant factors, sometimes accompanied by a decrease of anti-coagulation and fibrinolytic factors [47]. In other words, coagulation enzyme activity seems to be age-dependent, which may explain the additional stroke risk during AF.

To monitor the effect of AF and/or age on the goat coagulation system, we made use of a thrombin generation assay (CAT method) to assess the clotting potential at baseline and at 4 weeks of AF [D'Alessandro et al. 2021 / chapter 3]. As reported in literature the effect of lone AF on the coagulation system is still unclear [8, 12-16].

In our previous study, 16 weeks of lone AF in goats (aged between 1 and 5 years) did not change the clotting potential [unpublished data / chapter 4]. This may be explained by the hypothesis that lone AF induces a "pre-thrombotic state", but needs a second pro-coagulant hit to trigger thrombin generation [48]. In the current study, all groups showed similar coagulation profiles at baseline, indicating that the age difference did not affect thrombin generation. However, after 4 weeks of AF old goats showed a significant increase in clotting potential while this was not the case in young goats. This points out to a synergistic effect of age and AF on the goat coagulation system, suggesting that advanced age, as "second procoagulant hit", may potentiate the effect of AF leading to a pro-thrombotic state.

ETP and peak height values of the TG curves depend on the levels of multiple coagulation factors. One example is fibrinogen, which positively correlates with age [49, 50]. Plasma levels of fibrinogen are also increased in AF patients [51, 52]. In our study, neither age (O-SR), nor (lone) AF (Y-AF) affected the TG parameters, suggesting that none of the conditions alone was sufficient to induce increased coagulation activity. However, we propose that when AF and advanced age coexist, levels of pro-coagulant factors, such as fibrinogen, may further rise and thereby lead to increased clotting potential.

Limitations

For the purpose of this study, the largest possible age difference between young and old groups was desired. For practical reasons of animal availability, we could not include animals approaching the estimated maximal life-span. Nevertheless, the age difference present in this study was sufficient to detect significant age-related differences between the groups.

In case of paired analyses, missing values limited the sample size of comparison within the groups (e.g. TG parameters in Y-AF, O-SR and O-AF). Also, for the gene expression analysis, the sample size was limited by poor RNA quality of some samples. The quality of the ECG recordings during AF stabilization assessment was insufficient to quantify the number of AF paroxysms in 3 out of 7 Y-AF animals. Nevertheless, the entity of the difference between Y-AF and O-AF was large enough to detect a statistically significant difference.

The effect of increased atrial myocyte size on atrial-specific weight could not be determined as atria and ventricles were not weighed separately.

Finally, our coagulation analysis is based on thrombin generation only. This was due to a lack of goat-specific coagulation assays. Therefore, besides describing the overall clotting potential of these animals, we cannot further elaborate on specific changes of pro-coagulant or anti-coagulant pathways.

Conclusions

This study shows the synergism between advanced age and AF, which increases susceptibility to early AF stabilization, increases atrial structural remodeling processes (i.e. myocyte hypertrophy and LA epicardial endomysial fibrosis) and enhances coagulation potential. Moreover, AF complexity increased as response to AF, but not as a result of age.

Acknowledgments

We are grateful to Gwenola Tosser of the VarGoats consortium for providing us with the SNPS locations on target genes reported in this manuscript.

Funding

This study was supported by grants of the Netherlands Heart Foundation (CVON2014-09, RACE V Reappraisal of Atrial Fibrillation: Interaction between hyperCoagulability, Electrical remodeling, and Vascular Destabilisation in the Progression of AF) and the European Union (ITN Network Personalize AF: Personalized Therapies for Atrial Fibrillation: a translational network, grant number 860974; CATCH ME: Characterizing Atrial fibrillation by Translating its Causes into Health Modifiers in the Elderly, grant number 633196; MAESTRIA: Machine Learning Artificial Intelligence Early Detection Stroke Atrial Fibrillation, grant number 965286; REPAIR: Restoring cardiac mechanical function by polymeric artificial muscular tissue, grant number 952166).

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Supplementals

Supplemental Table 1. Target genes and primer sequences used for RT-PCR analysis.

Gene symbol	Target gene	Forward primer (5'->3')	Reverse primer (5'->3')
ACTA2	Actin alpha 2	GGGCCGTTTTCCCGTCTATC	TCATCACCCACGTAGCTGTC
ANGPT2	Angiopoietin 2	GACGGACGTTGAAGCCCAA	TCCAGGAAGCTGTTCTTATCTTG
CAV1	Caveolin 1	GGGGGCAAATACGTAGACTCA	GGGCTTGTAGATGTTGCCCT
CCL2	C-C motif chemokine 2	CTCGCTCAGCCAGATGCAAT	TGTCAGCCTCTGTATGGGGA
COL1A1	Collagen type I alpha 1 chain	TGTCCTAACGCCAAAGTCCC	TGGTCCGTGGTTGATTCCTG
F2R	Coagulation factor II thrombin receptor	GAAGGACGTTTGTGGGTCC	AGAAGGACGTTTGTGGGTCC
F2RL2	Coagulation factor II thrombin receptor like 2	GCTTCCTGTCAAAGCGGCAT	TTGGTGGTTTCTGTCCAGCC
F3	Coagulation factor III	GCACTAGCCACGAGAAAGGT	GAGTCACAGACAGGACGACG
IL6	Interleukin 6	CTTCACAAGCGCCTTCAGTC	GCTTGGGGTGGTGTCATTCT
NPPA	Natriuretic peptide A	TTTCAAGAATTTGCTGGACCGTT	ATACTTGTGAGGGCACAGCC
NPPB	Natriuretic peptide B	CTCTTCTTGCACCTGTCGCT	CCAGCAGCTCCTGTAACCCA
VEGFA	Vascular endothelial growth factor A	CCTTCACCATGCCAAGTGGT	GTCTCAATGGGACGGCAGAA
VWF	Von Willebrand factor	TTGATGGGGAGGTGAACGTG	TTCAGGAACACGGAGACAGC
Housekeeping gene			
HPRT	Hypoxanthine phosphoribosyltransferase 1	CGACTGGCTCGAGATGTGAT	ATCCAACAGGTCGGCAAAGA

CHAPTER 7

Ventricular tachypacing-induced heart failure decreases thrombin generation and promotes AF substrate development in goats

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In preparation for publication.

Abstract

Introduction

Heart failure (HF) and atrial fibrillation (AF) are associated with each other via numerous mechanistic interactions. In addition, HF increases the risk of thrombus formation and stroke in patients with AF. The mechanistic interactions of AF and HF in the development of an AF substrate, and of hypercoagulability, are incompletely understood. We hypothesize that HF induces a substrate that is favorable during the onset and progression of AF, potentially through alterations in coagulation activity.

Methods

Four groups of goats were studied: SHAM-operated goats (n=8), goats with 4 weeks of AF (n=7), goats with 4 weeks of HF (n=10) and goats with 4 weeks of HF with 1 week of AF in the final week (n=8). AF was initiated with 5 days of automated induction and stabilization monitoring. HF was induced by ventricular tachypacing with ejection fraction assessed at least once a week. Blood samples were taken at week 0 (baseline), 3 and 4 to determine coagulation activity using thrombin generation analysis. Finally, AF complexity was assessed by atrial contact mapping. Left atrial tissue was used for histological analysis.

Results

HF, induced by ventricular tachypacing, produced a progressive reduction of the left ventricular pump function and an increase in atrial pressures. In addition, HF resulted in faster AF stabilization compared to AF only ($p<0.01$). Moreover, AF complexity parameters were increased in HF and HF+AF, which was associated with elevated levels of left atrial endomysial fibrosis. At week 3 and 4, a significant reduction in clotting potential was observed in HF and HF+AF groups, while AF was comparable to SHAM.

Conclusion

This study shows that HF, induced by ventricular tachypacing in goats, produces a highly favorable substrate for the onset and progression of AF with enhanced endomysial fibrosis and AF complexity. Moreover, HF reduces the clotting potential, while AF has no effect.

Introduction

Atrial fibrillation (AF) is the most common form of sustained cardiac arrhythmia seen in clinical practice [1]. Currently, the prevalence of AF in adults is estimated to be approximately 3% and due to ageing of the general population it is expected to increase by 2.3-fold in the coming decades [2]. An important risk factor for AF is heart failure (HF) [3].

AF and HF are often encountered together [4], and their coexistence can be explained by shared underlying risk factors such as age, hypertension, valvular disease and myocardial infarction [5]. Furthermore, the coexistence of HF and AF leads to a higher risk of stroke as compared to AF alone [6].

Several mechanisms have been hypothesized by which HF can promote the onset and progression of AF. For example, increased cardiac pressures and altered signaling found in HF can lead to atrial fibrosis that may alter atrial conduction, thereby facilitating the progression of AF [7-10].

HF is also associated with endothelial dysfunction, reduced atrial contractility, lower atrial blood flow velocities and abnormalities in blood constituents, resulting in a tendency towards a pro-thrombotic state [11-13]. As a consequence, HF further increases the risk of thrombosis in patients with AF [2]. The activation of coagulation during AF has been linked to atrial structural remodeling and AF substrate development [14]. However, how HF exactly predisposes to AF and alters coagulation activity has not been investigated yet.

We hypothesized that HF induces a substrate that is favorable during the onset and progression of AF, potentially through alterations in coagulation activity. In this study we evaluated the effect of HF on coagulation status, atrial remodeling and AF progression in a goat model of HF with and without AF.

Methods

Goat models

The experimental protocol was approved by the local ethics committee and complies with the Dutch and European directives. Four study groups were included in this study; (i) SHAM (n=8), (ii) goats with 4 weeks of AF (n=7), (iii) goats with 4 weeks of HF (n=10) and (iv) goats with 4 weeks of HF and 1 week of AF in the final week (HF+AF, n=8). Goats were matched for age and body weight. Nevertheless, goats in the HF+AF group were somewhat younger than SHAM goats, 23.9 ± 6.3 vs. 32.8 ± 6.3 months ($p<0.05$), respectively.

All goats were anesthetized for surgery (induction: sodium thiopental 10 mg/kg, maintenance: sufentanyl 6 μg/kg/h, propofol 10 mg/kg/h). In the HF and HF+AF groups, before opening of the chest, the carotid artery and jugular vein were cannulated to determine cardiac output by means of the thermodilution method (7.5 F / 110 cm, Edward Lifesciences) and cardiac pressures were measured with a pressure-tip catheter (7F, Sentron Europe BV, Roden, The Netherlands).

In all groups custom-build electrode patches were in implanted, one on the left atrial (LA) pericardium (10 electrodes) and one patch on the left ventricular (LV) pericardium (4 electrodes). In the AF and HF+AF groups, an additional pericardial bipolar lead (Medtronic, Minneapolis, USA, Model 4968) was implanted on the LA pericardium for AF maintenance and connected to an implantable neurostimulator (Itrel 3 or 4, Medtronic). In the HF and HF+AF groups an additional screw-in lead (Medtronic, Model 5071) was placed on the epicardium at the LV basal anterior wall and connected to an implantable neurostimulator (Itrel 3 or 4, Medtronic).

Study protocol

After 2 weeks of recovery from surgery, the 4-week protocols were initiated (Figure 1):

- SHAM goats remained in sinus rhythm (SR) without any intervention for 4 weeks.
- AF goats started with 5 days of automated, monitored AF induction (10 mA 1-second 50 Hz burst after SR detection) at the LA. This allowed the determination of the number and duration of induced AF paroxysms required to maintain AF. During the remaining time, an implanted stimulator-maintained AF by pacing once every 10 s with a burst of 50 Hz (10 V, 1 s).
- HF goats were paced on the LV at 4.2 Hz in the first week of the protocol and at 3.9 Hz in the remaining 3 weeks. Atrial and ventricular rhythm and ejection fraction were assessed at least weekly. Pacing frequency was adjusted to target an ejection fraction of about 30%. Pacing frequency was adjusted in case animals showed signs of decompensation (shortness of breath, cyanosis, loss of appetite resulting in cachexia) or a rapid decline in ejection fraction, but was not decreased below 3.1 Hz.
- HF+AF goats were brought into HF with the same protocol as the HF group. At week 3, monitored AF induction (10 mA 1-second 50 Hz burst after each SR detection) was started. The number of paroxysms and AF paroxysm length were documented.

Blood samples were collected from the jugular vein at week 0, 3 and 4 in all groups. Blood was collected using a needle and holder (18G, PrecisionGlide, Becton,

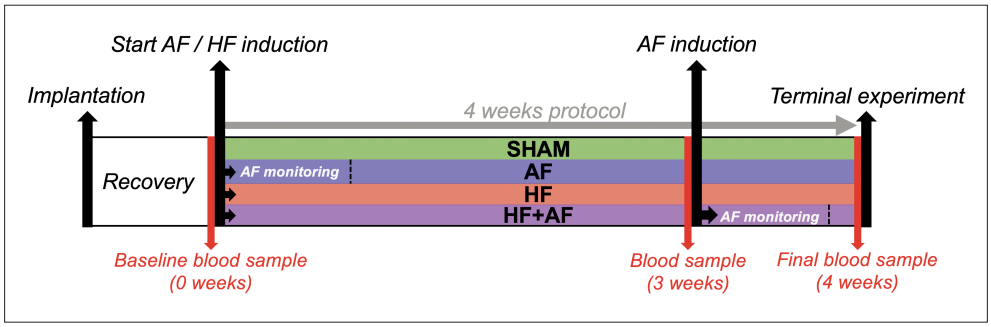

Figure 1. Experimental time line of the 4 experimental groups.

Dickinson and Company [BD], Franklin Lakes, USA) and blood was pooled in 3.2% (w/v) citrate (vacutainer, BD). Subsequently, the blood was processed into platelet poor plasma and stored at -80˚C [15].

Terminal experiment

At the end of the 4-week period, goats underwent hemodynamic and electrophysiological measurements under general anesthesia (induction: sodium thiopental 10 mg/kg, maintenance: sufentanyl 6 μg/kg/h, propofol 10 mg/kg/h and rocuronium 0.3 mg/kg/h). First, atrial and ventricular pressures and cardiac output were determined before opening of the chest, as described above. Next, a left-sided thoracotomy was performed and two mapping electrode arrays, each consisting of 249 unipolar electrodes (2.4 mm inter-electrode distance), were placed on the right atrial (RA) and LA free wall. Unipolar electrograms were recorded at a sampling rate of 1 kHz, 16-bit resolution, filter bandwidth 0.56-408 Hz.

AF was recorded for 5 minutes, in files of up to 60 seconds. During these 5 minutes, AF was monitored and reinduced with an automated algorithm (see the description above) once SR occurred. The number of AF paroxysms were documented per animal ("AF inducibility with open chest and under general anesthesia"). If AF did not terminate spontaneously after 5 minutes of AF measurements, it was cardioverted by internal defibrillation (10-20 J). The effective refractory period was measured using an S1S2 protocol (4X threshold) at a cycle length of 250 ms.

After completion of all measurements, all sensors were removed and the heart was left untouched for 30 minutes for stabilization. Goats were euthanized by excision of the heart. Hearts and other organs were weighted. Tissue samples from both atria and ventricles were taken and stored at -80 ºC.

Thrombin generation assay

The clotting potential was assessed by means of calibrated automated thrombography method (Thrombinoscope software, Stago, Paris, France) The initiation phase (lag time), endogenous thrombin potential (ETP) and highest thrombin concentration (peak height) were derived from the thrombin generation (TG) curve[16]. TG was triggered with goat brain protein extraction as source of tissue factor (TF) and procoagulant phospholipids (D'Alessandro et al. 2021 / chapter 4). Correction for inner filter effects and substrate consumption was performed by calibrating the results from each thrombin generation analysis against the fluorescence curve obtained from the same plasma with a fixed amount of calibrator. Fluorescence was measured in an Ascent Reader (Thermolabsystems, Maharashtra, India) equipped with a 390/460 nm filter set, and TG curves were calculated using Thrombinoscope software (Stago).

Analysis of pressures and electrograms

IDEEQ software (Instrument Development Engineering and Evaluation, Maastricht University, Maastricht, The Netherlands) was used to analyze RA, LA, right ventricular and aortic pressures. Maximal and minimal slopes of ventricular pressures were determined from the derivative (differentiation width of 5 ms) of the pressure signals.

