Portofoliu de 10 lucrări științifice

Prof. dr. Alina SCRIDON

1. **Scridon A**, Balan AI. *Challenges of anticoagulant therapy in atrial fibrillation. Focus on gastrointestinal bleeding.* Int J Mol Sci 2023; 24(8):6879. doi: 10.3390/ijms24086879. FI 2022: 5,600 (Q1).
2. **Scridon A**. *Autonomic imbalance and atrial ectopic activity – a physiological and clinical view*. Front Physiol 2022; 13:1058427. doi: 10.3389/fphys.2022.1058427. FI: 4,000 (Q2).
3. **Scridon A**, Somkereki C, Nicoară TR, Oprica M, Demian L. *Neutrophil gelatinase-associated lipocalin monitoring reveals persistent subclinical kidney injury following intraarterial administration of iodinated contrast agents*. Sci Rep 2022; 12:19464. doi 10.1038/s41598-022-24169-7. FI: 4,600 (Q2).
4. **Scridon A**. *Platelets and their role in hemostasis and thrombosis – from physiology to pathophysiology and therapeutic implications*. Int J Mol Sci 2022; 23(21): 12772. doi 10.3390/ijms232112772. FI: 5,600 (Q1).
5. **Scridon A**, Balan AI. *Targeting myocardial fibrosis – a magic pill in cardiovascular medicine?* Pharmaceutics 2022; 14(8):1599. doi: 10.3390/pharmaceutics14081599. FI: 5,400 (Q1).
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International Journal of
Molecular Sciences

MDPI

Review

Challenges of Anticoagulant Therapy in Atrial Fibrillation—Focus on Gastrointestinal Bleeding

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Abstract: The rising prevalence and the complexity of atrial fibrillation (AF) pose major clinical challenges. Stroke prevention is accompanied by non-negligible risks, making anticoagulant treatment an ongoing challenge for the clinician. Current guidelines recommend direct oral anticoagulants (DOACs) over warfarin for stroke prevention in most AF patients, mainly due to the ease of their use. However, assessing the bleeding risk in patients receiving oral anticoagulants remains—particularly in the case of DOACs—highly challenging. Using dose-adjusted warfarin increases threefold the risk of gastrointestinal bleeding (GIB). Although the overall bleeding risk appears to be lower, the use of DOACs has been associated with an increased risk of GIB compared to warfarin. Accurate bleeding (including GIB-specific) risk scores specific for DOACs remain to be developed. Until then, the assessment of bleeding risk factors remains the only available tool, although the extent to which each of these factors contributes to the risk of bleeding is unknown. In this paper, we aim to provide a comprehensive review of the bleeding risk associated with oral anticoagulant therapy in AF patients, with a highlight on the latest insights into GIB associated with oral anticoagulation; we emphasize questions that remain to be answered; and we identify hotspots for future research.

Keywords: atrial fibrillation; direct oral anticoagulants; gastrointestinal bleeding; thrombosis; vitamin K antagonists

Citation: Scridon, A.; Balan, A.I. Challenges of Anticoagulant Therapy in Atrial Fibrillation—Focus on Gastrointestinal Bleeding. *Int. J. Mol. Sci.* **2023**, *24*, 6879. https://doi.org/10.3390/ijms24086879

Academic Editor: Isabella Russo

Received: 11 March 2023
Revised: 2 April 2023
Accepted: 4 April 2023
Published: 7 April 2023

1. Introduction

With the continuous aging of the population and the increasing survival of patients with chronic cardiovascular diseases, atrial fibrillation (AF) has become the most common sustained cardiac arrhythmia encountered in clinical practice, affecting more than 43 million people worldwide [1]. Regardless of the presence of symptoms and the duration of the arrhythmia, the vast majority of AF patients require lifelong oral anticoagulation to prevent ischemic stroke. Although highly efficient, oral anticoagulation brings with it an increased risk of bleeding, particularly in certain categories of patients [1]. In this paper, we aim to provide a comprehensive review of the bleeding risk associated with oral anticoagulant therapy in AF, with a highlight on the latest insights into gastrointestinal bleeding (GIB) associated with oral anticoagulation.

2. Atrial Fibrillation—A Global Epidemic

The prevalence of AF has consistently increased over the past decades, positioning this arrhythmia as the most common sustained cardiac arrhythmia encountered in clinical practice [1]. Although the global prevalence of AF is estimated to be <1%, it increases to 3.7–4.2% in people aged 60–70 years, and to 10–17% in people aged ≥80 years [2,3]. Despite the major advancements in cardiovascular prevention strategies, AF prevalence is estimated to continue to increase in the following decades and to reach 10 million cases

in the United States [4], 17 million in Europe [5], and up to 72 million in Asia [6] by 2050. Given the high morbidity, mortality, and considerable health care costs [6] associated with AF, this perspective represents a worrisome global epidemiological problem.

3. Ischemic Stroke—The Hidden Enemy of the Atrial Fibrillation Patient

One of the main concerns in AF patients is their increased risk of systemic and mainly cerebral embolism. This risk is present regardless of whether the arrhythmia is paroxysmal, persistent, or permanent [7]. In addition, patients with AF-related strokes have worse outcomes and more serious disability than those with strokes that are not related to AF [8].

All three components of Virchow's triad (i.e., endothelial dysfunction, abnormal blood stasis, and altered hemostasis) contribute to intracardiac thrombus formation in AF patients, with hypercoagulant status being seen as the main culprit for the increased risk of stroke in this population [9]. Vascular and structural heart disease are compatible with Virchow's criterion for endocardial dysfunction, blood stagnation in the left atrium meets the criterion for blood stasis, and abnormal blood constituents (e.g., abnormal procoagulant platelet factors) are compatible with the abnormal coagulation and fibrinolysis criterion [9,10].

Many of the factors that contribute to thrombus formation in the fibrillating atria are well-established stroke risk factors, even in the absence of AF (Figure 1) [9,11,12]. Although AF has been associated with stroke even after adjustment for shared risk factors [9,11,12], there is no direct evidence that AF alone is sufficient to induce the occurrence of stroke. In fact, in AF patients that lack additional risk factors, the adjusted stroke rate appears to be essentially negligible [13]. This highlights the critical role of coexisting risk factors in stroke occurrence in the AF patient.

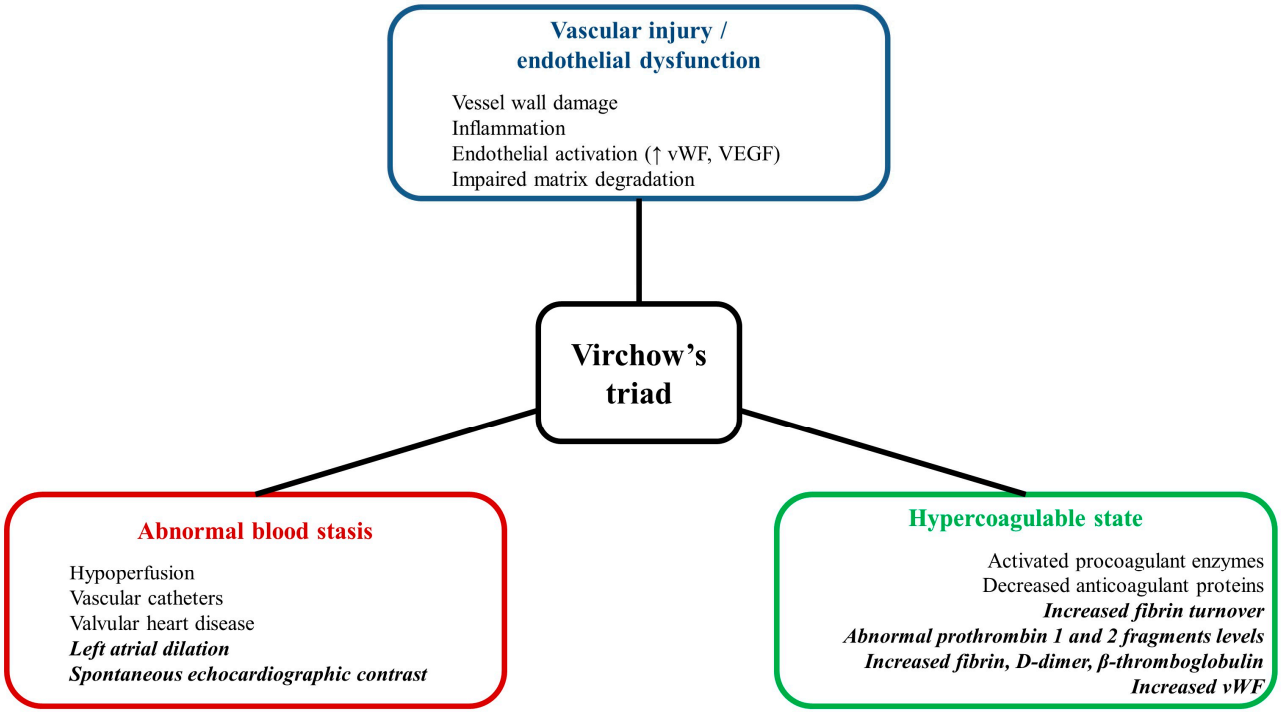

Figure 1. Virchow's criteria as risk factors for ischemic stroke in the presence and absence of atrial fibrillation. Factors specific for the presence of atrial fibrillation are written in bold font. VEGF—vascular endothelial growth factor; vWF—von Willebrand factor. ↑ indicates an increase in biomarkers levels.

Indeed, several scores that incorporate various combinations of risk factors have been developed to stratify the risk of stroke in AF patients. Among these, the CHA_2DS_2-VASc score is currently the most widely used in clinical practice. More recently, the Age, Biomarkers, Clinical history (ABC)-stroke score, which includes age, cardiovascular biomarkers (i.e., NT-proBNP and high-sensitivity cardiac troponin T), and clinical history (i.e., prior stroke/transient ischemic attack), has been shown to outperform the CHA_2DS_2-VASc score in its ability to predict stroke in patients with AF and Congestive heart failure, Hypertension, Age $\geq$ 75 years, Diabetes mellitus, Stroke (doubled) ($CHADS_2$) score $\geq$2 [14]. The GARFIELD-AF tool, which simultaneously calculates the risks of death, stroke, and bleeding, has also been shown to accurately predict stroke even in low-risk patients [15].

4. Stroke Prevention in Atrial Fibrillation

Despite the development of increasingly efficient arrhythmia detection strategies and of friendlier stroke prophylaxis regimens, the incidence of stroke remains high among AF patients, particularly if multiple risk factors are present [7]. Changes in guideline recommendations and the availability of newer, 'friendlier' drugs have led to a substantial increase in the proportion of AF patients that receive lifelong oral anticoagulation [16,17]. Throughout time, pharmacological strategies to prevent stroke in AF included antiplatelets, vitamin K antagonists (VKAs), and, over the past decade, direct oral anticoagulants (DOACs).

4.1. Antiplatelet Therapy—Less Effective than Oral Anticoagulation, but Just as 'Bloody'

Due to its potent antithrombotic effects, aspirin, the most widely used antiplatelet agent, was an obvious candidate for stroke prevention in AF. Compared with placebo, aspirin therapy was associated with ≈20% reduction in the relative risk of stroke [18], and adding clopidogrel to aspirin further reduced this risk in patients with AF [19]. However, in large clinical trials, aspirin and even dual antiplatelet therapy were clearly outperformed by warfarin in their ability to prevent ischemic stroke [20,21]. Moreover, both single and dual antiplatelet therapies were associated with similar bleeding risk to that associated with warfarin [20,21]. The accumulating data thus placed antiplatelet agents as not only less efficient (by ≈40%) [22], but also as similarly hazardous as warfarin, leading to rapid and complete withdrawal of antiplatelet drugs as stroke prevention strategies in AF [23,24].

4.2. Warfarin—The 'Blessed Poison'

With its ability to reduce the risk of stroke by more than two-thirds and mortality by one-quarter compared with control (aspirin or no therapy), warfarin is one of the most efficient prophylactic strategies that medicine has ever known [22]. Due to its wide availability, low cost, and easy-to-administer antidote, warfarin is widely used for stroke prevention in AF patients [22] and is the preferred oral anticoagulant in patients with mitral stenosis, valve replacement, or advanced renal disease [23]. However, its complex pharmacokinetics makes warfarin the target of numerous interactions. The manufacturer itself provides a list of over 200 drugs that can interfere with the anticoagulant effect of warfarin, and the list of food interactions is almost as generous [25]. Genetic polymorphisms, particularly in the CYP2C9 and VKORC1 genes, can also significantly affect the response of AF patients to warfarin therapy [26,27]. These interactions are even more important considering that the VKAs have narrow therapeutic window. Together, these factors make VKA anticoagulation extremely capricious and 'sentence' the AF patient to an endless stream of laboratory monitoring and dose adjustments. Meanwhile, the most optimistic statistics show that, even with extremely rigorous monitoring, patients receiving VKAs are adequately anticoagulated less than 70% of the time [28].

4.3. Direct Oral Anticoagulants—The 'New Kids on the Block'

The numerous challenges associated with warfarin usage have emboldened researchers and pharmaceutical companies in their search for newer, 'friendlier' oral anticoagulants. To be considered 'ideal', an anticoagulant will have to be as effective as or even more effective than warfarin, and as safe as or even safer than warfarin. It will have to be available for oral administration, in fixed doses, have few food and drugs interactions, and have rapid onset and offset of action. It should have a predictable effect, without the need for monitoring, and it should have a widely available and safe antidote [29].

After more than 50 years of VKA monopoly, two classes of direct, non-VKA oral anticoagulants—direct thrombin inhibitors and direct factor Xa inhibitors—have been approved for stroke prevention in AF [23]. These new drugs are easier to administer, for both the doctor and the patient, and, due to their more stable pharmacokinetic and pharmacodynamic properties, shorter half-lives, fewer food and drug interactions, and larger therapeutic windows, they do not require routine monitoring or dose adjustments [29]. Large clinical trials have shown these agents to be non-inferior to warfarin in ischemic stroke prevention and to associate lower risk of fatal and intracranial hemorrhage than warfarin [30], which placed them as the preferred oral anticoagulants in the vast majority of AF patients [23,30]. Because of numerous reasons, however, the evaluation of anticoagulation status, at least in selected categories of DOAC-treated patients, is not entirely futile.

Dabigatran plasma levels have been shown to vary considerably with age, sex, bodyweight, kidney function [31], and, at least in experimental settings, with plasma lipid levels [32]. In turn, these varying levels have been shown to significantly affect both the thrombotic and the hemorrhagic risk of dabigatran-treated patients [31]. Important variations have also been reported for the direct factor Xa inhibitors, even in healthy individuals, although the clinical impact of such variations remains unknown to date [33,34]. Although DOACs have fewer interactions than the VKAs, they are also not free from interactions. The CYP3A4 enzyme, involved in factor Xa inhibitors metabolism, interacts with more than half of the commercially available drugs [35]. Many other drugs interfere with the P-glycoprotein, one of the main modulators of DOACs transport [36]. The implications of these interactions are extremely important considering that these proteins are the substrate of many cardioactive drugs that are widely used in AF patients, including digoxin, calcium channel blockers, and antiarrhythmic drugs [29]. The European Society of Cardiology has already issued a number of recommendations regarding such drug associations, and, for many other combinations, data are not yet available [36]. The anticoagulant effect of DOACs is also not immune to genetic variability. Polymorphisms in the CES1 gene have been shown to increase dabigatran plasma levels and to affect bleeding risk in these patients [37]. Genetic variations have also been shown to influence factor Xa inhibitors levels, although the polymorphisms identified to date do not appear to exhibit significant clinical impact [38,39].

5. Good Things Come with a Cost—Bleeding Risk in the Anticoagulated Atrial Fibrillation Patient

In the AF patient, oral anticoagulation is the sole therapy with proven benefits on survival [30,40]. Unfortunately, this benefit does not come without a cost. Altered sense of taste and gastrointestinal side effects (nausea, vomiting, abdominal pain, bloating, and flatulence) are not uncommon in patients undergoing warfarin therapy [41]. In certain patients, skin necrosis, purple toe syndrome, osteoporosis, calciphylaxis, and valvular and vascular calcification can also occur [41]. However, the single most common warfarin side effect is bleeding. When VKA anticoagulation is carefully monitored, the risk of major bleeding increased by ≈0.3% per year (from 1.0% in control to 1.3% in warfarin-treated patients), with an increase in the risk of intracranial hemorrhage by ≈0.2% per year (from 0.1% in control to 0.3% in patients treated with warfarin) [42].

All patients should therefore undergo thorough evaluation of their bleeding risk prior to and during oral anticoagulant therapy [23]. Numerous factors have been shown to increase the risk of bleeding in patients receiving anticoagulants, and several bleeding risk scores that incorporate various combinations of those factors (Table 1) have been developed in the attempt to reduce bleeding in this population. One of the most widely used bleeding risk scores is currently the HAS-BLED score [43,44]. Other scores, such as ABC, HEMORR$_2$HAGES, or ATRIA, although have high specificity, have been shown to have only modest sensitivity [14]. Whereas certain factors (e.g., labile International Normalized Ratio [INR], anemia, malignancy, genetic background) are specific for different risk scores, others, such as advanced age, hypertension, or history of bleeding, are Incorporated in the vast majority of them [43,44]. Other factors, such as congestive heart failure or diabetes mellitus, although not generally included in the risk scores, have also been related to increased bleeding risk in different clinical studies [45]. In patients undergoing VKA therapy, the quality of anticoagulation, with a time in therapeutic range (TTR) > 70%, is regarded as a major player in the thrombotic/hemorrhagic risk balance [23,45]. Both an INR outside the recommended range and a TTR < 70% have been shown to increase the risk of bleeding, an INR > 3 being associated with a doubling in the incidence of major bleeding [45].

Table 1. Most common bleeding risk scores used in clinical practice for hemorrhagic risk assessment in patients with atrial fibrillation undergoing oral anticoagulation.

	Bleeding Risk Scores			
	HAS-BLED	**ABC**	**HEMORR$_2$HAGES**	**ATRIA**
Age	>65 years	Age	>75 years	≥75 years
Biomarkers	Labile INR	Hemoglobin Troponin T Growth differentiation factor-15	Reduced platelet count or altered platelet function Anemia	Anemia
Clinical history	Hypertension Abnormal kidney and liver function Stroke Bleeding Drugs Alcohol	History of bleeding	Liver or kidney disease Alcohol abuse Malignancy Bleeding Genetic factors Excessive fall risk Stroke	Bleeding Severe renal disease Hypertension
Maximum score	**9 points**	**>15%**	**12 points**	**10 points**

INR—International Normalized Ratio.

In their landmark clinical trials, most of the novel, non-VKA oral anticoagulants have been associated with lower risk of major bleeding, and all have shown significantly lower risk of intracranial hemorrhage compared to warfarin [46–49]. In a meta-analysis combining all four clinical trials, the use of DOACs was associated with similar rates of major bleeding, but with a significant, more than 50% reduction in the risk of intracranial hemorrhage compared with warfarin [30]. This does not mean, however, that DOACs are free of hemorrhagic risk. Despite their rapid onset and offset of action, more predictable pharmacodynamics, and fewer food–drug and drug–drug interactions, DOACs still associate a significant risk of major, including potentially life-threatening bleeding [50]. In different studies, fatal bleeding rates associated with the use of DOACs varied from 0.06% to 0.30%, while the rates of major bleeding ranged from 1.1% to 4.0% [50,51]. Data from the clinical trials and from real-life observational studies suggest that between-drug differences could also exist in regard to their safety. Whereas apixaban and low-dose (i.e., 110 mg bid) dabigatran appeared to reduce the risk of major bleeding compared to warfarin, this was not the case for rivaroxaban, nor for the high (i.e., 150 mg bid) dose of dabigatran [30,52]. However, given that no randomized clinical trial has performed so far a head-to-head com-

parison of the different DOACs, these data should not be used as a criterion for choosing between them.

All DOACs show, however, considerably better safety profiles than the VKAs, although there are certain considerations that temper the enthusiasm. In line with the suboptimal TTR achieved in the DOAC trials [30], bleeding rates in the warfarin groups were higher in those trials than those reported in the previous (warfarin vs. aspirin) trials [20]. The cutoff creatinine clearance of 30 mL/min utilized in the DOAC trials could also have influenced these results. Moreover, accumulating data clearly indicate that food and drug interactions, genetic variations, and phenotypic features can affect DOAC anticoagulation and can increase the bleeding risk in these patients [29]. Factors such as male sex, advanced age, hypertension, impaired renal function, diabetes, history of stroke or bleeding, anemia, and use of antiplatelet or anti-inflammatory drugs have all been associated with increased risk of major bleeding in AF patients treated with DOACs [53,54]. Patients with severe liver dysfunction are also at increased risk of bleeding secondary to coagulopathy. However, since patients with active liver disease were excluded from all DOAC trials, bleeding rates in these patients remain to date elusive [30]. Since all DOACs are partly eliminated via the kidneys, altered renal function can have a major impact on patients' risk of bleeding. This is particularly the case for dabigatran, for which ≈80% of the dose is eliminated via the kidneys [55]. In a subgroup analysis of the Randomized Evaluation of Long-Term Anticoagulation Therapy with Dabigatran Etexilate (RE-LY) trial, patients with moderate kidney dysfunction had 2–3-fold higher dabigatran plasma concentrations and were more likely to develop bleeding complications than those with normal renal function [31]. Administration of DOACs without the need for monitoring could thus provide a false sense of safety, at least in certain categories of patients, in whom laboratory evaluation would seem desirable. Unfortunately, the laboratory techniques specific to these agents are not readily available in most laboratories, and, even if they were available, we do not know, for the moment, how effective DOAC anticoagulation should look like. And, even if we did, our options would still be extremely limited. If, for the VKAs, an INR outside the therapeutic window is followed by dose adjustment, such a possibility does not exist for the DOACs, for which only specific doses have been approved, and not the drugs per se.

Careful selection of patients for DOAC treatment is therefore essential, and thorough bleeding risk evaluation should be performed in all candidates for DOAC therapy prior to and during anticoagulation [23]. A score comprising age, history of bleeding, and non-bleeding-related hospitalizations within the last 12 months has been proposed by Rutherford et al. to assess bleeding risk in AF patients treated with DOACs [56]. Other scores have also been developed to assess the risk of bleeding in patients receiving anticoagulants, but not all have been validated in patients undergoing DOAC therapy. Among those evaluated for validation, the HAS-BLED, HEMORR$_2$HAGES, RIETE, and CHEST scores did not seem to reach good diagnostic accuracy for predicting bleeding in patients receiving DOAC therapy [57].

In addition, the fact that many of the factors known to increase patients' propensity to thrombosis also increase their bleeding risk (Figure 2) further complicates the management of anticoagulant therapy. One should keep in mind, however, that, although bleeding is a major concern in anticoagulated patients, the risk of thrombosis most often exceeds that of bleeding, including in the elderly, in those with cognitive impairment, or those with frequent falls [23]. Bleeding risk scores should therefore be used to identify and correct modifiable bleeding risk factors [23] and not as a pretext for abstention from oral anticoagulation.

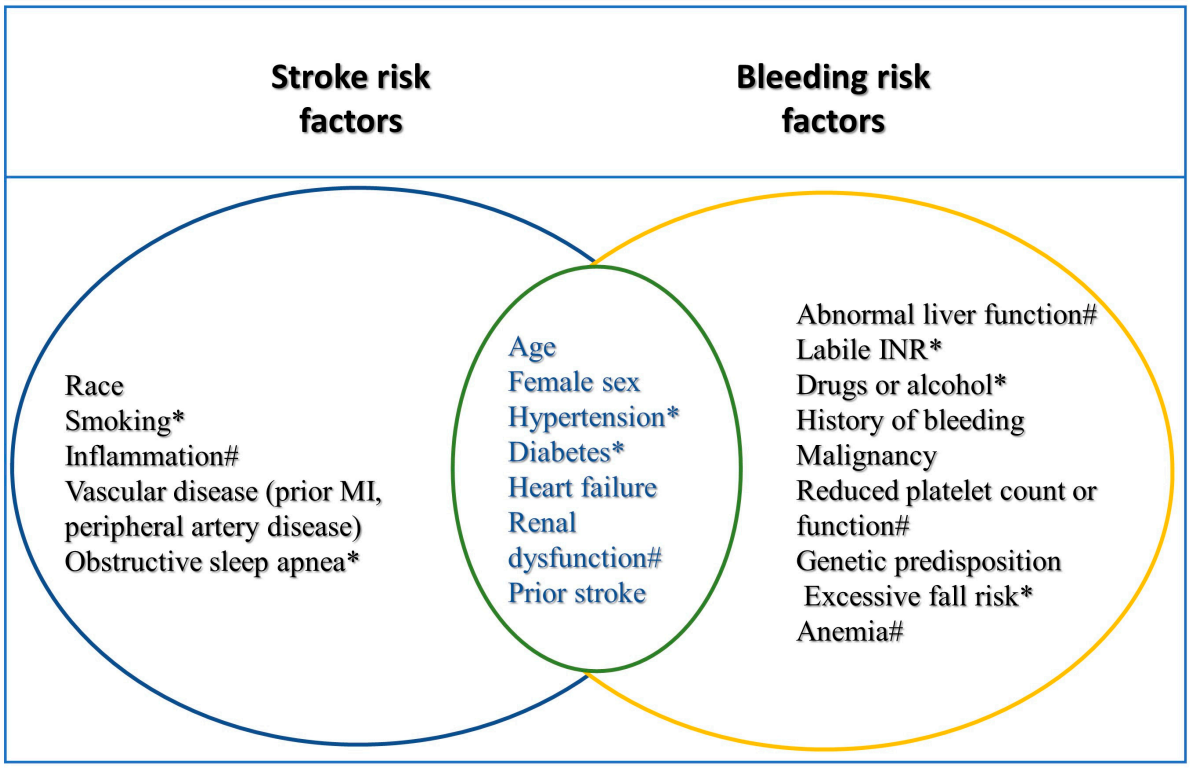

Figure 2. Risk factors for stroke, bleeding, and shared risk factors in patients with atrial fibrillation. *—indicates modifiable bleeding risk factors. #—indicates potentially modifiable bleeding risk factors. INR—International Normalized Ratio; MI—myocardial infarction.

6. Gastrointestinal Bleeding in the Anticoagulated Atrial Fibrillation Patient

Bleeding risk scores have been developed to allow the estimation of major bleeding risk. However, none of them allows differentiation of the type of hemorrhage [44], and, although it has been suggested that the HAS-BLED score can be used to estimate GIB risk in VKA-treated patients [43], scores specific for GIB risk have not been developed to date.

6.1. Gastrointestinal Bleeding in Patients Treated with Vitamin K Antagonists

Intracranial hemorrhage is the most feared anticoagulation-related complication. However, bleeding in the digestive tract is by far the most common type of hemorrhage in VKA-treated patients, leading to significant increase in hospitalization rates and important resource utilization [58,59]. Thirty-day mortality rates of up to 15.5% have been reported in patients with VKA-related GIB, although this high mortality is likely to be mainly due to comorbidities, rather than to VKA therapy per se [60]. In the study by Coleman et al., GIB incidence in patients treated with warfarin was 3.9% per patient-year, three times higher than in the general population [61]. In patients with a history of GIB, resumption of warfarin therapy resulted in recurrence of bleeding in 27.3% of cases [61]. The upper digestive tract appears to be the most common site for bleeding in patients receiving VKA therapy (8–15%), while hemorrhages in the lower digestive tract are found in ≈7% of patients [62].

Factors such as advanced age, increased INR, history of GIB, and liver cirrhosis have all been identified as independent predictors of GIB in warfarin-treated patients [58]. Previous history of gastrointestinal ulcer has also been related to a more than 6-fold increase in the risk of GIB [62]. The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is well recognized for its negative effects on GIB risk [62]. Interestingly, however, not all studies have reported a significant difference in NSAIDs usage between patients with and without GIB [63].

When GIB does occur, the risk–benefit ratio of oral anticoagulation should guide the clinician's decision to continue, halt, or reverse the effects of VKA therapy [64]. The clinical severity of the hemorrhage, the INR value, the duration of a potential endoscopic hemostatic procedure (using adrenaline injection, argon plasma coagulation, endoscopic hemoclips, unipolar or bipolar electrocoagulation, or, more rarely, sclerotherapy), and the thrombotic risk of the patient must be taken into account in the decision-making process [64,65]. Reversal of VKA effects is not urgent if GIB is self-limited GIB [64,65]. In patients with clinically significant GIB and supratherapeutic INR in whom the life-threatening risk of continuous bleeding outweighs the risk of thrombosis, discontinuation of anticoagulant therapy is justified [64]. To decrease the INR before urgent endoscopy, VKA reversal can be obtained using vitamin K, fresh frozen plasma (FFP), prothrombin complex concentrates (PCC), or recombinant activated factor VIIa (rFVIIa) [64]. No target INR value has been established so far for safe endoscopic therapy. However, obtaining an INR of 1.5–2.5 by VKA reversal appears to be effective for endoscopic therapy [66]. Data regarding the benefit of early correction of VKA-associated coagulopathy on clinical outcomes in patients undergoing endoscopic therapy is limited. Warfarin therapy at presentation does not appear to have a negative impact on bleeding-related mortality, and the INR value measured prior to endoscopic treatment did not correlate with the need for transfusion, duration of hospitalization, or mortality [67]. Moreover, in patients in whom the INR was in the therapeutic range, rebleeding rates did not differ substantially compared with those in whom the INR was supratherapeutic (i.e., $\geq$4) [67]. However, since coagulopathy was identified as the strongest clinical predictor of failed endoscopic hemostasis and data on patients without VKA reversal have not been reported to date, experts recommend that endoscopy be delayed until an INR value of 2.5 is achieved [65].