Custom-made software (The MathWorks, Inc., Natick, Massachusetts, USA) was used for the identification of local activation times [17]. Waves were defined as clusters of activation times that are connected in space and time by an apparent CV of $\geq$ 20 cm/s. If potential wavefronts overlapped > 90%, the wavefronts were merged to a single wave. Within waves, the CV was calculated for each activation by fitting a plane through the spatially neighboring activations. Waves of < 3 electrodes were excluded from the analysis.

Histological analysis

A frozen sample of the LA free wall was transmurally cut in cryosections of 7 μm thickness. Cell membranes and extracellular matrix was visualized by the fluorogenic wheat germ agglutinin (WGA) staining, as previously described by Winters et al. [18]. A Leica DM4B microscope and an MC170 HD camera (Leica microsystems, Wetzlar, Germany) were used to take images at 400 times magnification of the stained sections. Per goat, at least 5 images of both the epi- and endocardial region were taken. Analysis of overall fibrosis (percentage of WGA-positive red-stained pixels per image), endomysial fibrosis (cell to cell distances between myocytes) and myocyte size (minimal ferret per myocyte) was performed using the automated analysis software "JavaCyte" [18].

Statistical analysis

All data are expressed as mean ± standard deviation (SD), unless stated otherwise.

Group comparisons for normally distributed data (hemodynamics and organ weights) were done by one-way ANOVA with Šidák's post-hoc test. Hemodynamic comparisons in HF and HF+AF were done by a paired samples t-test. In non-normally distributed data (AF stabilization), two-group comparisons were done by the Mann-Whitney test and four-group comparisons by the Kruskal-Wallis test. LA structural remodeling was statistically assessed by a mixed model with goat and picture as random variables and treatment and cardiac wall as fixed factors. For AF complexity, a mixed effects model for repeated measures (restricted maximum likelihood) was fitted to the data using subject (goats) as random factor and atrium and treatment as fixed factors. A two-way ANOVA, with factors time (0, 3 and 4 weeks) and treatment was used to test the between and within group differences for the results of the thrombin generation analysis, as well as for the evaluation of the ejection fraction with factors time and treatment (HF vs. HF+AF). *P*-values < 0.05 were considered to be significant. SPSS (Version 26, IBM) and PRISM (version 9.0.0, GraphPad) were used to perform the statistical analyses.

Results

Heart failure development and hemodynamics

In the HF and HF+AF group, the decline in ejection fraction followed a similar pattern. During the first week, at a pacing frequency of 4.2 Hz, the decline of the ejection fraction was larger (week 0 to week 1 $p<0.001$) than during the weeks thereafter. At slower pacing rates in the subsequent weeks, the ejection fraction gradually decreased to 38 ± 6% in HF and 30 ± 11% in HF+AF ($p=0.42$, Figure 2). Tachypacing of the ventricles was never observed to retrograde to the atria and cause atrial tachycardia.

At the moment of the final experiment at 4 weeks, hemodynamics were affected by all treatments (AF, HF and HF+AF) as compared to SHAM (Table 1). The stroke volume and left-ventricular contractility (dP/dt_{max}) were significantly reduced in all treatment groups, while left-ventricular end-diastolic pressure was significantly elevated in HF and HF+AF as compared to baseline and SHAM, but not in AF versus SHAM (Figure 3; A-C, Table 1). Furthermore, both the HF and HF+AF group showed increased central venous, right atrial and right ventricular pressures (Figure 3; D-F, Table 1), while this was not the case in the AF group.

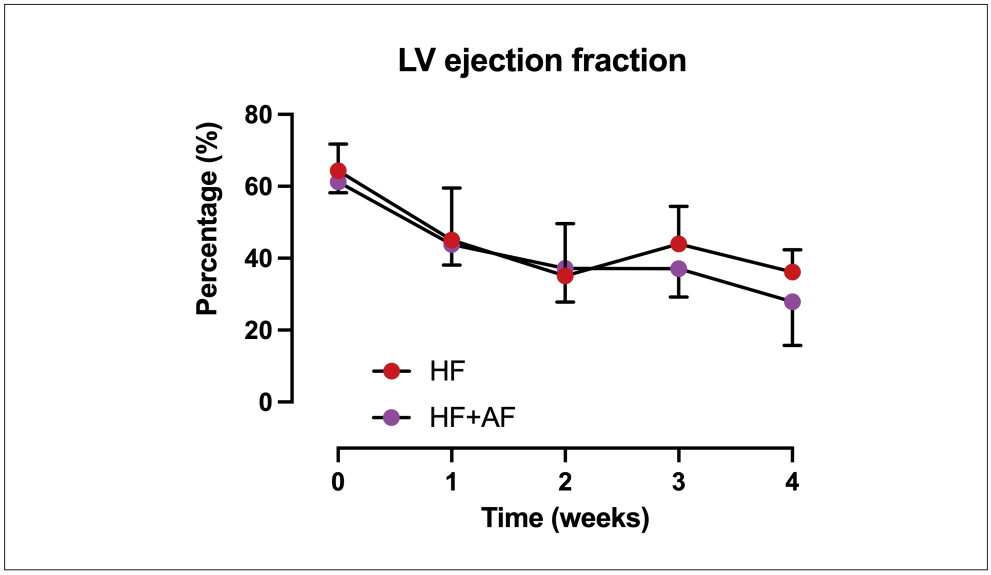

Figure 2. Development of heart failure with reduced ejection fraction over a period of 4 weeks of ventricular tachypacing (HF n=10) and with induction of AF in the 4th week (HF+AF n=8, p=0.42.)

Table 1. Hemodynamics and cardiac pressures indicated per group at baseline (HF and HF+AF) and 4 weeks (final, all groups).

	SHAM	AF	HF		HF+AF		ANOVA
			Baseline	Final	Baseline	Final	*P*-value
Heart rate (/min)	76±16	111±28*	65±11$^{\#\#\#\#}$	102±19	70±23$^{\#\#\#}$	139±19$^{****\$}$	<0.001
Cardiac output (l/min)	4.9±0.8	3.3±1.3	5.4±0.7	4.8±0.8	5.1±1.1$^{\#}$	3.9±0.9	0.09
Stroke volume (ml)	64±17	30±11***	84±15$^{\#\#\#\#}$	45±16*	77±21$^{\#\#}$	28±6****	<0.0001
Left ventricle							
Systole (mmHg)	121±16	85±17**	116±16$^{\#}$	94±12*	122±10	106±24	<0.01
end diastolic pressure (mmHg)	10.5±5.4	7.9±4.8	9.2±3.5$^{\#}$	16.7±4.4*	12.9±4.6$^{\#\wedge\wedge}$	17.3±2.5$^{\wedge\wedge}$	<0.001
dP/dt_{max} (mmHg/s)	2037±220	1318±419**	1822±307$^{\#\#\#}$	1050±294****	1800±239$^{\#\#}$	1246±474***	<0.0001
dP/dt_{min} (mmHg/s)	-2182±533	-1679±529	-2953±807$^{\#\#\#}$	-1412±428*	-3271±466$^{\#\#\#}$	-1604±733	0.06
Right ventricle							
Systole (mmHg)	39.0±7.1	26±6*	30±3.7	37±7.3	33.0±4.7$^{\#}$	39±8.5$^{\wedge}$	0.01
end diastolic pressure (mmHg)	10.1±4.1	8.0±2.9	10.2±1.9	16.1±7.7	12.9±3.5	15.5±2.1	0.03
dP/dt_{max} (mmHg/s)	704±151	408±158*	440±163	420±194*	439±135	491±212	0.03
dP/dt_{min} (mmHg/s)	-430±141	-294±40	-351±151	-401±84	-381±108	-445±142	0.05
Right atrium, mean (mmHg)	8.2±3.3	8.2±2.4	7.9±1.6$^{\#}$	13.7±3.8$^{**\wedge}$	9.9±3.5$^{\#\#}$	17.1±2.4$^{****\wedge\wedge\wedge}$	<0.0001
Central venous, mean (mmHg)	7.6±3.7	6.5±2.3	6.1±1.5$^{\#}$	11.7±4.1$^{\wedge}$	9.1±3.2$^{\#\#}$	16.1±3.5$^{****\wedge\wedge\wedge}$	<0.0001

Group comparisons were done by one-way ANOVA and Šidák's post-hoc test. The baseline vs. final comparison was done by paired samples t-test. Symbols indicate significance of different comparisons: $^{\#}$ baseline vs. final, * vs. SHAM, $^{\wedge}$ vs. AF, $^{\$}$ vs. HF. *: p<0.05 / **: p<0.01 / ***: p<0.001 / ****: p<0.0001. SHAM n=8; AF n=7; HF baseline n=10, final n=9; HF+AF baseline n=8, final n=7. The heart rate in the HF and HF+AF was measured in sinus rhythm.

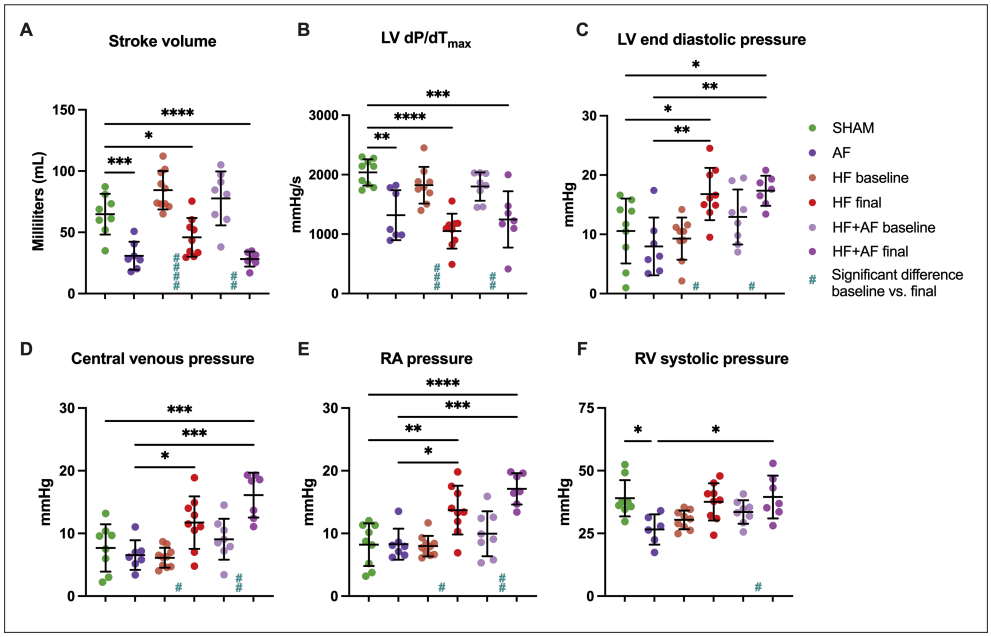

Figure 3. (A) Stroke volume, (B) left ventricular dP/dt$_{max}$ (velocity of the pressure rise during systole), (C) left ventricular end diastolic pressure, (D) central venous pressure, (E) right atrial pressure and (F) right ventricular systolic pressure. Significance of group comparisons at 4 weeks (final) is indicated by the asterisks (*; one-way ANOVA with Šidák's post-hoc test). The baseline vs. final comparison was tested with a paired sample t-test (#) The number of symbols determine the significance level: #/*: $p<0.05$ / **: $p<0.01$ / ***: $p<0.001$ / ****: $p<0.0001$.

Organ weights and macroscopic observations

At the final experiment several organs were collected and weighed. The weight of the lungs was found to be significantly higher in HF as compared to SHAM, also after correction for tibial length (Table 2).

On the macroscopic level we often observed enlarged and discolored (bluish) livers in the HF and HF+AF goats, but this was not systematically quantified and the liver weights were not significantly different between these groups. Two HF goats also showed infarcted areas in the kidneys. There was no significant difference in kidney weight between groups.

AF inducibility and stabilization

The AF stabilization rate was significantly faster in HF+AF goats (after 3 weeks of HF) when compared to AF-only goats (Figure 4, panel A-C). During the first 12

Table 2. Organ weights indicated per group.

Organ weights (grams)	**SHAM**	**AF**	**HF**	**HF + AF**	**ANOVA** *P*-value
Heart	326±49	356±29	392±161	322±70	0.43
Atrial	48±11	70±3	70±31	71±8	0.28
Ventricular	289±39	284±7	321±139	251±66	0.54
Lung	475±92	544±53	682±207*	576±84	0.02
Liver	1041±213	980±114	1304±398	1248±405	0.14
Kidney, right	85±18	90±10	78±17	73±13	0.19
Kidney, left	89±18	91±9	79±25	76±14	0.32
Normalized to tibial length					
Heart	14±1.9	14.0±1.2	16.0±6.5	14.0±3.1	0.59
Atrial	2.1±0.5	2.9±0.1	2.9±1.2	3.0±0.4	0.33
Ventricular	12.0±1.8	11.0±0.7	13.0±5.7	10.0±2.9	0.60
Lung	20.0±3.3	22.0±2.6	27.0±8.3*	24.0±3.4	0.04
Liver	45.0±8.6	40.0±3.9	53.0±16.4	52.0±17.8	0.19
Kidney, right	3.7±0.8	3.7±0.5	3.2±0.7	3.1±0.6	0.14
Kidney, left	3.9±0.8	3.8±0.5	3.2±1.0	3.2±0.6	0.17

Group comparisons were done by one-way ANOVA with Šidák's post-hoc test. The asterisk (*: $p<0.05$) indicates a significant difference between SHAM and HF. SHAM n=8; AF n=7; HF n=10, HF+AF n=8.

hours of AF initiation, 5 out of 7 HF+AF goats required only 1 burst to maintain AF for 5 days. One goat required 2 bursts and 1 goat required 5 bursts (on average 1.6±1.4 bursts), while the AF goats required on average 4939±3890 bursts ($p<0.01$). During the subsequent 12 hours, the AF-only goats required on average an additional 2699±3499 bursts to maintain AF, indicating progression of AF persistence. At the day 5 of atrial pacing, the durations of the last documented AF paroxysm in HF+AF goats were significantly longer than in AF-only goats ($p<0.001$). Interestingly, the AF inducibility during the open-chest surgery under general anesthesia did not differ between HF and SHAM goats, while AF and HF+AF goats showed a pronounced substrate for AF (Figure 4, panel D).

Analysis of electrophysiological parameters and AF complexity

The atrial effective refractory period (AERP) and AF cycle length (AFCL) showed a similar pattern of differences between the groups at 4 weeks. This was mainly significant in the RA. AERP and AFCL were shortened in the AF group. HF alone and HF+AF showed an increase compared to SHAM in AERP and AFCL, respectively (Figure 5, panel A-B).

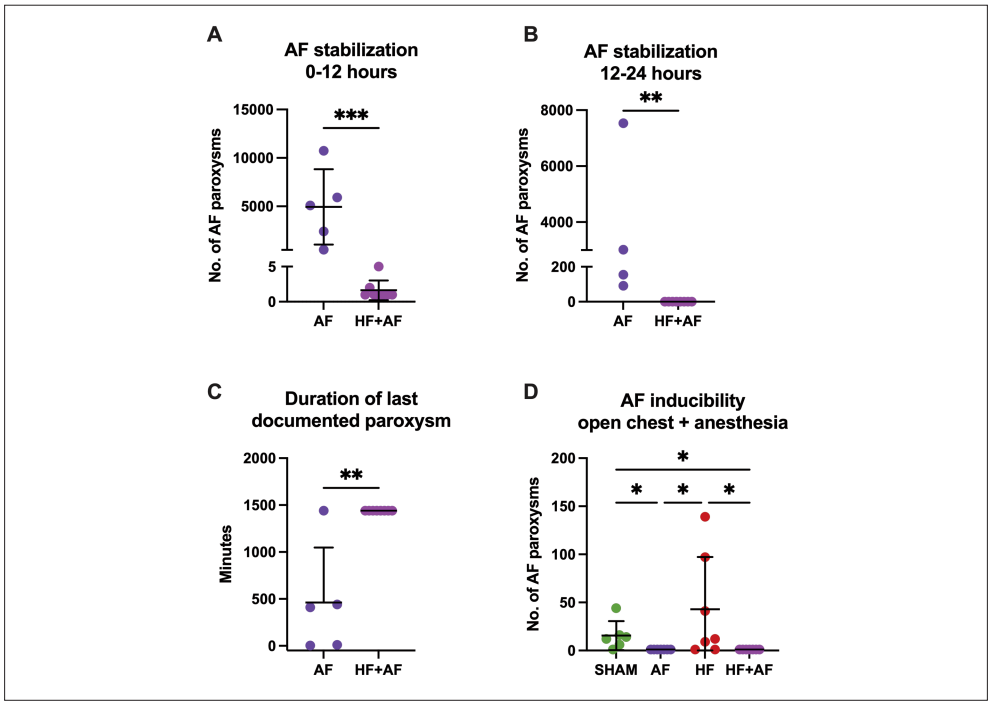

Figure 4. AF stabilization. (A) AF stabilization (number of AF paroxysms) of the first 12 hours after AF initiation (AF: n=5, HF+AF n=8), (B) and during the subsequent 12 hours (AF: n=4, HF+AF: n=8). (C) The duration of the last documented AF paroxysm at day 5. (D) AF inducibility during the open-chest experiment with anesthetic treatment after 4 weeks. Group comparisons in panel A-C were done by the non-parametric Mann-Whitney test. Group comparisons in panel D were done by the non-parametric Kruskal-Wallis test. *: $p<0.05$ / **: $p<0.01$ / ***: $p<0.001$.

Additionally, HF with and without 1 week of AF caused an increase in AF complexity. As compared to SHAM, in the HF and HF+AF group the number of waves per AF cycle in the LA was larger than in the respective control groups. Similarly, in the RA, the number of waves per AF cycle was significantly higher in HF+AF compared to SHAM and AF, and a trend in the same direction was observed between HF and AF ($p=0.09$, Figure 5, panel C). Furthermore, RA maximal activation time differences (electrical dissociation) was significantly larger in HF+AF compared to SHAM and AF, and in HF compared to AF (Figure 5, panel D).

Left atrial structural remodeling

Histological analysis of LA samples revealed that endomysial fibrosis, quantified as the inter-myocyte distance, was increased in HF and HF+AF groups when com-

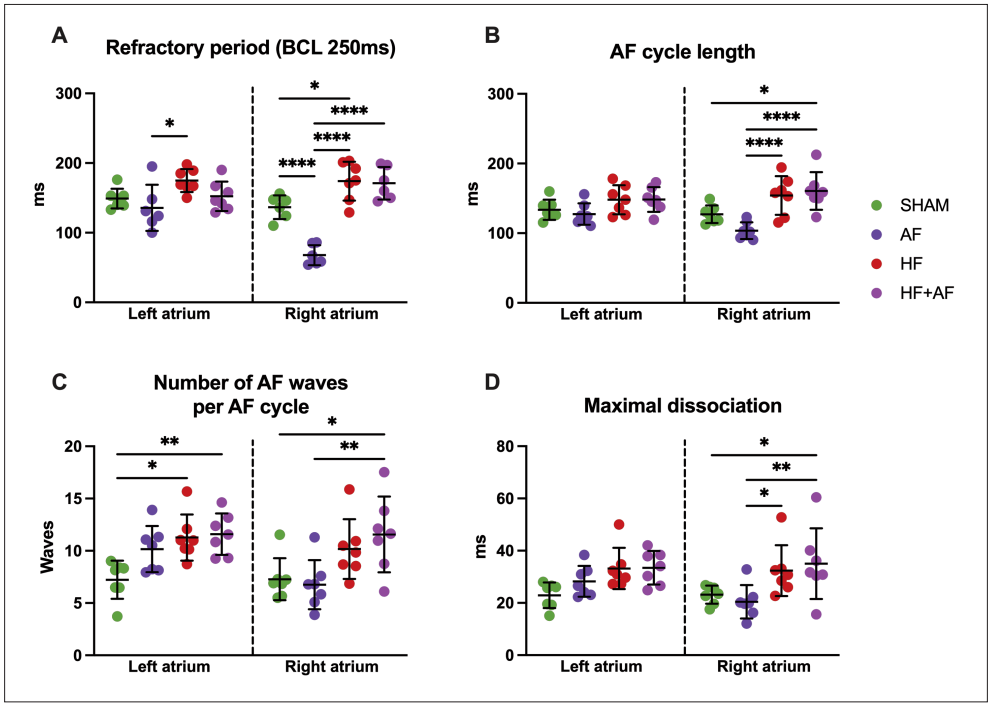

Figure 5. Electrophysiological and AF complexity analysis. (A) Refractory period, (B) AF cycle length, (C) number of waves per AF cycle and (D) maximal dissociation, indicated per group and atrium. Group comparisons were done by a mixed-effects model and Šidák's post-hoc test. *: $p<0.05$ / **: $p<0.01$ / ***: $p<0.001$ / ****: $p<0.0001$.

pared to SHAM. The HF group also showed significantly increased levels of endomysial fibrosis when compared to AF. A trend towards increased endomysial fibrosis was found in HF+AF goats when compared to AF-only animals. This pattern of differences was found in both the epicardium and endocardium of the LA wall (Figure 6, panel A-B).

Analysis of atrial myocyte size in the LA showed that myocyte diameters were comparable between the SHAM and treatment groups, in both the epicardium and endocardium (Figure 6, panel C-D).

Thrombin generation assay

At baseline (week 0), the clotting potential was comparable among groups (Figure 7). Interestingly, heart failure led to a significant reduction of clotting potential. In fact, thrombin generation analyses performed at 3 weeks indicated that the HF and HF+AF group showed a significant decrease in Peak height compared to SHAM

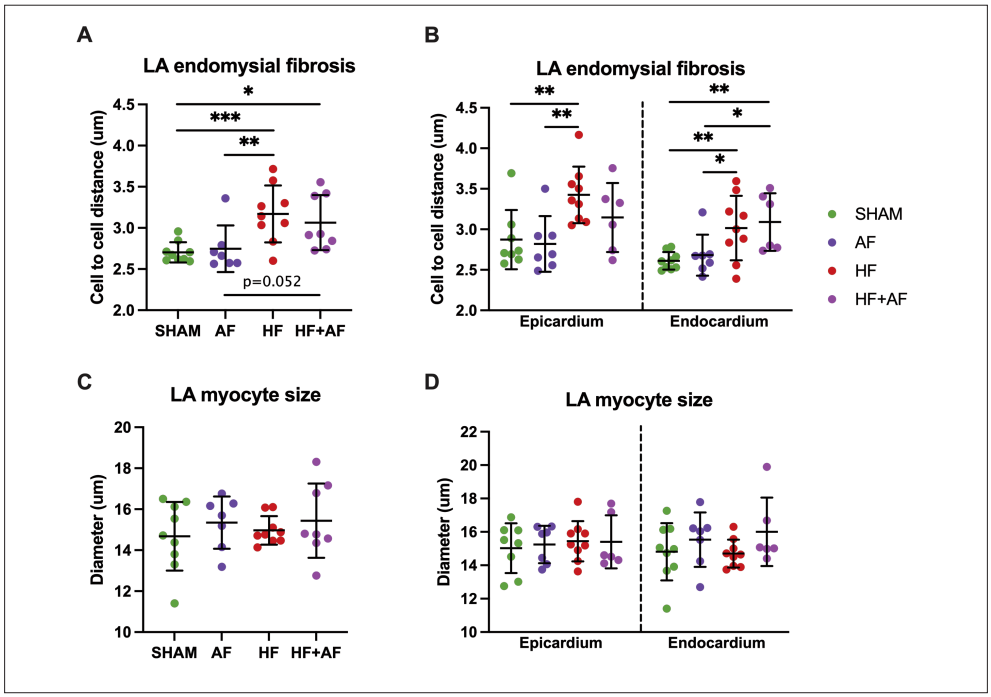

Figure 6. Histological analysis of tissue samples from left atrium. (A-B) Endomysial fibrosis, quantified as inter-myocyte distance. (C-D) The myocyte size, quantified as cellular diameter. The values are indicated per group for the epicardium and endocardium. Group comparisons were done by a mixed model analysis. *: $p<0.05$ / **: $p<0.01$ / ***: $p<0.001$.

and AF (Figure 7, panel A). ETP values were also significantly decreased in these groups compared to SHAM (Figure 7, panel B). Within group comparison of TG measured at 3 weeks, revealed that the presence of HF led to a decline in coagulation activity compared to baseline and that this reduction was also present at 4 weeks and did not change with one additional week of AF. Furthermore, we observed that AF alone did not affect thrombin gene-ration compared to baseline or SHAM.

Discussion

In this study we investigated the effect of HF on AF progression and characterized various forms of substrate remodeling. In the current goat model of tachypacing-induced HF, AF became extremely stable. Often only 1 AF provoking event was required to exhibit persistent AF, while goats without HF exhibited AF paroxysms even after 5 days and needed thousands of AF inductions. Our data suggest that HF

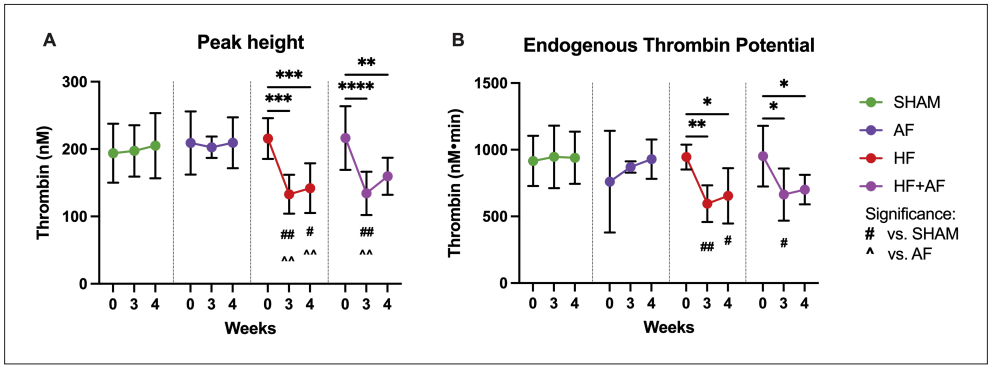

Figure 7. (A) Peak height and (B) ETP at week 0 (baseline), 3 and 4 (final), indicated per group. Within and between groups comparisons are done by a two-way ANOVA and Šidák's post-hoc test. The number of symbols determine the significance level: #/^/*: p<0.05 / **: p<0.01 / ***: p<0.001 / ****: p<0.0001. Week 0: SHAM n=8; AF n=6; HF n=8; HF+AF n=8. Week 3: SHAM n=8; AF n=7; HF n=7; HF+AF n=8. Week 4: SHAM n=7; AF n=7; HF n=7; HF+AF n=8.

in the goat indeed leads to an altered substrate that severely facilitates AF stabilization. This was characterized by left atrial endomysial fibrosis, atrial pressure overload and complex conduction during AF. Surprisingly, we found that 3 weeks of HF decreased the coagulation potential.

Hemodynamic changes caused by heart failure

In line with hemodynamic changes that were previously reported in other animal models of (congestive) HF, the HF and HF+AF groups showed a significant increase in central venous, RA, RV and LV end-diastolic pressures compared to SHAM [10, 19]. The increased central venous pressure and LV end-diastolic pressure (as a surrogate indicator for LA pressure) in goats with HF suggested the presence of systemic and pulmonary congestion, as also indicated by the increased lung weight and changes in liver color[20]. Furthermore, elevated RA and LV end-diastolic pressures indicated increased stretch affecting both atria of the HF groups.

Atrial structural remodeling

We previously reported that elevated atrial pressures in goats with AF were associated with atrial myocyte hypertrophy (Chapter 5 and 6). In this study, histological analysis of the LA did not show a difference in atrial myocyte size between groups. These contrasting findings could be explained by the fact that in our previous studies, myocyte hypertrophy was analyzed only after longer periods of AF (4 months), and in significantly older animals.

A study by Li et al. reported that 5 weeks of ventricular tachypacing increased left atrial interstitial fibrosis and strongly promoted the induction of AF in a dog model of HF [19]. In line with these findings, our study shows that both HF groups exhibited a significant increase in endomysial fibrosis in the LA, which was accompanied by increased atrial pressures. This observation is in accordance with previous reports on animal models of HF and human data that report on elevated atrial pressures and concomitant atrial dilation and stretch, leading to atrial fibrosis in HF [19, 21, 22].

Stabilization and complexity of AF

We show that HF led to an extremely fast AF stabilization rate when compared to AF-only animals, suggesting the presence of a strong substrate for AF after 3 weeks of HF. Since electrical remodeling (e.g. shortening of AERP and AFCL) was not observed in HF, the fast AF stabilization rate may be attributed to the ongoing endomysial fibrotic changes in the atria. Atrial endomysial fibrosis has been shown to lead to conduction disturbances and might thereby contribute to the stabilization of AF during HF [19, 23, 24]. Another contributing factor could be HF-related abnormal atrial Ca^{2+} handling, which was shown by Yeh et al. in a dog model of ventricular tachypacing [25]. They reported cellular Ca^{2+} overload and increased diastolic Ca^{2+} concentrations, predisposing to spontaneous Ca^{2+} release and triggered activity [25]. In our model, triggered activity potentially served as focal drivers that maintained AF after it was initiated by burst pacing.

Interestingly, the AF inducibility during open-chest procedure under general anesthesia did not differ between SHAM and HF, while AF and HF+AF goats showed a higher AF inducibility rate. These results may be explained by the fact that there were signs of reduced atrial refractoriness (electrical remodeling) in the AF group, whereas in the SHAM and HF group AERP tended to be higher. The HF+AF group did not show signs of electrical remodeling, but still exhibited a high AF inducibility. Since we showed that the level of left atrial endomysial fibrosis was increased in this group at 4 weeks, it is likely that the fibrotic changes were ongoing at 3 weeks already and contributed to the AF inducibility and stability. However, this does not explain why the HF group lost the effect on AF inducibility under general anesthesia.

Li and colleagues showed that the class III antiarrhythmic agent, dofetilide, terminated AF in HF dogs, but failed to do so in rapid atrial paced dogs [26]. In line with this observation, a possible explanation to our findings may be that AF and HF produced distinct experimental substrates for AF. In this context, anesthesia may have an antiarrhythmic effect to which the HF-only substrate may be more sensitive.

Alternatively, acute effects coming from the autonomous nervous system (ANS), which can significantly contribute to AF persistence, are potentially reduced by the anesthetics (propofol and sufentanyl) [27-29].

Furthermore, we found that HF strongly increased AF complexity (i.e. number of waves per AF cycle and maximal dissociation), possibly caused by the presence of atrial endomysial fibrosis, which increases separation of individual myocytes within muscle bundles [24]. As proposed by Schoonderwoerd et al., these findings suggest that the high ventricular rate, during HF, plays a major role in atrial structural remodeling (e.g. fibrosis), which has been shown to increase the complexity of fibrillatory conduction [21, 24].

Coagulation potential

Previously, our group demonstrated a link between activation of coagulation and AF progression [14]. In fact, inhibition of coagulation was found to prevent atrial endomysial fibrosis in goats with AF [30]. Moreover, in a recent study we found that increased thrombin generation in old goats with AF was accompanied by atrial myocyte hypertrophy (Chapter 6). These results suggest that increased activity of coagulation factors during AF may contribute to atrial structural remodeling and thereby facilitate stabilization of AF. In addition, HF has also been linked to mechanisms leading to thromboembolic events and further increases the risk of thrombosis in patients with AF [31-33].

Few studies have investigated coagulation activity in relation to HF in the absence of comorbidities. In a dog model of congestive HF, no changes in coagulation profile were found compared to controls [34]. Surprisingly, in our study, thrombin generation analysis revealed a significant decrease in clotting potential in goats with HF. The reduction in coagulability may be explained by disrupted expression of coagulation factors in the liver due to elevated atrial pressures and congestion in the hepatic veins [35].