In order to reduce the INR before urgent endoscopy, vitamin K, FFP, PCC, and rFVIIa can be used [68]. Vitamin K, by promoting the synthesis by the liver of new functional coagulation factors II, VII, IX, and X, lowers the INR value in 2–4 h after administration of 5–10 mg in slow IV infusion (minimum 30 min) [68]. Given that the effect occurs late, vitamin K is not ideal for emergency reversal of anticoagulation [59,68]. However, its administration provides a sustained correction of coagulopathy that extends beyond the correction provided by FFP and PCC. Another method for VKA reversal is the use of FFP, consisting of the fluid portion of human blood containing vitamin-K-dependent clotting factors [59,68]. The recommended dose is an IV infusion of 15 mL/kg (approximately 3–4 units of 250 mL plasma for an adult weighing 70 kg). A more rapid correction of the INR can be obtained with PCCs, which are pharmacological products containing inactivated concentrates of factors II, IX, and X, and varying amounts of factor VII [59,68]. These are administered according to their factor IX content and initial INR, usually at a dose of 25–50 IU of factor IX/kg. Although not licensed for VKA reversal, rapid correction of elevated INR values and treatment of VKA-associated bleeding using rFVIIa has been described in case reports and small case series [59,68]. However, its routine use should be avoided until sufficient evidence to attest its effectiveness and safety is available.

6.2. Gastrointestinal Bleeding in Patients Treated with Direct Oral Anticoagulants

Based on the results of large clinical trials and observational real-life registries, a consensus seems to have been reached regarding the lower overall risk of bleeding associated with DOACs compared with warfarin. Intriguingly, however, this does not seem to apply to GIB. In fact, the use of DOACs was associated with a 25% higher risk of GIB than warfarin in the clinical trials. Both major and minor GIB were included in that analysis, and the results seem to have been mainly driven by the occurrence of minor hemorrhages [69]. Furthermore, not all DOACs and not all DOAC doses have been associated with higher risk of GIB compared with warfarin. While the 150 mg dose of dabigatran has been shown to increase the risk of GIB, the 110 mg dose showed a risk similar to that of VKA therapy [46]. Apixaban did not show significant differences in the risk of major GIB compared with warfarin, while non-major bleedings, including non-major GIB, were significantly lower in

the apixaban group [30,53]. Edoxaban 60 mg daily was associated with increased major GIB rate compared with warfarin [47]. The 30 mg dose showed, however, lower rates of GIB than warfarin [47]. In a population-based study comparing bleeding rates for dabigatran, rivaroxaban, and apixaban, apixaban showed the best safety profile for GIB compared with both rivaroxaban and dabigatran, and rivaroxaban appeared to have a less favorable safety profile than dabigatran [70]. Similar results were also reported in a meta-analysis of 43 randomized clinical trials [69]. Differences in the site of hemorrhage have also been reported between certain DOACs and the VKAs. In the case of dabigatran, the upper and lower parts of the gastrointestinal tract appeared to be similarly affected (i.e., 53% of GIB occurred in the upper, and 47% in the lower gastrointestinal tract), whereas, in the warfarin-treated patients, three-quarters of GIBs occurred in the upper gastrointestinal tract [71].

No definitive mechanism has been proposed to date to explain the increased risk of GIB associated with DOAC compared with VKA use, or the differences that appear to exist between DOACs [72]. In the case of dabigatran, the tartaric acid coating, which has caustic effect on the intestinal mucosa, could provide at least partial explanation. Incomplete absorption of DOACs into the intestinal mucosa may also lead to a local anticoagulant effect. For rivaroxaban, administration in a single dose may lead to greater variance in drug plasma concentration, and thus to increased GIB risk.

Similarly to what was seen in VKA-treated patients, advanced age, particularly >75 years, has also been associated with increased GIB risk in DOAC-treated patients, and Chinese patients appear to be more prone to GIB than non-Asian individuals [73]. Due to drug accumulation, renal dysfunction has also been associated with increased risk of GIB in DOAC- and particularly in dabigatran-treated patients [69]. Patients with severe hepatic impairment have been excluded from the clinical trials. Data on GIB risk in DOAC-treated patients with liver disease are therefore extremely limited. Recent studies do not appear to indicate, however, a significant increase in bleeding in patients with advanced liver disease undergoing DOAC therapy [72,74]. Other factors, such as the use of gastrotoxic agents, alcohol consumption, and Helicobacter Pylori infection, have also been associated with increased GIB risk in DOAC-treated patients [75]. In the absence of specific GIB risk scores, all these risk factors should be considered when initiating chronic DOAC therapy. The presence of colonic diverticulosis, angiodysplasia, and history of peptic ulcer should also alert the clinician and all modifiable bleeding risk factors should be identified and corrected prior to initiation of anticoagulant therapy [72].

If bleeding does occur in DOAC-treated patients, discontinuation of treatment may be considered only after weighing the risks and benefits [23,76]. Given the short half-lives of DOACs, the risk of thrombotic events increases substantially even if they are stopped for a short period [23]. Stopping anticoagulation is therefore not recommended if bleeding is minimal [23]. If halting DOAC therapy is required, DOAC plasma levels return to normal within 12–24 h [76]. To prevent further absorption, particularly in the case of a recent dabigatran overdose, gastric lavage and oral charcoal may be considered if DOACs have been ingested within the last 2–3 h [77]. Non-specific pro-hemostatic agents such as activated and non-activated PCCs and rFVIIa have not been studied in large studies. However, in a few small case series, bleeding has been effectively controlled with PCC and, as a result, it is indicated in major hemorrhages [78]. On the other hand, there is no place for the use of vitamin K or FFP as antidotes against DOACs. Other, non-specific strategies, such as antifibrinolytics (tranexamic acid, epsilon-aminocaproic acid), and desmopressin can also be used in DOAC-treated patients with bleeding [76]. Specific DOAC reversal agents (i.e., idarucizumab for dabigatran; andexanet alfa for factor Xa inhibitors) are also available [23]. Idarucizumab (5 g IV) is only recommended if the use of dabigatran is certain and if thrombin time is prolonged [76]. When using andexanet (low-dose protocol—400 mg dose given at a rate of 30 mg/min, followed by an infusion of 4 mg/min for up to 2 h; high-dose protocol—800 mg administered at a rate of 30 mg/min, followed by an infusion of 8 mg/min for up to 2 h), clinicians should also consider its prothrombotic potential

and perform careful monitoring [76]. Hemodialysis and hemoperfusion may be useful in patients treated with dabigatran, but not with factor Xa inhibitors, which are highly protein-bound [76].

Methods specific to GIB can also be applied, regardless if bleeding is VKA- or DOAC-related. Patients with GIB undergoing anticoagulation therapy should be evaluated endoscopically and the timing until endoscopy should be decided according to the severity of the hemorrhage [65,72]. Whenever possible, postponing the endoscopic procedure can improve detection of bleeding and facilitate endoscopic therapy [65]. Hemodynamically unstable patients, however, should undergo rapid, symptoms-guided endoscopic evaluation [65,72]. In the case of endoscopic treatment failure, radiological and surgical interventions can be used as alternatives [79].

6.3. Resuming Anticoagulation after Major Gastrointestinal Bleeding

In the case of GIB occurrence, temporary discontinuation of anticoagulant therapy is often required to reduce the risk of bleeding. However, permanent discontinuation of anticoagulant therapy may increase the risk of stroke and death in patients with AF. Therefore, it is important to consider the risks and benefits of both continuing and discontinuing anticoagulant therapy in patients with AF who have experienced an episode of GIB. Masiero et al. evaluated over 11,000 patients with AF and GIB and found that temporary discontinuation of anticoagulants was not associated with an increased risk of stroke or death within 30 days [80]. However, in a study evaluating more than 3000 patients with AF and GIB, temporary discontinuation of anticoagulants was associated with an increased risk of stroke and death 90 days after the bleeding event. In addition, continued anticoagulant therapy was not associated with increased risk of recurrent bleeding [81], and restarting oral anticoagulation was associated with lower risk of all-cause mortality and thromboembolic events [82]. There is limited evidence regarding the ideal timing of resuming anticoagulant therapy. The optimal timing for restarting anticoagulant therapy may vary depending on the severity of GIB and individual risk of thromboembolic events. In the study by Qureshi et al., restarting therapy 7–30 days after GIB was associated with a decreased risk of thromboembolic events and mortality without an increased risk of recurrent GIB [82].

The advantages and disadvantages of restarting VKA, as well as the appropriate timing to resume VKA therapy in patients with GIB, have not been sufficiently evaluated. The risk of recurrent GIB was significantly greater if warfarin was resumed within the first week of major GIB [83]. The cause of the bleeding dictates the probability of rebleeding after successful hemostasis. However, risk factors for GIB must be assessed and corrected before restarting VKA treatment. In a study by Chen et al., 27.3% of patients who resumed warfarin therapy experienced recurrent GIB, while thromboembolic events were seen in 16.7% of patients who did not continue warfarin therapy [58]. A warfarin dose adjusted to maintain an INR of 2.0 or less could be an alternative anticoagulation strategy to prevent thromboembolic events while minimizing the risk of GIB. Long-term acid suppressants may also be considered, especially in patients with peptic ulcer bleeding [84].

The timing for restarting anticoagulant therapy with DOACs was not evaluated. However, considering that restarting oral anticoagulation among patients with AF and GIB was associated with lower risk of all-cause mortality and thromboembolism, these agents should be started as soon as possible after weighing the hemorrhagic risk [82]. There are studies suggesting that restarting DOACs after a GIB episode may be safe and effective in preventing stroke in patients with AF. Chan et al. showed that the risk of GIB recurrence was similar in patients who received DOACs, compared to those in whom anticoagulant therapy was stopped [85]. However, it should be kept in mind that there is no standardized approach for restarting DOACs after a GIB episode and that the decision must be individualized for each patient. Dose reduction could be considered for patients with an increased risk of GIB recurrence. Moreover, changing the DOAC to another one with a lower risk of GIB could be an option.

7. Clinical Implications

Studies have shown that AF patients receiving anticoagulant therapy are at increased risk of GIB. Identification of risk factors and use of existing risk scores, although not specific for GIB, can help prevent hemorrhages through appropriate monitoring and management of patients. Identifying patients who are most susceptible to GIB contributes to an appropriate management of anticoagulant therapy to minimize risk. Anticoagulant therapy may need to be adjusted to reduce the risk of bleeding. Once GIB occurs, identifying the best bleeding control strategies are necessary to minimize the risk of death. Continuous evaluation of the effectiveness of different therapeutic options for GIB in patients with AF will lead to improved treatment and patient outcomes and contribute to the development of updated clinical guidelines for the management of AF and GIB.

8. Gaps in Evidence and Future Research

Despite the numerous clinical trials and observational studies conducted to date, numerous questions remain regarding the risk of bleeding associated with anticoagulant and particularly with DOAC therapy. The lack of easily available laboratory parameters to show the efficacy and safety of DOAC therapy makes it difficult, if not impossible, to detect inappropriate anticoagulation, which is likely to remain unnoticed until a thrombotic or bleeding complication occurs [86,87]. Monitoring the effects of DOACs would certainly be useful at least in specific clinical settings, such as suspicion of overdose, acute thrombotic or bleeding events, surgery, or acute renal failure [88]. Although activated partial thromboplastin and prothrombin time may be used to estimate anticoagulation with dabigatran or certain factor Xa inhibitors, both tests provide only qualitative information about the presence (but not the absence) of the drug [88]. More complex laboratory tests, such as the diluted thrombin time and the ecarin chromogenic assay, for dabigatran, and the chromogenic anti-factor Xa assays, for the Xa inhibitors, have been developed. However, none of them are routinely available in clinical practice. Development of widely available, easy-to-use tools to evaluate DOACs in various clinical settings is therefore imperative.

Accurate bleeding (including GIB-specific) risk scores for DOAC-treated patients also remain to be developed. Until then, the assessment of proven risk factors for bleeding is the only available tool, although the extent to which each of these factors contributes to the risk of bleeding remains unknown to date. Finally, the need for DOAC dose adjustment, at least in selected categories of patients, remains a pending issue [23]. Despite their more stable pharmacokinetics compared to warfarin, concerns exist that the 'one dose fits for all' strategy may not be entirely appropriate [89]. Plasma concentrations of DOACs have been shown to be affected by parameters such as drug interactions, renal impairment, or bodyweight, and evidence on the optimal dose of DOACs in 'sensitive' categories of patients is still lacking [89].

9. Conclusions

The prevalence of AF is constantly increasing globally, making AF an epidemic condition associated with increased morbidity and mortality. The increased risk of stroke associated with this arrhythmia is considerably reduced by chronic oral anticoagulant therapy. The advent of DOACs has changed the landscape of oral anticoagulation and has reduced the risk of major and intracranial hemorrhage compared to warfarin. Intriguingly, however, this does not seem to apply to the risk of GIB. Numerous other questions also remain regarding the risk of bleeding associated with DOAC therapy. The assessment of proven risk factors in DOAC-treated patients is essential, but the extent to which each factor contributes to the global risk of bleeding remains unknown to date. Given the expanding usage of DOACs, future studies will have to clarify these issues.

Funding: This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS-UEFISCDI, project number PN-III-P1-1.1-TE-2019-0370, within PNCDI III.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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frontiers | Frontiers in Physiology

TYPE Review
PUBLISHED 02 December 2022
DOI 10.3389/fphys.2022.1058427

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Cardiac Electrophysiology, a section of the journal Frontiers in Physiology

RECEIVED 30 September 2022
ACCEPTED 22 November 2022
PUBLISHED 02 December 2022

CITATION
Scridon A (2022), Autonomic imbalance and atrial ectopic activity—a pathophysiological and clinical view. *Front. Physiol.* 13:1058427.
doi: 10.3389/fphys.2022.1058427

Autonomic imbalance and atrial ectopic activity—a pathophysiological and clinical view

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The heart is one of the most richly innervated organs and the impact of the complex cardiac autonomic network on atrial electrophysiology and arrhythmogenesis, including on atrial ectopy, is widely recognized. The aim of this review is to discuss the main mechanisms involved in atrial ectopic activity. An overview of the anatomic and physiological aspects of the cardiac autonomic nervous system is provided as well as a discussion of the main pathophysiological pathways linking autonomic imbalance and atrial ectopic activity. The most relevant data on cardiac neuromodulation strategies are emphasized. Unanswered questions and hotspots for future research are also identified.

KEYWORDS

atrial arrhythmias, atrial ectopy, atrial fibrillation, autonomic nervous system, neuromodulation

Introduction

According to Coumel's triangle concept, abnormal triggers, arrhythmogenic substrate, and modulating factors are needed to ensure the occurrence of sustained cardiac arrhythmias (Figure 1), including atrial fibrillation (AF) (Coumel, 1993). Autonomic imbalance is currently regarded as the most important pro-arrhythmic modulating factor, favoring both atrial ectopic activity and reentry (Scridon et al., 2018). In the presence of extensively remodeled atria, even infrequent ectopic activity can initiate sustained, persistent or permanent AF. However, in the absence of significant substrate that can support reentry, triggers sensitive to autonomic modulation will generate only unsustained atrial arrhythmias, including isolated or more complex

Abbreviations: AF, atrial fibrillation; AHRE, atrial high rate episodes; ANS, autonomic nervous system; HRV, heart rate variability; $I_{Ca\text{-}L}$, L-type calcium current; I_f, pacemaker current; I_{KAch}, acetylcholine-gated potassium current; I_{Ks}, slow delayed rectified potassium current; I_{Kur}, ultra-rapid delayed rectified potassium current; I_{to}, transient outward potassium current; NCX, Na^+/Ca^{2+} exchanger; PAC, premature atrial contraction; RyR2, ryanodine-receptor channels; SR, sarcoplasmic reticulum.

premature atrial contractions (PACs), or short, self-terminating episodes of clinically overt or subclinical AF (Figure 2). (Scridon et al., 2018)

The aim of this review is to discuss the main mechanisms involved in atrial ectopic activity. An overview of the anatomic and physiological aspects of the cardiac autonomic nervous system (ANS) is provided as well as a discussion of the main pathophysiological pathways linking autonomic imbalance and atrial ectopic activity. The most relevant data on atrial neuromodulation strategies are emphasized.

Ectopic automaticity and afterdepolarizations–the players behind the atrial ectopy scene

Physiologically, the electrical activity of the heart arises as a result of spontaneous diastolic depolarization of sinoatrial node pacemaker cells (Figure 3A). Three main mechanisms have been shown to underlie the advent of abnormal triggers and of atrial ectopy: enhanced ectopic automaticity, early (phase 2 or 3) and delayed (phase 4) afterdepolarizations (Figure 3B–D). In AF, the trigger is often related to ectopic foci located at or in the close vicinity of the pulmonary venous ostia, although other areas, such as the right atrium or the left atrial posterior wall, the ostium of the superior vena cava or of the coronary sinus, crista terminalis, or the ligament of Marshall can also act as origin of abnormal atrial electrical activity (Wu et al., 2001).

Similarly to pacemaker cells, normal cardiac myocytes also express the pacemaker current–I_f. These cells do not behave as pacemaker cells because the activity of I_f is much lower and because they concomitantly express the inwardly rectifying Kir current, I_{K1}, that opposes I_f, preventing the spontaneous depolarization of these cells. However, a decrease in the activity of I_{K1}, an increase in the activity of I_f, or both, can easily transform these cells into pacemaker cells that will generate abnormal, ectopic electrical impulses (Figure 3B). Indeed, in rats with spontaneous AF one of the most upregulated genes was *HCN4*, encoding for proteins of I_f (Scridon et al., 2014), whereas *in vitro* studies performed in canine AF models have shown a significant increase in I_f activity and spontaneous diastolic depolarization rate, which were both counteracted by the I_f

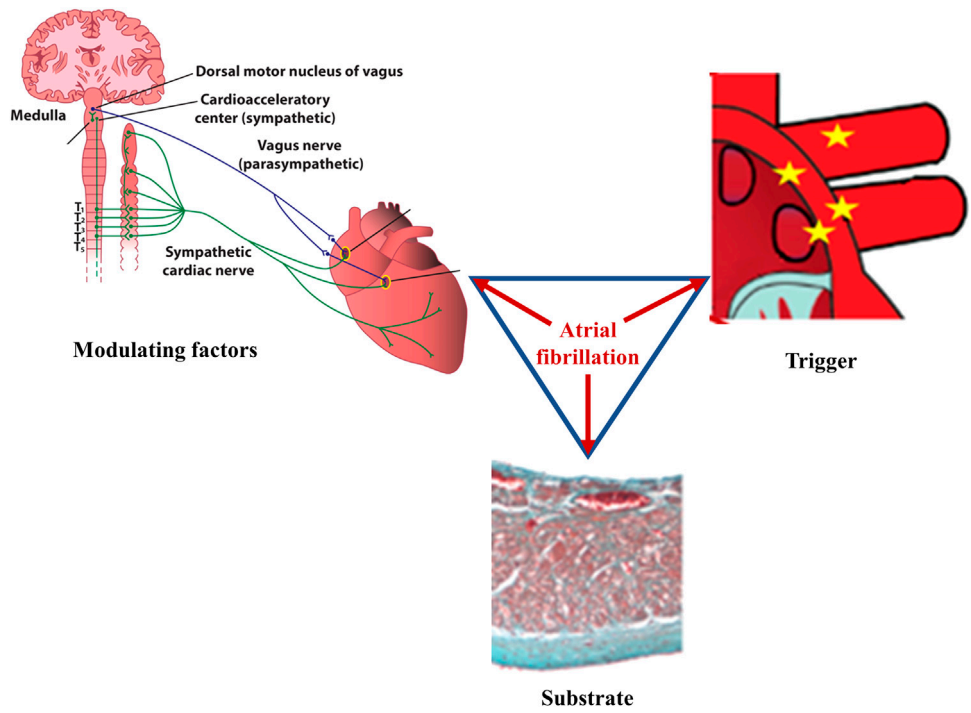

FIGURE 1
Main factors underlying atrial fibrillation. According to Coumel, three factors are needed for sustained cardiac arrhythmias to occur: abnormal triggers, substrate, and modulating factors. In atrial fibrillation, ectopic foci, most commonly located at the junction between the left atrium and the pulmonary veins, generate rapid, chaotic, and non-coordinated electrical impulses that initiate multiple reentry circuits; this component represents the arrhythmic trigger. If present, arrhythmogenic electrical and/or structural changes of the atria will act as the substrate that ensures persistence of the arrhythmia. Autonomic imbalance is currently recognized as the main pro-arrhythmogenic modulating factor, favoring both the initiation and the maintenance of the arrhythmia. Once it begins, atrial fibrillation favors its own perpetuation ("atrial fibrillation begets atrial fibrillation") by inducing pro-arrhythmic electrical, structural, and autonomic remodeling of the atria.

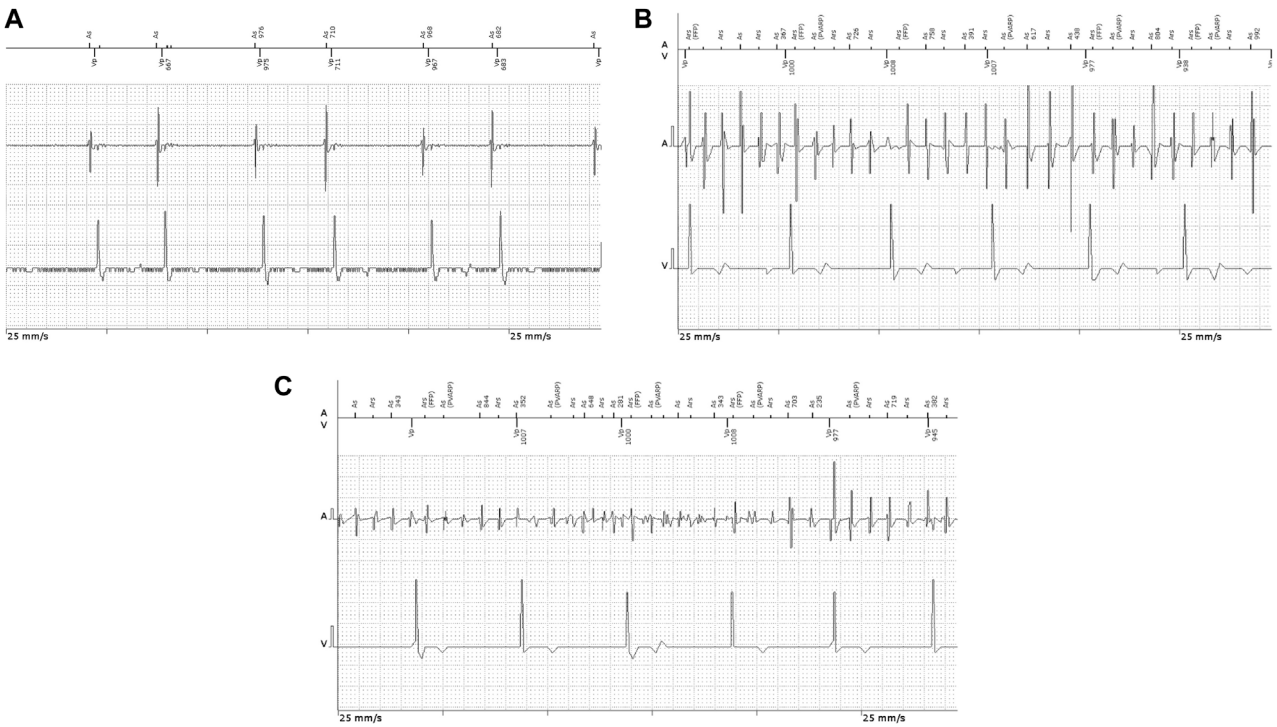

FIGURE 2
Intracavitary ECG recordings in patients implanted with permanent pacemakers showing **(A)** atrial couplets and **(B,C)** atrial high-rate episodes.

blocker ivabradine (Li et al., 2015). In normal rats, chronic ivabradine administration induced a significant increase in atrial *HCN4* mRNA expression levels (Scridon et al., 2021), suggesting that I_f blockade may exert not anti-, but rather proarrhythmic effects over the long term, as also indicated by the modest, yet significant increase in AF rates recorded in the clinical studies that evaluated ivabradine (Martin et al., 2014; Cammarano et al., 2016). Human patients with sinus node dysfunction may also develop AF (tachy-brady syndrome).

Ectopic activity can also arise when cardiomyocytes develop spontaneous, progressive depolarizations, that interrupt phases 2, 3, or 4 of the action potential. Excessive action potential prolongation, most commonly due to decreased repolarizing potassium currents activity, allows calcium channels to recover their activatability, creating inward cation movement during action potential phases 2 or 3, thus generating early afterdepolarizations and ectopic atrial activity (Figure 3C). Although early afterdepolarizations can act as triggers in certain cases, delayed, phase 4 afterdepolarizations seem to be the most important source of atrial ectopic activity. Spontaneous release of calcium from the sarcoplasmic reticulum (SR) can occur through cardiac ryanodine-receptor channels (RyR2) during phase 4 of the action potential due to SR calcium overload, decreased binding of calcium to calsequestrin (the main calcium-binding protein of the SR), or to increased RyR2 activity (Scridon et al., 2018). The consequent intracellular calcium overload further activates the Na^+/Ca^{2+} exchanger (NCX), which creates a net inward movement of positive charges, thus setting the basis for delayed afterdepolarizations and for ectopic atrial activity (Figure 3D).

An anatomic and physiological view on the autonomic innervation of the atria

Anatomy of the cardiac autonomic nervous system

The heart contains very rich intrinsic and extrinsic autonomic innervation that provides physiological regulation of the heart rate and hemodynamic parameters, as well as cellular and subcellular properties of individual cardiac cells (Shen and Zipes, 2014). The extrinsic cardiac ANS mediates connections between the heart and the nervous system (Figure 4), whereas the intrinsic ANS of the heart consists of a local network of ganglionated plexi, interconnecting ganglia, and autonomic nerve axons (Hou et al., 2007).

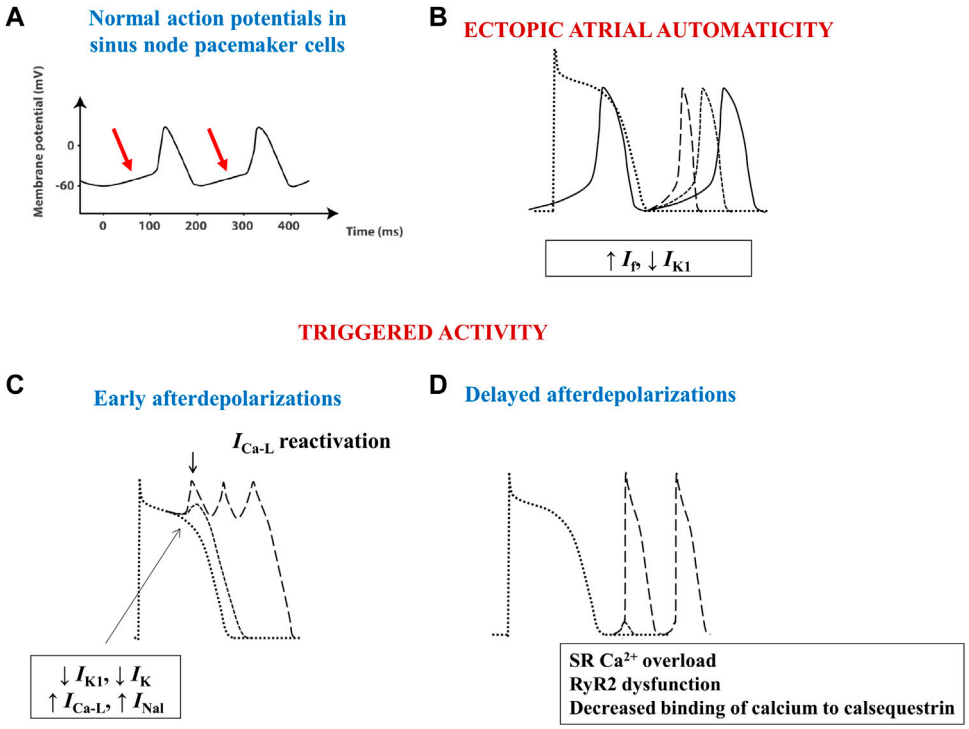

FIGURE 3
Schematic images of normal and arrhythmia-related atrial electrical activity. **(A)** Normal slow-response action potential in cardiac pacemaker cells. The arrows indicate the spontaneous diastolic depolarization phase. **(B)** Ectopic automaticity arises as a result of an increase in pacemaker current (I_f) activity, a decrease in inwardly rectifying Kir current (I_{K1}) activity, or a combination of both, capable of transforming atrial myocytes into pacemaker-like cells. **(C)** Excessive action potential prolongation due to a decrease in outward potassium currents (I_{K1} and I_K) activity, an increase in inward (L-type calcium–I_{Ca-L}–or late sodium - I_{Nal}) current activity, or a combination of both, allows calcium channels to recover their ability to activate, creating inward cation movement during action potential phases 2 or 3, thus generating early afterdepolarizations and ectopic atrial activity. **(D)** Spontaneous release of calcium from the sarcoplasmic reticulum can occur during phase 4 of the action potential due to sarcoplasmic reticulum calcium overload, decreased binding of calcium to calsequestrin, or to increased cardiac ryanodine-receptor channel activity. The consequent intracellular calcium overload further activates the Na^+/Ca^{2+} exchanger, creating a net inward cation movement, thus providing the basis for delayed afterdepolarizations and for ectopic atrial activity. RyR2—cardiac ryanodine-receptor channels; SR–sarcoplasmic reticulum.

Extrinsic sympathetic fibers mainly derive from the sympathetic (i.e., superior and middle cervical, stellate, mediastinal, and thoracic) ganglia (Dacey et al., 2022). The axons of the neurons located herein will give rise to the superior, middle, and inferior cardiac sympathetic nerves, whose terminal branches travel along the coronary vessels from the epicardial regions towards the endocardium of the atria and the ventricles. Extrinsic parasympathetic fibers originate mainly from the nucleus ambiguus of the medulla oblongata and are carried almost entirely within the vagus nerves (Kapa et al., 2010), whose cardiac fibers converge at the "third fat pad", located between the superior vena cava and the aorta, before arriving to the sinoatrial and atrioventricular nodes (Chiou et al., 1997). Extrinsic cardiac innervation is much more tangled than a simple sympathetic-parasympathetic duet, and there are multiple points within the intrinsic cardiac nervous system where sympathetic and parasympathetic nerves converge (e.g., the vagus nerves also contain sympathetic fibers, and parasympathetic fibers are also found in sympathetic nerves). (McGuirt et al., 1997; Randall et al., 2003; Beaumont et al., 2013; Rajendran et al., 2016; Khan et al., 2019).

The afferent signals involved in cardiac autonomic modulation originate from baro-, chemo-, and multimodal receptors located within the heart and the walls of the great vessels. Afferent neural signals are transmitted from the heart to integration centers located within the intrinsic nervous system, extracardiac intrathoracic ganglia, the spinal cord, and the brain stem, which further regulate the neural output to the heart *via* the sympathetic and parasympathetic nerves. Activation of high-pressure baroreceptors located in the carotid sinus and the aortic arch generate the main input for sympathetic stimulation and parasympathetic withdrawal *via* the arterial baroreceptor reflex, whereas chemoreceptor activation within the carotid and aortic bodies as well as the medulla (by hypoxemia, hypercapnia,

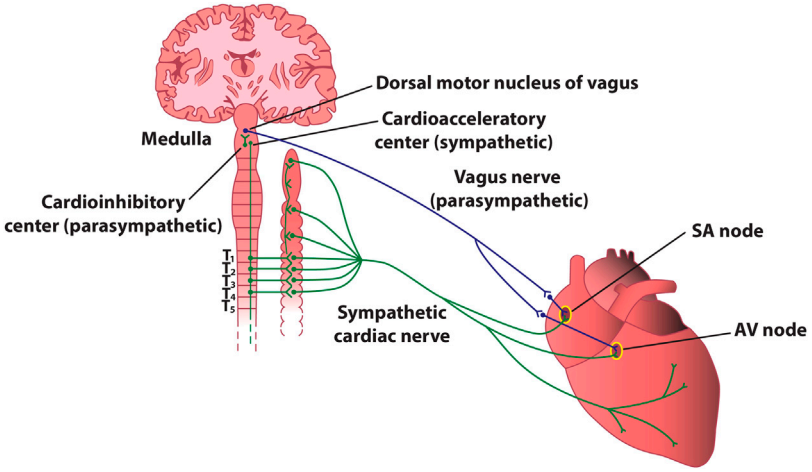

FIGURE 4
Stylized diagram of the anatomy of cardiac sympathetic and parasympathetic innervation. The figure presents schematically some of the most important connections between the autonomic nervous system and the heart. In reality, sympathetic and parasympathetic projections are less spatially distinct, and overlap at various levels. For instance, the parasympathetic innervation of the atrioventricular node and of the inferior atrium is primarily *via* fibers originating in the inferior vena cava-right atrium, and that of the ventricles is primarily *via* fibers originating in the ventral interventricular artery ganglionated plexi. AV–atrioventricular; SA–sinoatrial.

various neuropeptides such as bradykinin, substance P, or the calcitonin gene-related peptide) drives sympathetic tone *via* direct signaling to the nucleus of the tractus solitarius and medulla (Floras and Ponikowski, 2015). This also initiates local inflammatory and vascular reactions important in cardiac remodeling (Wang et al., 2014). Low-pressure baroreceptors are mainly located at the veno-atrial convergence and react in response to atrial filling and contraction. When activated, they cause inhibition of the cardiac and peripheral sympathetic ANS (Linz et al., 2019). However, at each level of integration, the system can modulate cardiac activity with numerous efferent feedback loops (Goldberger et al., 2019). In addition, both the heart and the blood vessels are densely innervated by sensory nerve endings that express chemo-, mechano-, and thermos-sensitive receptors. Activation of the receptors by a nociceptive stimulus in settings such as myocardial ischemia has been shown to reduce vagal tone and exhibit ventricular proarrhythmic effects (Premkumar and Raisinghani, 2006; Salavatian et al., 2022). The role of nociceptors activation in atrial arrhythmias is less clear.

The exquisitely complex intrinsic ANS of the heart contains clusters of intrinsic autonomic ganglia that form a complex network of ganglionated plexi, located in the fat pads found at the surface of the atria and the ventricles, interconnecting ganglia, and postganglionic autonomic fibers' axons (Armour et al., 1997). Despite the tangled nature of the intrinsic ANS of the heart, different ganglionated plexi appear to modulate specific cardiac anatomic regions (Sampaio et al., 2003). Damage to the projections of the parasympathetic sinoatrial node nerves that penetrate the epicardium at the pulmonary vein antrum could explain, for instance, the increased heart rate, that is, recorded following pulmonary vein ablation procedures (Armour et al., 1997). Activation of the neurons located in any of the major ganglionated plexi has been shown to affect not only adjacent tissue, but all cardiac chambers (Yuan et al., 1994). Moreover, a large gradient appears to exist between different atrial regions in the density of cholinergic and adrenergic neurons, forming a highly intricate heterogeneously distributed atrial autonomic neural network (Scridon et al., 2018). In addition, the cardiac ANS displays a cross-linked structure of interconnected ganglionated plexi. These autonomic structures provide fine regional regulation of various cardiac functions (e.g., cardiac automaticity and conductivity) and act as integrating centers that process both centripetal and centrifugal information, coordinate the sympathetic and parasympathetic inputs received from the rest of the cardiac ANS, and modulate the complex interactions between the extrinsic and the intrinsic systems (Hou et al., 2007; Scridon et al., 2018). They are mainly located at the surface of the right (e.g., on the right atrium, at the junction between the inferior vena cava and the right atrium) and the left (at the pulmonary veins ostia) atria, whereas ventricular ganglionated plexi are mainly located at the origins of the aorta and the main branches of the coronary system (Armour et al., 1997). Ganglionated plexi located at the junction between the inferior vena cava and the right atrium have been shown to control the function of the atrioventricular node and inferior atrial tissues (O'Toole et al., 1986; Cardinal et al., 2009).

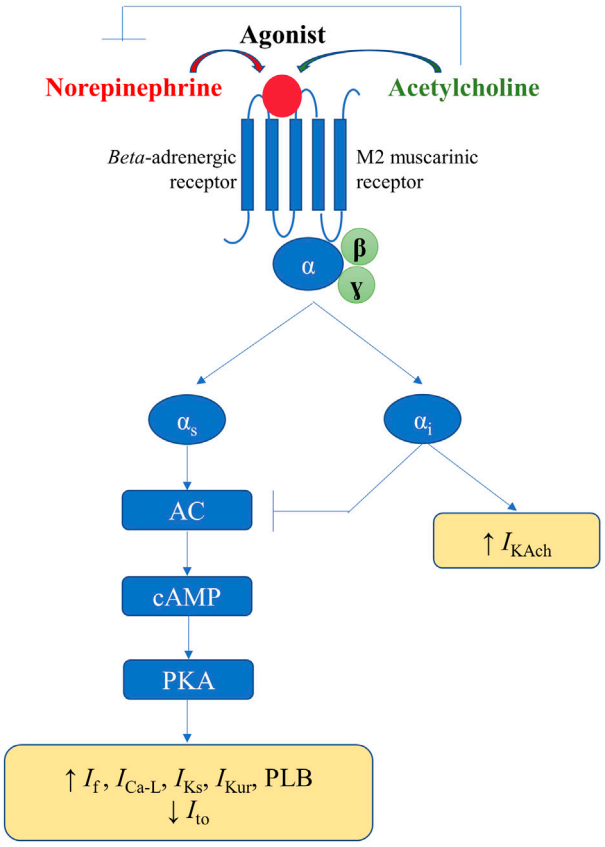

FIGURE 5
Physiological mechanisms involved in the autonomic modulation of cardiac electrical activity.

Effects of autonomic inputs on cardiac electrophysiology

In response to the appropriate stimuli, postganglionic sympathetic fibers release norepinephrine, thus activating the cardiac *beta*-adrenergic receptors (particularly *beta*-1), coupled with stimulatory G proteins (Figure 5). Subsequent activation of protein kinase A, *via* the adenylyl cyclase/cAMP signaling pathway, increases the activity of the L-type calcium current ($I_{Ca\text{-}L}$) and phospholamban, thus increasing calcium inflow, as well as calcium uptake by the SR. Additionally, sympathetic stimulation enhances the activity of the pacemaker current (I_f), inhibits the cardiac transient outward potassium current (I_{to}), and stimulates the slow delayed rectified potassium current (I_{Ks}) and the ultra-rapid delayed rectified potassium current (I_{Kur}), the latter expressed exclusively at the level of the atria (Schotten et al., 2011). Overall, the net result of these effects will be an increase in cardiac chronotropy, excitability, and dromotropy, in parallel with unaffected or only slightly abbreviated action potential, both at the level of the atria and of the ventricles (Figure 5). (Zipes et al., 1974; Vaseghi et al., 2013)

Activation of the parasympathetic nervous system will lead to release of acetylcholine from the nerve endings and to consequent activation of muscarinic (particularly M2) receptors within the heart, triggering effects that counteract those of the cardiac sympathetic nervous system. Alongside the interactions that occur within the intrinsic cardiac nervous system, parasympathetic-sympathetic interferences also occur at other–presynaptic, receptor, and intracellular–levels (Figure 5). Presinaptically, acetylcholine inhibits norepinephrine release. Acetylcholine inhibits the adenylyl cyclase/cAMP system (*via* M2 receptors coupled with inhibitory G proteins), thereby reducing the activity of $I_{Ca\text{-}L}$ and I_f (Figure 5). Muscarinic receptors activation and the consequent stimulation of inhibitory G proteins also inhibit, *via* a direct, adenylyl cyclase/cAMP system-independent mechanism, the acetylcholine-gated potassium current (I_{KAch}), expressed almost exclusively at the level of the atria (Linz et al., 2019). Overall, the net result will be a decrease in cardiac chronotropy,

excitability, and dromotropy, in parallel with a significant and heterogeneous shortening in atrial action potential and refractoriness (due to inhomogeneous spatial distribution of atrial M2 receptors and parasympathetic nerve endings) (Figure 5). (Liu and Nattel, 1997; Schotten et al., 2011) Accumulating data also indicate a potential role for neuromediators such as the neuropeptide Y, in control of cardiac function, particularly in progressive cardiac pathology (Armour et al., 2005; Herring et al., 2008; Tan et al., 2018; Ajijola et al., 2020).

Simplistically, sympathetic and parasympathetic nervous systems essentially work in a 'ying-yang' fashion, displaying opposite effects on cardiac electrical parameters. However, constant communication exists between the two systems that complicates their effects, and an intricate balance exists between sympathetic and parasympathetic inputs. Greater absolute reductions in heart rate are recorded when parasympathetic stimulation is applied in the presence of higher sympathetic tone, an interaction known as accentuated antagonism (Stramba-Badiale et al., 1991). Similar effects are recorded regarding calcium handling and cardiac electrophysiology parameters (Brack et al., 2004). Meanwhile, both chronic and acute afferent vagus nerve stimulation have been shown to reflexively inhibit efferent sympathetic nerve activity (Schwartz et al., 1973; Shen et al., 2011). Furthermore, in diseased hearts, autonomic stimulation may exert effects opposite to those seen in the normal heart, and vagal (but not sympathetic) stimulation has different effects on atrial and ventricular myocytes (Shen and Zipes, 2014).

Autonomic imbalance and ectopic atrial activity

A plethora of intricate mechanisms links atrial arrhythmias risk factors with autonomic imbalance

A wide array of cardiac and non-cardiac conditions act as major risk factors for atrial arrhythmias, particularly AF, by promoting atrial proarrhythmic electrical and structural remodeling (Şerban and Scridon, 2018; Scridon and Balan, 2022). Numerous systemic (e.g., obesity, diabetes mellitus, hypertension, obstructive sleep apnea, aging, sustained endurance training) and cardiac (e.g., heart failure, cardiomyopathies, acute and chronic ischemic heart disease) conditions have also been shown to induce structural and/or functional autonomic alterations, further promoting atrial arrhythmogenicity (Figure 6). (Scridon et al., 2018) Increased sympathetic tone is a major pathophysiological feature of most of these conditions, although spontaneous unsustained AF has also been associated with sympathetic withdrawal and relative parasympathetic activity in aging, spontaneously hypertensive rats (Scridon et al., 2012). Moreover, in that model, sympathetic stimulation exhibited marked atrial antiarrhythmic effects, whereas parasympathetic stimulation induced a significant increase in atrial arrhythmic burden (Scridon et al., 2012; Sayin et al., 2015). Long-term vigorous endurance training has also been related to vagally-mediated AF (Figure 6). (Elliott et al., 2017) Meanwhile, transient exposure to occasional intensive exercise has been shown to favor atrial arrhythmias *via* a complex sympathetic-parasympathetic interplay. Adaption of the cardiac output to exercise relies on progressive sympathetic activation and concomitant parasympathetic withdrawal. Upon cessation of exercise, sustained sympathetic hyperactivity coupled with rapid parasympathetic reactivation result in a transient, proarrhythmogenic state of sympathetic-parasympathetic coactivation that favors the occurrence of atrial arrhythmias (Elliott et al., 2017). A similar sympatho-vagal coactivation has also been reported in patients with obstructive sleep apnea, in whom increased atrial transmural pressure gradients lead to parasympathetic activation, *via* the diving reflex, whereas pulmonary stretch and hypoxia prompt sympathetic surge (Linz et al., 2018).

The pathophysiological bases of cardiac autonomic neuropathy have not yet been fully elucidated and are likely to vary widely depending on the underlying pathology. Among them, inflammation, oxidative stress, and neurohormonal alterations [particularly catecholamine release and renin-angiotensin-aldosterone system (RAAS) activation], encountered in various degrees in the vast majority of cardiac diseases, appear to play central roles in the development of cardiac autonomic neuropathy and in the pathophysiology of atrial arrhythmias (Figure 6). (Şerban and Scridon, 2018) Additional factors, such as increased production of advanced glycation end-products, hyperglycemic activation of the polyol and protein kinase C pathways, and neurovascular insufficiency, as well as sudden sympathetic activation in response to transient episodes of hypoglycemia may contribute to cardiac autonomic neuropathy and atrial arrhythmia occurrence in the presence of diabetes mellitus (Şerban and Scridon, 2018). Meanwhile, proinflammatory cytokines, released in a wide range of cardiac conditions (Scridon et al., 2015), have been shown to promote restructuring of the cardiac autonomic network, stimulate nerve sprouting and growth, favor autonomic variations, and promote cholinergic transdifferentiation of cardiac sympathetic nerves (Kanazawa et al., 2010). Inflammation is also seen as a link between epicardial adipose tissue (a major local source of cytokines, hormones, and vasoactive substances), autonomic dysfunction, and atrial arrhythmias (Scridon et al., 2015). Proinflammatory adipocytokines have been shown to stimulate ganglionated plexi located within the epicardial fat pads, promoting both sympathetic and parasympathetic overactivation, thus favoring the occurrence of atrial arrhythmias (Scridon et al., 2015). Sympathetic activation has also been shown to induce

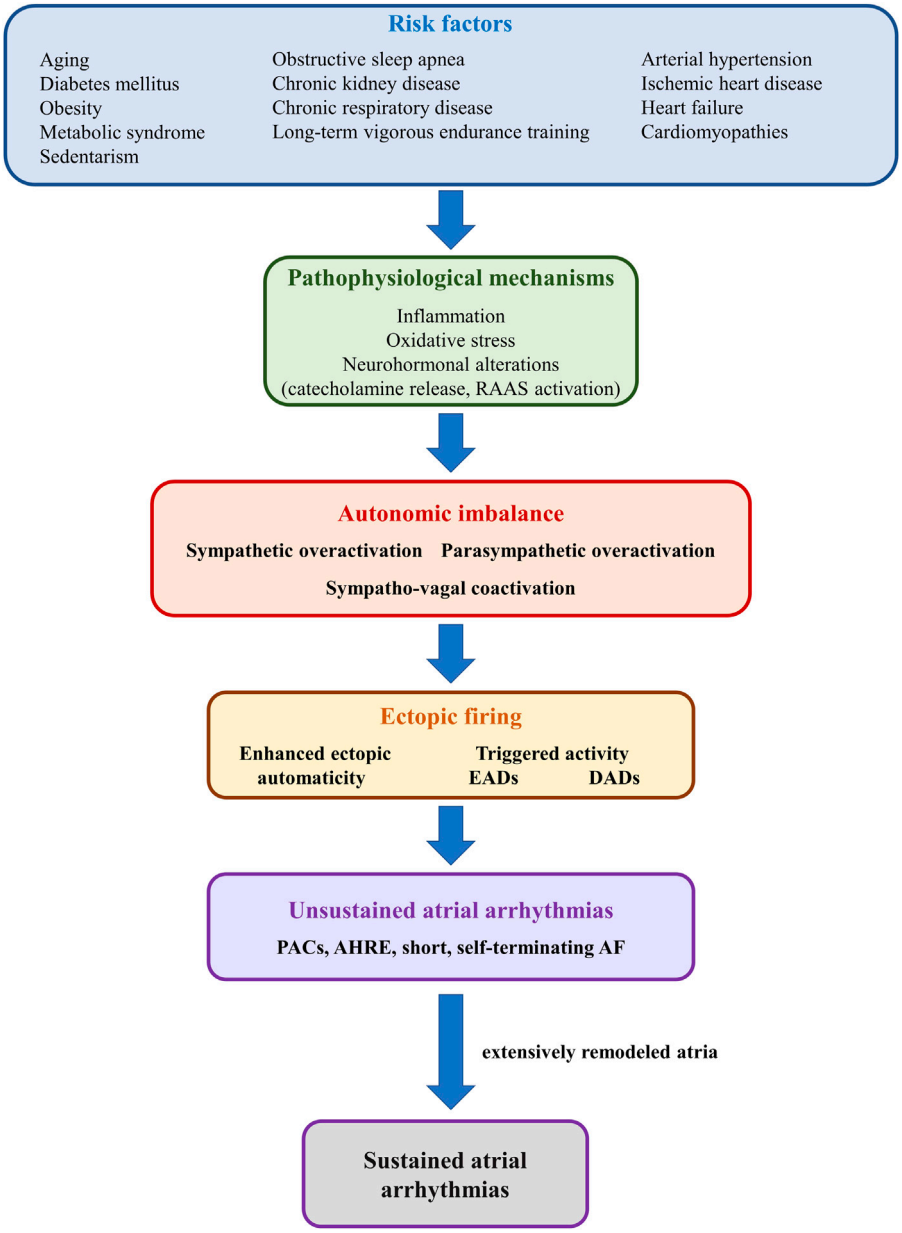

FIGURE 6
Schematic representation of the mechanisms linking risk factors with autonomic imbalance and atrial ectopic activity. Risk factors such as diabetes mellitus, obesity, or heart failure (*upper panel*) promote, *via* multiple mechanisms (*second panel*), sympathetic-parasympathetic imbalance (*third panel*). In turn, sympathetic or parasympathetic overactivation, or a combination of both, promotes enhanced ectopic automaticity or triggered activity (*fourth panel*), and atrial ectopic activity (*fifth panel*). In the absence of adequate substrate, ectopic activity translates into unsustained atrial arrhythmias, such as premature atrial contractions or short, self-terminating episodes of clinically overt or subclinical atrial fibrillation (*sixth panel*). However, in the presence of extensively remodeled atria, even reduced ectopic activity can initiate sustained episodes of atrial arrhythmia.

proinflammatory cytokine expression in the atrial myocardium (Acampa et al., 2018), thus generating a highly proarrhythmic vicious circle at the level of the atria. A similar reciprocal relationship has also been reported between increased adrenergic drive and oxidative stress, as well as between increased adrenergic drive and activation of the RAAS (Acampa et al., 2018).

Additionally, AF itself has been postulated to cause substantial autonomic imbalance, leading to a feedback loop in which autonomic dysfunction contributes to the pathogenesis

of AF, whereas AF promotes autonomic dysfunction, further perpetuating and facilitating the progression of the arrhythmia (Figure 6). (Scridon et al., 2018) Long coupling intervals between ventricular contractions during AF have been shown to cause beat-to-beat variations in pulse pressure, causing sympathetic activation and blunting baroreflex sensitivity (Segerson et al., 2007; Malik et al., 2019). In animal models, pacing-induced AF has been shown to cause an increase in both sympathetic and parasympathetic innervation of the atria, while also promoting deafferentation of the atria (Yu et al., 2014; Malik et al., 2019). In addition, during AF, decreased arterial blood pressure and the consequent unloading of arterial baroreceptors, coupled with increased cardiac filling pressures with consequent activation of cardiac baroreceptors "confuse" the ANS, leading to simultaneous, conflicting sympatho-excitatory and sympatho-inhibitory signals (Scridon et al., 2018).

Role of autonomic dysfunction in the genesis of atrial ectopic activity

Activation of the extrinsic ANS of the heart is seen prior to AF onset in 73%–100% of cases (Tan et al., 2008; Choi et al., 2010), with a predominance of vagal activity in young patients with "idiopathic AF" and structurally normal hearts, and sympathetic dominance in patients with "organic AF" or following cardiac surgery (Zhong et al., 2022). Diurnal prevalence, increased sympathetic indices at heart rate variability (HRV) analysis, and the presence of frequent PACs that produce a short-long-short RR intervals pattern provide indirect evidence for a strong adrenergic stimulation-atrial ectopy-AF relationship (Lombardi et al., 2004). Oppositely, nocturnal prevalence and increased parasympathetic indices at HRV analysis underscore the implication of the vagal system in arrhythmia onset (Herweg et al., 1998). Meanwhile, a direct implication of the intrinsic cardiac ANS in atrial arrhythmias occurrence is much less clear. Studies performed in dogs using direct nerve activity recordings from both intrinsic and extrinsic autonomic components showed that although the vast majority of AF episodes were preceded by simultaneous firings from both the intrinsic and extrinsic autonomic systems, ≈10% of the episodes were triggered by activation of the intrinsic cardiac ANS alone (Yu et al., 2014). An increase in PAC burden has been noted following increased sympathetic activity (Honjo et al., 2003), whereas the contribution of autonomic dysfunction to atrial high rate episodes (AHRE) is much less clear. The sole study performed to date showed no correlation between HRV indices and the burden of AHRE (Khan et al., 2021). However, that study was small, with only 22 patients included in each group, and there was no control group. Models of neurally-induced arrhythmias have also been developed and have been very useful in defining effects of surgical, pharmacological, and bioelectric interventions (Armour et al., 1972; Cardinal et al., 2006; Richer et al., 2008; Gibbons et al., 2012; Salavatian et al., 2016).

Vagal activation exerts different electrophysiological effects at the level of the atria and the ventricles. At ventricular level, vagal stimulation prolongs action potential duration and refractoriness, exhibiting antiarrhythmic effects. Meanwhile, at the atrial level, vagal stimulation causes opposite effects, abbreviating atrial action potentials and refractoriness in a spatially heterogeneous fashion, thus setting the background (dispersion of refractoriness) for reentry and atrial arrhythmias (Shen and Zipes, 2014). In addition, vagally-released non-cholinergic molecules such as the vasoactive intestinal peptide further shorten the atrial action potential and generate intraatrial conduction delays. Vagal stimulation has been shown to promote not only reentry, but also atrial ectopic firing, by promoting early afterdepolarization toward the end of phase 3 of the action potential and allowing ectopic automaticity to arise (Figure 6). (Burashnikov and Antzelevitch, 2003) In an early study by Scherf et al. (1950) local acetylcholine application was followed by rapid action potentials firing and AF, whereas arrhythmias offset was recorded when the parasympathetic source was removed. In dogs, high-frequency stimulation of the cervical vagal trunk and of the atrial epicardial ganglionated plexi also significantly increased AF occurrence, effect that could be eliminated by blocking vagus nerves function with atropine (Lu et al., 2009; Qin et al., 2019). The exact mechanisms by which parasympathetic activation triggers atrial ectopic activity remain to date unclear. However, by decreasing I_f activity in the sinus node, vagal stimulation can alter the normal pacemaking hierarchy of the heart and can thus enable ectopic activity to arise (Scridon et al., 2018). In addition, by activating I_{KAch}, vagal stimulation can also shorten the action potential and thus promote the occurrence of late-phase 3 afterdepolarizations (Figure 6), particularly if accompanied by an increase in the calcium transient (Burashnikov and Antzelevitch, 2003). The impact of vagus nerve stimulation on atrial arrhythmias is highly dependent on stimulation parameters. Both vagus nerve and spinal cord stimulation have been shown to exhibit stabilizing effects on intrinsic cardiac nervous system function, which affects ANS contribution to arrhythmia formation (Cardinal et al., 2006; Gibbons et al., 2012; Salavatian et al., 2016).

Sympathetic activation is recognized as the most relevant culprit in autonomic dysfunction-related atrial ectopy (Coumel, 1994), and has been associated with increased risk of PACs, AF, and, more recently, with the presence of AHRE (Yılmaz, 2021). Whereas stimulation of cardiac *beta*-adrenoreceptors augments I_f, *alpha*-receptor stimulation has been shown to decrease I_{K1} activity. Together, these responses can augment automaticity in non-pacemaker atrial cells, leading to atrial ectopic activity, as demonstrated in rat pulmonary veins (Figure 6). (Maupoil et al. 2007) In

parallel, by increasing the activity of $I_{Ca\text{-}L}$ as well as that of phospholamban, sympathetic stimulation increases both calcium inflow through sarcolemmal voltage-gated calcium channels and calcium uptake by the SR, thereby promoting SR calcium overload. These changes, accompanied by simultaneous sympathetic-induced increase in RyR2 activity, leads to excessive intracellular calcium accumulation, with consequent activation of the sarcolemmal NCX. Due to its 3:1 stoichiometry, with inflow of three sodium ions for each ion of calcium expelled from the cell, NCX functioning is electrogenic, generating a net inward cation current that can underlie delayed afterdepolarizations-related ectopic activity (Linz et al., 2019). *Alpha*-adrenergic-induced inhibition of I_{K1} coupled with sympathetic-induced cardiomyocyte calcium overload thereby ensure both action potential prolongation and a net gain of cations, favoring the occurrence of early afterdepolarizations in atrial cells (Scridon et al., 2018).