Previously, our group showed that anticoagulation treatment in goats with four weeks of AF, reduced AF-related atrial endomysial fibrosis [14]. In light of the current findings, we conclude that the observed atrial endomysial fibrosis during HF might not be driven by increased coagulation activity, at least not by systemic hypercoagulability.

Limitations

At this stage, we do not have information on the coagulation status during the first weeks of HF. Thrombin generation analysis in the blood samples that were collected during the early onset of HF will be relevant for the interpretation of our findings.

As has been shown in a rabbit model of ischemia-reperfusion injury, activation of the coagulation may take place in the interstitial space [36]. Investigation of these aspects in atrial tissue may help us to gain insight on local signaling pathways triggered by coagulation factors within the tissue during HF.

Macroscopic alterations of the collected organs were encountered at a later stage of the study and not systematically recorded. Therefore, we can only use our observations for the global interpretation of the results. Additional histological analysis of the liver and the kidney, accompanied by screening for organ failure markers in plasma, should be performed to further evaluate the effect of the different interventions.

Conclusion

This study shows that HF, induced by ventricular tachypacing in goats, produces a highly favorable substrate for the onset and progression of AF with enhanced endomysial fibrosis and AF complexity. Moreover, HF reduces the clotting potential, while AF has no effect.

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CHAPTER 8

General discussion

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General discussion

Atrial fibrillation is often associated with increased blood coagulation [1]. However, little is known about the effect of activated coagulation in the blood or in the interstitial space on atrial remodeling potentially leading to stabilization of AF.

Activated coagulation factors have been described to elicit pleiotropic effects and contribute to pathological processes such as inflammation and cardiac remodeling via activation of protease-activated receptors (PARs) [2, 3]. Based on this knowledge, a bidirectional interaction between AF and hypercoagulability has recently been hypothesized[4]. However, the effect of AF on coagulation activity and how this can lead to atrial remodeling and AF progression, has not been fully clarified. A better understanding of the bidirectional mechanistic link between AF and coagulation could help to characterize disease processes that take place during the pathogenesis of AF.

In **chapter 2** we reviewed the most important and recent findings regarding the non-hemostatic signaling pathways initiated by the tissue factor (TF):Factor (F)VIIa complex, with an emphasis on the heart and blood vessels. In this chapter, we also described some of the pleiotropic effects elicited by two other key regulators of the coagulation cascade, thrombin and FXa, and their cellular signaling through activation of PARs.

Thrombin and FXa have been implicated in the activation of cardiac fibroblasts (CF) and promotion of cardiac remodeling [4, 5]. In **chapter 3** we showed that these coagulation factors upregulated the gene expression of pro-fibrotic genes in CF, supporting the relation between pathological activation of the coagulation system and cardiac remodeling. Moreover, we found that FXa induces pro-inflammatory responses in CF through activation of PAR-1.

To answer our research questions *in vivo,* we made use of the goat model of AF. This model allows the investigation of changes in the heart and blood that occur within days to months of AF. In **chapter 4** we described how we customized the Calibrated Automated Thrombography (CAT) assay to provide a global view of the goat coagulation profile by assessing thrombin generation (TG) in goat plasma.

Our central hypothesis is that the hypercoagulable state during AF contributes to the development of an AF substrate through activation of PARs (Figure 1). In **chapter 5** we investigated the effect of FXa-inhibition, by rivaroxaban treatment, on AF substrate development in the goat model of AF. We found that four months of AF led to atrial myocyte hypertrophy, which was fully prevented by FXa-inhibition. Interestingly, AF did not cause a hypercoagulable state, as assessed by the TG assay. One possible explanation is that AF without the presence of other comorbidities, or risk factors for stroke, might not be sufficient to trigger a pro-coagulant response in

goats. As age is the most important risk factor for AF, as well as for stroke in patients with AF, we studied the effect of advanced age in the goat model of AF. In **chapter 6** we reported that age and AF synergistically increased coagulation potential, early AF stabilization and promoted atrial structural remodeling (Figure 1).

Another important risk factor, but also a comorbidity of AF, is heart failure (HF). HF can promote the onset and progression of AF and increases the risk for stroke during AF [6, 7]. In **chapter 7** we demonstrated that HF created a favorable sub-

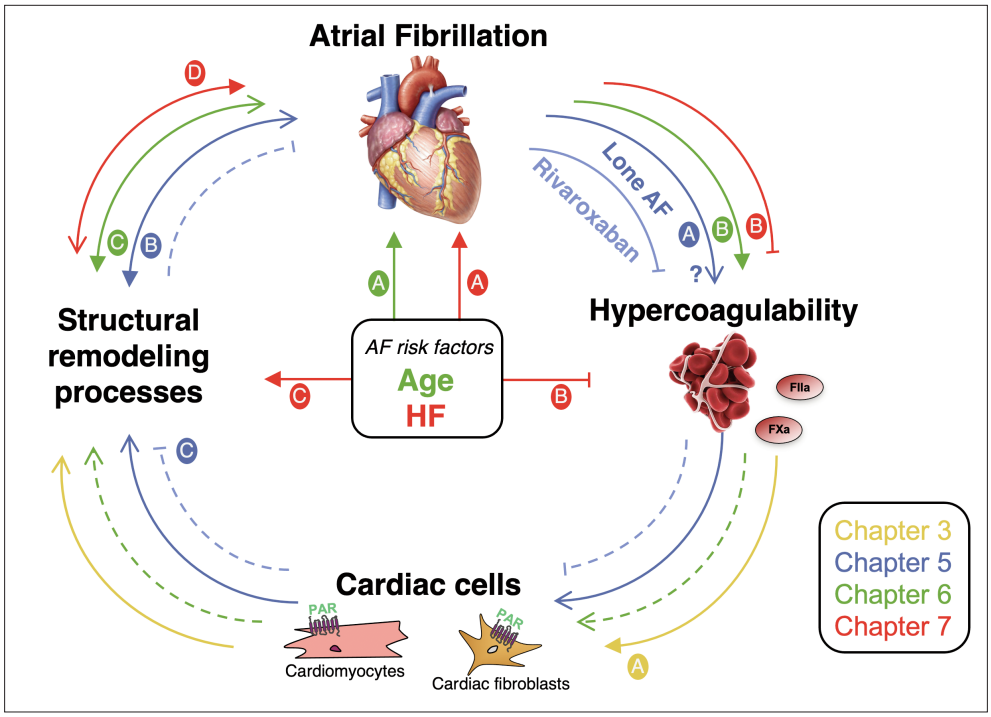

Figure 1. Schematic representation of the main findings in this thesis.
The color of the arrows describes the chapter content. Yellow refers to chapter 3, blue to chapter 5, green to chapter 6 and red to chapter 7. Chapter 3: (A) Thrombin and FXa upregulated the gene expression of pro-fibrotic genes in CF. Also, FXa induced pro-inflammatory responses in CF through activation of PAR-1. Chapter 5: (A) lone AF may induce local activation of coagulation in the atria; (B) lone AF led to myocyte hypertrophy, which may impair atrial conduction; (C) rivaroxaban treatment during AF inhibited thrombin generation and prevented AF-related myocyte hypertrophy. Chapter 6: (A) advanced age led to faster AF stabilization; (B) advanced age and AF synergistically increased thrombin generation; (C) advanced age and AF synergistically accelerated myocyte hypertrophy. Chapter 7: (A) HF led to extremely fast AF stabilization; (B) HF±AF was associated to reduced thrombin generation; (C) HF led to increased endomysial fibrosis; (D) HF-related fibrosis led to increased AF complexity. Dashed lines and open arrows refer to literature and the thesis hypothesis. Closed arrows and uninterrupted lines refer to the findings of this thesis.

strate for the onset of AF, which was associated with a decrease in coagulation activity and increased atrial fibrosis (Figure 1).

The aim of this general discussion is to review and explore the biological meaning of these findings, and to discuss them in relation to each other and to relevant literature.

Coagulation changes during AF

AF is associated with a five-fold increased risk of thromboembolic stroke. This risk is not homogeneous and depends on stroke risk modifiers, as summarized in the CHA_2DS_2-VASc score (Table 1) [8, 9]. The pathogenesis of thrombus formation during AF is multifactorial and results from changes in physiological processes such as reduced blood flow and stasis in the fibrillating atria, endothelial dysfunction (and structural changes) and hypercoagulability. Blood stasis in fibrillating atria results both from loss of organized contraction and atrial dilation. The latter is an independent risk factor for thromboembolism that previously has been associated to atrial contractile dysfunction by our group [10-12]. Hypercoagulability is related to increased platelet reactivity, activation of the coagulation cascade and reduced fibrinolysis[13]. Activation of the coagulation cascade, with subsequent thrombin formation, is the leading cause of a prothrombotic state. Estimation of the thrombotic state can be assessed by thrombin generation tests (e.g. CAT assay) [14].

To investigate the main changes in the coagulation system related to AF in the goat model, the CAT assay has been chosen as primary tool for hemostatic assessment (**chapter 4**). This assay measures the plasma thrombin generation upon *in vitro* activation of coagulation. TF is one of the most common triggers used to initiate thrombin generation in the CAT assay. At low TF concentrations, the CAT assay is equally sensitive to pro- and anti-coagulant forces and therefore offers an important tool to estimate the overall coagulation profile. However, TF and other reagents commonly used in the CAT assay are human-derived and/or optimized for human plasma. In **chapter 4** we described the customization of the CAT assay for goat plasma. We developed two distinct methods to measure thrombin generation in goats. In the first method, we utilized goat brain protein extraction (GBP) as a source of goat TF. In the second method, the Russell's viper venom-FX activator (RVV-X) was used as alternative trigger to activate the goat common pathway and trigger thrombin generation. Both the GBP and the RVV-X method were found equally effective to reliably measure the goat clotting potential.

Other valuable coagulation assays are ELISA-based assays for activated coagulation factors. These assays can estimate the levels of specific activated coagulation

proteins in plasma and therefore can help to understand which coagulation pathways are mainly affected during AF or other conditions. However, anti-goat antibodies targeting activated coagulation factors are currently not commercially available and ELISAs, designed for human plasma, appeared unsuitable.

Unlike ELISAs, the CAT assay does not require many species-specific reagents, which makes this test easier to be performed in goat plasma. Moreover, the CAT assay can be modified to evaluate the contribution of both pro- and anti-coagulant pathways. In fact, depending on the type and concentration of the trigger, and presence of specific inhibitors in the reaction mixture, the sensitivity of the CAT can be adjusted to study specific changes of the coagulation system. Due to time restraints and prioritization, these assay modifications could not be accomplished and included in this thesis. Nevertheless, preliminary data (not reported) showed promising results for future applications in goats.

Although the CAT assay presents limitations due to inter-laboratory variation, it correlates well with the most commonly used prothrombotic indices. For example, in plasma of AF patients treated with warfarin, ETP and peak height correlated with prothrombin fragment 1 + 2 and D-dimer [15]. Moreover, it has been shown to be sensitive to anticoagulation with vitamin K-antagonists (VKA) and direct oral anticoagulants (DOACs) in human plasman[15-18].

Using the CAT-assay described in **chapter 4**, we show in **chapter 5** that FXa-inhibition via the DOAC rivaroxaban, substantially decreased the thrombin generation potential in the goat model with four months of AF. Interestingly, the presence of AF alone did not induce a systemic increase in thrombin generation in the goat model.

It is important to note that the CAT assay measures the overall potential of a plasma sample to generate thrombin and not the actual ongoing coagulation activity. Therefore, in "lone AF" goats, the absence of increased thrombin generation cannot exclude the presence of underlying prethrombotic processes (e.g. endothelial dysfunction, inflammation) which can confer a thrombotic tendency.

Moreover, our results cannot exclude activation of coagulation in the atria or in the interstitial space of the atrial myocardium. As reported in the literature, local activation of the coagulation system was increased in the atria of patients in AF, or as a consequence of rapid atrial pacing (RAP) in animal models, while this was not observed in the peripheral circulation [19, 20]. In our study we attempted to compare thrombin generation in blood that was collected either locally (left atrium) or systemically (femoral vein). However, we were not able to detect differences (data not shown). This procedure took place under general anesthesia and was performed in the presence of heparin, which was required to prevent formation of thrombi during cardiac catheterization. Heparin impaired the detection of thrombin generation dif-

ferences. Detecting the acute effect of AF on local blood coagulation in the atria remains a relevant aspect for future investigation. For this purpose, a goat coagulation assay that is less sensitive to heparin would be desirable.

Furthermore, as shown by Hobbelt et al, in young very-low-risk patients with paroxysmal AF, the levels of specific coagulation factors may be increased without complete activation of the coagulation cascade and subsequent thrombin generation [21]. This pre-thrombotic state may be similar to our study in which AF was induced in young, healthy goats, in the absence of comorbidities ("lone AF").

In this setting, a second pro-coagulant hit may be needed to provoke a systemic pro-thrombotic response during AF. Among the stroke risk factors included in the CHA_2DS_2-VASc score (Table 1), advanced age is one of the strongest predictors. In **chapter 6** we report that AF and advanced age together led to an increase in the goat thrombin generation potential. This phenomenon might contribute to the synergistic interaction between AF and age on stroke risk in patients.

Another important stroke risk determinant in AF is HF (Table 1, CHA_2DS_2-VASc). In **chapter 7** we found that HF, characterized by decreased left ventricular ejection fraction, was surprisingly associated with a significant decrease in ETP and peak height of the thrombin generation curve. This hypocoagulable state remained during the addition of one week of AF.

Generally, chronic HF is associated with an increased risk of thrombosis due to fulfillment of Virchow's n]. Although HF increases the risk of stroke in AF, no clear consensus exists on the role of anticoagulation in chronic HF[22]. In fact, clinical studies found no beneficial effect in preventing stroke by anticoagulation in HF patients that were in sinus rhythmn [23-25]. Moreover, HF was recently identified as a non-modifiable bleeding risk factor in AF patients taking rivaroxaban[26]. In light of these observations, the decrease in thrombin generation found in our goat study may suggest the presence of hypocoagulant changes due to HF. A possible explanation could be that HF caused liver failure and led to hepatic coagulopathy [27]. In fact, a study on patients with acute liver failure reported reduced ETP and peak height levels compared to controls, seemingly as a consequence of protein C deficiency [28]. To test this hypothesis in future studies, possibly using samples from our experiments, thrombin generation analysis could be performed in the presence of thrombomodulin to assess the contribution of the protein C pathway in the test assay. Furthermore, analyzing blood samples collected during the early onset of HF (i.e. in the first two weeks), would provide more insight on the time course of the HF-related coagulation changes.

Table 1. The CHA_2DS_2-VASc score: a stroke risk stratification score for patients in AF

Risk factor	Score
Congestive heart failure / LV dysfunction	1
Hypertension	1
Age ≥75 y	2
Diabetes mellitus	1
Stroke / TIA / TE	2
Vascular disease (prior myocardial infarction, peripheral artery disease, or aortic plaque)	1
Age 65-74 y	1
Sex **c**ategory (i.e. female gender)	1

LV = left ventricular; TIA = transient ischemic attack; TE = thromboembolism.
In general, patients without clinical stroke risk factors do not need antithrombotic therapy, while patients with stroke risk factors (i.e. a score of 1 or more for men, and 2 or more for women) are likely to benefit from OAC [8].

Development of the AF substrate

As in most of the AF patients, the goat model of AF is characterized by a progressive increase in the duration of AF episodes, until AF becomes persistent. During the first 1-2 days, electrical remodeling takes place. This includes shortening of the atrial refractory period and the AF cycle length [29]. In parallel, a decrease in atrial contractility occurs (contractile remodeling) [10]. In the weeks to months thereafter, a much slower remodeling process takes place. This involves structural changes which have been related to the stabilization of AF [30]. In parallel with these structural alterations, the success rate of pharmacological cardioversion by antiarrhythmic drugs decreases [31, 32].

The forms of atrial remodeling described above promote the onset and maintenance of the arrhythmia and together form a substrate for AF. Recently, thrombin and FXa have been implicated in activation of cardiac fibroblasts and promotion of structural remodeling involved in AF substrate development. In principle, these non-hemostatic effects can be mediated by activation of PAR [4].