Complex interactions exist between the two ANS limbs, and atrial ectopic firing is rarely purely sympathetic or vagally mediated. Studies have shown that sympathetic and parasympathetic coactivation often precedes atrial ectopic activity, in both clinical and experimental settings. An increase in cardiac sympathetic modulation minutes before, followed by parasympathetic activation immediately prior to arrhythmia onset have been reported in paroxysmal AF patients (Bettoni and Zimmermann, 2002). Simultaneous sympathetic and parasympathetic discharges have also been shown to precede paroxysmal AF onset in dog models of rapid atrial pacing (Tan et al., 2008) and heart failure (Ogawa et al., 2007), whereas bilateral cryoablation of the stellate ganglia and of the superior cardiac branches of the left vagus nerve eliminated all AF episodes (Bettoni and Zimmermann, 2002; Ogawa et al., 2007). Similarly, AF onset was preceded by synchronous sympathetic-parasympathetic activation in the study by Tan et al. (2008) Moreover, AF inducibility was significantly higher in that study when a combination of isoprenaline and acetylcholine was administered compared to acetylcholine alone (Tan et al., 2008), suggesting that sympathetic-vagal coactivation is more arrhythmogenic than vagal activation alone. The pathogenic pathway connecting sympatho-vagal coactivation with atrial ectopy is rather intuitive. Increased calcium transient caused by sympathetic activation accompanied by vagal-induced atrial effective refractory period shortening disrupts the balance between action potential duration and intracellular calcium transient, cardiac electrical features that are normally closely coupled (Amar et al., 2003). This further leads to increased NCX activity, thus favoring early afterdepolarizations and atrial triggered activity.

Gaps in evidence and future research

The clinical impact of AF is well known (Hindricks et al., 2021). Meanwhile, that of PACs, and particularly of AHRE, remains elusive. Electroanatomical mapping of AF recurrence following spontaneous or electrical restoration of sinus rhythm indicates PACs as the most common trigger of recurrent AF episodes (Haïssaguerre et al., 2000). Frequent PACs and runs of non-sustained atrial tachyarrhythmias have also been associated with increased risk of incident AF (Dewland et al., 2013; Nguyen et al., 2016; Christensen et al., 2017; Kerola et al., 2019) and of AF-related complications such as stroke and heart failure (O'Neal et al., 2017). In a rat model of spontaneous AF, short, non-sustained runs of AF have been associated with increased endocardial von Willebrand factor expression and intraatrial thrombosis (Scridon et al., 2013). Very short (i.e., ≤10–20 s/day) episodes of AHRE do not appear to be clinically relevant. Longer episodes (i.e., ≥5–6 min) have been associated with higher risk of clinically overt AF, stroke or systemic embolism, and cardiovascular events, including death. A linear relationship has been described between AHRE duration and risk of stroke (Hindricks et al., 2021). Whether AHRE are only a risk marker (not causally associated) or a risk factor of stroke *per se* remains a matter of debate. The potential clinical benefit of oral anticoagulation in these patients also remains unclear.

The role of autonomic imbalance in atrial ectopy occurrence has been well established over the years. Moreover, autonomic imbalance has been related to the presence and severity of symptoms (i.e., dizziness, presyncope, and syncope) in these patients (Linz et al., 2019). Pharmacologic or interventional ANS modulation could thus emerge as a viable target for maintenance of sinus rhythm as well as for symptoms control. Numerous questions still remain to be answered regarding the autonomic imbalance-atrial ectopy relationship.

Data regarding autonomic imbalance in animal models and patients with atrial ectopy indicate high inter- and intra-individual variability in the magnitude, the contribution, and even the direction of autonomic imbalance in this setting. Observational clinical studies yielded conflicting results, likely due to the inclusion of heterogeneous and insufficiently well characterized populations, usage of different assessment techniques, analysis of different parameters, and different criteria used to diagnose autonomic imbalance and atrial ectopic activity. Furthermore, although observational studies can provide invaluable insights into the ANS-atrial arrhythmias relationship, they cannot ascribe causality.

Animal studies can generally provide much more robust mechanistic insights. More complex and more accurate methods can be used in preclinical studies and, unlike in clinical studies, selective and specific sympathetic and parasympathetic manipulation can be applied in experimental settings (Scridon et al., 2012; Sayin et al., 2015; Scridon et al., 2021). However, caution should be used when extrapolating animal data to human patients. Interspecies differences should obviously be considered. More importantly, currently available experimental atrial arrhythmia models may not be the most appropriate for ANS assessment. Whereas most atrial arrhythmia patients are of

TABLE 1 Tests of cardiac autonomic function with potential usefulness in patients with atrial arrhythmias.

Test	Physiological meaning
Heart rate	Autonomic impact on the sinus node
Heart rate variability	Autonomic modulation of the sinus node
Heart rate recovery	Parasympathetic reactivation following cessation of physical exercise
Baroreflex sensitivity	Response of the sinus node to baroreceptor activation
Autonomic reflex testing (Ewing's tests)	Heart rate responses to physiological manipulations (i.e., breathing, handgrip, tilting, Valsalva maneuvers)
Plasma/urinary catecholamines levels	Total catecholamine spillover to plasma or urine
Transcardiac norepinephrine spillover	Cardiac spillover of norepinephrine
Sympathetic nerve recordings by microneurography	Evaluation of regional (muscle or skin) sympathetic output
PET/CT evaluation of cardiac autonomic nerves	Distribution and function of cardiac sympathetic nerves

TABLE 2 Cardiac neuromodulation interventions with potential applications in patients with atrial arrhythmias.

Pharmacological and biological interventions	Interventional neuromodulation techniques	
	Ablative/inhibition-based strategies	Stimulation-based strategies
Beta-adrenoreceptor blockade (metoprolol)	Surgical ablation of GP	Spinal cord stimulation
Central sympathetic inhibition (moxonidine)	Transcatheter endocardial and/or epicardial ablation of GP (with or without concomitant PVI)	Low-level cervical vagus nerve stimulation
RAAS blockade	Selective atrial vagal denervation	Low-level transcutaneous tragus stimulation
Colchicine	Ablation of extrinsic cardiac nerves	Carotid baroreceptor stimulation
Statins	Transcutaneous blockade of the stellate ganglia	—
Long-chain n-3 polyunsaturated fatty acids	Catheter-based renal sympathetic denervation	—
Probucol	—	—
Botulinum toxin	—	—
G-protein inhibitory peptides	—	—
Gene therapy (Gαi2 C- and Gα01 C-terminal peptide delivery)	—	—

GP, ganglionated plexi; PVI, pulmonary vein isolation; RAAS, renin-angiotensin-aldosterone system.

advanced age, have coexisting conditions, such as hypertension, heart failure, obesity, or ischemic heart disease, and are often on different cardioactive medications, experimental studies most commonly use juvenile, healthy, and medication-free animals. Such differences raise questions regarding the translational value of these preclinical models. Moreover, rapid atrial pacing is generally employed for mimicking atrial arrhythmias in experimental settings. Although highly efficient, pacing the atria at 1,000 bpm or more will inevitably produce autonomic changes, hindering our ability to accurately quantify the intensity of autonomic activation, the sympathetic-parasympathetic interactions, and their role in the occurrence of atrial arrhythmias. Future experimental studies will therefore have to employ more clinically relevant atrial arrhythmia models in order to allow better translation of the results to the clinical setting.

Contrary to the experimental settings, accurate evaluation of the ANS is highly challenging in clinical settings. Baroreflex sensitivity, heart rate recovery, heart rate responses to physiological manipulations (i.e., Ewing's tests), and particularly HRV analysis using continuous ECG recordings are techniques that are non-invasive and relatively easy to use (Table 1). However, the results may be affected by the presence of an implanted pacemaker, of AF, or other cardiac arrhythmias, and they provide only indirect information regarding ANS activity. One common feature of all these methods is that they all imply evaluation of ANS modulation of the sinus node, whereas the adverse effects of autonomic imbalance in atrial arrhythmias are mainly driven by its direct impact at the level of the atria. In addition, the results are affected by the presence of concomitant sinus node disease, which is rather common with advancing age or in patients with obesity, hypertension, obstructive sleep apnea, or heart failure (Piccirillo et al., 2009; Floras and Ponikowski, 2015; Linz et al., 2019). Moreover, the complex anatomic/physiological relationships within the cardiac

ANS, the complex interplay within this multilevel system (including multiple levels of feedback and excitatory/inhibitory control), regional autonomic effects that likely exist in the presence of cardiac autonomic neuropathy as well as possibly present in healthy subjects, raise questions about the ability of these markers to accurately quantify cardiac sympathetic/parasympathetic modulation or guide therapeutic management. These challenges explain why none of these techniques has entered the realm of clinical evaluation even after many decades of evaluation.

A plethora of other ANS evaluation methods have been developped over the years (Table 1), including measurement of circulating and urinary catecholamines levels, direct muscular or skin sympathetic nerve recordings by microneurography, measurement of transcardiac norepinephrine spillover, and PET/CT evaluation of autonomic nerves activity. Although such methods could provide a more precise estimation of ANS activity, these techniques are complex and difficult to use in routine clinical practice, require dedicated costly equipment, and are unavailable in most centers. Moreover, the clinical value of routine assessment using these methods in improving patient phenotyping and therapeutic management remains unclear. None are capable of assessing the ANS in its entirety. Future studies need to establish the potential value of these techniques and/or identify other non-invasive, easy to use markers to evaluate cardiac autonomic modulation, similarly to what has been achieved in other clinical settings (Scridon and Șerban, 2016; Delinière et al., 2019).

Finally, studies will have to establish the clinical impact of pharmacologic and interventional neuromodulation for rebalancing cardiac autonomic function in patients with atrial ectopy (Table 2). Modification of risk factors known to be associated with cardiac autonomic neuropathy, such as obesity, sedentarism, hypertension, diabetes mellitus, or obstructive sleep apnea has been shown to efficiently reduce AF burden, particularly when a global approach of risk factor modification is employed (Lau et al., 2017).

Beta-adrenoreceptor blockade using metoprolol, central sympathetic inhibition using moxonidine, RAAS blockade, colchicine, statins, long-chain n-3 polyunsaturated fatty acids, and probucol have all been shown to modulate sympathetic activity and to decrease atrial arrhythmogenicity in some clinical and preclinical settings (Kühlkamp et al., 2000; Naccarelli et al., 2007; Fauchier et al., 2008; Gong et al., 2009; Van Wagoner, 2011; Giannopoulos et al., 2014; Kerola et al., 2019), although none of them has, to date, demonstrated sufficient efficacy to become an established part of clinical practice for atrial arrhythmias prevention and/or treatment. More novel pharmacological and biological methods such as injection of botulinum toxin, which interferes with cholinergic neurotransmission, in the ganglionated plexi at the time of open-heart surgery and selective disruption of parasympathetic signaling using G-protein inhibitory peptides, have also shown promising effects in preclinical studies (Aistrup et al., 2009; Pokushalov et al., 2015), but confirmation in clinical settings is required. Alternative techniques, such as acupuncture, have also been tested, with some data indicating a potential benefit (Lombardi et al., 2012). Delivery to the posterior left atrium of plasmids containing cDNA for the Gαi2 C-terminal peptide, particularly when coupled with Gα01 C-terminal peptide delivery, led to massive, atrial-selective attenuation of vagal signaling, opening the way for genetic therapy in atrial arrhythmias and neuropathy (Bauer et al., 2004).

Surgical and transcatheter ablation of endocardial and/or epicardial ganglionated plexi, selective atrial vagal denervation, extrinsic cardiac nerves ablation, transcutaneous stellate ganglia blockade with lidocaine, spinal cord stimulation, renal sympathetic denervation, low-level cervical vagus nerve or tragus transcutaneous stimulation, and carotid baroreceptor stimulation using implantable devices have reduced AF burden in clinical and preclinical studies. Nevertheless, many results remain scarce or highly controversial, and long-term outcomes remain questionable (Lemery et al., 2006; Ogawa et al., 2009; Sakamoto et al., 2010; Bernstein et al., 2012; Zhao et al., 2012; Katritsis et al., 2013; Driessen et al., 2016; Goldberger et al., 2019). The long-term efficacy and safety, the optimal ablation/stimulation protocols, as well as the criteria for selecting the most adequate target population, for establishing the optimal time to intervene, and for confirming successful autonomic modification also remain to be defined. In addition, the long-term impact of these approaches on 'hard' clinical endpoints (e.g., stroke, heart failure, or mortality) remains unknown. Moreover, the impact of such approaches has never been tested in patients with frequent PACs or AHRE. If efficient, these techniques may reduce AF prevalence and, consequently, the burden that AF and AF-related complications impose on the healthcare systems. Adequately powered, well-conducted randomized controlled trials are needed to clarify all these issues.

Conclusion

The heart is one of the most richly innervated organs and the extrinsic and intrinsic cardiac ANS provides fine tuning of cardiac electrophysiology. Atrial ectopic activity can arise *via* multiple mechanisms promoted by sympathetic or vagal fluxes, or by a combination of both. Furthermore, a bidirectional relationship contributes to the pathogenesis of atrial arrhythmias, whereby autonomic imbalance promotes atrial arrhythmic events, which, in turn, promote atrial autonomic imbalance. Autonomic modulation has thus become a major target of investigation over the past decades. Combining aggressive risk factors management together with adjunct pharmacological or interventional strategies may provide a solution for rebalancing autonomic tone and for reducing atrial arrhythmic burden. Numerous questions need to be answered before reaching this

milestone. Techniques that are accurate, widely available, non-invasive, and easy-to-use remain to be developed and implemented in routine clinical practice. With very few exceptions, data regarding pharmacological and interventional neuromodulation strategies are limited, controversial, and mostly derived from small-scale studies. The cardiac ANS is unique in each individual and the autonomic imbalance-atrial arrhythmia relationship is highly complex. A personalized approach, using patient-specific, targeted correction of autonomic abnormalities may thus be needed to reduce the atrial arrhythmia burden. Future studies from basic and clinical laboratories will have to clarify the exact role of autonomic imbalance in atrial arrhythmogenesis, to elucidate whether interventions targeting specific components of cardiac autonomic innervation can improve atrial arrhythmias management, and to identify the optimal time and means for intervention.

Author contributions

AS contributed to conception and design of the work and drafted the manuscript.

Funding

This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS-UEFISCDI, project number PN-III-P1-1.1-TE-2019-0370, within PNCDI III.

Conflict of interest

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

Publisher's note

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Neutrophil gelatinase-associated lipocalin monitoring reveals persistent subclinical kidney injury following intraarterial administration of iodinated contrast agents

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Clinically overt contrast-induced nephropathy (CIN) is one of the most feared complications in patients exposed to iodinated contrast media and has been extensively studied over the years. Meanwhile, the incidence and evolution of subclinical contrast-induced kidney injury remain elusive. With the continuous increase in the number of patients that are repeatedly exposed to contrast media, elucidating these issues is of critical importance. Accordingly, we aimed to evaluate the incidence and the evolution of clinical and subclinical kidney injury in patients exposed to contrast media. A total of 178 patients who underwent elective percutaneous angioplasty procedures were evaluated prospectively. Serum creatinine and neutrophil gelatinase-associated lipocalin (NGAL) levels were evaluated pre-procedurally, 48 h and 1 month after administration of contrast media. The evolution of creatinine and NGAL levels was analyzed at the three time points, and the potential predictors of contrast-induced clinical and subclinical renal injury were evaluated. Clinically overt CIN occurred in 10 (5.6%) patients. Baseline serum creatinine and the volume of contrast media were the only independent predictors of CIN and in all 10 patients creatinine levels returned to baseline by 1 month (p = 0.32). Subclinical contrast-induced kidney injury was much more common, affecting 32 (17.9%) patients, was only predicted by the baseline serum creatinine, and persisted in 53.1% of patients after 1 month. This study showed that whereas clinically overt CIN is rather rare and regressive, subclinical contrast-induced kidney injury is considerably more frequent, affecting almost 18% of patients that receive intraarterial contrast media. More importantly, subclinical kidney injury persisted after 1 month in more than 50% of the initially affected patients, who may thus be at increased risk for further renal impairment, particularly if exposed to nephrotoxic agents or repeated administration of contrast media.

Abbreviations

CIN	Contrast-induced nephropathy
eGFR	Estimated glomerular filtration rate
NGAL	Neutrophil gelatinase-associated lipocalin

In parallel with the major advancements in diagnostic and therapeutic imaging techniques, the number of patients exposed to iodinated contrast agents has tremendously increased over the past decades[1]. One of the

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most feared complications in these patients is contrast-induced nephropathy (CIN). The use of less nephrotoxic agents has significantly reduced this risk. Yet, the risk of CIN remains considerable (2–25%) in this population, with up to 0.5% of patients requiring dialysis[2].

Currently, the diagnosis of CIN relies on a relative and/or absolute increase in serum creatinine following contrast administration, reflecting an acute impairment in kidney function[3]. The same criteria, based on altered creatinine levels, suggest that CIN is most commonly a transient process that is usually followed by full restoration of kidney function within 7–14 days. However, the exact effects of contrast administration on the kidneys remain incompletely elucidated, particularly over the long term.

Serum creatinine alone provides only a rough estimation of the impact of contrast agents on the kidneys and is incapable to exclude the persistence of a degree of subclinical kidney injury over the long term[4]. Pathophysiologically, persistence of at least a certain degree of tubular damage following contrast administration seems highly plausible. The occurrence of CIN has been linked mechanistically to vasoconstriction, followed by renal hypoperfusion and hypoxia, cytotoxicity caused by increased local production of reactive oxygen species, and direct tubular toxicity, characterized by vacuolization and necrosis of kidney tubular cells[5]. Episodes of ischemia–reperfusion similar to those seen in CIN have also been related to subsequent loss of peritubular capillaries and progressive tubular fibrosis[6]. Together, these observations strongly suggest that renal injury associated with contrast administration may not be entirely transient and that biomarkers more sensitive than creatinine may be needed to fully understand the impact of contrast agents on the kidneys.

Neutrophil gelatinase-associated lipocalin (NGAL), a glycoprotein that is rapidly released into the bloodstream in response to renal tubular injury[7], appears to be a promising candidate in this regard. Unlike serum creatinine, which provides a rather late and strictly functional reflection of renal injury, NGAL is an early marker of tubular damage that can unmask the presence and the evolution of renal injury even in the absence of a significant functional impairement[4]. With the widespread use of procedures that rely on contrast administration, many patients are likely to receive repeated doses of contrast agents throughout their lives. If contrast-induced kidney injury proves to be persistent, even at a subclinical level, such an effect could become highly relevant in the following decades.

Accordingly, in the present study, we aimed (1) to evaluate the incidence of clinical and subclinical kidney injury in patients exposed to iodinated contrast agents and (2) to assess the evolution of clinical/subclinical kidney injury in these patients over the long term.

Methods

Study population. Consecutive patients who underwent elective angioplasty procedures in our center between January 2020 and November 2021 were evaluated in a prospective observational study. All patients included in the study were ≥ 18 years of age, were admitted to hospital for an elective angioplasty procedure, and had an estimated glomerular filtration rate (eGFR) > 30 mL/min/1.73 m^2. Exclusion criteria included acute severe conditions, regardless of their nature, ongoing non-steroidal antiinflammatory or antibiotic therapy, exposure to contrast media over the past 6 months, dialysis or history of kidney transplantation, multiple myeloma, and lymphoplasmacytic lymphoma. The research protocol complied with the Declaration of Helsinki and was approved by the local Ethics Committees of the Emergency Institute for Cardiovascular Diseases and Transplantation Târgu Mureș (approval number 7545) and of the University of Medicine, Pharmacy, Science and Technology "George Emil Palade" of Târgu Mureș (approval number 230). All patients gave written informed consent to participate in the study.

Evaluated parameters. *Baseline evaluation.* Age, gender, body mass index, left ventricular ejection fraction assessed by transthoracic echocardiography, associated conditions (i.e., arterial hypertension, diabetes mellitus, heart failure), and ongoing therapy with potentially nephrotoxic (i.e., angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, metformin) and/or nephroprotective (i.e., methylxanthines, statins, ascorbic acid, N-acetyl cysteine, dihydropyridine calcium channel blockers) drugs were recorded for each patient on admission. Arterial hypertension was considered present if a diagnosis of hypertension was present in the patient's medical records, was established during hospital stay, in accordance with current guidelines, or if the patient was under ongoing antihypertensive therapy. Pre-procedural hydration status was evaluated in all patients based on the maximum inferior vena cava diameter measured by echocardiography in subcostal 4-chamber view. Venous blood samples were collected from each patient prior to the angioplasty procedure and total blood count, hemoglobin, plasma glucose and lipids, total protein and albumin, and uric acid levels were evaluated using standardized laboratory tests. Serum creatinine was measured using a modified buffered kinetic Jaffe reaction and eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation. Serum levels of NGAL were measured using an enzyme-linked immunosorbent assay (Biovendor, Czech Republic; intra- and inter-assay variation coefficients 7.0% and 9.8%, respectively) on the Elisa Dynex DSX fully automated Elisa analyzer (DYNEX Technologies, Inc.; Chantilly, VA). The volume and type (i.e., iomeprol or ioversol) of contrast agent administered were also recorded.

Evaluation at 48 h and at 1 month following the angioplasty procedure. The Mehran score[8] was calculated for each patient 24 h after the angioplasty procedure. Administration of potentially nephrotoxic and/or nephroprotective drugs, as listed above, during the 48 h after the angioplasty procedure was recorded for each patient. Occurrence of death, cardiogenic shock, acute pulmonary edema, the need for dialysis and/or positive inotrope agents during the 48 h after the procedure, and the length of hospital stay were also recorded. A second venous blood sample was collected from each patient 48 h after the angioplasty procedure and the serum levels of cre-

atinine, total protein and albumin, uric acid, sodium, and NGAL were measured as described above. The eGFR was calculated using the MDRD equation.

Administration of potentially nephrotoxic and/or nephroprotective drugs and the occurrence of death, interventions requiring repeated contrast administration, hospitalizations, and the need for dialysis during the first month following the angioplasty procedure were also recorded. A third blood sample was collected from each patient 30 days after the angioplasty procedure and serum creatinine, total protein and albumin, uric acid, and NGAL levels were measured as described above. The eGFR was calculated using the MDRD equation.

Outcomes definitions. In accordance with current recommendations[9], CIN was defined as an increase in serum creatinine by at least 25% 48 h following contrast administration *vs*. baseline. Early subclinical kidney injury was defined as an increase in NGAL by at least 25% 48 h following contrast administration compared to baseline. Kidney dysfunction/injury was considered persistent if serum creatinine or NGAL levels, respectively, remained at least 25% higher 1 month following contrast administration compared to baseline in patients in whom early CIN or subclinical kidney injury was initially detected.

Statistical analysis. Continuous variables are presented as mean values ± standard deviation or median and interquartile range, as appropriate. Categorical data are summarized using frequencies and percentages. The occurrence of early (i.e., at 48 h after contrast administration) kidney dysfunction/injury was assessed by comparing renal parameters (i.e., serum creatinine and NGAL) measured at 48 h after contrast administration *vs*. baseline using the paired *t*-test or the Wilcoxon matched-pairs signed-ranks test, as appropriate.

The patients were divided into groups depending on the presence or absence of CIN and on the presence or absence of subclinical kidney injury, respectively, and all parameters were compared between groups using the unpaired *t*-test or the Mann–Whitney *U* test (for continuous variables), or Fisher's exact test (for categorical data). Logistic regression analysis was used to assess predictors of CIN and of early subclinical kidney injury. The models were adjusted for parameters that differed significantly between the groups. For the continuous variables, the cutoff values were established using receiver operating characteristic analysis. The evolution of kidney dysfunction/injury at 1 month was evaluated in patients who presented CIN or early kidney injury by comparing renal parameters (i.e., serum creatinine and NGAL) measured at 1 month vs. those measured at 48 h after contrast administration using the paired *t*-test or the Wilcoxon matched-pairs signed-ranks test, as appropriate. All tests were two-sided, and a p-value of less than 0.05 was considered statistically significant. All data were computed using MedCalc for Windows, version 12.4.3.0 (MedCalc Software; Ostend, Belgium).

Results

Study population characteristics. A total of 178 patients (mean age 63.2 ± 9.2 years; 69.1% male) were included in the present study. Pre-procedural intravenous hydration (saline solution, 1 ml/kg/h, 12 h before and 12 h after the procedure) was used in 35 (19.6%) of the study patients. All patients were evaluated at baseline and at 48-h follow-up and 63 of the 178 study patients completed the 1-month follow-up. Patients' characteristics at the three time points are summarized in Table 1.

Early (48 h) and late (1 month) post-contrast administration kidney dysfunction/injury. There was no significant difference in serum creatinine levels measured 48 h (1.01 ± 0.39 mg/dL) or 1 month (0.96 ± 0.24 mg/dL) following contrast administration vs. baseline (1.05 ± 0.36; both $p > 0.05$). Consequently, no significant difference was recorded in eGFR in neither of the two moments (i.e., 48 h and 1 month after contrast administration) vs. baseline (both $p > 0.05$). The same results were obtained when NGAL levels were assessed comparatively at the three time points (both $p > 0.05$).

Overall, 10 (5.6%) of the 178 study patients fulfilled the criteria for CIN 48 h after contrast administration and in all of them creatinine levels returned to baseline values by the 1-month follow-up ($p = 0.32$). Compared to patients who did not develop CIN, those who presented CIN (Table 2) had higher serum creatinine levels ($p = 0.03$), lower eGFR ($p = 0.02$), and lower left ventricular ejection fraction ($p < 0.01$) on admission, were more often anemic ($p = 0.001$) and on diuretic treatment (RR 29.3 [95%CI 1.6–50.9]; $p < 0.001$) on admission, received higher volumes of contrast media ($p = 0.01$), and had longer duration of hospital stay ($p < 0.01$). However, in the logistic regression analysis, only a baseline serum creatinine > 1.62 mg/dL (and a baseline eGFR ≤ 63 mL/min/1.73 m^2; Supplementary material) and a volume of contrast agent administered > 180 mL remained independent predictors of CIN (Table 3).

Early subclinical kidney injury, defined as ≥ 25% increase in NGAL levels at 48 h after contrast administration compared to baseline, was identified in 32 (17.9%) of the 178 study patients. Only 3 of these 32 patients presented CIN, defined as ≥ 25% increase in serum creatinine levels 48 h after contrast administration compared to baseline, whereas in the other patients there was no significant change in serum creatinine ($p = 0.32$). One month following contrast administration, NGAL levels remained stationary (79.75 ± 31.91 ng/mL *vs.* 76.64 ± 39.64 ng/mL, $p = 0.14$) and significantly positively correlated ($r = 0.73$, 95%CI 0.61–0.86; $p < 0.01$) with those measured 48 h after contrast administration. Subclinical kidney injury was still present 1 month after contrast administration in 17 (26.9%) of the 63 patients that underwent the 1-month follow-up. All those 17 patients were among the 32 patients who presented early subclinical kidney injury (53.1%); in 9 (28.1%) of those 32 patients the renal injury regressed after 1 month, and 6 (18.7%) of those patients were lost of follow-up.

Compared to patients who did not develop early subclinical kidney injury, those who presented an increase in NGAL levels ≥ 25% 48 h after contrast administration *vs.* baseline (Table 4) had higher serum creatinine and lower eGFR on admission (both $p < 0.01$), and higher total protein levels (both on admission [$p = 0.04$] and at the 48-h follow-up [$p = 0.001$]), had higher Mehran scores ($p = 0.02$) and lower sodium levels at the 48 h follow-up

Baseline characteristics (n = 178)	
Age (years)	63.2 ± 9.2
Male gender (n, %)	123 (69.1%)
Body mass index (kg/m^2)	28.8 ± 4.1
Left ventricular ejection fraction on admission (%)	50.8 ± 9.1
Comorbidities	
Arterial hypertension (n, %)	162 (91.0%)
Diabetes mellitus (n, %)	80 (44.9%)
Heart failure (NYHA class)	2 (2–2)
Chronic kidney disease (n, %)	32 (17.9%)
Ongoing therapy with potentially nephrotoxic drugs	
ACEI (n, %)	116 (65.1%)
ARB (n, %)	39 (21.9%)
Diuretic (n, %)	80 (44.9%)
Metformin (n, %)	43 (24.1%)
Ongoing therapy with potentially nephroprotective drugs	
Methylxanthine (n, %)	0 (0%)
Statin (n, %)	176 (98.8%)
Ascorbic acid (n, %)	0 (0%)
N-acetyl cysteine (n, %)	0 (0%)
Dihydropyridine calcium channel blocker (n, %)	73 (41.0%)
Inferior vena cava diameter (cm)	1.9 ± 0.2
Laboratory parameters on admission	
White blood cells (/mm^3)	7669 ± 1928
Platelets (/mm^3)	234,662 ± 67,053
Hemoglobin (g/dL)	13.6 ± 1.6
Glucose (mg/dL)	141.4 ± 63.7
Total cholesterol (mg/dL)	162.0 ± 48.5
Triglycerides (mg/dL)	161.0 ± 101.7
Total protein (g/dL)	66.0 ± 4.8
Albumin (g/dL)	4.3 ± 0.2
Uric acid (mg/dL)	6.2 ± 1.7
Serum creatinine (mg/dL)	1.05 ± 0.36
eGFR (mL/min/1.73 m^2)	76.31 ± 23.43
NGAL (ng/mL)	75.79 ± 26.46
Contrast agent administered	
Type-iomeprol (n, %)	153 (85.9%)
Volume (mL)	148.5 ± 68.2
Pre-procedural intravenous hydration (n, %)	35 (19.6%)
Parameters at 48 h after contrast administration (n = 178)	
Mehran score (points)	4 (1–6)
Therapy with potentially nephrotoxic drugs during the past 48 h	
ACEI (n, %)	114 (64.0%)
ARB (n, %)	43 (24.1%)
Diuretic (n, %)	84 (47.1%)
Metformin (n, %)	43 (24.1%)
Therapy with potentially nephroprotective drugs during the past 48 h	
Methylxanthine (n, %)	0 (0%)
Statin (n, %)	176 (98.8%)
Ascorbic acid (n, %)	0 (0%)
N-acetyl cysteine (n, %)	0 (0%)
Dihydropyridine calcium channel blocker (n, %)	80 (44.9%)
Complications during the past 48 h	
Death (n, %)	0 (0%)
Cardiogenic shock (n, %)	0 (0%)
Acute pulmonary edema (n, %)	0 (0%)
Dialysis (n, %)	0 (0%)
Continued	

Positive inotrope agents (n, %)	0 (0%)
Length of hospital stay (days)	4 (3–6)
Laboratory parameters 48 h after contrast administration	
Total protein (g/dL)	65.5 ± 5.1
Albumin (g/dL)	4.3 ± 0.3
Uric acid (mg/dL)	6.0 ± 1.6
Serum creatinine (mg/dL)	1.01 ± 0.39
eGFR (mL/min/1.73 m^2)	80.17 ± 23.49
NGAL (ng/mL)	76.64 ± 39.64
Parameters 1 month after contrast administration (n = 63)	
Therapy with potentially nephrotoxic drugs during the past month	
ACEI (n, %)	47 (74.6%)
ARB (n, %)	11 (17.4%)
Diuretic (n, %)	24 (38.0%)
Metformin (n, %)	16 (25.3%)
Therapy with potentially nephroprotective drugs during the past month	
Methylxanthine (n, %)	0 (0%)
Statin (n, %)	60 (95.2%)
Ascorbic acid (n, %)	0 (0%)
N-acetyl cysteine (n, %)	0 (0%)
Dihydropyridine calcium channel blocker (n, %)	32 (50.7%)
Complications during the past month	
Death (n, %)	0 (0%)
Repeated contrast administration (n, %)	0 (0%)
Hospitalization (n, %)	0 (0%)
Dialysis (n, %)	0 (0%)
Laboratory parameters 1 month after contrast administration	
Total protein (g/dL)	70.7 ± 5.9
Albumin (g/dL)	4.5 ± 0.2
Uric acid (mg/dL)	5.7 ± 1.2
Serum creatinine (mg/dL)	0.96 ± 0.24
eGFR (mL/min/1.73 m^2)	72.6 ± 16.3
NGAL (ng/mL)	79.75 ± 31.91

Table 1. Characteristics of patients included in the study. eGFR was calculated using the Modification of Diet in Renal Disease equation. Quantitative data are expressed as mean values ± standard deviation or median and interquartile range, as appropriate. Categorical data are expressed as number (percentage). *ACEI* angiotensin converting enzyme inhibitor, *ARB* angiotensin II receptor blocker, *eGFR* estimated glomerular filtration rate, *NGAL* neutrophil gelatinase-associated lipocalin, *NYHA* New York Heart Association.

($p = 0.02$), but there was no significant between-groups difference in the volume of contrast media administered ($p = 0.67$). In the logistic regression analysis, a baseline serum creatinine > 1.37 mg/dL (and a baseline eGFR ≤ 54 mL/min/1.73 m^2; Supplementary material) remained the only independent predictor of early subclinical kidney injury (Table 5). None of the tested parameters was significantly different between patients who presented and those who did not present late subclinical kidney injury at the 1-month follow-up (all $p > 0.05$).

Discussion

The main findings of the present study (Fig. 1) were that (1) clinically overt CIN was rather rare, affecting 5.6% of the study population, (2) its occurrence was only affected by the baseline kidney function and the volume of contrast administered, and that (3) it was entirely regressive at the 1-month follow-up. Meanwhile, (4) subclinical kidney injury was considerably more common, affecting almost 18% of the study patients at the 48-h follow-up, (5) was independently predicted only by the baseline kidney function, and (6) was persistent at the 1-month follow-up in more than 50% of the initially affected patients.

In the era of modern iodinated contrast media, clinically overt contrast-induced nephropathy is rather rare and entirely regressive after 1 month. With the progressive increase in the prevalence of atherosclerotic disease, the rate of angioplasty procedures is on a continuous rise worldwide[1]. One of the most feared complications of such procedures is CIN. However, our data indicate that in the era of modern iodinated contrast agents, the incidence of CIN is rather low, affecting 5.6% of the study patients.

Several large-scale studies have even questioned the concept of CIN and the relationship between contrast media administration and acute kidney injury[10]. Indeed, studies have shown similar rates of acute kidney injury

Parameter	CIN (n = 10)	No CIN (n = 168)	p-value
Baseline characteristics			
Age (years)	66.7 ± 5.5	63.4 ± 9.3	0.49
Male gender (n, %)	8 (80.0%)	115 (68.4%)	0.72
Body mass index (kg/m^2)	28.3 ± 6.7	29.0 ± 4.0	0.93
Left ventricular ejection fraction on admission (%)	37.5 ± 19.3	51.4 ± 7.8	< 0.01
Comorbidities			
Arterial hypertension (n, %)	10 (100.0%)	152 (90.4%)	0.60
Diabetes mellitus (n, %)	5 (50.0%)	75 (44.6%)	0.75
Heart failure (NYHA class)	2 (1.5–2.5)	2 (2.0–2.0)	0.64
Ongoing therapy with potentially nephrotoxic drugs			
ACEI (n, %)	8 (80.0%)	108 (64.2%)	0.49
ARB (n, %)	2 (20.0%)	37 (22.0%)	1.00
Diuretic (n, %)	10 (100.0%)	70 (41.6%)	< 0.001
Metformin (n, %)	2 (20.0%)	41 (24.4%)	1.00
Ongoing therapy with potentially nephroprotective drugs			
Statin (n, %)	10 (100.0%)	166 (93.2%)	1.00
Dihydropyridine calcium channel blocker (n, %)	2 (20.0%)	71 (42.2%)	0.20
Inferior vena cava diameter (cm)	1.8 ± 0.1	2.0 ± 0.2	0.37
Laboratory parameters on admission			
White blood cells (/mm^3)	8430 ± 977	7696 ± 1991	0.19
Platelets (/mm^3)	179,250 ± 95,175	239,053 ± 65,638	0.23
Hemoglobin (g/dL)	13.3 ± 0.9	13.6 ± 1.7	0.62
Anemia (n, %)	8 (80.0%)	45 (26.7%)	0.001
Glucose (mg/dL)	172.7 ± 72.0	140.9 ± 63.1	0.19
Total cholesterol (mg/dL)	148.5 ± 36.1	162.9 ± 49.0	0.70
Triglycerides (mg/dL)	153.0 ± 69.4	156.7 ± 94.1	0.76
Total protein (g/dL)	70.4 ± 4.1	65.6 ± 4.8	0.09
Albumin (g/dL)	4.6 ± 0.4	4.3 ± 0.2	0.19
Uric acid (mg/dL)	7.7 ± 3.2	6.1 ± 1.5	0.39
Serum creatinine (mg/dL)	1.43 ± 0.15	1.02 ± 0.34	0.03
eGFR (mL/min/1.73 m^2)	58.25 ± 26.84	78.68 ± 23.19	0.02
Pre-procedural intravenous hydration (n, %)	3 (30.0%)	32 (19.0%)	0.41
Contrast agent administered			
Type-iomeprol (n, %)	8 (80.0%)	145 (86.3%)	0.63
Volume (mL)	255.0 ± 108.4	140.0 ± 61.6	0.01
Parameters at 48 h after contrast administration			
Mehran score (points)	7 (2.5–13.0)	4 (1.0–6.0)	0.27
Therapy with potentially nephrotoxic drugs during the past 48 h			
ACEI (n, %)	8 (80.0%)	106 (63.0%)	0.33
ARB (n, %)	2 (20.0%)	41 (24.4%)	1.00
Diuretic (n, %)	10 (100.0%)	74 (44.0%)	< 0.001
Metformin (n, %)	2 (20.0%)	41 (24.4%)	1.00
Therapy with potentially nephroprotective drugs during the past 48 h			
Statin (n, %)	10 (100.0%)	166 (93.2%)	1.00
Dihydropyridine calcium channel blocker (n, %)	2 (20.0%)	78 (46.4%)	0.19
Length of hospital stay (days)	10.0 ± 3.5	4.7 ± 2.1	< 0.01
Laboratory parameters 48 h after contrast administration			
Total protein (g/dL)	65.5 ± 8.4	65.3 ± 5.1	0.82
Albumin (g/dL)	4.3 ± 0.6	4.2 ± 0.3	0.57
Uric acid (mg/dL)	7.7 ± 1.6	5.9 ± 1.6	0.07

Table 2. Characteristics of patients who developed *versus* those who did not develop contrast-induced nephropathy. Contrast-induced nephropathy (CIN) was defined as an increase in serum creatinine by at least 25% 48 h following contrast administration *vs.* baseline. Anemia was considered present when hemoglobin was < 13 g/dL and/or the hematocrit was < 39% in males, and when hemoglobin was < 12 g/dL and/or the hematocrit was < 36% in females, respectively. Quantitative data are expressed as mean values ± standard deviation or median and interquartile range, as appropriate. Categorical data are expressed as number (percentage). p-values refer to comparisons between patients with and without CIN based on the unpaired *t*-test or the Mann–Whitney *U* test, as appropriate, for continuous variables, and Fisher's exact test for categorical variables. *ACEI* angiotensin converting enzyme inhibitor, *ARB* angiotensin II receptor blocker, *CIN* contrast-induced nephropathy, *eGFR* estimated glomerular filtration rate, *NYHA* New York Heart Association.

Parameter	OR (95%CI)	p-value
Left ventricular ejection fraction on admission ≤ 45%[a]	3.80 (0.24–58.84)	0.58
Diuretic therapy on admission	1.48 (0.27–88.53)	0.99
Anemia on admission	2.31 (0.11–45.61)	0.45
Volume of contrast agent administered > 180 mL[a]	4.78 (2.06–20.53)	0.001
Serum creatinine on admission > 1.62 mg/dL[a]	6.40 (2.50–25.20)	0.03

Table 3. Logistic regression analysis of predictors of contrast-induced nephropathy. Contrast-induced nephropathy was defined as an increase in serum creatinine by at least 25% 48 h following contrast administration *vs*. baseline. Anemia was considered present when hemoglobin was < 13 g/dL and/or the hematocrit was < 39% in males, and when hemoglobin was < 12 g/dL and/or the hematocrit was < 36% in females, respectively. [a]Cutoff values for left ventricular ejection fraction on admission, volume of contrast agent administered, and serum creatinine on admission were established using receiver operating characteristic analysis.

in patients undergoing computed tomography scanning with and without intravenous administration of contrast media, and suggested that the risk of CIN may essentially be nonexistent in patients with normal baseline renal function[11]. The relationship between contrast media administration and CIN appears to be, however, rather solid, when the contrast agent is administered intraarterially[12]. Several hypotheses have been proposed to explain this discordance. Patients that undergo angiography procedures have more severe arterial disease than those who require intravenous administration of contrast media and are therefore at higher risk of acute kidney injury. Manipulation of the aorta could lead to dislodgement of cholesterol crystals, atherosclerotic plaque fragments, or thrombi, and to renal microembolization and could therefore promote kidney injury during intraarterial procedures[13]. Moreover, intraarterial administration of contrast media, particularly in the abdominal aorta, above the level of the renal arteries, is associated with higher contrast concentrations in the renal vasculature[12]. Together, these data suggest that the risk of CIN is therefore not directly linked to the route of contrast administration, but rather to the patients' comorbid conditions, the characteristics of the procedure, and the volumes of contrast media administered.

Indeed, several risk factors have been associated with an increased risk of CIN. Among them, preexisting renal disease has been proposed as the most relevant risk factor for CIN. A linear relationship has been shown to exist between baseline serum creatinine and the risk of CIN, with an incidence of contrast-induced acute kidney injury of up to 62% in patients with preexisting chronic kidney disease and serum creatinine ≥ 2 mg/dL[14]. Due to the major impact that diabetes mellitus exhibits on the renal and cardiovascular systems[15], this condition was also seen as a major non-modifiable risk factor for CIN[16]. Several other parameters, such as advanced age[16], female gender[17], history of congestive heart failure[18], anemia[19], hyperuricemia[20], hypercholesterolemia[14], and the use of nephrotoxic drugs[21], have also been linked to an increased risk of CIN in various clinical studies, although their impact remains highly controversial[12]. Multiple sources have also reported a dose-dependent relationship between the volume of contrast administered and the risk of CIN[22]. In the present study, only basal renal function, as reflected by the baseline serum creatinine, and an increased volume of contrast administered were independently associated with an increased risk of CIN. For other parameters that were associated with CIN in univariate analysis (i.e., left ventricular ejection fraction, ongoing diuretic treatment, and anemia), the association was lost in the multiple logistic regression analysis.

In accordance with previous studies[23], clinically overt CIN was a transient, reversible event in the present study, and was associated with more prolonged hospital stay.

Subclinical kidney injury is rather common after administration of iodinated contrast agents and persists in more than half of patients at 1-month follow-up. Clinically overt renal dysfunction related to contrast administration therefore appears to be entirely regressive in one month. However, mechanistically, it seems unlikely that contrast-induced renal injury could be fully devoid of any long-term impact. The pathophysiology of CIN remains incompletely elucidated at this point. The most accepted theory involves contrast-induced vasoconstriction, leading to renal hypoxia[24], increased production of oxygen-free species, and subsequently to renal injury[25]. Other factors, including a rise in blood viscosity, changes induced by the contrast media on the renal blood supply[26], ischemia–reperfusion injury, release of angiotensin II, dopamine, and vasopressin, and a direct cytotoxic effect of contrast agents on the renal tubular cells have also been shown to contribute to the deleterious effects exhibited by contrast media on the kidneys[16]. These mechanisms strongly suggest that contrast agents may not be entirely innocuous over the long term and that, similar to other clinical settings[27,28], measurement of biomarkers more sensitive than serum creatinine may be required to detect subtle renal changes in this setting. The diagnosis of CIN relies at present on measurement of serum creatinine. This approach has, however, several limitations, including the delayed and non-linear response to renal impairment of serum creatinine and its sensitivity to numerous non-renal factors, such as age, gender, diet, medication, muscle mass, hydration status, and volume of intravascular fluid[29]. Moreover, creatinine is a marker of glomerular filtration, and not a marker of tubular damage, which is the injury typical for CIN. Meanwhile, NGAL has been proposed as one of the most promising biomarkers of renal structural injury[30]. Unlike serum creatinine, NGAL is produced by the distal nephron and rapidly released into the bloodstream, which makes NGAL a much more sensitive marker of kidney injury[31]. Studies have also pointed NGAL as an earlier marker of kidney injury than

Parameter	Early injury (n = 32)	No early injury (n = 146)	p-value
Baseline characteristics			
Age (years)	65.5 ± 10.3	63.5 ± 9.2	0.51
Male gender (n, %)	24 (75.0%)	99 (67.8%)	0.52
Body mass index (kg/m^2)	29.9 ± 3.4	27.9 ± 3.2	0.10
Left ventricular ejection fraction on admission (%)	49.1 ± 11.2	50.8 ± 9.2	0.73
Comorbidities			
Arterial hypertension (n, %)	29 (90.6%)	133 (91.0%)	1.00
Diabetes mellitus (n, %)	16 (50.0%)	64 (43.8%)	0.56
Heart failure (NYHA class)	2 (1.5–2.0)	2 (2.0–2.0)	0.60
Ongoing therapy with potentially nephrotoxic drugs			
ACEI (n, %)	21 (65.6%)	95 (65.0%)	1.00
ARB (n, %)	11 (34.3%)	28 (19.1%)	0.09
Diuretic (n, %)	19 (59.3%)	61 (41.7%)	0.07
Metformin (n, %)	11 (34.3%)	32 (21.9%)	0.17
Ongoing therapy with potentially nephroprotective drugs			
Statin (n, %)	31 (96.8%)	145 (99.3%)	0.32
Dihydropyridine calcium channel blocker (n, %)	13 (40.6%)	60 (41.0%)	1.00
Inferior vena cava diameter (cm)	1.9 ± 0.1	2.0 ± 0.1	0.42
Laboratory parameters on admission			
White blood cells (/mm^3)	7525 ± 2052	7653 ± 1915	0.89
Platelets (/mm^3)	230,583 ± 73,775	227,698 ± 66,697	0.89
Hemoglobin (g/dL)	13.6 ± 1.6	13.3 ± 1.6	0.52
Anemia (n, %)	11 (34.3%)	42 (28.7%)	0.52
Glucose (mg/dL)	147.9 ± 70.8	141.5 ± 63.6	0.88
Total cholesterol (mg/dL)	163.7 ± 53.6	162.5 ± 49.6	0.92
Triglycerides (mg/dL)	146.4 ± 55.2	162.3 ± 112.9	0.98
Total protein (g/dL)	68.7 ± 5.9	65.6 ± 4.3	0.04
Albumin (g/dL)	4.3 ± 0.3	4.3 ± 0.2	0.94
Uric acid (mg/dL)	6.3 ± 1.6	6.1 ± 1.7	0.73
Serum creatinine (mg/dL)	1.30 ± 0.25	1.00 ± 0.24	< 0.01
eGFR (mL/min/1.73 m^2)	66.58 ± 23.50	77.40 ± 19.49	< 0.01
Pre-procedural intravenous hydration (n, %)	9 (28.1%)	26 (17.8%)	0.21
Contrast agent administered			
Type-iomeprol (n, %)	29 (90.6%)	124 (84.9%)	0.57
Volume (mL)	153.3 ± 88.8	149.2 ± 62.1	0.67
Parameters at 48 h after contrast administration			
Mehran score (points)	6 (3.5–8.0)	4 (1.0–6.0)	0.02
Therapy with potentially nephrotoxic drugs during the past 48 h			
ACEI (n, %)	21 (65.6%)	93 (63.6%)	1.00
ARB (n, %)	11 (34.3%)	32 (21.9%)	0.17
Diuretic (n, %)	19 (59.3%)	65 (44.5%)	0.17
Metformin (n, %)	8 (25.0%)	35 (23.9%)	1.00
Therapy with potentially nephroprotective drugs during the past 48 h			
Statin (n, %)	31 (96.8%)	145 (99.3%)	0.32
Dihydropyridine calcium channel blocker (n, %)	13 (40.6%)	67 (45.8%)	0.69
Length of hospital stay (days)	5.3 ± 3.0	4.9 ± 2.4	0.79
Laboratory parameters 48 h after contrast administration			
Sodium (mEq/L)	139.7 ± 1.7	141.5 ± 2.2	0.02
Total protein (g/dL)	69.6 ± 5.8	64.6 ± 4.4	0.001
Albumin (g/dL)	4.4 ± 0.4	4.2 ± 0.3	0.30
Uric acid (mg/dL)	6.5 ± 1.9	5.9 ± 1.6	0.22

Table 4. Characteristics of patients who developed *versus* those who did not develop early subclinical kidney injury. Early subclinical kidney injury (early injury) was defined as an increase in NGAL by at least 25% 48 h following contrast administration *vs.* baseline. Anemia was considered present when hemoglobin was < 13 g/dL and/or the hematocrit was < 39% in males, and when hemoglobin was < 12 g/dL and/or the hematocrit was < 36% in females, respectively. Quantitative data are expressed as mean values ± standard deviation or median and interquartile range, as appropriate. Categorical data are expressed as number (percentage). p-values refer to comparisons between patients with and without early subclinical kidney injury based on the unpaired *t*-test or the Mann–Whitney *U* test, as appropriate, for continuous variables, and Fisher's exact test for categorical variables. *ACEI* angiotensin converting enzyme inhibitor, *ARB* angiotensin II receptor blocker, *eGFR* estimated glomerular filtration rate, *NYHA* New York Heart Association.

Parameter*	OR (95%CI)	p-value
Total protein on admission > 68.2 g/dL	5.68 (0.87–36.76)	0.06
Mehran score > 4	4.22 (0.25–15.37)	0.12
Sodium level at the 48-h follow-up < 140 mEq/L	2.50 (0.38–16.18)	0.33
Serum creatinine on admission > 1.37 mg/dL	10.92 (5.27–13.64)	0.02

Table 5. Logistic regression analysis of predictors of early subclinical kidney injury. Early subclinical kidney injury was defined as an increase in NGAL by at least 25% 48 h following contrast administration vs. baseline. *Cutoff values for the evaluated parameters were established using receiver operating characteristic analysis.

serum creatinine in various clinical settings, including in patients with normal renal function, with septic shock, or post-cardiac surgery[4,32,33], causing NGAL to be seen as a 'kidney troponin'[34]. Based on these data, the Acute Dialysis Quality Initiative proposed a combination of kidney functional (i.e., serum creatinine) and structural (e.g., NGAL) damage markers to stratify the risk of acute kidney damage[30].

In line with these data, in the present study, repeated NGAL evaluation demonstrated that acute renal injury was much more common than reflected by serum creatinine, affecting almost 18% of the study patients. Moreover, our data indicate that unlike CIN, which was regressive at the 1-month follow-up, subclinical kidney injury was still present after 1 month in more than half of patients in whom the kidneys were initially affected by the contrast media. In addition, similarly to what was seen for clinically overt CIN, the occurrence of subclinical kidney injury was also independently associated in the present study with the basal renal function, as reflected by the baseline serum creatinine. Moreover, the risk of developing subclinical kidney injury following contrast administration was related to a lower degree of baseline kidney dysfunction than the risk of clinically overt CIN.

Clinical implications. In line with previous studies, our data indicate that the occurrence of clinically overt CIN is favored not only by non-modifiable (baseline kidney function), but also by modifiable factors—the volume of contrast administered. Technological innovations and technical adjustments, such as lowering the X-ray voltage or using the latest generation imaging platforms could thus be of use for CIN prevention by reducing the amount of contrast media that is being administered[35]. However, such approaches may not be efficient for reducing the risk of contrast-induced subclinical kidney injury. Unlike CIN, contrast-induced subclinical kidney injury was not affected by contrast volume in the present study, but only by a non-modifiable factor—the baseline kidney function. Oral and intravenous hydration, and pharmacologic strategies such as methylxanthines, statins, ascorbic acid, N-acetyl cysteine, or dihydropyridine calcium channel blockers have all been proposed as potentially efficient interventions for the prevention of CIN[16]. None of the patients included in the present study was receiving methylxanthines, ascorbic acid, or N-acetyl cysteine, and almost all patients (i.e., 98.8%) were undergoing statin therapy. Thus, the potential impact of such strategies on the risk of CIN could not be evaluated in the present study. Dihydropyridine calcium channel blockers did not appear to affect, however, the risk of CIN or of subclinical kidney injury in the present study. Previous studies have also suggested a higher risk of CIN with low-osmolar than with high-osmolar contrast media in patients with pre-existing chronic kidney disease[36], whereas the benefit of iso-osmolar over low-osmolar contrast agents remains debatable[37,38]. In the present study, there was no significant difference in the type of contrast media used between patients with and without CIN or subclinical kidney injury. However, all patients in this study received low-osmolar contrast agents and more than 85% of them received the same contrast agent (i.e., iomeprol).

Strengths and limitations. The effects of contrast media at the renal level were assessed in a prospective study, using both functional (i.e., serum creatinine) and structural (i.e., NGAL) renal damage markers, providing a comprehensive view on contrast-induced kidney injury. In addition, to the best of our knowledge, this is the first study to evaluate the long-term effects of contrast media on subclinical kidney injury, as reflected by the levels of NGAL. These analyses demonstrated that subclinical kidney injury was still present after 1 month in more than half of patients in whom the kidneys were initially affected by the contrast media, suggesting that these patients may be at increased risk for further, potentially clinically significant renal impairment, particularly if exposed to nephrotoxic agents or repeated administration of contrast media. Studies with longer-term follow-up of renal function, including after repeated administration of contrast agents, will have to clarify this issue. The long-term impact of the renal changes identified in the present study on 'hard' clinical endpoints (e.g., dialysis, death) also remains to be clarified. In the present study, only 35.3% of the study patients underwent, however, the 1-month follow-up. The high rate of loss of follow-up was mainly related to the fact that the angioplasty procedures took place in a tertiary center, and the patients' subsequent follow-ups were mainly performed in their home centers. Future studies will have to further elucidate the impact of contrast media on subclinical kidney injury over the long term. The prospective nature of the present study allowed us to evaluate the renal impact of a large series of parameters associated with contrast-induced kidney injury in previous studies. Yet, only baseline kidney function and the volume of contrast administered were identified as independent predictors of CIN, whereas subclinical contrast-induced kidney injury was only independently predicted by the baseline serum creatinine. The relatively low number of patients included in the present study may have affected our ability to detect other potential predictors of contrast-induced acute kidney injury. However, with the exception of baseline kidney function, which has been related to CIN in the vast majority of previous studies, the role of the

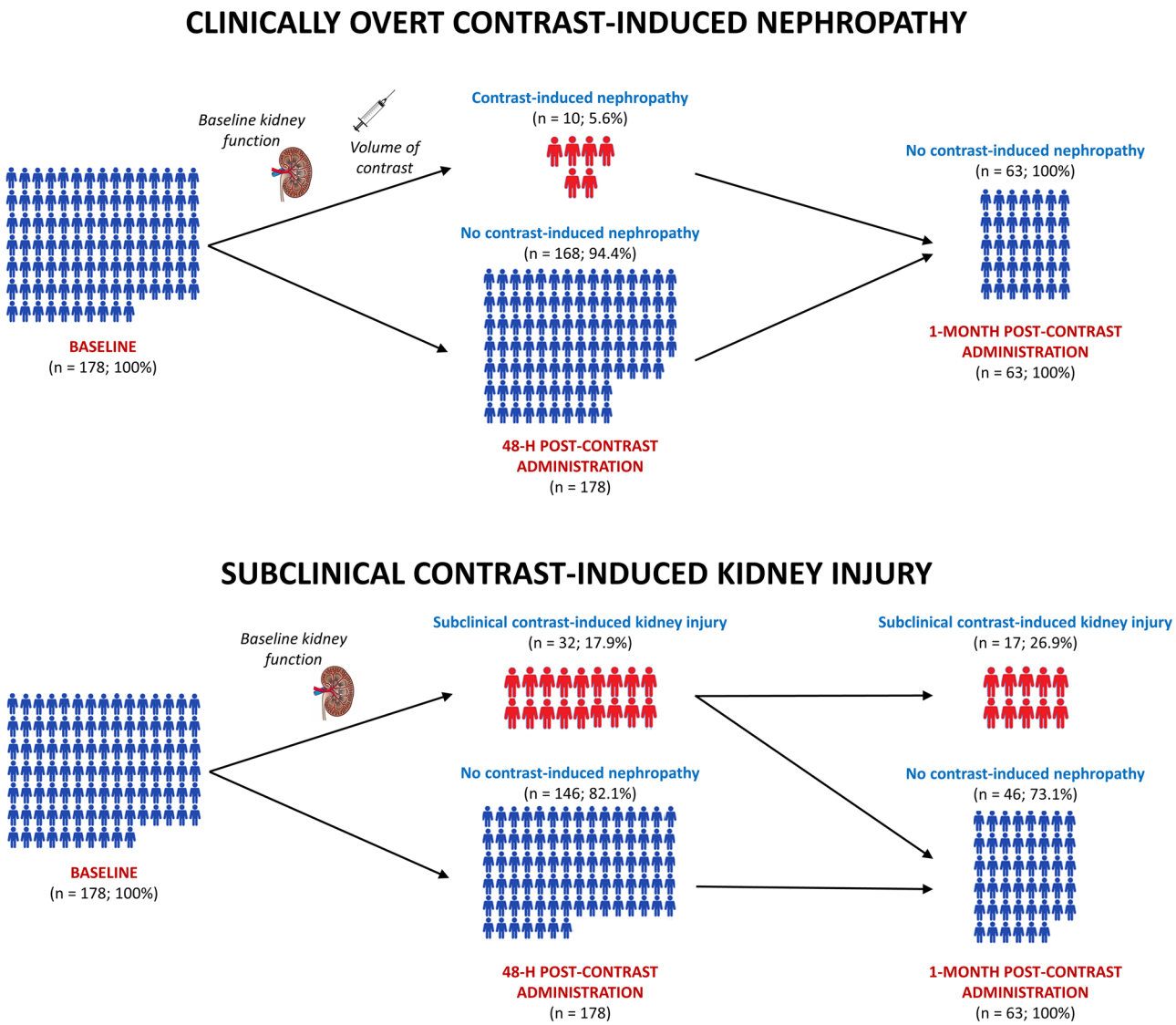

Figure 1. Overview of the main study findings.

other tested factors is highly controversial in the literature[12]. Although NGAL is clearly a valuable biomarker of contrast-induced subclinical kidney injury, one should be aware that NGAL is not specific for this condition and that low levels of NGAL can also originate from other sources, such as neutrophils, cardiomyocytes, prostatic cells, or respiratory and gastrointestinal epithelia[4]. None of the patients evaluated at the 1-month follow-up presented anamnesis, clinical signs or symptoms of inflammatory disease. However, no specific laboratory analyses were performed at that time to this end. To date, there is no official definition for early subclinical kidney injury. In the present study, subclinical kidney injury was defined by analogy with clinically overt CIN. Future long-term studies will have to establish the most appropriate NGAL cut-off values for the diagnosis of subclinical contrast-induced kidney injury. Finally, the impact of contrast media on the kidneys was evaluated in the present study using serum creatinine and NGAL. Evaluation of additional parameters, such as urinary NGAL, molecule-1, or cystatin C would also have been of interest to fully elucidate the renal effects of contrast media.