In **chapter 3** we demonstrated that FXa is a strong inducer of pro-inflammatory responses in cardiac fibroblasts, causing the upregulation of two well-known mediators of inflammation, *IL6* (interleukin 6) and *CCL2* (monocyte chemoattractant protein 1). In contrast with the existing literature, we demonstrated that FXa elicited its pro-inflammatory activity mainly via activation of PAR-1 [33].

Inflammatory processes can also contribute to the development of a substrate for AF [34, 35]. AF patients indeed show increased cardiac and systemic signs of inflammation [36-39]. Inflammation has been shown to play a role in supporting pro-fi-

brotic processes [40, 41]. Indeed, some pro-inflammatory stimuli can contribute to proliferation and activation of cardiac fibroblasts and therefore support tissue fibrosis [42, 43] Fibrosis, together with myocyte hypertrophy, are two main features of atrial structural remodeling that have been linked to conduction abnormalities related to AF [44-46].

In our experiments (**chapter 3**), thrombin and FXa mediated fibrotic responses in CF. We found that both coagulation factors upregulated the expression of two key-regulating genes of pro-fibrotic responses, *ACTA2* (alpha smooth muscle actin) and *TGFB1* (transforming growth factor beta 1). These results are in line with other *in vitro* and *in vivo* studies and show that these coagulation factors can affect structural remodeling processes such as fibrosis, indirectly (through promotion of inflammation) and directly via activation of PAR expressed on cardiac cells (e.g. fibroblasts) [33, 47].

In our *in vivo* study (**chapter 5**) we investigated whether inhibition of coagulation, via rivaroxaban treatment, would inhibit the progression of AF by protecting against atrial structural remodeling induced by four months of AF in goats. In this study, based on the low success rate of the flecainide cardioversion experiments already at early time points, no difference in AF stability was found. Moreover, AF, with and without rivaroxaban treatment, did not affect atrial endomysial fibrosis, although upregulation of pro-fibrotic markers was observed in the atria. Interestingly, we found that AF led to a significant increase in atrial myocyte size, which was fully prevented by rivaroxaban.

Myocyte hypertrophy can be induced by different stimuli during AF. One example is atrial stretch as a consequence of AF-related increased atrial pressures. However, the protective effect of FXa-inhibition in our study suggests that activated coagulation factors (e.g FXa and thrombin) may contribute to myocyte hypertrophy. Although we could not elucidate the underlying mechanism in the goat model, this observation agrees with a recent report by Guo et al. [47], who observed that exposure of rat neonatal cardiomyocytes to FXa induced hypertrophy and increased expression of *NPPA* (atrial natriuretic peptide) via activation of either PAR-1 or PAR-2 [47].

In **chapter 6** and **7** we observed that the substrate for AF can be affected by the coexistence of AF and its related risk factors, or by the presence of AF-related comorbidities alone.

In **chapter 6**, 4 weeks of AF increased atrial myocyte hypertrophy in old goats, but not in young goats. Moreover, we showed that old goats with AF presented signs of epicardial endomysial fibrosis. This was not found in the goat model of "lone AF" (**chapter 5**) in which only changes in myocyte size were observed. These observations suggest that advanced age as an AF risk factor may accelerate the AF-related atrial myocyte growth and endomysial fibrosis. Moreover, we observed an increased coagulation potential in AF goats with advanced age, while this was not the case in

young AF goats. Whether this contributed to the accelerated development of the observed structural changes cannot be proven with the current data. Nevertheless, it remains an interesting finding that emphasizes the potential link between structural remodeling and coagulation activity.

Another possible cause for the faster development of structural remodeling processes in old goats with AF can be the larger increase in atrial pressures compared to young animals. Hemodynamic overload of the atria has been shown to lead to stretch conditions (elongation of cardiomyocytes) and could thereby contribute to atrial structural changes [48]. The AF-related structural changes in goats with advanced age were associated with increased AF complexity, possibly due to transverse propagation delays that can lead to discontinuous conduction between hypertrophic myocytes [45, 49, 50].

Advanced age also resulted in faster AF stabilization during the first 24 hours after pacing was initiated. This could be due to the fact that the aged atrial myocardium is less tolerant to the acute onset of AF and the accompanying pressure increase. Age-related cardiac stress intolerance, characterized by reduced contractility, has indeed been linked to molecular alterations such as Ca^{2+} handling abnormalities and metabolic dysfunction [51]. For instance, in the ageing heart it has been shown that reduced expression of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase2 (SERCA2a) and overexpression of the ryanodine receptors significantly prolong the SR CA^{2+} transient leading to attenuation of cardiomyocyte contraction [52, 53]. Moreover, age-related reduction of adenosine triphosphate (ATP) production in the heart, as a consequence of mitochondrial dysfunction, has also been shown to be involved in reduced cardiac contractile function[54] (Barton et al. 2017). During changes in energy metabolism, such as in atrial tachycardia, the aged heart shows less adaptive capacity and might therefore be more vulnerable to AF stabilization.

Another important risk factor for AF, that can be both a cause and a consequence of AF, is HF. In **chapter 7** we showed that three weeks of HF led to a strong substrate for AF. In contrast to the type of substrate that is induced by AF, the HF-induced substrate did not show electrical remodeling. In fact, the atrial effective refractory period (AERP) and AF cycle length were found to be increased in HF at 4 weeks. This is in agreement with previous reports on experimental HF [55, 56]. Schoonderwoerd et al. reported a dynamic behavior of the AERP in AV-tachypaced (atrial and ventricular 1:1) goats [56]. At first, the AERP decreased upon AV-pacing in a similar way as in AF or RAP. However, when HF developed, AERP progressively prolonged and the atria dilated. AV-pacing-induced HF resulted in a substantial increase of the atrial diameter, which was not found in A-paced goats. In our study, we did not measure atrial size, but we did observe strongly increased atrial pressures in HF. This was accompanied by significant atrial endomysial fibrosis, without signs

of myocyte hypertrophy. By contrast, previously both fibrosis and myocyte hypertrophy have been reported in other animal models of heart failure [55, 57, 58].

Moreover, we found that the AF vulnerability and stabilization during RAP were strongly accelerated in the presence of HF, compared to the substrate that was created by RAP alone. These findings suggest that HF and RAP-induced AF lead to two distinct substrates for the stabilization of AF. This was also reported by Li et al., who found a higher efficacy of dofetilide, a class III antiarrhythmic compound, in CHF compared to AF dogs, which was associated to less complex AF patterns [55, 59].

The coexistence of AF and HF is expected to impair cardiac function more than HF alone, thereby leading to worsening of HF symptomsb [60, 61]. In our model, the addition of AF in the presence of HF, as described in **chapter 7**, seemed to worsen the hemodynamic state of the goats. This, however, was not significant and also did not lead to additional electrical or structural remodeling, nor to increased AF complexity.

A two-way street with many side-roads

In the previous paragraphs we have tried, on the basis of our findings, to explain some of the possible mechanisms by which AF and changes in the coagulation system could interact and affect each other; we have hypothesized a bidirectional crosstalk. However, as our results also suggest, the causal relation between AF and activation of coagulation (and stroke) is difficult to establish and may involve a third component.

Clinically, AF and stroke share many risk factors, whose underlying pathological processes can reinforce the development and progression of both cardiovascular conditions. Therefore, the association between AF and stroke appears to be more complex than a bidirectional crosstalk. In fact, the association between AF and stroke might also be due to common causes and shared etiologies of both conditions.

For example, coronary artery disease can lead to ischemia in the atrial myocardium.

AF can also lead to atrial supply-demand ischemia, as found in a pig model [62]. Ischemic conditions have been shown to enhance interstitial expression and activity of coagulation factors in the myocardium. In a rabbit model of ischemia reperfusion injury, both TF mRNA levels and pro-coagulant activity were increased in the at-risk ischemic regions of the myocardium compared to control [63]. This, together with vascular leakage, may create the favorable conditions for the local activation of the coagulation system within the myocardial interstitial space during AF. Moreover, ischemia can lead to inflammatory responses in the atria which may also con-

tribute to endothelial dysfunction and damage [64]. As a consequence, endothelial cells increase expression of the pro-thrombotic protein von Willebrand factor, which facilitates platelet adhesion to the activated endothelium [65, 66]. Furthermore, exposure of the TF-expressing subendothelium to the blood stream, as a consequence of endothelial denudation, may support activation of the coagulation cascade within the atrial cavity [67]. Interestingly, ischemia-mediated pro-inflammatory processes can also influence the progression of AF. In addition, inflammatory stimuli play an important role in the activation of cardiac fibroblasts and their differentiation into myofibroblasts. As a consequence, the increased collagen deposition mediated by cardiac myofibroblasts may promote fibrosis and lead to a higher likelihood for reentry and progression of AF [68, 69].

HF is another example of a shared etiology of AF and stroke. During HF, increased atrial pressures lead to atrial stretch which has been shown to activate the renin angiotensin system (RAS) in the atrial wall [70, 71]. Local RAS activation, and the resulting increase in angiotensin II can trigger fibrotic changes in the atria (e.g. differentiation of cardiac fibroblasts into myofibroblasts) which may further support pro-arrhythmic electrophysiological abnormalities leading to AF [72].

In parallel, increased atrial stretch and the loss of atrial contractile function in HF can be a potential trigger for thrombotic events [65]. Reduced shear stress (resulting from the loss of atrial contractility) has been proven to downregulate the endothelial production of nitric oxide, which mediates vasodilation and has antithrombotic properties [73]. Downregulation of atrial nitric oxide would therefore not only increase aggregation of platelets, but also increase expression of the protein plasminogen activator inhibitor, resulting in impaired fibrinolysis [65].

These are only a few examples of the molecular and cellular mechanisms triggered by shared risk factors of AF and stroke within the atria. Because their pathophysiological mechanisms often result in structural remodeling of the atrial myocardium and prothrombotic alterations of the atrial endothelium, these risk factors are associated with both AF and stroke. As a consequence, the causal relation between AF and stroke should be addressed as the result of a multidirectional network between AF, stroke and their shared comorbidities rather than a pure bidirectional interaction.

Future perspectives

The principal hypothesis of this thesis is that the AF-associated hypercoagulable state contributes to atrial remodeling and AF substrate development through PAR-activation. Based on our experimental findings, we proved that the coagulation fac-

tors thrombin and FXa elicit direct non-hemostatic functions on CF (**chapter 3**). CF are one of the most important cell types in the heart, as they regulate its hemostasis and can mediate structural remodeling. We have demonstrated that thrombin and FXa activate CF and promote pro-fibrotic processes in this cell type. Moreover, we have shown that FXa has a remarkable pro-inflammatory effect on CF, which appears to be mediated by PAR-1 activation and inhibited by the FXa-direct inhibitor rivaroxaban.

The possible link between pathological activation of the coagulation system and cardiac remodeling was further supported by our *in vivo* study on the goat model of four months of AF (**chapter 5**). We have shown that anticoagulation treatment via the DOAC rivaroxaban prevented atrial cardiomyocyte hypertrophy induced by four months of AF.

Furthermore, we hypothesized that the AF-related coagulation state can be modulated by the coexistence of AF with its related risk factors, or by the presence of AF-related comorbidities alone, thereby altering AF substrate development. In this context, we have shown that AF and its risk factor (advanced) age synergistically promote an increase in clotting potential in goats. Along with this observation, our results suggest that advanced age may also accelerate the AF-related atrial myocyte hypertrophy and endomysial fibrosis (**chapter 6**). Finally, in **chapter 7**, we showed that the presence of the AF-risk factor HF strongly enhanced AF vulnerability and promotes a substantial decrease of clotting potential, also after the addition of (one week of) AF.

Despite these relevant findings, some important research questions remain unanswered in our study. One example is about the role of PARs in the atrial structural remodeling processes that take place in the goat model of AF. Although our group and others have demonstrated the involvement of PARs *in vitro*, it was not possible to assess the role of these receptors in our *in vivo* studies. Gene expression analysis of the goat atria confirmed that also in this animal model, atrial tissue mainly expressed PAR-1 and -3 isoforms (**chapter 5**). Moreover, this analysis indicated changes in PARs expression mRNAs in goats with AF compared to control. However, these findings were not sufficient to demonstrate a link between PARs and cardiac remodeling.

To elucidate this aspect, a possible interesting approach could be the *in vivo* inhibition of PAR signaling during AF. A recent study has shown that pharmaceutical inhibition of PAR-1 via a selective antagonist was effective in blocking PAR signaling and attenuating cardiac remodeling in renin-overexpressing hypertensive mice [74]. Applying such a pharmaceutical inhibition of PAR signaling in the goat model might be a valid strategy to shed light on the role of PAR activation in cardiac remodeling processes induced by long term AF.

Another interesting aspect that was not fully addressed in our study is whether AF causes local (atrial) activation of the coagulation system. As already mentioned in this chapter, coagulation assays with lower sensitivity to heparin might be helpful to compare local vs. systemic blood samples and therefore should be developed for the goat model.

Furthermore, it would be valuable to improve the development of staining protocols and other methods to detect interstitial signs of activation of coagulation in the goat atrial tissue, as well as to investigate the presence of hypoxic markers, which may contribute to pro-thrombotic processes in the atria during AF. These protocols would be useful to gain additional data from the current studies through evaluation of the stored tissues, but also in the development of future studies. In this context, a more profound analysis of pro-inflammatory changes in atrial tissue and systemic blood samples would be helpful to characterize the inflammatory state during AF, and for the evaluation of possible anti-inflammatory properties of FXa-inhibition by DOACs.

In **chapter 6** we reported that, compared to young goats, old goats with four weeks of AF showed increased thrombin generation which was accompanied by atrial myocyte hypertrophy and fibrosis. We hypothesized that advanced age, as an AF risk factor, may accelerate the AF-related atrial myocyte hypertrophy and endomysial fibrosis. However, a causal relation between increased clotting potential and accelerated AF-related cardiac remodeling could not be proven in our study. Thus, a possible strategy to clarify this aspect could be to assess whether anticoagulation in the goat model of AF with advanced age could protect against the pleiotropic effects of coagulation factors in the myocardium, or whether pharmaceutical inhibition of PAR signaling could also protect against other forces causing cardiac remodeling in this model, such as AF-related atrial stretch induced by increased atrial pressure.

Additionally, as mentioned before, a striking finding in our HF goat study (**chapter 7**), was the observed decrease in thrombin generation. Based on literature, we speculated on HF-related hepatic changes that caused coagulopathy through alterations in protein C. To test this hypothesis, the thrombin generation analysis could be performed in the presence of thrombomodulin to challenge the protein C pathway in the test assay and compare the HF plasma samples to control samples.

Although important research questions remain unanswered, we believe that the knowledge acquired in this thesis can be the basis for future investigations. **Chapter 4**, for example, represents the starting point for further development of the thrombin generation assay in goats. The assay turned out to be an essential tool for the assessment of the coagulation status in the goat model of AF, which was not possible before. Future modifications of the assay may contribute to unravel more complex aspects of AF-related hypercoagulable state which may go beyond the quantification of generated thrombin.

Furthermore, the findings reported in **chapter 3** and **5** may be important for clinical considerations. In fact, they provide insights on the pleiotropic effects of activated coagulation factors in the atrial myocardium and on whether their direct inhibition via DOACs may not only prevent the risk of stroke in AF patients but also prevent cardiac remodeling processes (e.g. inflammation, fibrosis and myocyte hypertrophy).

Finally, **chapter 6** and **7** highlight the important impact that AF comorbidities and risk factors have on coagulation status and AF progression. Our observations may become relevant for future (clinical) studies on AF therapy and stroke/bleeding prevention.

Taken together, the results of this thesis lay the basis for future experimental investigation and important translational considerations. Our findings confirm the presence of a crosstalk between AF and coagulability, which should be addressed as a multidirectional network between AF, stroke and their shared comorbidities rather than purely as a bidirectional interaction.

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Appendix

Summary

Atrial fibrillation (AF) is the most common form of sustained cardiac tachyarrhythmia with an estimated prevalence of approximately 3%. AF is characterized by high-frequency, irregular activation of the atria, leading to hemodynamic impairment. AF often starts with short, self-terminating episodes. However, the progressive nature of the arrhythmia is characterized by prolongation of the AF episodes until they become persistent.

Epidemiologically, advancing age is the predominant risk factor for AF. AF is associated to increased cardiovascular mortality due to sudden death, heart failure (HF) and stroke. The risk of developing thromboembolic stroke increases by five-fold after patients have developed AF.

Recently, hypercoagulability has been described to play a role in the progression of AF. Activated coagulation factors, such as thrombin and coagulation factor (F) Xa, are able to modulate physiological and pathological processes, such as inflammation and fibrosis, which may contribute to cardiac remodeling and progression of AF. These extravascular (non-hemostatic) functions affect different cell types via activation of protease activated receptors (PARs).

The general aim of this thesis was to evaluate how AF progression is linked to hypercoagulability by activated blood coagulation (through thrombin and FXa) and consequent PAR activation. Furthermore, we have investigated the effect of the risk factor age, and the AF comorbidity HF, on AF substrate development and coagulation activity.

In **chapter 2** we reviewed the most important and recent findings regarding the tissue factor (TF):Factor (F)VIIa complex, an essential coagulation trigger, with the emphasis on the heart and blood vessels. TF facilitates the activation of FVII into activated FVII (FVIIa), thereby initiating the extrinsic coagulation pathway followed by the activation of FX (FXa) and thrombin formation. The so-called non-hemostatic functions of TF:VIIa play a role in diverse processes such as inflammation, atherosclerosis and remodeling of vessels and myocardium. We also described some of the pleiotropic effects elicited by thrombin and FXa, and their cellular signaling through activation of PAR.

Thrombin and FXa have been implicated in the activation of cardiac fibroblasts (CF) and promotion of cardiac remodeling. In **chapter 3** we showed that these coagulation factors upregulated the gene expression of pro-fibrotic genes in CF, supporting the link between pathological activation of the coagulation system and cardiac remodeling. Moreover, we found that FXa induces overexpression of proinflammatory genes in human CFs via PAR-1, which was found to be the most abundant PAR isoform in this cell type.

The central hypothesis of this thesis is that the hypercoagulable state during AF contributes to the development of an AF substrate through activation of PAR. To test this hypothesis *in vivo*, we made use of the goat model of AF. This model allows the investigation of changes in the heart and blood that occur within days to months of AF.

In **chapter 4** we described how we customized the Calibrated Automated Thrombography (CAT) assay to provide a global view of the goat coagulation profile by assessing thrombin generation (TG) in goat plasma. Since TG assessment with human reagents appeared not suitable for goat plasma, we reported on two distinct methods using either goat brain proteins (GBP) or Russell's viper venom-factor X activator (RVV-X) as successful triggers of TG in goat plasma. Moreover, we showed that both methods were able to detect the decrease in clotting potential induced by FXa-inhibition.

In **chapter 5** we investigated the effect of FXa-inhibition, by rivaroxaban treatment, on AF substrate development in the goat model of AF. Although rivaroxaban treatment did not prevent the progression of AF, we found that four months of AF led to atrial myocyte hypertrophy, which was fully prevented by FXa-inhibition. Interestingly, AF did not cause a hypercoagulable state, as assessed by the TG assay. One possible explanation is that AF without the presence of other comorbidities, or risk factors for stroke ("lone AF"), might not be sufficient to trigger a thrombotic response in goats. As age is the most important risk factor for AF, as well as for stroke in patients with AF, we studied the effect of advanced age in the goat model of AF. In **chapter 6** we reported that age and AF synergistically increased coagulation potential, early AF stabilization and promoted atrial structural remodeling.

Another important comorbidity of AF is heart failure (HF), which can promote the onset and progression of AF and can increase the risk for stroke during AF. In **chapter 7** we demonstrated that HF created a favorable substrate for the onset of AF, which was associated with a decrease in coagulation activity and increased atrial fibrosis. Moreover, HF led to a strong increase in complex fibrillatory conduction.

Taken together, the results of this thesis lay the basis for future experimental investigations and important translational considerations. Our findings confirm the presence of a crosstalk between AF and hypercoagulability, whose causal link can be described as a multidirectional network between AF, stroke and their shared comorbidities.

Impact

Atrial fibrillation (AF) is the most common form of sustained cardiac tachyarrhythmia. It often starts asymptomatically, which allows it to progress until it causes adverse cardiovascular events (e.g. thromboembolic stroke), before it is even detected. AF prevalence increases with age. Due to ageing of the general population in the coming decades, the burden on the worldwide health care systems is expected to increase significantly. New insights on mechanisms that affect the initiation and progression of AF are therefore highly needed.

Previously, it has been demonstrated that inhibition of specific coagulation factors may reduce pathological processes that are involved in AF substrate development. In this context, the general aim of our thesis was to evaluate how hypercoagulability is linked to AF progression. Furthermore, we investigated the effect of two main AF risk factors (age and heart failure [HF]) on AF substrate development and coagulation activity.

Our *in vitro* experimental work as described in chapter 3 focused on one of the most important cell types in the heart, cardiac fibroblasts (CF). We proved that the coagulation factors thrombin and FXa activate CF and promote pro-fibrotic processes. Moreover, we have shown that FXa has a pronounced pro-inflammatory effect on CF, which appears to be mediated by PAR-1 activation and inhibited by the FXa-direct inhibitor rivaroxaban. These findings again demonstrate that coagulation factors elicit direct non-hemostatic functions. This observation could also support other researchers to extend the study of the pleiotropic effects of these coagulation proteins not only in the heart but also in other organs of the body.

A major part of this thesis was based on *in vivo* investigations making use of the goat model. This model is a well-established and extensively researched model for the pathogenesis of AF. Because AF can be maintained in the goat for several months, this animal model also represents an interesting tool to investigate the long-term effects of AF on the coagulation system. However, suitable coagulation assays for goat plasma are not available from commercial sources. In chapter 4 we described how we customized the Calibrated Automated Thrombography (CAT) assay to provide a global view of the goat coagulation profile by assessing thrombin generation (TG) in goat plasma. To the best of our knowledge, this is the first study that reports the customization of the CAT assay for goats. In future applications of the AF goat model, in fact for any (experimental) goat model, the main changes in the coagulation system can now be included for evaluation. For instance, other interesting research questions, which can be now answered, may focus on the effect that the presence of other AF-related comorbidity, such as hypertension, in combination with AF, would have on the coagulation system, or on how coagulation activity

would be affected by paroxysms of AF and/or restoration of sinus rhythm after a prolonged period of AF. Moreover, additional modifications of the CAT assay can give insights on the contribution of both pro- and anti-coagulant pathways.

The possible link between pathological activation of the coagulation system and cardiac remodeling was further supported by chapter 5. Using the customized CAT assay, we showed that anticoagulation treatment via the DOAC rivaroxaban, prevented atrial cardiomyocyte hypertrophy induced by four months of AF. These findings are potentially important for clinical considerations. In fact, they provide insights on the pleiotropic effects of activated coagulation factors in the atrial myocardium and on the fact that their direct inhibition via (clinically approved) DOACs (in this case rivaroxaban), may not only prevent the risk of stroke in AF patients but also prevent cardiac remodeling processes (e.g. inflammation, fibrosis and myocyte hypertrophy). Clinical trials on the effect of DOACs on outcome in patients with AF (e.g. stroke, cognitive decline, cardiovascular outcome) offer opportunities to test this hypothesis. For example, the ongoing NOAH-AFNET 6 trial investigates the effect of DOACS (edoxaban) on stroke and cardiovascular complication in low risk patients with atrial high rate episodes but without documented AF. The control group does not receive anticoagulation and using the implanted devices an effect of the DOAC on progression to AF can be quantified.

The findings of chapter 6 highlighted the importance of the synergistic effect of AF and its risk factors (in this case age) on coagulation activity and AF substrate development, while in chapter 7 we showed that the lone presence of the AF risk factor HF strongly increased the AF substrate, which was associated with a substantial decrease of clotting potential. Although a causal relation between the clotting potential and atrial remodeling could not be proven in the studies, we believe that the knowledge acquired can be the basis for future investigations, nonetheless for important translational considerations.

Taken together, our findings support the relation between pathological activation of the coagulation system and atrial remodeling. The methods and findings described in this thesis are of potential value to all researchers involved in AF, its risk factors or coagulation and can be of importance to clinical considerations. In fact, based on the effect that activated coagulation factors elicit on the atrial myocardium, our data support the thought that early anti-coagulation therapy in AF may not only mitigate the risk of stroke but also slow down AF progression.

Table of contributions

- **Chapter 1** **General introduction**
  Authors: E. D'Alessandro*, B. Scaf*
  **Contributed equally*

- **Chapter 2** **Tissue factor (:Factor VIIa) in the heart and vasculature: More than an envelope**
  (*Thrombosis Research*, 2018)
  Authors: E. D'Alessandro, J. Posma, H.M.H. Spronk, H. ten Cate.
  Contributions: E. D'Alessandro was the main responsible person for writing the manuscript, all authors were involved in writing of the manuscript, data discussions and decision making.

- **Chapter 3** **Coagulation Factor Xa induces pro-inflammatory responses in cardiac fibroblasts via activation of Protease-Activated Receptor-1**
  (*Cells*, 2021)
  Authors: E. D'Alessandro, B. Scaf, C. Munts, A. van Hunnik, C. Trevelyan, S. Verheule, H.M.H. Spronk, N.A. Turner, H. ten Cate, U. Schotten, F.A. van Nieuwenhoven.
  Contributions: E. D'Alessandro was responsible for the experimental work and writing of the manuscript, and B. Scaf was mainly involved in the finalization of the manuscript. F. van Nieuwenhoven was the main supervisor and all authors were involved in data discussions and decision making.

- **Chapter 4** **Optimization of the Thrombin Generation Assay using Calibrated Automated Thrombography in goat plasma**
  (*Research and Practice in Thrombosis and Haemostasis*, 2021)
  Authors: E. D'Alessandro, B. Scaf, R. van Oerle, F.A. van Nieuwenhoven, A. van Hunnik, S. Verheule, U. Schotten, H. ten Cate, H.M.H. Spronk.
  Contributions: E. D'Alessandro was the main responsible person for the in vitro experimentation and writing of the manuscript, B. Scaf was responsible for the goat specimens and involved in finalization of the manuscript, H.M.H. Spronk was the main supervisor and all authors were involved in data discussions and decision making.

- **Chapter 5** **Rivaroxaban prevents atrial myocyte hypertrophy in a goat model of persistent atrial fibrillation**

(*In preparation for publication*)

Authors: B. Scaf[*], E. D'Alessandro[*], D. Opačić, A. van Hunnik, M. Kuiper, J. Simons, J. Winters, A. Isaacs, S. Heitmeier, M. Stoll, R. van Oerle, F.A. van Nieuwenhoven, H. ten Cate, H.M.H. Spronk, S. Verheule, U. Schotten.

[*]*Contributed equally to the writing of the manuscript*

Contributions: B. Scaf was the main responsible person for the goat experiments and analyses, E. D'Alessandro was also involved in goat experimentation and the main responsible person for the coagulation tests. Both first authors were equally responsible for the remaining experimental aspects and analyses, and for the representation of the study to the scientific community. All authors were involved in animal experimentation or in data discussions and both first authors were equally responsible for the writing of the manuscript.

- **Chapter 6** **Atrial fibrillation and age synergistically increase clotting potential and promote atrial structural remodeling in goats**

(*In preparation for publication*)

Authors: E. D'Alessandro[*], B. Scaf[*], A. van Hunnik, V. Sobota, M. Kuiper, R. Peeters, J. Winters, R. van Oerle, H.M.H. Spronk, F.A. van Nieuwenhoven, H. ten Cate, S. Verheule2, U. Schotten.

[*]*Contributed equally to the writing of the manuscript*

Contributions: B. Scaf was the main responsible person for the goat experiments and analyses, E. D'Alessandro was also involved in goat experimentation and the main responsible person for the coagulation tests. Both first authors were equally responsible for the remaining experimental aspects and analyses, and for the representation of the study to the scientific community. All authors were involved in animal experimentation or in data discussions and decision making. Both first authors were equally responsible for the writing of the manuscript.

- **Chapter 7** **Ventricular tachypacing-induced heart failure decreases thrombin generation and promotes AF substrate development in goats**

  (*In preparation for publication*)

  Authors: B. Scaf[*], E. D'Alessandro[*], A. van Hunnik[*], V. Sobota, M. Kuiper, R. Peeters, H.M.H. Spronk, F.A. van Nieuwenhoven, H. ten Cate, S. Verheule, U. Schotten.

  [*]*Contributed equally to the writing of the manuscript*

  Contributions: A. van Hunnik, B. Scaf, E. D'Alessandro and V. Sobota were responsible for the goat experiments and writing of the manuscript. B. Scaf and E. D'Alessandro were the main responsible persons for the coagulation tests and histological analysis. All authors were involved in animal experimentation or in data discussions and decision making.

- **Chapter 8** **General discussion**

  Authors: E. D'Alessandro[*], B. Scaf[*]

  [*]*Contributed equally*

- **Appendix** **Summary**[*]
  Impact[*]

  Authors: E. D'Alessandro[*], B. Scaf[*]

  [*]*Contributed equally*

Note from the authors

This thesis is the result of a joint effort of two PhD candidates. For this reason, it may appear quite unique and/or unusual. Here, we would like to give you a short insight on behind the scenes of our journey and briefly explain, how our collaboration naturally evolved and resulted in this joint thesis.

As our PhD trajectory started, we became responsible for an exciting multidisciplinary large animal intervention study (chapter 5) which, due to changes in animal legislation and an ending application, had to be finalized within our first year.

Thanks to that, we found ourselves in a unique and lucky situation. In fact, we were able to immediately start experimenting and gain our first data while, for many fellow PhD students, this would not happen before the second year.

Although the precious support from the group, our little experience as first-year PhD students, and the limited amount of time, were challenging. Therefore, we took advantage of our different backgrounds and worked in parallel on separate aspects of the same study (hemostasis and electrophysiology). Nevertheless, we also worked together: brainstorming, performing experiments, treating animals and doing a lot of manual work. To give you an idea, in our first year, together we prepared by hand an equal amount of rivaroxaban pills, as the amount to treat a man for more than 80 years!

Over time, we realized that this collaboration was highly productive. In fact, ultimately, we were able to finalize 3 multidisciplinary goat studies, visit an external university to perform in vitro experiments and establish novel techniques.

Most importantly, we came to understand that the research questions of our projects were intertwined. In other words, our collaboration became "a two-way street with many side-roads".

Of course, we could have split the content of this thesis in half. However, this would have been an unnatural choice and it would have not reflected the real essence of this collaborative work.

We are proud of our colleagues in the two beautiful departments of Physiology and Biochemistry, and grateful for the support of our supervision team.

About the authors

Elisa D'Alessandro was born on the 20th of August 1992 in Potenza, Italy. She obtained her secondary school diploma at Liceo Scientifico Galileo Galilei in Potenza in 2011. Subsequently, she moved to Siena, Italy, where she discovered her passion for cellular biology. She graduated in Biology (2014) and later in Molecular and Cellular Biology (2016). In Siena, Elisa was a tutor at the Office for Students with Learning Disabilities, where she provided support to students with specific learning difficulties. During her last year of her master's, she obtained the Erasmus for Traineeship Scholarship that allowed her to undertake a 6-months internship on RNA methylation in diabetic vascular complications at the Bristol Heart Institute, United Kingdom, under the supervision of Professor Costanza Emanueli.

In 2017, Elisa became a shared PhD candidate between the Departments of Biochemistry and Physiology at Maastricht University. In this privileged position, she joined the RACE V (Reappraisal of Atrial Fibrillation: Interaction between hypercoagulability, Electrical remodeling, and Vascular Destabilization in the Progression of AF) consortium and she worked under the supervision of Professor Hugo ten Cate, Professor Ulrich Schotten, Dr. Henri Spronk, Dr. Frans van Nieuwenhoven and Dr. Sander Verheule. Since the very beginning of her PhD trajectory, she worked in close collaboration with her fellow PhD candidate and friend, Billy Scaf. By making use of their different expertise, they investigated the relation between Atrial fibrillation and Hypercoagulability and they had a lot of fun. The result of this collaboration and the main findings of their doctoral work are presented in this thesis.