Conclusions

The present study showed that in the era of modern contrast media, clinically overt CIN is rather rare, regressive, and that its occurrence is affected by only the baseline renal function and the amount of contrast media administered. Subclinical kidney injury was, however, considerably more frequent in patients receiving intraarterial contrast media. More importantly, subclinical contrast-induced kidney injury persisted after 1 month in more than 50% of the initially affected patients. Pending confirmation in future studies, these data suggest that patients who develop subclinical contrast-induced kidney injury may be at increased risk for further, potentially clinically significant renal impairment, particularly if exposed to nephrotoxic agents or repeated administration of contrast media.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Received: 23 June 2022; Accepted: 11 November 2022
Published online: 14 November 2022

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Author contributions

A.S. designed the study and performed the statistical analyses. A.S., C.S., and T.R.N. analyzed and interpreted the patient clinical data. M.O. and L.D. performed and interpreted the laboratory analyses. A.S. and C.S. drafted the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS–UEFISCDI, project number PN-III-P1-1.1-TE-2019-0370, within PNCDI III. The funding source had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-24169-7.

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International Journal of
Molecular Sciences

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Review

Platelets and Their Role in Hemostasis and Thrombosis—From Physiology to Pathophysiology and Therapeutic Implications

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Abstract: Hemostasis is a physiological process critical for survival. Meanwhile, thrombosis is amongst the leading causes of death worldwide, making antithrombotic therapy one of the most crucial aspects of modern medicine. Although antithrombotic therapy has progressed tremendously over the years, it remains far from ideal, and this is mainly due to the incomplete understanding of the exceptionally complex structural and functional properties of platelets. However, advances in biochemistry, molecular biology, and the advent of 'omics' continue to provide crucial information for our understanding of the complex structure and function of platelets, their interactions with the coagulation system, and their role in hemostasis and thrombosis. In this review, we provide a comprehensive view of the complex role that platelets play in hemostasis and thrombosis, and we discuss the major clinical implications of these fundamental blood components, with a focus on hemostatic platelet-related disorders and existing and emerging antithrombotic therapies. We also emphasize a number of questions that remain to be answered, and we identify hotspots for future research.

Keywords: antiplatelet agents; anticoagulants; hemostasis; platelets; thrombosis

Citation: Scridon, A. Platelets and Their Role in Hemostasis and Thrombosis—From Physiology to Pathophysiology and Therapeutic Implications. *Int. J. Mol. Sci.* **2022**, *23*, 12772. https://doi.org/10.3390/ijms232112772

Academic Editor: Isabella Russo

Received: 1 October 2022
Accepted: 21 October 2022
Published: 23 October 2022

1. Introduction

From their initial description in the 1870's, it had already become clear that platelets were exceptional cells that will never stop amazing the scientific community. It soon became clear that although platelets circulate in isolation in the blood, they can rapidly form aggregates at the sites of vascular injury [1], contributing to both hemostasis and thrombotic disease. However, at that point, the saga of platelets was only in its infancy. Observations that span more than half a century demonstrate that, besides their classical roles in clot formation, platelets play extremely versatile functions in many areas of physiology. Platelets' α-granules express receptors that facilitate adhesion with other vascular cells and release a broad variety of mediators that participate in and regulate functions such as chemotaxis, stem cell homing, cell migration, proliferation and differentiation, inflammation, angio- and lymphangiogenesis, the maintenance of lymphatic and blood systems as separate entities, the deposition of matrix proteins, host defense, viral replication, the transport of information, vasomotor function, and many others. Via this plethora of functions, platelets' α-granules contribute to a wide range of physiologic and pathologic processes [2]. Activated platelets release a vast array of growth and angiogenic factors, cytokines, and chemokines (Table 1). Once released, these molecules act to regulate the chemotaxis, inflammation, and vasomotor function critical for restoring the integrity of injured vascular walls, for angiogenesis, and for the growth of new blood vessels in injured tissue areas. These features provide platelets with tremendous potential for wound healing and tissue regeneration, as already demonstrated in settings such as diabetic ulcers, bone or tendon defects, maxillofacial and dental surgery, and corneal diseases [3]. It has also been suggested that platelets could promote rejuvenation and the reversal of aging by acting

as a 'fountain of youth' [4], although many of these applications are not yet supported by adequate evidence from clinical trials.

Table 1. Main classes of substances present in platelet granules and cytosol.

***Alpha*-Granules**
Growth and angiogenic factors
(e.g., platelet-derived growth factor, fibroblast growth factor, vascular endothelial growth factor, connective tissue growth factor, epidermal growth factor, transforming growth factor β, insulin-like growth factor 1)
Cytokines and chemokines
(e.g., interleukin-1β, CD40 ligand, CCL2, CCL3, CCL5, CXCL1, CXCL4, CXCL12, CXCL16, platelet factor 4)
Adhesion molecules
(e.g., P-selectin, fibronectin, vitronectin, von Willebrand factor)
Coagulation factors
(e.g., factors V, XIII, von Willebrand factor, high-molecular-weight kininogen, fibrinogen)
Anticoagulation factors
(e.g., tissue factor pathway inhibitor, protein S)
Fibrinolytic factors
(e.g., plasmin, plasminogen)
Antifibrinolytic factors
(e.g., α2-antiplasmin, thrombin activatable fibrinolysis inhibitor, plasminogen activator inhibitor-1)
Other molecules
(e.g., albumin, calcitonin, angiotensinogen, thrombospondin)
Dense granules
Serotonin
Histamine
Adenine nucleotides (ADP, ATP)
Cations (e.g., calcium, magnesium), polyphosphate
Adhesion molecules (e.g., P-selectin)
Lysosomal granules
Cathepsin D, E
Carboxypeptidase A, B
Acid phosphatase
Arylsulfatase
Cytosol
Adhesion molecules
(e.g., P-selectin, fibronectin, vitronectin, fibrinogen, thrombospondin, von Willebrand factor)
Coagulation factors
(e.g., factors V, von Willebrand factor)
Platelet activators
(e.g., platelet-activating factor, TxA2)
Vasoconstrictors
(e.g., TxA2, 12-hydroxyeicosatetraenoic acid)

ADP—adenosine diphosphate; ATP—adenosine triphosphate; GP—glycoprotein; TxA2—thromboxane A2.

However, in parallel, activated platelets also cause alterations in endothelial and white blood cells and release inflammatory mediators, thereby promoting atherosclerosis and atherothrombosis, vascular intima proliferation and restenosis post-angioplasty, and tumor growth, metastasis, and immune evasion, as well as amplifying inflammatory and infectious states [2]. Studies have also shown that platelets release bioactive, antimicrobial peptides and kinocidins and exhibit antimicrobial host defense properties, possessing the unambiguous structural and functional characteristics of immune cells [5]. Platelets thus play central roles as part of the intravascular innate immune system and coordinate adaptive antimicrobial host defense, thereby bridging anti-infectious innate and adaptive immunity. There is evidence that platelets play complex roles in malarial infections, dengue fever, sepsis, and rheumatic diseases, and deficiencies in platelet quantity or quality are increasingly recognized as correlates of infection risk and severity [6]. In addition,

complex interactions have been shown to exist between platelets' inflammatory and hemostatic functions. A prothrombotic platelet phenotype (involving platelet activation and platelet consumption leading to thrombocytopenia) can be seen in conditions with genetic deficiency in complement regulation such as atypical hemolytic uremic syndrome or paroxysmal nocturnal hemoglobinuria [7].

Platelets, therefore, display a plethora of multifaceted functions. However, the main role of platelets remains maintaining normal hemostasis, in conjunction with the coagulation system. The classical theory of hemostasis (Figure 1A) describes a three-step process during which: (1) immediately after vascular injury, the injured vessel undergoes vasoconstriction to limit blood loss at the site of the injury; (2) platelets adhere to the injured vessel wall, activate, and form aggregates, i.e., the platelet plug; which (3) is eventually stabilized by a dense fibrin mesh formed via the coagulation cascade. In reality, however, this process is much more complex. The three phases that ensure hemostasis are by no means independent and their activation is not precisely sequential. Rather, the three main processes that entail normal hemostasis activate simultaneously and continuously potentiate one another throughout the hemostatic process (Figure 1B). Particularly, the contribution of platelets to hemostasis far exceeds their simple participation in 'primary hemostasis', and the multiple interactions that exist between platelets, the vessel wall, and the coagulation system complicate the fundamental process of hemostasis.

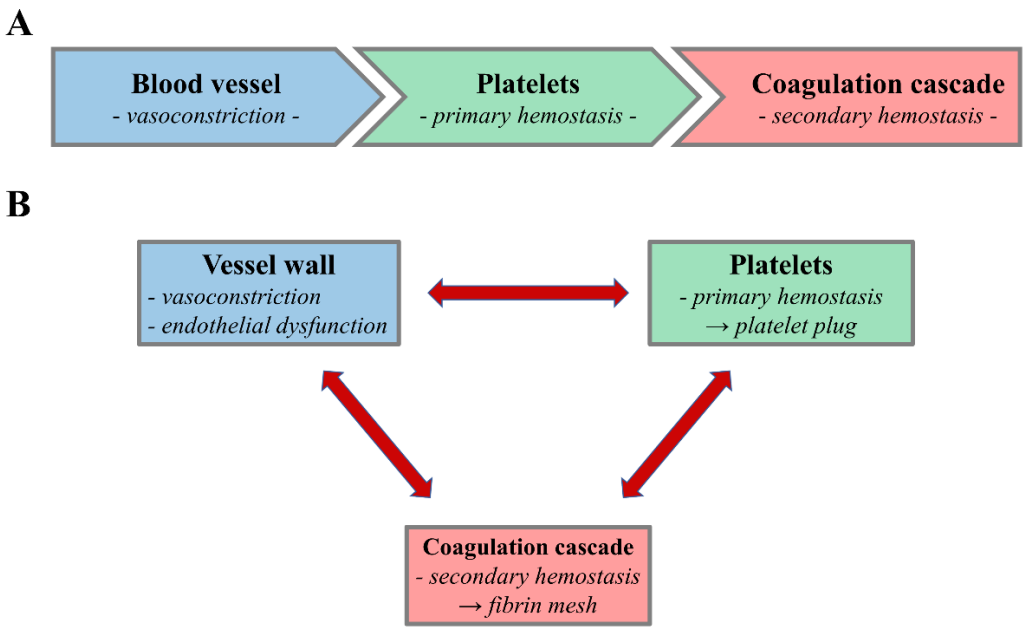

Figure 1. Schematic image of the hemostatic process. (**A**) Classic view of hemostasis as a three-step process involving vasoconstriction and primary and secondary hemostasis as independent and sequential events. (**B**) Current view of hemostasis as a complex process in which the vessel wall, the platelets, and the coagulation system collaborate and continuously influence one another.

In this review, we aim to provide a comprehensive view of the complex role that platelets play in hemostasis and thrombosis, and to discuss the major clinical implications of these fundamental blood components, with a focus on hemostatic platelet-related disorders and existing and emerging antithrombotic therapies. We also emphasize a number of questions that remain to be answered, and we identify hotspots for future research.

2. Platelet Shape and Structure at Rest and during Activation

Platelets are small (≈2–4 μm), short-living (≈8–10 days), anucleated cell fragments derived from the megakaryocyte lineage. Most platelets circulate in the bloodstream in isolation in a resting, discoid form, without interacting with the vessel wall, but continuously

monitoring their surrounding environment via a wide array of receptors and adhesion molecules and are ultimately cleared from the blood at the end of their lifespan. Therefore, continuous platelet production is required to maintain normal platelet counts (i.e., 150–400 $\times$ 10^9 /L in a healthy adult).

In response to vessel injury, platelets activate and rapidly reveal their highly dynamic nature: they undergo massive shape and ultrastructure changes, including the 'ruffling' of their plasma membranes due to the emergence of cytoplasmic projections, causing them to take an 'amoeboid form'. In parallel, platelets undergo granule centralization and discharge. These critical ultrastructural changes mediated by the platelet cytoskeleton allow platelets to adhere to the site of vessel damage, to spread over the injured area, release the content of intracytoplasmic granules, and aggregate with other activated platelets to form the platelet plug, while also promoting fibrin formation and vessel wall repair. The constituents critical for hemostasis are located both on the surface of the platelet membrane and in the cytoplasm, particularly within the granules (Figure 2).

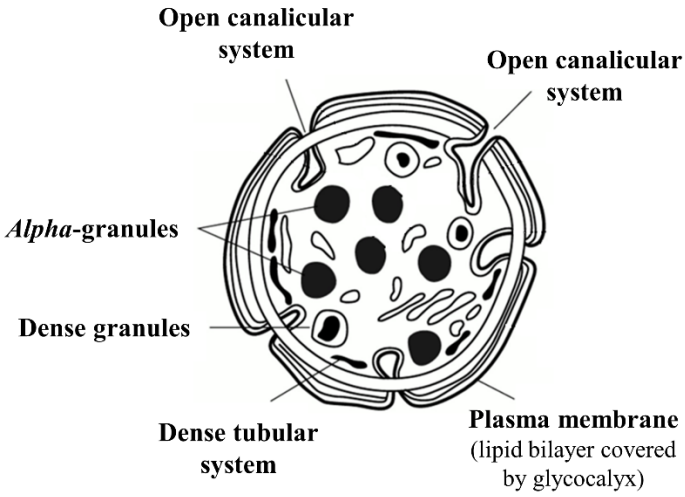

Figure 2. Schematic image of platelets. Resting platelets have asymmetrical distribution of phospholipids: the anionic phospholipids phosphatidylserine and phosphatidylethanolamine, responsible for binding many of the blood coagulation proteins, are sequestered in the inner leaflet of the membrane lipid bilayer facing the cytosol, whereas the electrically neutral phosphatidylcholine and sphingomyelin are exposed on the outer membrane leaflet. This arrangement prevents the membranes of resting platelets from supporting coagulation. When platelets become activated, the function of the phospholipid transporters is altered, leading to transfer of anionic phospholipids on the outer membrane leaflet. Loss of this asymmetry provides a procoagulant surface for sequential activation of coagulation enzymes. The outer surface of resting circulating platelets is covered by a prominent glycocalyx that prevents spontaneous platelet aggregation. Platelets possess a plasma membrane-based open canalicular system connected with the extracellular space through a multitude of small pores that increases the platelet membrane's surface area. A second platelet canalicular system that is not connected to the platelet's exterior—the dense tubular system—serves as a store for calcium and for various enzymes involved in platelet activation. Platelet alpha and dense granules contain a large number of substances critical for hemostasis, as well as for vasomotor function and immunity.

The outer surface of resting circulating platelets is covered by a layer of glycolipid and glycoprotein molecules that form a prominent **glycocalyx**. With its negative net electric charge, the glycocalyx provides a repulsive surface that prevents spontaneous platelet aggregation with other platelets or other blood or endothelial cells [8], while also playing a role in calcium-signaling regulation [9] and in platelet turnover [10].

A wide variety of glycoproteins are embedded in the **platelet membrane**, acting as receptors for various soluble (i.e., platelet activators) and fixed (i.e., platelet adhesion molecules) ligands that mediate platelets' adhesion to the vessel wall, activation, spreading,

and aggregation [11]. Although the platelet plasma membrane does not differ considerably from those of other cells, it does possess some critical features, including the presence of a plasma membrane-based open canalicular system (i.e., a network of membrane invaginations that penetrate the platelet interior) connected with the extracellular space through a multitude of small pores that provides platelets with a membrane surface area much larger than that expected for such small cells (Figure 2). A second platelet canalicular system (i.e., the dense tubular system), derived from the smooth endoplasmic reticulum of megakaryocytes, is not connected with the extracellular fluid, and serves as a store for calcium and various enzymes involved in platelet activation [11].

The platelet plasma membrane contains numerous adhesion and signaling integrin molecules, leucine-rich glycoproteins, immunoreceptors, prostanoids, and G protein-coupled receptors (Table 2).

Table 2. Platelet receptors critical for the hemostatic function.

Function in Hemostasis	Receptors	Receptor Family	Main Ligands
Platelet adhesion to the vessel wall	GPIb-IX-V *	Leucine-rich repeat	vWF, thrombospondin-1, thrombin, factors XI, XII, P-selectin
	GPVI	Immunoreceptors	Collagen, laminin
	$\alpha 2\beta 1$	Integrins	Collagen, laminin
	$\alpha 6\beta 1$		Laminin
	$\alpha 5\beta 1$		Fibronectin
	$\alpha V\beta 3$		Vitronectin, vWF, fibronectin, fibrinogen
	$\alpha IIb\beta 3$ (i.e., GPIIb/IIIa) **		Fibrinogen
Platelet activation	PAR-1, PAR-4	G protein-coupled receptors	Thrombin
	P2Y1, P2Y12		ADP
	TPα, TPβ		TxA2
	PGE_2 receptor		PGE_2
	5-HT2A		Serotonin
	P2 × 1	Ion channel	ATP
Platelet aggregation	Activated $\alpha IIb\beta 3$ (i.e., GPIIb/IIIa)	Integrins	Fibrin, vWF, thrombospondin-1, fibronectin

* —affected in Bernard–Soulier syndrome; ** —affected in Glanzman thrombasthenia. ADP—adenosine diphosphate; ATP—adenosine triphosphate; GP—glycoprotein; HT—hydroxytryptamine; PAR—protease-activated receptor; PG—prostaglandin; TP—thromboxane receptor; TxA2—thromboxane A2; vWF—von Willebrand factor

Adhesion molecules expressed on the platelets' surface are critical for platelets' adhesion to the vessel wall and to platelets' interactions with other cells such as leukocytes and endothelial cells [11]. Many of these molecules are expressed in small amounts in the membrane of resting, unstimulated platelets, and their expression increases rapidly upon platelet activation via the fusion of granule membranes, which also express these molecules, with the platelet membrane. Such molecules include P-selectin, glycoprotein (GP) Ib-IX-V, the main platelet receptor for von Willebrand factor (vWF), and GPIIb/IIIa, critical for platelet aggregation by binding vWF, fibronectin, vitronectin, and, particularly, fibrinogen. Platelet **collagen receptors** are essential for platelets' adhesion to the vessel wall (Table 2). They are mainly represented by GPVI, a member of the immunoglobulin superfamily, and by α/β integrin, the primary collagen receptor, with critical hemostatic roles. Finally, the **G-protein-coupled receptors** (Table 2) ensure platelet activation through several ligands such as thrombin, adenosine diphosphate (ADP), adenosine triphosphate (ATP), and prostanoids. Thrombin, a key constituent of the coagulation cascade, is among the most powerful platelet activators. Platelet activation by thrombin is mediated by protease-activated receptors (PARs) expressed in the platelet plasma membrane. The activation

of PARs occurs via proteolytic cleavage by thrombin and specific ligand unmasking [12]. Thrombin and various synthetic compounds known as thrombin-receptor agonist peptides (TRAPs) bind to and activate PAR-1 and PAR-4, leading to platelet activation, shape change, granule release, and aggregation. However, platelet activation by TRAPs does not involve receptor cleavage. While PAR-1 activation can occur at low thrombin concentrations, higher agonist concentrations are required for the activation of PAR-4. Contrary to human platelets, rodent platelets do not express PAR-1 [13]. Meanwhile, ADP and ATP are known to evoke platelet responses different than those induced by thrombin. Low ADP concentrations have been shown to initiate reversible platelet aggregation, with no granule release, whereas higher ADP concentrations trigger granule release and prostaglandin synthesis, leading to a typical, biphasic, irreversible aggregation process [1]. Platelets' response to ADP is mediated by P2Y (P2Y1 and P2Y12) G protein-coupled receptors. Although less potent than ADP, ATP can also trigger, via P2 × 1 receptors, platelet activation, shape change, granule release, and can increase platelets' sensitivity to other agonists (e.g., collagen) [14]. Platelet activation by thromboxane A2 (TxA2), a product of arachidonic acid metabolism under the effect of cyclooxygenase (COX) 1, occurs via TxA2 receptors α and β activation, with TxA2 receptor α being the predominant isoform in human platelets. The activation of these receptors is rapidly followed by platelet activation, shape change, degranulation, aggregation, and increased sensitivity to other agonists, leading to the amplification of the platelet activation response (Figure 3). Receptors for other prostanoids, such as prostaglandin E and prostacyclin, a platelet aggregation inhibitor, are also expressed on the platelet plasma membrane.

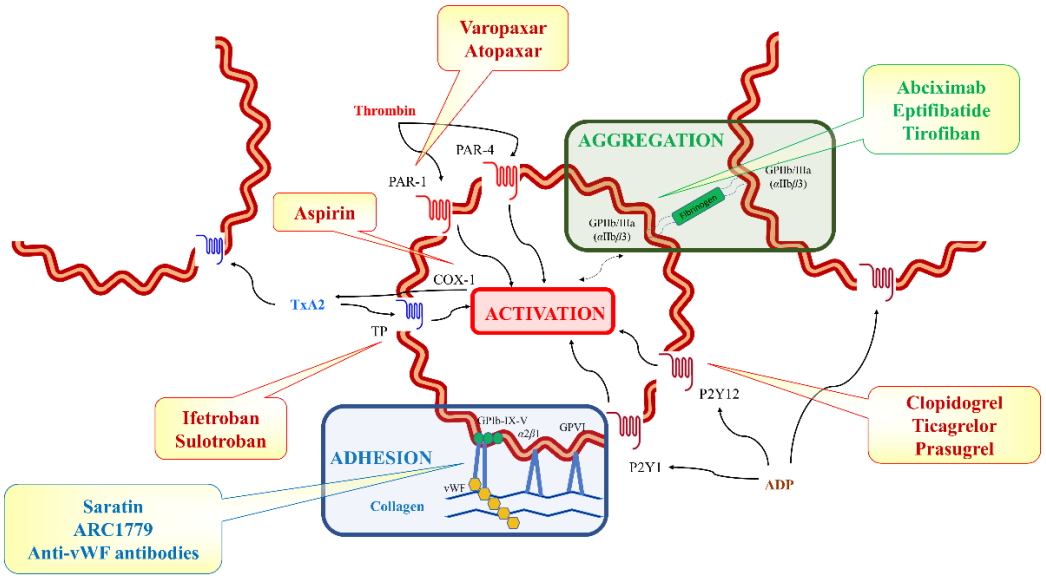

Figure 3. Main receptors and ligands involved in platelets' adhesion, activation, and aggregation. The boxes indicate some examples of existing or emerging antiplatelet therapies. ADP—adenosine diphosphate; COX-1—cyclooxygenase-1; GP—glycoprotein; PAR—protease-activated receptors; TP—thromboxane receptor; TxA2—thromboxane A2; vWF—von Willebrand factor.

Meanwhile, numerous intracytoplasmic **platelet granules** (Figure 2) serve as secretory vesicles capable of releasing their content into the extracellular fluid, and also as 'guides' that direct molecules to the plasma membrane during exocytosis. Platelet degranulation is a complex process that involves granules' merging and fusion in the platelet center, as well as their fusion with the open canalicular system and with the plasma membrane. Three main types of granules (i.e., *alpha*, dense [δ], and lysosomal granules) are present in the cytoplasm of quiescent, non-activated platelets, each displaying specific content, ultrastructure, function, and exocytosis kinetics, and each releasing their content in response to different agonists and at different degrees of stimulation (e.g., α- and δ-granule release

occurs in response to low levels of thrombin or ADP, whereas lysosomes' degranulation requires much higher concentrations of these agonists).

***Alpha* granules** are the largest (≈200–500 nm), most abundant (≈40–80/platelet), and heterogeneous platelet granules [15], being responsible for the granulated platelet cytoplasm's appearance on peripheral blood smears stained with Romanowsky-type dyes. The vast majority of platelet factors involved in hemostasis—including β-thromboglobulins, platelet factor 4, thrombospondin, and P-selectin; numerous coagulation, anticoagulation, fibrinolytic, and antifibrinolytic factors; and a number of molecules involved in platelets' adhesion to vessel walls such as fibronectin, laminin, and vitronectin—are contained by these granules (Table 1). Factors involved in inflammation, cell growth, and host defense, such as cytokines, chemokines, and growth factors, as well as microbicidal proteins and immune mediators, are also contained in the α-granules (Figure 3), which also display a series of membrane-bound receptors such as αIIbβ3, GPVI, the GPIb-IX-V complex, and P-selectin. Hence, the content of α-granules pertains to two main functions: hemostasis and immunity. Proteomic studies have identified over 300 soluble proteins released by α-granules [16] and it has been postulated that α-granules can release specific molecules in response to different agonists [17], although no conclusive evidence has been provided so far in this regard.

Opposed to the α-granules, **dense granules** are the smallest platelet granules (≈150 nm), are less abundant (≈3–8/platelet), and, due to their high calcium and polyphosphate content, appear highly electron dense in osmium-stained and whole-mount electron microscopy [11]. High concentrations of serotonin and adenine nucleotides are also present within the dense granules, as well as serotonin, histamine, cations, small GTP-binding proteins, and adhesion molecules such as GPIb, GPIIb/IIIa, and P-selectin (Table 1). Upon platelet activation, the content of δ-granules is released into the extracellular fluid via exocytosis, contributing to platelet recruitment and aggregation (via calcium, polyphosphates, and adenine nucleotides), as well as to local vasoconstriction (via serotonin). The third category of platelet granules, **lysosomes**, have an intermediate size between the α- and the δ-granules (≈200–250 nm). Their acidic internal environment, with hydrolytic enzymes active against several substrates, including some of the extracellular matrix, contribute to thrombus dissolution and extracellular matrix remodeling [11].

3. Role of Platelets in (Primary) Hemostasis

Primary hemostasis can be seen as a three-step sequence of events involving (1) platelets' adhesion to the vessel wall, (2) platelet activation, and (3) the formation of platelet aggregates, which has as an objective the formation of a blood clot that seals a breach formed in a vessel wall.

Seconds after a vessel injury, stationary (e.g., collagen) and mobile (e.g., thrombin, ADP, and TxA2) platelet agonists accumulate locally. Platelets start to **adhere** to the proteins of the subendothelial matrix via their collagen receptors, with more or less involvement of vWF, depending on the local conditions (i.e., local shear rate of flow). Platelets' contact with the damaged vessel wall is dependent on a unique membrane receptor complex: GPIb-V-IX (Figure 3). The loss of endothelial integrity, such as that occurring during atherosclerotic plaque erosion or rupture, exposes vWF and the collagen of the subendothelial matrix to contact with the circulating platelets. At low shear rates (i.e., 100–1000/s), such as those typical for the venous system or the interior of the atria, platelets can interact directly with the collagen, laminin, and fibronectin molecules of the extracellular matrix (via GPVI; α2β1 and α6β1; and αIIbβ3, αVβ3, and α5β1, respectively). The Von Willebrand factor's contribution becomes critical in areas with high shear stress (i.e., 1000–4000/s) such as those seen in the arterial system and particularly in areas with stenotic lesions, where circulating vWF becomes immobilized on the exposed subendothelial collagen, binds to GPIbα receptors, and unfolds, exposing multiple binding sites for the GPIb-IX-V complex, thereby facilitating the additional, direct binding of GPVI with the subendothelial collagen (via integrin α2β1) and fibronectin (via integrin α5β1). Initial platelet tethering to exposed

vWF-collagen complexes can thus remain in place long enough for the platelets to become activated by collagen. This process leads to the formation of a platelet monolayer that will further support the adhesion of additional activated platelets. Although vWF-GPIb-mediated platelets' adhesion to vessel walls can resist high shear stress, this interaction is transient, making this latter phase a mandatory step for stable platelet adhesion. The interaction between vWF and GPIbα facilitates the deceleration of circulating resting platelets and allows them to 'roll over' the vessel wall, thereby allowing other platelet receptors to interact with components of the exposed extracellular matrix and/or locally generated soluble agonists such as thrombin. The fibrinogen, fibronectin, vitronectin, and vWF released from the platelet α-granules further strengthen platelets' adhesion to the vessel wall by forming cross-bridges between platelet GPIIb/IIIa receptors and the endothelial αVβ3 integrin or the intercellular adhesion molecule (ICAM) 1 [18]. In turn, integrin α/β-induced adhesion induces, through phospholipase C-dependent GTPase Rap1b stimulation, platelet GPIIb/IIIa receptors' activation [19]. Together, these features explain why platelets' adhesion to the vessel wall is stronger in areas with high local shear stress, why platelets play more important roles in arterial than in venous thrombosis, and why, despite the high flow velocities encountered in areas with vascular stenosis, blood clots generally form precisely in those areas.