As of November 2021, Elisa is a postdoctoral researcher at the Department of Physiology at Maastricht University in the group of Dr. Ed Eringa. There she investigates the role of perivascular adipose tissue in organ function and metabolism.

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Scaf B., **D'Alessandro E.**, The role of Factor Xa in Atrial Fibrillation-related cardiac remodeling, *Dutch Physiology Day, Nederlandse Vereniging voor Fysiologie, Utrecht*, *the Netherlands*, **2020** (Oral presentation).

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Billy Scaf was born on the 19th of April 1990 in Vilt, the Netherlands. He received his first (VMBO-TL in 2006) and his second (HAVO in 2008) high school degree at the Sint Maartens-college in Maastricht. Billy followed his fascination for Biology (in particular for animals) and continued to study at the HAS Hogeschool in 's-Hertogenbosch. A 6-months internship at the Veterinary University of Vienna in 2011 resulted in his first co-authorship of a scientific paper. In 2012, he graduated as a Bachelor of Science in Applied Biology. He continued to study at Wageningen University and became a Master of Science in Animal Sciences in 2014.

His interest in doing research with laboratory animals had grown during his internship at the Biochemistry department of Maastricht University in the group of Prof. dr. Chris Reutelingsperger and Prof. dr. Leon Schurgers. There, he wrote his MSc thesis on (different types of) anticoagulants and their effects on the atherosclerotic plaque phenotype in mice.

After a period of working as a biotechnician at Charles River Laboratories in 's-Hertogenbosch, where he gained experience in working with different types of laboratory animals (small and large), he decided to apply for a PhD position.

In 2017, Billy started his PhD-trajectory at Maastricht University. The project was a collaboration between the departments of Physiology and Biochemistry and involved also a second PhD-candidate, Elisa D'Alessandro. Both PhD-candidates were supervised by Prof. dr. Ulrich Schotten, Prof. dr. Hugo ten Cate, dr. Sander Verheule, dr. Henri Spronk and dr. Frans van Nieuwenhoven. The project was called RACE-V (Reappraisal of Atrial fibrillation: interaction between hyperCoagulability, Electrical remodeling, and Vascular destabilization in the progression of atrial fibrillation), which was a consortium of the universities of Leiden, Groningen and Maastricht.

Elisa and Billy worked on the pre-clinical part of the project, involving large animal experiments and complex laboratory techniques, investigating the relation between Atrial Fibrillation and Hypercoagulability. From the very first beginning, they worked closely together and shared tasks, thoughts, responsibilities, worries, strengths, frustrations, energy, and laughter. The result of this unique collaboration resulted in this shared doctoral thesis which contains the highlights of their experimental work between 2017 and 2021.

From November 2021, Billy has started to work at the Clinical Trial Center Maastricht (CTCM) as a Project Quality Specialist, performing clinical data management and monitoring.

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Acknowledgments by Elisa D'Alessandro

Collaboration is the force that drives big changes and achievements. In many ways, this thesis is an example of how collaboration is essential to accomplish great things. In fact, many people have collaborated with me throughout my journey and many more have supported me by sharing their friendship, experience and advice. For these reasons, I would like to spend a few words to thank them all.

First, I would like to express my gratitude and affection to all my supervisors. I would like to thank Prof. ten Cate and Prof. Schotten for being my promotors. Thank you both for giving me the opportunity to be part of your groups and learn from you. Dear **Hugo**, thank you for your unbendable kindness and good mood. Before moving to the Netherlands, it was unimaginable for me to call a professor by name and feel comfortable with it. You made that easy, and fun!

Dear **Uli**, thank you for always being present. I am especially grateful for your subtle and discreete way of pointing out the right direction to follow, while always encouraging the development of critical and independent thinking.

I also would like to express my gratitude to Dr. van Nieuwenhoven. Dear **Frans**, thank you for your daily guidance and for our lively brainstorming sessions. They contributed substantially to my scientific growth. Thank you for agreeing with me, but especially for disagreeing.

Further, I would like to thank Dr. Spronk. Dear **Henri**, thank you for supporting and guiding me, but also for teasing and challenging me. Besides being essential for the realization of this thesis, you contributed a lot to my self-growth during these years.

Finally, I would like to express my gratitude to Dr. Verheule. Dear **Sander**, thank you for always finding the time to share your advice and suggestions. I have sincerely valued and appreciated your input on my research and career path.

I would like to thank my travel companion, **Billy**. Dear Billy, I am pretty sure you will probably hate me for this, but I am going to be a bit emotional. In fact, this was one of the biggest differences between us. You, a very discreet, controlled and organized Dutch guy, and me, a very extrovert, emotional, and sometimes forgetful Italian girl. No surprise that this led to some friction between us! Many people, especially our office neighbors, would say that we had many fights, and to some extent, they are right. However, the truth is that we became good friends, and good friends can have "animated disagreements". In all honesty, working with you was a lot of fun. I am grateful for that because I could always count on your support when I needed it and because I knew that I would always get an honest opinion from you. I really hope I succeeded in doing the same for you. Thank you for giving your word and always maintaining it and for those times you took the role of the big (small) brother I have never had. I wish you all the best for what awaits ahead of you.

My gratitude goes also to the members of the Assessment Committee **Prof. Tilman Hackeng**, **Prof. Andreas Götte**, **Prof. Kevin Vernooy**, **Prof. Philippe Wenzel** for taking the time to read and review my thesis.

Further I would like to thank all the members of the RACEV consortium. Dear **Harry**, **Isabelle**, **Michiel**, **Anton-Jan**, **Hetty**, **Sophie**, **Monika**, **Aaron** and others, I am proud and grateful for being part of this amazing consortium and for the opportunity to collaborate with you during these years, thank you.

My journey was particularly blessed by the fact that I could be part of two extraordinary departments, the Physiology, and the Biochemistry department. From both sides, many people have contributed to making every day of my journey an exciting and precious opportunity to learn and progress.

Dear **Arne**, I still remember the warm and friendly smile you gave me the first time we met, during my first lab meeting in your group. Thank you for making me feel welcomed, for supporting and guiding me and Billy with our experiments, for always being critical and honest with me (us).

Dear **Chantal**, **René**, **Simone**, **Stefanie**, **Diane** and **Patricia**, thank you all for your kindness, for sharing your knowledge, and for being of great help. My work would have not been possible without you. I have sincerely appreciated your support and valued every single moment I spent learning from you.

Dear **Marion**, thank you for all those long hours of hard work in the OR. You were of precious help to me and Billy. A great part of this thesis would have not been possible without you.

I also would like to thank the CPV staff members: **Huub**, **Richard**, **Saskia**, **Petra** and **Rachelle**. Thank you all for always being there and for helping us take care of our animals.

My gratitude also goes to all the colleagues that collaborated with me and filled my days with friendship and laughter. Dear **Giulia**, thank you for helping me to settle down in Maastricht. You were my reference point during my first days in the Netherlands. Thank you for being an exceptional colleague, but most of all thank you for being my partner in crime for many adventures and pranks!

Another big thanks goes to one of the kindest colleagues I ever met, **Vladimir**. Dear Vlady, thank you for being such a polite and fun victim of me and Giulia's pranks! I am grateful for all the help you have given to me and Billy with the experiments, for your input and for your friendship.

I also would like to thank **Dragan**, **Stef**, **Patrick**, **Joris**, **Martijn** and **Matthias**. Especially when Giulia and I were the only girls in the group, I have always seen you as "big brothers". In fact, I want to express how grateful I am for all the times you shared your advice and suggestions with me. Thank you for making me feel welcome in your group.

Over time other amazing colleagues joined the physiology group. Dear **Toni**, **Ben**, **Michal**, **Ozan**, **Victor** and **Zarina** thank you for being such fun and wonderful colleagues to work with.

Dear **Luuk**, **Moedi** and **Jolijn**, you have contributed to creating a welcoming atmosphere for me in the department. I always enjoyed our coffee break chats and I had a lot of fun with you enjoying work trips and tasty meals.

My gratitude and affection go also to Nienke and Meike. The laughs and giggles coming out from your office filled our corridor with warmth and good vibes. Thank you for being such amazing colleagues and great friends. Dear **Nienke**, thank you for your good mood and extreme kindness. I am particularly grateful also for the time we spent together outside the office and for introducing me to PhD academy. It was a great honor to organize with you the PechaKucha night 2020 and be your co-host.

Dear **Meike**, I will never forget all those times that we worked in the same lab. One look was enough to tell each other that although we were working on different experiments, we were also sharing the same excitement and… frustrations! Thank you for our brainstorms, for taking over and helping me whenever I needed it, but especially for being a caring friend in the good and in the bad times.

Dear **Jens** and **Bram**, I had the time of my life sharing the office with you two for some time. It was fun seeing you two interacting and learning from you some Limburg words. Thank you for all the time you helped when I needed it.

I also would like to thank **Arina**, **Magdi**, **Paola** and the entire **CTH group**, for the precious feedback and constructive discussions we had during our lab meetings. Being able to share my work with such an amazing group was fundamental for me to explore different strategies and the interpretations of my findings. Together with others like **Stella**, **Elisabetta**, **Alicia**, **Isabella**, and many more, you are great scientists and wonderful people with whom I share nice and fun memories.

During my journey, I met many more extraordinary and high-quality scientists who became important figures for me during my journey. One of these is **Prof. Sandro Gelsomino**. Caro Sandro, desidero ringraziarti per la tua amicizia, per l'incoraggiamento che mi hai sempre dimostrato e per tutti i confronti costruttivi. Sono lieta che tu abbia fatto parte di questo mio percorso, grazie.

In February 2020 I had the opportunity to visit Leeds University to carry out part of my study. It was a critical moment as the COVID-19 pandemic had just spread out extensively in Italy and was about to do so in the UK and the Netherlands as well. Despite those events, I was lucky to visit Leeds and collaborate with Dr. Neil Turner and Chris Trevelyan. Dear **Neil**, thank you for your hospitality, guidance, and interest in my research. It has been a pleasure to work in your group.

Dear **Chris**, thank you for helping me to settle down in the lab. Thanks for introducing me to the Yorkshire tea & milk, that was a revelation!

Now I would like to thank the secretaries of the Biochemistry and Physiology department. A special group of women without whom I would have not gone too far in this journey.

Dear **Bianca** and **Vivian**, thank you so much for your support and your patience, especially during my first days in Maastricht.

Dear **Lidewij** and **Trees**, thank you for your precious help with all the administrative work during my journey I am particularly grateful for the sweet and pleasant chat we had any time I would stop by your office. Your door was always open for me, you are my true reference point at the Biochemistry department.

Finally, I would like to express my profound gratitude to **Jolanda**. Dear Jo, what to say that I have not told you already? On many occasions, you proved to me that you have some "magical powers". You made impossible things happen! Thank you so much for your support, professionalism, and for your dedication.

As of November 2021, I have joined another amazing group at the physiology department of Maastricht University. Therefore, I would like to express my gratitude to Dr. Ed Eringa. Dear **Ed**, thank you for the warm welcome in your group and for providing me with all the time I needed to finalize this thesis. I look forward to continuing the collaboration with you and Geertje!

Dear **Geertje**, thank you for all the support you have already given me during these months, I cannot wait to accomplish great things together.

The support that I have received over these years come also from my close friends outside academia.

Dear **Gabie**, **Felix**, **Sammy**, **Malu**, **Kahina** and **Massi** thank you for being my friends and family in Maastricht. I am grateful for all the laughs we had together and for all the times that you patiently heard my frustrations and shared with me advice and suggestions. I could not imagine my journey without you guys.

Cara **Roberta**, non ci sono parole per esprimere l'affetto che provo per te. Grazie per i consigli, le risate, i nostri gossip, lo shopping, lo yoga e le nostre cenette.

Dear **Delia** and **Tate**, I feel lucky to have you guys in my life. Our spinning sessions, nights out, your kindness and friendship have been priceless to me.

A special thanks goes also to my closest Italian friends who, although the distance that separated us, were always by my side.

Caro Ndri (**Andrea**), grazie per le nostre lunghe telefonate e per esserci sempre quando ho bisogno di te.

Cara Caprapall (**Roberta**), grazie per sopportarmi anche dopo tutto questo tempo e grazie per aver accettato di disegnare la copertina di questa tesi.

Caro Uccio (**Domenico**), grazie per esserci stato durante le prime fasi di questo percorso. Il mio primo colloquio l'ho fatto a casa tua. Ricordo che eri così in apprensione per me, che per scaricare l'ansia durante quella mezz'ora di colloquio, hai puli-

to tutta casa da capo a fondo. Grazie anche per l'aiuto che mi hai "mandato" quelle volte in cui tutto sembrava andare storto, ha funzionato!

Cara Cri (**Cristina**), grazie per i tuoi consigli e per avermi indicato la strada che mi ha portata a scegliere Maastricht e questo progetto. Auguro a te, Tiago e Sofia tutto il meglio e non vedo l'ora di rivedervi presto.

Finally, I would like to express my gratitude, love, and affection to my family. Dear **Luc**, thank you for inspiring me. I am blessed to have you in my life and be able to count on your support and extreme patience. I will always look up to you as I truly believe you are a great example of intelligence and dedication to what you love.

Un caro ringraziamento va anche a tutti i miei **zii e cugini**, e a quella forza della natura che è la mia nonnina. **Nonna**, "Elisa ne tien un' e nun la vol' da' a nisciun".

Infine, voglio ringraziare voi, **Mamma e Papà**. Il mio amore per voi è infinito. Siete la forza motrice di ogni mia azione e la ragione di ogni mio successo.

Dankwoord van Billy Scaf

Inmiddels is het bijna 6 jaar geleden dat ik aan dit PhD-avontuur begon. Ik heb het ook echt als een avontuur ervaren, omdat ik vanaf het eerste moment de uitdaging heb gevoeld, onzekerheden heb gekend en die (vaak) heb overwonnen, bijzondere en soms ongemakkelijke situaties heb meegemaakt, en bovenal omdat ik zeer veel heb geleerd. En leren doe je niet alleen. Ik heb van veel mensen geleerd. Niet alleen door kennisoverdracht, maar ook door de vele adviezen en zienswijzen die ik van veel mensen heb meegekregen, of heb opgepikt.

Meer dan bewust ben ik mij van het feit dat een groot aantal personen heeft bijgedragen aan het bereiken van deze persoonlijke mijlpaal. Daarom wil ik jullie oprecht bedanken voor alle steun en vertrouwen.

Prof. dr. Schotten, beste **Uli**, ik ben ontzettend dankbaar dat ik deel uit mocht maken van uw AF-groep en uw vakgroep, Fysiologie. Ondanks uw zeer drukke agenda, vol met reizen en vergaderingen, wist u toch altijd tijd vrij te houden voor onze vaste meetings. Tijdens deze meetings konden wij ons hart op vele vlakken luchten. Van praktische, experimentele struggles en (soms zeer) verrassende data, tot bijzondere situaties op inter-menselijk vlak. Door uw oplossingsgerichtheid liepen wij altijd weer met een plan én met vertrouwen naar buiten. Ook uw uitspraak "never worry about data" is nog vaak door mijn hoofd geschoten en gelukkig heb ik die ook weten toe te passen. Het feit dat hier nu een gedeelde thesis ligt, met de input van vijf officiële begeleiders, is voor een groot deel aan u te danken, omdat u de juiste personen bij elkaar wist te brengen en te houden. Bedankt daarvoor.

Dr. Verheule, beste **Sander**, op je eigen unieke manier ben je een zeer belangrijke mentor voor mij geweest. Het feit dat je veel op de afdeling aanwezig was, mee ging lunchen en altijd tijd vrijmaakte als ik aan je deur stond was zeer prettig. Ook jouw (opbouwende) kritiek met een vleugje humor heeft mij veel geleerd op het gebied van het maken van posters, presentaties en het schrijven van stukken. Daarnaast ben ik je dankbaar voor de begeleiding en hulp bij de dierexperimenten en de vele weefselverzamelingen en analyses daarvan. Ook aan de geduldige uitleg bij de AF muisexperimenten (helaas geen onderdeel van deze thesis) en de perfect uitgevoerde koprol in Amsterdam, zonder ook maar een kabel te beschadigen, heb ik leuke herinneringen. Het willen opvoeden van een jonge kraai(achtige) was een idee van jou, maar inmiddels een doel van mij. Mocht het ooit zo ver komen, laat ik het je zeker weten!

Dr. Spronk, beste **Henri**, ik bewonder jouw creatieve manier van denken. Bij complexe vraagstukken, of andere onderzoeksgerelateerde onverklaarbaarheden, wist jij vaak met nieuwe ideeën te komen. Vooral toen duidelijk werd dat een groot deel van de bestaande coagulatie-assays niet bruikbaar bleek voor geitenbloed

hebben we veel aan jouw creatieve input gehad. Ook jouw directe en soms wat ongebruikelijke manier van grappen is iets wat me zal bijblijven. Zeker op PhD-cursussen of symposia, zoals het jaarlijkse NVTH in Koudekerke, waar nog niet iedere nieuwe PhD-student jou kende, leverde dat vaak bijzondere reacties op (haha)! Bedankt voor de leuke en leerzame momenten.

Prof. dr. ten Cate, beste **Hugo**, ondanks dat ik niet bij jullie op de Biochemie-afdeling gevestigd was voelde ik me er wel heel erg thuis. Vooral de wijze waarop u mij betrok bij uw onderzoeksgroep maakte dat ik me er helemaal onderdeel van voelde (ondanks onze ietwat afwijkende "geitenpraatjes"). U bent ook echt een voorbeeld van iemand waar de deur altijd openstaat. Steeds als we ergens vastliepen konden we bij uw kantoor aankloppen. Het feit dat u standaard rekening hield met ons een- of tweewekelijkse 'CVON-overleg' en de kalmte die u daarin uitstraalde, maar ook de welwillendheid om overal over mee te denken, heb ik als zeer prettig ervaren. Bedankt!

Dr. van Nieuwenhoven, beste **Frans**, jij bent als laatste van de begeleiders aangesloten in ons supervisieteam. Vanaf het moment dat je erbij kwam voelde ik meteen de meerwaarde. Over ieder onderwerp dacht je mee en je deed ook echt huiswerk om ons te kunnen begeleiden bij onze vraagstukken. Uiteindelijk ben je belangrijk geweest voor een groot deel van de hoofdstukken in deze thesis. Jij was altijd aanwezig op de afdeling en bereikbaar voor vragen en overleg. In de laatste fase kwamen we steeds regelmatiger met elkaar samen en ik heb die samenwerking altijd heel fijn gevonden. Bedankt!

Dear prof. dr. **Hackeng**, prof. dr. **Götte**, prof. dr. **Vernooy** and prof. dr. **Wenzel**, I would like to thank you, as Assessment Committee, for your willingness to critically read and review this thesis.

(Dr.) D'Alessandro, dear **Elisa**, who could have imagined in January 2017 that we would finish our PhD together, including a shared thesis and a combined defense. I do not think that I should spend too much words on thanking you, because everyone who goes through this thesis can clearly imagine how well we collaborated and how well we can get along. Nevertheless, many people will say that we fought a lot (which in fact is true). However, if you ask my friends or family, they will not describe me as someone that likes to fight. Therefore, I believe that it was just our way of debating, stress relief, playing and part of our communication. And I admit, that is unique. So, thank you for that and for the nice years of working together.

Dr. van Hunnik, beste **Arne**, zonder jou was veel in de soep gelopen en had ik veel minder geleerd. Jij was mijn onofficiële dagelijkse begeleider. Het feit dat jij al zo lang bij deze onderzoeksgroep betrokken bent en verschillende rollen vervult, maakt jou de ultieme vraagbaak, sparpartner en raadgever. Ik kan tal van voorbeelden noemen waarvoor ik weer eens met de handen in het haar aan jouw deur

stond. Altijd nam jij de tijd om mee te denken naar een oplossing, of om een een-op-een college te geven. Naast je onderzoeksinhoudelijke competenties was je ook een gezellige toevoeging aan de dagelijkse werkzaamheden. We hadden aardig wat raakvlakken. Variërend van schoenenkeuze, muzieksmaak en een fietshobby, tot dierenliefde. Bedankt voor al je input, geduld en aanwezigheid!

Dr. Opacic, dear **Dragan**, Elisa and I continued where you left. Especially during the first year of our PhD you were a great mentor and of major importance to us. I felt like a real freshman, but your encouraging words, and guidance in how to deal with the many facets of doing a PhD and large experimental work, made that we had a flying start. I honestly acknowledge your contributions to chapter 5 of this thesis. Unfortunately, since your own defense, we did not see each other anymore, but I will always remember you. Thanks!

Beste **Marion**, jij was onmisbaar bij alles wat te maken had met ons geitenwerk. Jij runde de OK en had altijd alles netjes voorbereid. Die zekerheidsfactor bracht veel rust op de toch al hectische OK-dagen. Vele uren hebben we samen doorgebracht in die blauwe kostuums. Het waren lange dagen: beginnend met meerdere bloedafnames, dan een lang OK-experiment en soms nog een cardioversie-experiment erachteraan. Met het foute uur van Qmusic op de OK was het trouwens wel gezellig! Ook ben ik je zeer dankbaar voor het steeds willen helpen bij het voorbereiden van de vele cupjes, potjes, buisjes en ontelbare labels. Bedankt!

Dear **Vladimir**, I really enjoyed working with you. Together we performed many animal experiments and especially during your last two years you helped us a lot. Thanks for that! I really have to laugh when I think back at the time that I brought the skates to the lab. You enjoyed it so much, you even did the "swan-lake" pose while skating through the lab. And yes, I still have the videos! Even though you are in Bordeaux now, I know we will stay in touch.

Beste **Joris**, je bent net iets eerder begonnen en ook net wat eerder dan klaar dan ik. We hebben veel samengewerkt op het gebied van histologie en ik ben dan ook trots dat ik onderdeel ben van jouw paradepaardje 'JavaCyte'. Ook hebben we leuke momenten gehad in o.a. Papendal, San Francisco en Kopenhagen. Bedankt!

Beste **Stef**, **Ben**, **Matthias** en **Martijn**, bedankt voor de fijne momenten binnen de AF-groep. Het waren altijd leuke en interessante gesprekken die we voerden en dat ging lang niet altijd over werk. Van ieder van jullie heb ik anekdotes in mijn hoofd die ik niet zal vergeten.

Beste **Nienke**, **Meike** en **Chantal**, bedankt voor de leuke tijd op de afdeling. Het was altijd weer gezellig met jullie als iemand op iets lekkers trakteerde in de koffiekamer omdat die jarig was!

Dear **Giulia**, **Patrick**, **Toni**, **Victor**, **Aaron**, **Ozan** and **Michal**, thank you all for being pleasant colleagues. Unfortunately, due to different circumstances, I

could not get to know you all equally well. But the moments that we spent together were nice and I enjoyed it a lot. Take care!

Beste **Vivian** en **Bianca**, jullie runden de afdeling tijdens mijn eerste jaren bij Fysiologie. Jullie waren altijd vrolijk en dat heb ik steeds als zeer prettig ervaren. Ik vond het dan ook jammer dat jullie weggingen.

Beste **Jolanda**, wat moet de afdeling toch zonder jou. En wat moest ik zonder jou. De laatste jaren was jij onze steun en toeverlaat en voor alles had jij een oplossing, of vond jij een oplossing. Heel erg bedankt voor de support bij tal van zaken. Ik waardeer dat enorm!

Beste **Luuk**, mijn kamergenoot van San Francisco 2019. Daar heb ik echt hele leuke herinneringen aan. Vooral de fietstocht over de Golden Gate Bridge, door het Golden Gate park met Moby op de speakers, langs de hippiewijk en het uiteindelijk spenderen van de fietshuurkosten in een outdoor winkel waren memorabel. Succes met jouw laatste loodjes!

Beste **Moedi**, toen jij op de afdeling kwam was ik al langzaam aan het afronden. Ondanks dat hebben we toch nog leuke momenten samen gehad. Eerder dit jaar zaten we nog samen in de zaal van het eerste echte grote congres sinds de COVID-perikelen, EHRA in Kopenhagen. Erg gezellig!

Beste **Bram** (Brem) en **Jens** (Jinz), de mede PhD-studenten uit de groep van Hugo. We hebben veel samen gelachen en vaak had ik niet eens door waarom. We hebben leuke tripjes gehad naar Koblenz en Koudekerke. Ik denk nog graag terug aan het blackjacken en de stoeimomenten op de gang van het hotel tijdens NVTH.

Beste **René**, **Simone**, **Stefanie**, **Diane**, **Marcel**, **Magdi** en **Patricia**, bedankt voor jullie warme welkom en support als wij steeds weer nieuwe buisjes geitenbloed kwamen afdraaien. De ontelbare cryos geitenplasma zullen nog vele jaren voortleven!

Beste **Arina** en **Paola**, bedankt voor jullie betrokkenheid tijdens de dinsdagochtend meetings als wij weer eens een geitenpraatje hielden.

Beste **Leon**, bij jou had ik mijn eerste UM en biochemie-ervaring. Al in 2013 liep ik stage bij jou en vanaf de eerste dag voelde ik me daar thuis. Je betrok mij als stagiair meteen bij je toenmalige PhD-studenten, Dennis, Brecht en Martijn en nodigde ons bij je thuis uit om microscoopglaasjes te gaan schrijven onder het genot van pizza en bier. Ook **Rick** en **Armand** wil ik bedanken. Niet alleen voor de gezellige Star Wars-avonden, maar ook voor de deur die altijd openstond voor advies of een minder serieus gesprek.

Beste **Peter**, in 2013 keek ik als stagiair al bij jou over je schouder mee hoe jij aortaboogjes van muizen in een mum van tijd wist te prepareren. Een waar vakman. Het heeft tot het laatste 1,5 jaar van mijn PhD moeten duren voordat ik zelf (AF-) muisexperimenten ging doen (helaas geen onderdeel van dit boekje). Die keren dat we samen op het lab stonden heb ik als zeer prettig ervaren.

Beste **Trees** en **Lidewij** ook jullie wil ik bedanken voor de fijne tijd bij biochemie. Al was ik niet dagelijks aanwezig op de afdeling, toch gaven jullie mij het gevoel dat ik erbij hoorde. Ook **Stella** wil ik bedanken. Jij bent altijd gezellig en vrolijk. Het was altijd leuk om jou weer even tegen te komen.

Beste **Guillaume**, dankzij u heb ik vanuit Wageningen een ingang in Maastricht gevonden. Wetende dat u "met muizen" werkte, en het feit dat ik naarstig op zoek was naar een stageplek op biomedisch gebied, deed mij in 2013 doen besluiten contact met u op te nemen. Daar heb ik tot de dag van vandaag geen spijt van. Ik kende u natuurlijk al vanuit mijn jonge jeugd toen mijn zus en uw dochter goede vriendinnen waren op de middelbare school, maar onze echte connectie is de voetbalclub. U als betrokken clubicoon van vv Berg '28 en ik als speler. Wekelijks treffen wij elkaar en, nog voordat ik het kleedlokaal in ga, spreken we even over de wedstrijd én over de uni. Bedankt.

Beste **Huub**, **Petra**, **Rachel** en **Inger**, ik wil jullie echt heel erg bedanken voor het zorgdragen voor de geiten. Ik heb altijd zeer prettig met jullie gewerkt en ik voelde dat de dieren in goede handen waren bij jullie. Ook bedankt voor jullie flexibiliteit als er opeens nog even bloed moest worden genomen, een kabelhok gereed moest worden gemaakt, een kar moest worden afgespoten, de kussens konden worden opgeruimd en nog vele andere handelingen!

Beste **Richard** en **Saskia**, bedankt voor de prettige samenwerking rond de geitexperimenten. Tijdens de piekmomenten hadden we zeer regelmatig contact over de dieren, zelfs in de avond of in het weekend. Dit verliep in mijn ogen altijd erg prettig. Bedankt daarvoor.

Beste **Harry**, **Isabelle**, **Michiel**, **Anton-Jan**, **Hetty**, **Sophie**, **Monika** en alle anderen van het RACE-V consortium, bedankt voor de interessante, leerzame en gezellige bijeenkomsten. RACE-V voelde als een hechte club en ik ben zeer trots dat ik er onderdeel van ben.

Een dank voor de steun gaat ook uit naar de leden en teamgenoten van de clubs waar ik meermaals per week te vinden was. Eerst **SV Vilt**, de club waar ik sinds 1994 speelde en die tijdens mijn eerste jaar als PhD-student, in 2017, helaas werd opgedoekt. En daarna **vv Berg '28**. De club die mij warm ontving en waar ik gedurende mijn hele PhD-periode, en nog steeds, trouw voor heb gestreden.

Ook wil ik mijn nieuwe werkplek, **CTCM**, en dan met name de **PQS'ers** bedanken voor het warme welkom, de fijne sfeer en de steun tijdens de afronding van dit boekje en de weg naar de verdediging.

Dennis, **Enrico** en **Nick**, mijn matties sinds de pubertijd (waar we trouwens nog steeds in lijken te zitten). Uiteraard moet ik jullie bedanken. Volgens mij hebben jullie nooit echt begrepen wat mijn PhD inhield, maar dat hoeft ook niet. Bij jullie kan ik namelijk ontspannen en hebben we het over hele andere dingen. Dat we nog maar veel lol mogen trappen samen!

Jullie zijn trouwens onderdeel van een groter vriendencollectief, maar dat wordt te veel om hier te bedanken. Vandaar dat ik de twee belangrijkste groepsapps wil benoemen: 'de normale nette vrienden' (A.K.A. 'Berg oost front', of 'Tuuteduukers') en 'de Pratsjpikken'. Bedankt!

Gaston, **Wolbert**, **Tom**, **Koen** en **Aerjen**, mijn Toegepaste Biologie-buddy's. Inmiddels ken ik jullie al 14 jaar en in die tijd zijn we een heel hecht groepje geworden. Ondanks dat we kriskras door het land wonen, zien we elkaar regelmatig en dat voelt steeds weer als thuiskomen. Ik wil jullie bedanken voor jullie steun, vertrouwen en groene avonturen.

Lieve zussen, **Gwenny** en **Curly**, en uiteraard ook **Raoul**, **Ronald**, **Sammie**, **Juul** en **??**, niets geeft zo'n goed gevoel als een fijne familie te hebben. We zien elkaar heus niet wekelijks, maar we weten wel wat we aan elkaar hebben. Als we elkaar zien is er altijd ruimte voor een luisterend oor en een lachend gezicht. Bedankt voor jullie steun. Maar ook bedankt voor jullie goede voorbeeld van werklust en discipline.

Mijn schoonouders, **Piet** en **Anka**, en jullie partners, ook jullie wil ik bedanken voor de steun de afgelopen jaren. Vaak genoeg ben ik bij jullie aan tafel geschoven na een lange werkdag. Dat ik steeds zo welkom was (ben), waardeer ik zeer.

Al mijn lieve **tantes** en **ooms**, heel erg bedankt voor jullie steun en de warmte die jullie steeds weer uitstralen.

Lieve mam en pap, **Romy** en **John**, als ik iemand moet bedanken voor waar ik nu sta, zijn jullie dat wel. Niet alleen voor de laatste paar jaar, maar voor altijd al. Het maakte jullie niet uit welke richting ik uitging, als ik er maar mijn best voor deed. En met jullie voor ogen kon ik dat steeds weer opbrengen. Jullie hebben mij ontzettend veel gesteund en geleerd, op welk gebied dan ook. Daar ben ik jullie echt heel erg dankbaar voor!!

Lieve **Sophie**, als laatste ben jij aan de beurt. Jou wil ik bedanken voor de fijne tijd die wij samen hebben en voor alle support die jij me door de jaren heen hebt gegeven om dit succesvol te kunnen afronden. Thuis hebben we veel verantwoordelijkheden met al die twee- en viervoeters (op het moment van schrijven 16 dieren, oftewel 50 voeten). Maar telkens als ik weer eens langer op het werk moest blijven, of als ik 's avonds flink moest doortrekken om te typen, dan kon ik met een gerust hart op jou terugvallen. Jij bent de beste dierenmoeder die ik ken! Bedankt x.