The activation of platelet GPVI collagen receptors rapidly triggers phospholipase C activation, the increase of cytosolic calcium, and the hydrolyzation of phosphatidylinositol-4,5-bisphosphate into inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, leading to platelet **activation** (Figure 3). In turn, inositol trisphosphate will mobilize calcium from the intracellular stores, with the consequent additional entry of calcium from the extracellular space, thus leading to the substantial amplification of the platelet calcium signal. In physiological conditions, this prolonged rise in intracellular calcium is most likely triggered by phospholipase C activation and subsequent calcium inflow induced by collagen (via the GPVI receptors) and by high thrombin/vWF concentrations (via PAR-1 and GPIb receptors) [20]. During activation, platelets undergo massive shape and ultrastructural changes and release a series of mediators from their α-granules, including TxA2 and ADP. Once released, these mediators will bind to their specific platelet receptors and will act as additional platelet activators via autocrine and paracrine mechanisms, thus amplifying platelet activation and recruiting novel platelets into the hemostatic process (Figure 3). Meanwhile, membrane-bound diacylglycerol, together with calcium, activates protein kinase C, resulting in further integrin activation and platelets' spreading and degranulation. Adenosine diphosphate-induced platelet activation is initiated by P2Y1 receptors' activation and is completed and amplified by the activation of the dominant P2Y12 receptors. Once activated, the G_i-coupled P2Y12 receptors trigger a sequence of intracellular events that lead to the inhibition of platelet adenylate cyclase, with a consequent decrease in cyclic adenosine monophosphate, thereby causing mild negative feedback upon platelet activation via the protein kinase A signaling pathway, coupled with the Akt pathway, and integrin's activation via Rap1b's inactivation [14]. The activation of the G_q-coupled P2Y1 receptors activates the phospholipase C pathway, leading to increased intracellular calcium, the activation of Rap1b, and, ultimately, to GPIIb/IIIa receptors' activation, which is involved in platelet aggregation (Figure 3). Similar platelet changes are induced by thrombin, which acts as an exogenous platelet agonist (Figure 3). The activation of PAR-1 and PAR-4 thrombin receptors also results in phospholipase C pathway activation, calcium mobilization, and protein kinase C activation, involving fast but reversible PAR-1, followed by sustained PAR-4 cleavage [1], whereas TxA2 receptors' activation results in phospholipase C and RhoGEF activation, with subsequent platelet degranulation, generation, and the release of lipid mediators, as well as GPIIb/IIIa receptors' activation [21]. Although extracellular matrix components are crucial for hemostatic mass formation, G protein-coupled receptors and their ligands are key to the hemostatic process. Soluble ligands can activate platelets located in the outer layers of a frowning thrombus, whereas extracellular matrix components cannot. The latter provide, however, a system that delicately adjusts the strength and duration of platelet

activation and are capable of triggering extremely fast intracellular signaling pathways necessary for platelets' adhesion under flow conditions [22].

In addition to the substantial shape changes and degranulation, platelet activation also entails the externalization of anionic phospholipids on the outer surface of the platelet membrane, providing a scaffold for the progress of the coagulation cascade. Resting, quiescent platelets typically expose an anticoagulant membrane due to an asymmetrical distribution of phospholipids, with only phosphatidylcholine and sphingomyelin being exposed on the outer membrane leaflet. Both molecules are electrically neutral and, therefore, cannot bind to clotting proteins with appreciable affinity, whereas the anionic phospholipids phosphatidylserine and phosphatidylethanolamine, responsible for binding to many of the blood coagulation proteins, are sequestered in the inner leaflet of the membrane lipid bilayer facing the cytosol. This arrangement is actively maintained by phospholipid transporters and prevents the membranes of resting, non-activated platelets from supporting coagulation [23]. However, when platelets become activated, the function of the phospholipid transporters is altered, resulting in the 'scrambling' of the membrane asymmetry and the transfer of phosphatidylserine and phosphatidylethanolamine on the outer membrane leaflet. The loss of this asymmetry, with anionic phospholipids moving toward the outer membrane's lipid bilayer, provides a procoagulant surface for the sequential activation of coagulation enzymes, providing the platelet plasma membrane with the critical ability to support the generation of thrombin. Furthermore, the exposure of phosphatidylserine on the outer surface of the platelet membrane prompts the platelets to continue to dramatically change their shape—they lose most of their cytoskeletal structure, rapidly swell, and become balloon-like structures, further increasing their procoagulant surface. Platelets' inability to expose phosphatidylserine on their outer plasma membrane, as seen in Scott syndrome, significantly diminishes platelets' procoagulant activity.

Phosphatidylserine's exposure on the platelet surface is triggered during platelet activation by strong agonists, particularly collagen plus thrombin. The strong exposure induced by these two agonists ensures that the procoagulant transformation of platelets occurs at sites where coagulation is desired (i.e., where the collagen of the vessel wall is exposed) or is already initiated (i.e., where thrombin is present in sufficiently large amounts) [24]. In parallel with the externalization of anionic phospholipids on the platelet plasma membranes, membrane blebs—phosphatidylserine-rich microvesicles—are generated and released into the bloodstream. Platelet microvesicles' formation occurs via the intervention of calpain, which degrades and dissociates the membrane skeleton from the plasma membrane, facilitating the protrusion of membrane patches, which will no longer interact with the platelet cytoskeleton [25]. Platelet-derived microvesicles contain platelet cytoskeletal proteins and membrane GP Ib, IIb, IIIa, and IV, which contribute to the clot-promoting activity of plasma [1]. Studies suggest that, in addition to their contribution to normal hemostasis, platelet microvesicles also play a role in the thrombotic risk associated with several diseases, particularly via the tissue factor pathway. Circulating platelet-derived microvesicles have been detected in patients with disseminated intravascular coagulation, antiphospholipid syndrome, thrombotic thrombocytopenic purpura, heparin-induced thrombocytopenia, and transient ischemic attacks [1].

Although the sources and the intracellular pathways triggered by the main platelet agonists are different, the consequence of platelet receptors' activation is common, to some extent, for all platelet agonists: they all lead to the amplification of platelet activation, the recruitment of additional platelets, and to the activation—via inside-out signaling—of the most abundant platelet adhesion molecules, the GPIIb/IIIa receptors, which are critical for platelet **aggregation** (Figure 3). The interaction of this integrin with immobilized ligands is the most critical step in stable hemostatic plug formation, alterations in GPIIb/IIIa activation leading to severe bleeding, and/or thrombotic disorders. In resting platelets, the GPIIb/IIIa receptors are in a closed conformation, with their heads being massively bent over in a compact *V*-shape. Once activated, GPIIb/IIIa receptors undergo important conformational changes. The α and β domains of the headpieces shift from the closed to an open,

high-affinity conformation, exposing the binding sites for their ligands. This shift allows GPIIb/IIIa receptors to pass from a low-affinity to a high-affinity state and to bind their ligands—vWF, fibronectin, vitronectin, and mainly fibrinogen—and thus form bridges with the neighboring activated platelets, forming platelet aggregates (Figure 3). Fibrinogen's binding to the GPIIb/IIIa receptors leads to receptor grouping, triggers additional platelet activation, and leads (via an outside-in signaling pathway that involves Src family kinases and Syk) to more stable, irreversible platelet aggregation and to clot retraction, limiting the growth of the hemostatic mass and stabilizing the clot structure [26]. In parallel, a number of inhibitory molecules released by the vascular endothelium, such as nitric oxide, prostacyclin, and CD39, prevent unwanted platelet activation and limit the hemostatic process at the site of vascular injury [27]. Furthermore, accumulating evidence indicates that, following the initial stimulation, hemostatic clot formation involves the limitation of platelet activation by providing a restricted environment where agonists can accumulate. This ensures that platelet activation has a heterogeneous, but not random, distribution. While certain platelets become fully activated, change their shape, and degranulate, others are only partially activated, with the more stable core of the clot containing the former, in a densely packed structure, and the less stable shell containing the latter, in a less tightly packed configuration. This platelet subpopulations' gradient is accurately mirrored by agonist distribution gradients [28]. Moreover, recent data suggest that thrombin activity is restricted to the core region, whereas ADP and TxA2 appear to be the main drivers for platelet accumulation in the shell of the hemostatic clot, explaining the limited impact that the TxA2 and ADP pathway inhibitors exhibit on the clot core [29].

4. Clinical Implications of Platelet (Patho)Physiology

4.1. Platelet Abnormalities Translate into Clinically Relevant Dysfunctions of (Not Only) Primary Hemostasis

Rare but clinically significant platelet disorders caused by GPIb and GPV (i.e., Bernard-Soulier syndrome) or GPIIb/IIIa (i.e., Glanzman syndrome) deficiencies are associated with bleeding diathesis, whereas platelet granule abnormalities are commonly associated with only mild bleeding. Other genetic platelet abnormalities, such as the naturally occurring Leu/Pro 33 variant of GPIIIa, the presence of the 807T allele of the α2 gene, and different polymorphisms in the GPIIb gene, have all been related to an increased susceptibility to arterial thrombosis and myocardial infarction in different clinical studies [30].

However, platelets' function extends far beyond their contribution to primary hemostasis. Platelets also act as a rich reservoir of coagulation factors, as well as a scaffold mandatory for the progress of the coagulation cascade. Hence, the impact of platelet abnormalities also extends far beyond primary hemostasis, affecting the process of coagulation as well. Tissue factor's interaction with coagulation factor VII is a critical initial step in the coagulation process. Since tissue factor is an integral membrane protein, tissue factor-activated factor VII interactions require that the tissue factor be expressed on the surface of the cell membrane. Traditionally, it was believed that tissue factor was only expressed by extravascular, subendothelial tissues such as smooth muscle cells, macrophages, and fibroblasts. Studies have shown, however, that tissue factor can also be expressed by endothelial and circulating cells, such as monocytes and platelets [31]. The expression of tissue factor by these latter cells could contribute to the increased risk of disseminated intravascular coagulation in patients with sepsis and to thrombosis in cancer patients. In addition, platelet-derived tissue factor microparticles have been shown to circulate in the blood, and the levels of these microparticles have been shown to be significantly higher in patients with diabetes or atherosclerosis, contributing to the increased risk of thrombosis seen in these populations [32]. The pool of platelet-released factor V also seems to be highly relevant. Residual factor V secretion by activated platelets appears to be sufficient to prevent severe bleeding in patients with congenital factor V deficiency, which present only mild bleeding disorders, whereas platelet factor V deficiency is associated with a bleeding phenotype in patients with Quebec platelet disorder [33]. Similarly, platelet vWF has been shown

to partially compensate for the lack of plasma vWF in pigs with severe von Willebrand disease [34]. The critical role of phosphatidylserine exposure in coagulation is exemplified by the bleeding phenotype seen in patients with Scott syndrome, an isolated deficiency in platelets' procoagulant activity caused by mutations in genes encoding for the phospholipid scramblase. In these patients, although the platelets aggregate normally in response to all agonists, they display a markedly decreased exposure of anionic phospholipids on their surface, have a reduced number of activated factors V and VIII binding sites, and fail to promote prothrombin and factor X activation [35].

Conversely, since thrombin is one of the most powerful platelet activators, coagulation abnormalities will also disturb normal platelet function. Indeed, patients with von Willebrand disease present not only coagulation, but also platelet disorders, as demonstrated by the markedly impaired thrombin generation time measured in the plasma of type 3 von Willebrand disease patients, which lack vWF in plasma, platelets, and endothelial cells, whereas this is not the case when thrombin generation time is measured in the presence of normal platelets [36]. In patients with Owren's parahemophilia (with congenital factor V deficiency), the existence of functional platelet-derived coagulation factor V is thought to support enough thrombin generation to prevent severe bleeding in patients with virtually undetectable plasma factor V [33]. Meanwhile, the increased tendency for bleeding in patients with Bernard–Soulier syndrome, which lack functional GPIb and GPV receptors, appears to be due to decreased platelet activation in response to thrombin [37].

4.2. The Functional Properties of Platelets Explain Their Stronger Implications in Arterial Than Venous Thromboses

All thrombi, regardless of their location, contain platelet aggregates, fibrin, and trapped red blood cells. However, the proportions of these elements are considerably different in the arterial and the venous territories. Arterial thrombosis, such as that occurring in myocardial infarctions, acute limb ischemia, or strokes, usually occurs as a result of an atherosclerotic plaque rupture in vascular areas with high wall shear stress and is generally characterized by obstructive, 'white thrombi' that are rich in platelets disposed in large aggregates, have a modest fibrin content, and relatively few trapped red blood cells (Figure 4A). Meanwhile, venous thrombosis occurs in areas with low shear stress and is generally characterized by 'redder' thrombi, with more trapped red blood cells and a higher fibrin and lower platelet content, the latter being mainly recruited as single cells (Figure 4B) [27]. Consequently, arterial thrombosis has generally been regarded as a platelet- and venous thrombosis as a coagulation-related disease. In line with this concept, anticoagulant agents have been shown to efficiently prevent venous thrombosis [38], whereas antiplatelet agents have been shown to be highly efficient for arterial thrombosis therapy and secondary prevention [39].

The more important role played by the platelets in the arterial than in the venous territory is mainly related to the high shear stress characteristic of the arterial circulation. In the arteries, and particularly in stenotic areas, where shear stress can reach values as high as 10,000/s, vWF-mediated platelets' adhesion to the vessel wall via the GPIb-V-IX complex increases in parallel with the wall shear rate, and hence with the flow rate. The same conditions exhibit opposite effects on the coagulation system, washing out coagulation factors and impeding thrombin accumulation, thus limiting coagulation cascade efficacy [40]. These mechanisms clearly explain why platelets play more important roles in the arterial circulation, whereas coagulation factors are more important in the venous system.

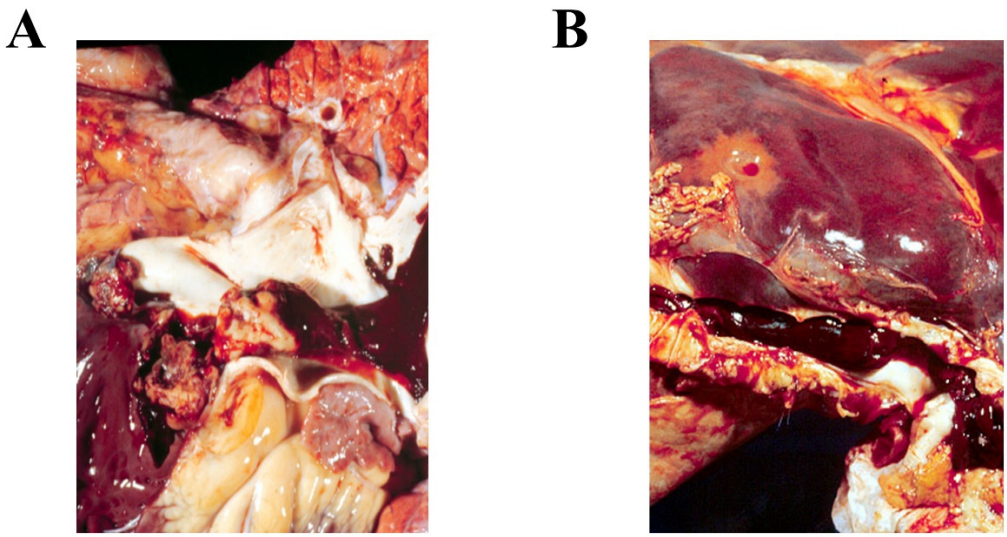

Figure 4. Macroscopic appearance of a (**A**) white thrombus in a patient with carotid artery occlusion and of a (**B**) red thrombus in a patient with deep vein thrombosis.

In reality, however, this view of thrombosis oversimplifies a very complex process that involves blood stasis, the activation of the vascular endothelium, innate immunity, platelets, and coagulation factors. There is strong evidence for systemic platelet activation in patients with acute venous thromboembolism [41]. In the same vein, platelets have been shown to play critical roles in the initiation and propagation of venous thrombi, and platelet inhibition or depletion suppressed thrombus formation in mouse models of venous thrombosis [42]. Furthermore, studies have shown aspirin and clopidogrel to be capable of attenuating the risk of venous thrombosis in murine venous stasis models [43], as well as in patients undergoing orthopedic surgery [44], and to reduce the risk of deep vein thrombosis recurrence by 32% compared with a placebo, without a significant increase in the risk of bleeding [45], although their effectiveness in venous thrombosis is clearly inferior to that of the oral anticoagulants [46]. Similar results were also reported for patients with atrial fibrillation [47,48]. The ongoing large pragmatic Comparative Effectiveness of Pulmonary Embolism Prevention after Hip and Knee Replacement (PEPPER) trial (NCT02810704)—designed to evaluate the effects of three upfront antithrombotic strategies (i.e., aspirin 81 mg BID, warfarin dose-adjusted to achieve a target international normalized ratio of 2.0, and rivaroxaban 10 mg once daily) on the composite of all-cause mortality and symptomatic venous thromboembolism [49]—is expected to yield more data regarding aspirin's efficacy in primary venous thromboembolism prevention.

In parallel, while thrombin clearly plays a critical role in venous thrombosis, this procoagulant enzyme is also the most potent physiological platelet activator, which places thrombin as a key element for not only venous but also arterial thrombosis. Indeed, thrombin has been shown to initiate arterial thrombosis and essentially all experimental thrombosis models have been shown to be sensitive to deficiencies in either platelets or coagulation factors [50]. Furthermore, in the landmark Cardiovascular Outcomes for Peoples Using Anticoagulant Strategies (COMPASS) trial, a combination of a low dose of the activated factor X inhibitor rivaroxaban and the antiplatelet drug aspirin was more effective than aspirin alone for the prevention of stroke, myocardial infarction, and cardiovascular death in patients with stable coronary or peripheral artery disease [51]. Similarly, in the Anti-Xa Therapy to Lower cardiovascular events in addition to Aspirin with or without thienopyridine therapy in Subjects with Acute Coronary Syndrome–Thrombolysis in Myocardial Infarction (ATLAS ACS 2-TIMI 51) study, the combined inhibition of platelets (with aspirin and a P2Y12 antagonist) and coagulation (with rivaroxaban) reduced the risk of in-stent thrombosis compared to dual antiplatelet therapy alone [52]. Early clinical trials also demonstrated a clinical benefit of vitamin K antagonists in arterial thrombosis,

although at the expense of increased bleeding [53]. Moreover, studies have shown that in patients with acute myocardial infarction, the platelet and fibrin content of coronary thrombi varies, with fibrin becoming more abundant, and platelet content decreasing in direct relationship with the duration of the ischemia [27], observations that may have critical implications on the optimal timing of antithrombotic therapy administration.

4.3. Antiplatelet (and Anticoagulant) Drugs Target Key Mechanisms of Platelets' Adhesion, Activation, and Aggregation

Hemostasis is a physiological process critical for survival. Meanwhile, thrombosis is amongst the leading causes of death worldwide, making antithrombotic therapy one of the most crucial aspects of modern medicine. This context explains the broad range of antiplatelet agents that clinicians have at their disposal. Four main classes of drugs (i.e., COX-1, P2Y12, PAR-1, and αIIbβ3 inhibitors) are currently in use, alone or in various combinations, to counteract platelet hyperreactivity and arterial thrombosis (Figure 3). Several other drug classes, such as activated αIIbβ3, αIIbβ3 outside-in signaling, novel PAR (e.g., parmodulins, pepducins, and PAR-4 inhibitors), phosphatidylinositol 3-kinase-β, and protein disulfide-isomerase inhibitors are currently under evaluation with the promise of providing a safer inhibition of thrombosis, with minimal perturbations of hemostasis [27]. A detailed description of these existing and emerging drugs is beyond the scope of the present paper.

Importantly, however, given the complex interactions that exist between platelets and the coagulation system, anticoagulant agents can also exhibit antiplatelet effects. The dual antiplatelet and anticoagulant effect of anticoagulant agents is particularly important in light of the contemporary trend toward combining direct oral anticoagulants with antiplatelet therapy, which could affect the risk of bleeding via a synergistic effect [54] and potentially influence the choice of treatment based on the patient's risk profile, particularly if potent high-dose anticoagulants and/or potent antiplatelet agents (such as PAR-1 antagonists) are considered for use. The same synergy could also explain, however, the increased efficiency obtained by combining COX-1 with activated factor X inhibitors compared with COX-1 inhibition alone in patients with stable atherosclerotic vascular disease [51]. Clinical studies have also shown that the direct thrombin inhibitor dabigatran decreases platelet aggregation, while also enhancing platelet-mediated fibrinolysis in patients with atrial fibrillation [55–57]. However, given that most clinical studies indicate a mild numerical increase, and not a decrease, in the frequency of myocardial infarction following direct thrombin inhibition [58,59], the clinical significance of these findings remains unclear. Moreover, several studies have associated dabigatran administration with an increase in platelet reactivity and this has been at least partly attributed to altered GPIbα-thrombin interaction in the presence of shear forces [60] as well as increased PAR-1 [61] and PAR-4 [62] expression on the platelets' surface following long-term thrombin inhibition [63]. This finding could be of particular importance since PARs can also be activated by serine proteases other than thrombin, including plasmin; activated protein C; matrix metalloproteases 1, 2, and 13; elastase; proteinase-3; granzyme; neutrophil-derived cathepsin G; or calpain [64].

Recent studies indicate that, in addition to thrombin, activated factor X can also act, via PARs, as a direct platelet activator, suggesting that activated factor X inhibition could affect platelet function via a thrombin-independent mechanism [64]. However, the data regarding the effects of direct activated factor X inhibitors on platelet function remain unclear. In some studies, rivaroxaban did not appear to affect platelet aggregation in response to ADP, collagen, TxA2, or thrombin [65]; in other studies, rivaroxaban exerted strong antiplatelet effects [66,67], whereas in others still rivaroxaban was associated with an increase in platelet aggregation [68]. In experimental settings, the direct inhibition of activated factor X using high but not low concentrations of apixaban has also been shown to reduce platelet aggregation as well as fibrin generation [69]. More importantly, studies suggest that apixaban at doses one fifth to one half of that recommended for stroke prevention in atrial fibrillation patients could reduce the formation of large platelet aggregates, while

still allowing fibrin formation, thus preserving its contribution to hemostasis [69]. Data concerning the effects of vitamin K antagonists on platelet function are also controversial, with some studies showing that platelet aggregation is not affected by warfarin [70], whereas others showed reduced platelet aggregation following vitamin K antagonists' administration [71]. Meanwhile, negatively charged activated factor X inhibitors have been shown to not exhibit inhibitory platelet effects. On the contrary, unfractionated heparin and fondaparinux are known to increase platelet functions via non-immune mediated mechanisms [72,73].

5. Gaps in Knowledge and Future Research

Advances in biochemistry, molecular biology, and the advent of 'omics' techniques have provided crucial data for our understanding regarding the complex structure and functions of platelets and their interactions with the coagulation system. Next-generation sequencing, RNA-sequencing, and platelet proteomics are expected to further unravel the complex processes involved in platelets' adhesion, activation, and aggregation; in signal transduction, granule secretion, and platelet cytoskeletal changes; and to provide valuable pharmacogenetic information. Advancements in laboratory testing could also provide the means to accurately evaluate platelet function and predict clinical events, similarly to what has already been achieved in other clinical settings [74,75].

Drugs that interfere with platelet function are under continuous development. Antagonists of PAR-1 have already been approved for clinical use and PAR-4 antagonists showed great promise in preclinical studies [76]. Other therapies are also expected to emerge in the near future. The next frontier in this area will probably be reached when antithrombotic drugs that manage to differentiate thrombosis from hemostasis and target them discriminately make their way into clinical practice, thereby offering the prospect of a safer antiplatelet therapy.

The exact short- and long-term effects of the oral anticoagulants on platelet function is another open area for research. This is particularly important given the expanding use of direct oral anticoagulants, especially in patients exposed to such agents for decades, such as those with atrial fibrillation [74,77]. If long-term direct thrombin and/or activated factor X inhibition induce a clinically-relevant increase in platelet reactivity, this effect may translate into an increased risk of thrombosis in certain high-risk populations [78–80], particularly following abrupt drug discontinuation [81,82], or could potentially interfere with patients' responses to concomitant antiplatelet therapy [83,84].

6. Conclusions

With their exceptional structural and functional features, platelets play critical roles in hemostasis, vasomotor function, and immunity. In hemostasis, their contribution far exceeds their simple participation in the formation of the 'platelet plug'. The multiple interactions that exist between platelets, the vessel wall, and the coagulation system complicate the fundamental process of hemostasis and particularly that of thrombosis. Antithrombotic drugs that interfere with platelet functions are under continuous development. The next frontier in this area will probably be reached when antithrombotic drugs that manage to differentiate thrombosis from hemostasis make their way into clinical practice, offering the prospect of safer antiplatelet therapy. Given the rapidly expanding use of the direct oral anticoagulants, establishing their exact impact on platelet functions is another open area for research.

Funding: This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS-UEFISCDI, project number PN-III-P1-1.1-TE-2019-0370, within PNCDI III.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Targeting Myocardial Fibrosis—A Magic Pill in Cardiovascular Medicine?

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Abstract: Fibrosis, characterized by an excessive accumulation of extracellular matrix, has long been seen as an adaptive process that contributes to tissue healing and regeneration. More recently, however, cardiac fibrosis has been shown to be a central element in many cardiovascular diseases (CVDs), contributing to the alteration of cardiac electrical and mechanical functions in a wide range of clinical settings. This paper aims to provide a comprehensive review of cardiac fibrosis, with a focus on the main pathophysiological pathways involved in its onset and progression, its role in various cardiovascular conditions, and on the potential of currently available and emerging therapeutic strategies to counteract the development and/or progression of fibrosis in CVDs. We also emphasize a number of questions that remain to be answered, and we identify hotspots for future research.

Keywords: antifibrotic strategies; cardiac fibrosis; cardiovascular diseases; fibrosis pathways; therapeutic strategies

Citation: Scridon, A.; Balan, A.I. Targeting Myocardial Fibrosis—A Magic Pill in Cardiovascular Medicine? *Pharmaceutics* **2022**, *14*, 1599. https://doi.org/10.3390/pharmaceutics14081599

Academic Editors: Ionut Tudorancea and Radu Iliescu

Received: 21 June 2022
Accepted: 28 July 2022
Published: 30 July 2022

1. Introduction

Cardiovascular diseases (CVDs) are the leading cause of death and morbidity, accounting for up to one-third of deaths worldwide. The prevalence of CVDs has seen a tremendous increase over the past decades, with a doubling of CVD cases between 1990 and 2019 [1]. In parallel, cardiovascular mortality has also gradually increased during this period, from 12.1 million in 1990 to 18.6 million in 2019 [1]. Metabolic, behavioral, environmental, and social factors have all been linked to increased cardiovascular risk. Whereas several of those factors are modifiable and their removal may lower the prevalence of CVDs, others, such as age, race, sex, or family history, continue to have a significant impact on the evolution of CVDs' prevalence [1].

Initially seen as an adaptive process designed to ensure wound healing and tissue repair following injury, myocardial fibrosis is now recognized as a major contributor to CVDs and CVD-related morbidity and mortality in many clinical settings [2]. Accumulating data show that most CVDs involve pathological myocardial remodeling characterized by cardiac fibrosis. In myocardial infarction, fibrosis develops as a repair mechanism for maintaining the integrity of the cardiac wall. However, over the long term, the lack of contractile capacity of the fibrous tissue along with the death of cardiac myocytes eventually lead to impaired cardiac function [2]. In many other CVDs (e.g., hypertensive heart disease, diabetic, dilated, and hypertrophic cardiomyopathy, heart failure, chronic ischemic heart disease, or cardiac arrhythmias), fibrosis is also recognized at present as a causative or at least as an aggravating factor [2]. In addition, the natural process of aging promotes cardiac fibrosis via countless pathophysiological pathways, even in the absence of concomitant heart disease [2]. Myocardial fibrosis has thus rose as a promising diagnostic and prognostic

marker in CVD patients, and strategies aiming to prevent, halt, or even reverse fibrosis have emerged as promising means to prevent and/or treat various forms of CVD.

This paper aims to provide a comprehensive view on cardiac fibrosis, with a focus on the main pathophysiological pathways involved in its occurrence and progression, its role in various cardiovascular conditions, on the techniques available for fibrosis identification and quantification, and on the potential of currently available and emerging therapeutic strategies to counteract the development and/or progression of fibrosis in CVDs.

2. The Extracellular Matrix—From Physiology to Pathophysiology

The cardiac muscle and the conduction system, supported by a fibrous skeleton, constitute the basic framework of the heart. The cardiac wall consists of three overlapped layers: the epicardium, composed of fibro-elastic and adipose tissue, the myocardium, consisting of cardiomyocytes arranged in layers and surrounded by a complex network of proteins that form the extracellular matrix (ECM), and the endocardium, made up of endothelium and subendothelial connective tissue [3]. At the level of the myocardium, the ECM works as a scaffold for cellular components and contributes to the transmission of the contractile force [4]. Tension strength is mostly provided by thick type I collagen, which accounts for ≈85% of the cardiac collagen, whereas type III collagen, present in smaller amounts (≈11%), is responsible for maintaining the elasticity of the ECM [4]. In addition to collagen, the ECM also contains elastic fibers, fibronectin, glycoproteins, glycosaminoglycans, proteoglycans, latent growth factors, and proteases [4].

2.1. Cellular Components Involved in Cardiac Fibrosis

The main cellular component involved in cardiac fibrosis is represented by the ***cardiac fibroblasts***, which are responsible for maintaining ECM integrity by regulating collagen turnover [5]. In contrast to cardiomyocytes, fibroblasts are non-excitable cells. Fibroblasts are connected, however, via gap junctions, to the neighboring cardiomyocytes, thereby contributing to optimal electrical conduction within the heart [6]. In settings favoring cardiac fibrosis, such as ischemic, hypertensive, or valvular heart disease, fibroblasts transdifferentiate into ***myofibroblasts*** (Figure 1), hybrid fibroblast/cardiomyocyte cells that express numerous ultrastructural and phenotypic characteristics of muscle cells, but not excitability [7]. Although most myofibroblasts originate in the myocardium, studies have shown that they can also have hematopoietic or endothelial origin [7]. Myofibroblasts play critical roles in both myocardial repair and fibrosis [8] and can be identified in the damaged myocardium already from the early stages of the fibrotic response by highlighting cytoplasmic actin-derived stress fibers and later α-smooth muscle actin [9]. Smooth muscle myosin heavy chain, paxillin, and tensin can also be used as myofibroblast biomarkers [10].

Monocytes and ***macrophages*** are also critical for both the initial and the chronic phase of the fibrotic response, but they also contribute to resolution of fibrosis [11]. Their ability to exert both pro- and antifibrotic effects can be explained by the large heterogeneity of these cells, which is related to the presence of several specific cell subpopulations and to their variable response to microenvironmental factors [11]. Certain monocyte and macrophage subpopulations have the ability to differentiate into myofibroblasts and to secrete numerous profibrotic cytokines (such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6), growth factors (e.g., transforming growth factor β (TGF-β), platelet-derived growth factors (PDGFs), and fibroblast growth factors (FGFs)), and proteases, thereby participating in ECM remodeling [11]. The removal of dead cells by macrophages via phagocytosis facilitates fibroblasts growth, further contributing to myocardial fibrotic remodeling [11]. In parallel, however, monocytes and macrophages also act to eliminate profibrotic stimuli via phagocytosis of apoptotic myofibroblasts, ECM cells, and residues [11]. Due to their remarkable functional plasticity, macrophages also regulate the secretion of cytokines and growth factors in response to changes in the microenvironment [11]. Other cells of hematopoietic origin, such as the ***mast cells***, have also been shown to play important roles in the fibrotic process related to myocardial infarction and various cardiomyopathies [12].

The role of mast cells in cardiac fibrosis seems to be primarily related to their increased content in granules rich in bioactive mediators, cytokines, and growth factors, including histamine and mast cell-specific proteases tryptase and chymase [12]. Although the role of Th2 ***lymphocytes*** in pulmonary fibrosis has been thoroughly reviewed [13], the involvement of lymphocytes in cardiac fibrosis is much less clear. A profibrotic effect of Th17 cells has been reported in experimental myocardial fibrosis models [14], but other subsets of T lymphocytes have been shown to act as fibrosis inhibitors [15]. ***Neutrophils***, the first cells that arrive at the site of a tissue injury, have also been shown to play critical roles in myocardial inflammation and consequent fibrosis. After acute myocardial infarction, neutrophils accumulate at the border between the healthy and the necrotic tissue and release inflammatory mediators and proteolytic enzymes that degrade necrotic myocardial cells and ECM residues [16]. Neutrophil persistence at the site of the injury appears to also cause, however, additional damage to viable cardiomyocytes [16]. Neutrophil inhibition one week after myocardial infarction has been shown to cause a paradoxical increase in fibroblast activity and excessive collagen deposition [17], suggesting that the moment of such a therapeutic intervention may be critical. Meanwhile, in a myocarditis animal model, neutrophil extracellular traps strongly correlated with the amount of collagen deposited and inhibition of cytokines responsible for neutrophil recruitment attenuated collagen deposition in that model [18].

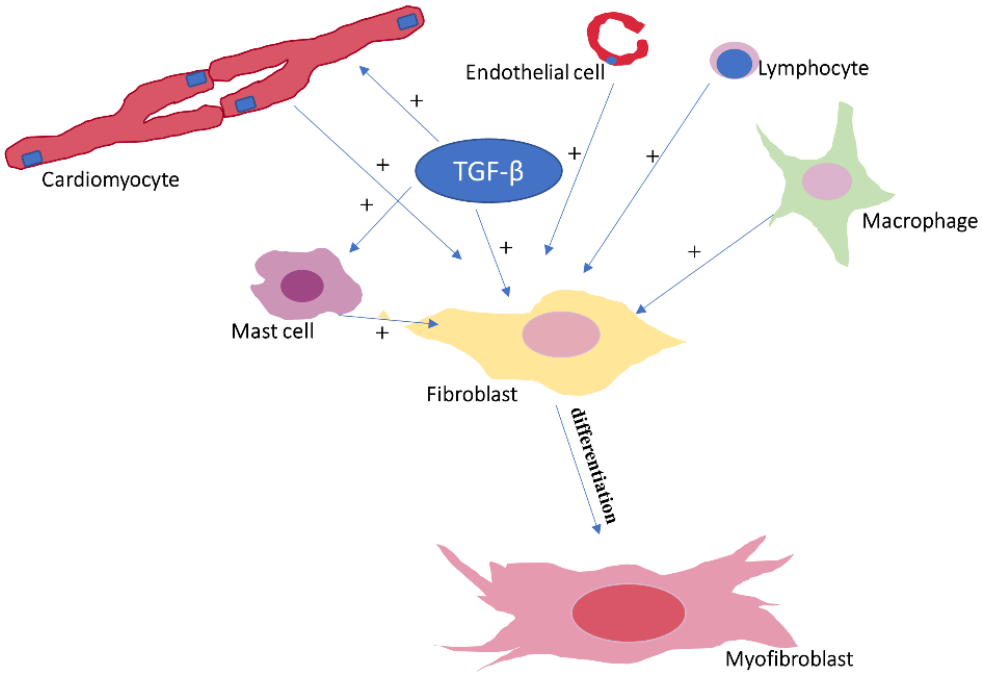

Figure 1. Interactions between different cardiac cells involved in the development of cardiac fibrosis. Cardiac cells (i.e., cardiac myocytes, macrophages, mast cells, lymphocytes, endothelial cells, and fibroblasts) regulate cardiac fibrosis in a coordinated manner. In the presence of cardiac injury, these cells release inflammatory mediators that stimulate fibroblast-to-myofibroblast differentiation, contributing to the development of fibrotic tissue. Transforming growth factor-*beta* (TGF-*β*) is among the most relevant of these profibrotic mediators. "+" designates a stimulatory effect.

The angiogenic response and the presence of perivascular fibrosis in settings associated with cardiac fibrosis have drawn attention toward a potential role of ***endothelial cells*** in cardiac remodeling and fibrosis, mainly via the secretion of endothelin, a major promotor of fibrotic matrix production, by these cells [19]. By releasing proinflammatory cytokines and chemokines, the endothelium has an important ability to recruit numerous types of fibrogenic cells [20]. In addition, endothelial cells can take, via mesenchymal transition, a mesenchymal cell phenotype, which enhances their invasiveness and migratory capacity,

resistance to apoptosis, and ability to produce ECM components [7]. However, similarly to other types of cells, endothelial cells also possess antifibrotic effects via the secretion of factors such as interferon-γ-inducible protein-10/chemokine (C-X-C motif) ligand 10 and hypoxia inducible factor-1, which have been shown to protect the murine heart and the aorta from pressure overload via suppression of TGF-β signaling [21].

Cardiac myocytes can also trigger, through their death, a profibrotic inflammatory response [22]. Moreover, in certain pathological settings, even viable cardiomyocytes can promote, via pannexin-1 channels-induced ATP release, the activation of interstitial fibroblasts [23]. Deoxycorticosterone/salt-sensitive cardiomiocyte mineralocorticoid receptors have also been shown to play important roles in cardiac inflammation and fibrosis, whereas loss of these receptors has been shown to attenuate the cardiac fibrotic response [24]. Cardiomyocyte-selective TGF-β receptor II (TβRII) blockade decreased interstitial fibrosis in response to pressure overload [25], whereas cardiomyocyte-specific overexpression of angiotensin II (Ang II) type 2 receptor (AT2) was shown to exhibit antifibrotic actions mediated by the activation of the kinin–nitric oxide system [26].

2.2. Extracellular Components Involved in Cardiac Fibrosis

The accumulation of excessive amounts of fibrillar and non-fibrillar ***collagen*** within the myocardium represents the landmark of cardiac fibrosis [27,28]. In the remodeled heart, fibrillar collagen is represented by type I and type III collagen, the ratio between the two depending on the context that favored cardiac fibrosis development [27,28]. Cardiac myofibroblasts are the main source of cardiac collagen. Once secreted, collagen is assembled and cross-linked into a network that provides mechanical support and structural integrity to bear the increased stress and load in the presence of myocardial injury [27,28]. Non-fibrillar collagen type IV, VI, and VIII is also present in cardiac fibrosis [29]. Of these, type VI collagen has been shown to activate cardiac fibroblasts and promote myofibroblast conversion, whereas its absence has been associated with a reduction in myocardial fibrosis [29]. Other ECM components, such as amino- and proteoglycans, elastin, fibronectin, and laminin, are also present, and they play critical roles in maintaining cardiac structural integrity. Elastin provides resilience and elasticity to the cardiac wall, fibronectin fibers, organized in a fibrillar network at the cell surface, influence the structural and mechanical properties of the ECM, whereas the structural role of laminin translates into ECM cells anchoring and binding to multiple other proteins present within the matrix [4].

2.3. Types of Cardiac Fibrosis

The ability of the cardiac muscle to regenerate in response to injury is extremely low. Cardiac repair therefore occurs mainly via fibroblasts activation and differentiation into myofibroblasts, which is followed by excessive collagen deposition, fibrosis, increased ECM stiffness, and impaired cardiac contractile function [30]. Three major types of fibrotic changes have been described in the heart: replacement, interstitial, and perivascular fibrosis [30]. Replacement fibrosis is characterized by the loss and consequent fibrotic replacement of cardiac myocytes. Interstitial fibrosis includes two subtypes: reactive and infiltrative fibrosis. Reactive fibrosis occurs in response to pressure overload and is characterized by excessive ECM, without significant loss of cardiomyocytes, whereas the accumulation of insoluble proteins in the heart cells, as seen in Fabry disease, is defined as infiltrative fibrosis [30]. Finally, perivascular fibrosis involves the deposition of connective tissue around the blood vessels, as often seen in patients with hypertensive heart disease [30].

Regardless of the underlying cause, the accumulation of ECM proteins within the cardiac interstitium initially occurs as a beneficial, protective mechanism that promotes wound healing and tissue regeneration. Later, alterations in ECM composition and quality lead to fibrosis progression beyond the physiological threshold and to negative consequences on myocardial excitation–contraction coupling [31]. The distorted cardiac architecture increases ventricular stiffness and alters the contraction and relaxation of the heart, resulting in cardiac systolic and diastolic dysfunction [31]. Concomitantly, fibrosis disturbs the normal

electrical activity of the heart, promoting both brady- and tachyarrhythmias [32]. Whereas conduction blocks in the sinoatrial and/or atrioventricular nodes caused by fibrosis favor bradyarrhythmias, tachyarrhythmias often occur due to the increased propensity to re-entry of the fibrotic myocardium [32].

2.4. Molecular Pathways of Myocardial Fibrosis

Extensive evidence links the activation of the ***renin–angiotensin–aldosterone system*** (RAAS) with the pathogenesis of cardiac fibrosis. The main effector of this system, Ang II, has a wide range of cardiac physiological and pathophysiological effects [33]. Cells present in the heart, particularly macrophages and fibroblasts, have been shown to produce both renin and the angiotensin-converting enzyme (ACE), which are required to generate Ang II. Once released, Ang II stimulates cardiac fibroblasts, directly and indirectly (via TGF-β), promoting collagen production by these cells (Figure 2) [34]. In parallel, AngII decreases the activity of matrix metalloproteinase (MMP)-1, thereby concomitantly reducing collagen degradation [33]. These profibrotic effects of Ang II occur mainly via the AT1 receptors and multiple subsequent intracellular signaling pathways [33]. Among them, the mitogen-activated protein kinase (MAPK) and the phosphoinositol-3 kinase/Akt pathways have been shown to regulate cardiac cells survival, apoptosis, and growth and to play critical roles in Ang II-induced cardiac remodeling [35,36].

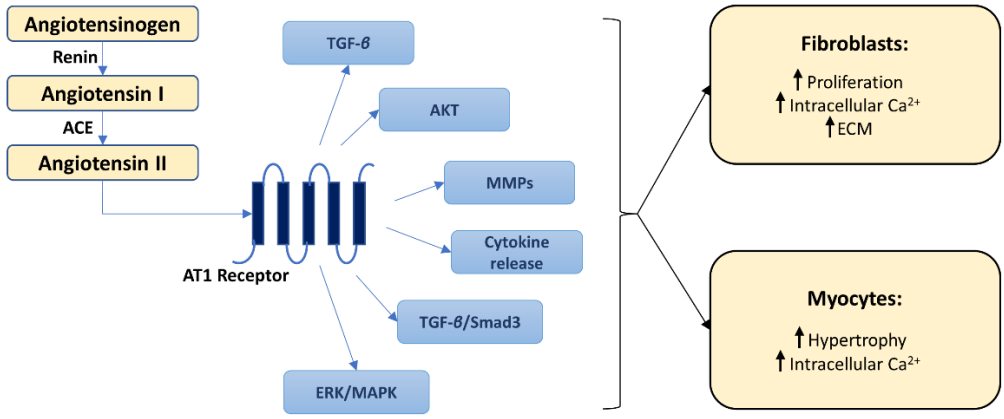

Figure 2. Pathways related to angiotensin II and their contribution to myocardial fibrosis. The figure describes the formation of angiotensin II (left part of the figure) and the consequent activation, via AT1 receptors, of numerous inflammatory and profibrotic pathways (middle part of the figure), which will eventually lead to profibrotic cardiac fibroblast and myocyte changes (right part of the figure). Myocyte hypertrophy has been shown to promote fibrosis by stimulating fibroblast activation via a complex network of downstream signal transduction pathways and by increasing the production of growth factors. "↑" designates an increase in profibrotic cardiac fibroblast and myocyte changes. ACE—angiotensin-converting enzyme; AKT—protein kinase B; AT1—angiotensin II type 1 receptor; ECM—extracellular matrix; ERK—extracellular signal-regulated kinase; MAPK—mitogen-activated protein kinase; MMPs—matrix metalloproteinases; TAK1—TGF-β-activated kinase 1; TGF-β—transforming growth factor-*beta*.

In contrast, AT2 receptor stimulation counteracts the profibrotic effects of AT1 by suppressing fibroblast proliferation and matrix synthesis [37]. Another component of the RAAS system, aldosterone, also contributes to excessive ECM accumulation by activating macrophages, cardiac myocytes and fibroblasts and increasing the expression of proinflammatory cytokines and chemokines [38].

G protein-coupled receptors (GPCRs) are cellular receptors that activate G-protein-dependent intracellular signaling pathways [39]. In parallel, several GPCR kinase- and β-arrestin2-mediated processes act as regulatory mechanisms to prevent excessive G protein activation (Figure 3) [39].

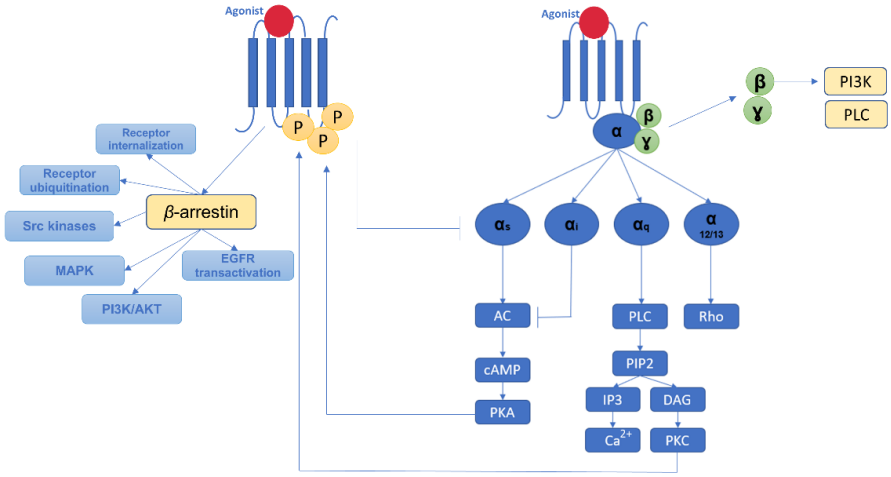

Figure 3. G protein-coupled receptors-related pathways and *β*-arrestin-mediated events. G-protein coupled receptors are transmembrane proteins embedded in the membrane of cardiomyocytes, fibroblasts, endothelial, and vascular smooth muscle cells that convert extracellular signals into intracellular responses. When activated by agonists (e.g., epinephrine, peptide hormones), inactive G protein heterotrimers dissociate into separate, active G*α* and G*βγ* subunits that differentially control downstream signal transduction. Intracellular mediators such as protein kinases A and C resulted from this process further phosphorylate the receptors and activate *β*-arrestin-mediated signaling, activating subsequent signaling cascades involved in cardiac fibrotic disease. AC—adenylyl cyclase; AKT—protein kinase B; cAMP—cyclic adenosine monophosphate; DAG—diacylglycerol; EGFR—epidermal growth factor receptor; IP3—inositol trisphosphate; MAPK—mitogen-activated protein kinase; PI3K—phosphoinositide 3-kinase; PIP2—phosphatidylinositol-4,5-bisphosphate; PKA—protein kinase A; PLC—phospholipase C; PKC—protein kinase C.

Several 'biased ligands' can activate signaling pathways independent of the G proteins but dependent on GPCR kinase/*β*-arrestin2 [39]. One such 'biased ligand' is metoprolol, which can therefore promote cardiac fibrosis and alter the cardiac diastolic function [39]. *Beta*-adrenergic receptors (*β*ARs) are the predominant GPCR subtype expressed within the heart [40]. In physiological conditions, *β*1ARs represent ≈80% of the total cardiac *β*ARs. However, in the setting of heart failure, *β*1ARs percentage can decrease to as low as 60%, with a concomitant increase in the proportion of *β*2ARs, whereas *β*3ARs are present in the heart in much smaller amounts [40]. Excessive *β*1ARs stimulation has been linked with myocyte apoptosis [40]. Meanwhile, the role of *β*2ARs in this setting remains controversial. Some studies suggested that *β*2Ars-mediated signaling could be cardioprotective [40], but in others, non-specific *β*ARs stimulation with isoproterenol and transgenic overexpression of *β*2ARs were highly profibrotic [41,42], leaving this topic an open area for future research. The role of *β*3AR in cardiac fibrosis remains even less understood, although recent studies suggest that *β*3AR-mediated signaling could modulate oxidative stress-dependent paracrine signaling and consequently exhibit antifibrotic effects [43].

Endothelin-1 (ET-1), a protein synthesized by the vascular endothelium, has also been shown to play key roles in cardiac remodeling and dysfunction by promoting ECM synthesis and decreasing collagenase activity [44]. In addition, in vitro studies have shown that ET-1 increases fibroblasts' resistance to apoptosis [45], whereas ET-1 antagonization has been shown to attenuate cardiac fibrosis related to hypertension and myocardial infarction [46].

Immediately after myocardial injury, inflammatory cells, fibroblasts, and cardiomyocytes release a vast amount of cytokines and growth factors [11]. Among them, ***TNF-α***, ***IL-1β***, and ***IL-6*** levels are particularly increased in response to the inflammatory process and strongly contribute to the future development of cardiac fibrosis [47]. The role of TNF-*α* in cardiac fibrosis is supported by numerous experimental and clinical studies [48,49]. Meanwhile, the absence of TNF-*α* reduced the inflammatory response and cardiac fibrosis

in mice [47], and TNF-α inhibition has been shown to improve left ventricular structure and function in patients with advanced heart failure [48]. In contrast to the vast majority of profibrotic stimuli, TNF-α does not exert its fibrotic effect by an increase but rather by a decrease in collagen synthesis, suggesting that the profibrotic effect of TNF-α is more likely to occur as a response to ECM degradation [49]. Additional mechanisms involved in TNF-α-induced fibrosis include synthesis of the matrix protein cellular communication network factor 4, favoring fibroblast proliferation, increased TGF-β1 expression, immune cell activation and proliferation, and promotion of AT1 receptors synthesis [50]. Data regarding the role of IL-1β in cardiac fibrosis are rather controversial. Some experimental studies suggested that IL-1β may promote cardiac fibroblast migration, while others reported the opposite [51]. In patients with rheumatoid arthritis, IL-1 inhibition led, however, to a significant improvement in left ventricular function [52]. In some, but not all studies, a relationship was found between low IL-6 levels and cardiac fibrosis [53,54].

The most studied fibrosis-related growth factor, ***TGF-β***, has been shown to play a central role in maladaptive cardiac remodeling in both myocardial infarction and heart failure (Figure 1) [55,56]. In gain-of-function studies, cardiac overexpression of TGF-β1 increased collagen deposition and promoted cardiac fibrosis, whereas TGF-β1 deficiency has been associated with a lower degree of aging-related cardiac fibrosis [55,57]. The stimulating effect of TGF-β on cardiac fibroblasts appears to be the basis for an increased synthesis of ECM proteins [57]. Its effects on monocytes, lymphocytes, and cardiomyocytes further contribute to the profibrotic effects of TGF-β [58] as well as the reversal of the fibrosis degradation/preservation balance toward a matrix-preserving pattern via inhibition of collagenases and induction of protease inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitors of metalloproteinases [57]. Meanwhile, in experimental studies, a loss of TGF-β receptors reduced cardiac fibrosis [59], further supporting the importance of TGF-β signaling cascades in cardiac fibrosis. The TGF-β signaling cascades exert their profibrotic effects through Smads, intracellular effector proteins, but also through Smad-independent pathways, both leading to fibroblast activation (Figure 4) [60].

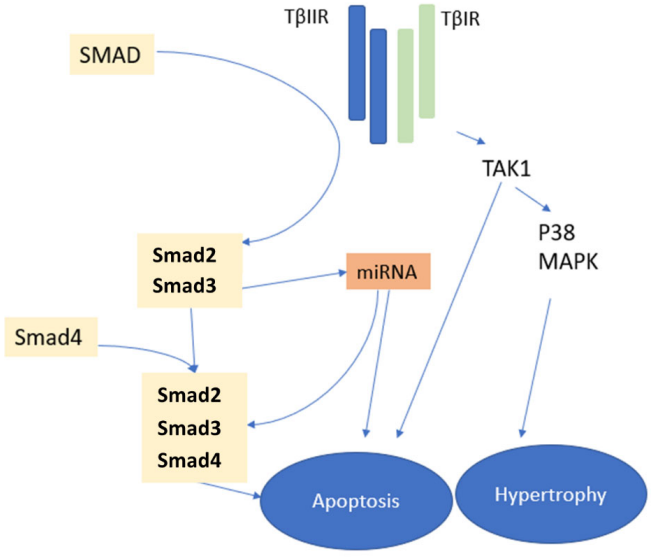

Figure 4. Transforming growth factor *beta*-related pathways and their contribution to myocardial fibrosis. Paracrine factors in fibroblasts, the most important of which is transforming growth factor-*beta*, induce profibrotic responses in cardiomyocytes. The activation of type I and II transforming growth factor-*beta* receptors regulates cell phenotypes by activating Smad- and non-Smad-related signaling pathways that eventually result in cardiomyocyte apoptosis and hypertrophy. MAPK—mitogen-activated protein kinase; TAK1—transforming growth factor-*beta*-activated kinase 1; TβIR—transforming growth factor-*beta* receptor type I; TβIIR—transforming growth factor-*beta* receptor type II.

Studies have shown Smad3 signaling to be critically involved in chronic fibrotic cardiac remodeling and to contribute to fibroblasts activation, α-smooth muscle actin expression, and synthesis of ECM [61]. In contrast, myofibroblast-specific Smad2 signaling appears to be only transiently implicated in early adverse remodeling and does not seem to play a major role in fibroblast activation [60]. Smad-independent profibrotic TGF-β-related pathways involve p38, MAPK, extracellular signal-regulated kinase, and TGF-β-activated kinase 1 (TAK1) signaling pathways activation (Figure 1) [62].

Exosomes are extracellular microvesicles that supply cells with RNA, proteins, lipids, and other biologically active signaling molecules, while acting as couriers for intercellular communication. Recent evidence indicates that in the presence of cardiac fibrosis, there is altered intercellular communication via the exosomes [63]. In addition, exosomes have been shown to alter the process of cardiac repair and to cause fibrosis via the modulation of fibroblast function [64]. During cardiac injury, exosomes promote the activation of naive fibroblasts to initiate the wound-healing process and contribute to fibroblast differentiation into myofibroblasts [64]. Injured endothelial cells have also been shown to secrete exosomes enriched with profibrotic, antiangiogenic factors, and microRNAs that will further contribute to cardiac fibrosis [65].

3. Identification and Quantification of Myocardial Fibrosis

Invasive and non-invasive methods have been developed over time in order to identify and quantify myocardial fibrosis (Table 1). Of these, myocardial biopsy is the most reliable method.

Table 1. Advantages and disadvantages of different techniques used in the evaluation and quantification of cardiac fibrosis.

Technology	Advantages	Disadvantages
Echocardiography	- favorable safety profile - non-invasive - acceptable to most patients - low cost - portable	- does not allow direct identification and quantification of fibrosis type and extent - cannot be used to measure and monitor the degree and progression of myocardial fibrosis - poor reproducibility - dependent on acoustic windows - affected by operator's skills
Cardiac magnetic resonance	- can identify macroscopic fibrosis - can identify different patterns of fibrosis - acceptable to patients - non-invasive	- potential artifacts in uncooperative patients and in the presence of tachyarrhythmias - contraindicated in patients with magnetic resonance-incompatible implants - high cost
Endomyocardial biopsy	- allows direct microscopic assessment of myocardial components and fibrotic changes	- risk of major complications - sampling error in cases of localized fibrosis - unreliable in detecting replacement fibrosis

3.1. Invasive Methods to Investigate Cardiac Fibrosis

A histological evaluation of myocardial tissue samples obtained during myomectomy, open heart surgery, or endocardial biopsy remains the gold standard for diagnosing and quantifying myocardial fibrosis [66]. Using appropriate staining methods, histological analysis of the collagen volume fraction (CVF; i.e., the ratio of the sum of connective tissue areas to the sum of all areas of connective and muscle tissue) is the most widely used method for quantitative evaluation of cardiac fibrosis [66]. No cut-off values have been defined so far; however, for CVF, the values varying considerably from one study to another. In addition, the use of this technique is strongly limited by the fact that it requires direct, invasive access to cardiac tissue samples, which carries inevitably the risk of several potentially major complications. Moreover, in settings with regional fibrosis, obtaining tissue samples does not guarantee a correct diagnosis.

3.2. Non-Invasive Methods to Evaluate Cardiac Fibrosis

Several ***blood biomarkers*** have been proposed to assess cellular and molecular changes that reflect the amount of fibrotic tissue of the heart. C-terminal propeptides of collagen I and N-terminal propeptides of collagen III highly correlated with total CVF, creating optimism about future clinical use [67]. However, both biomarkers have low specificity, and increased levels can also be observed in liver fibrosis [68]. High levels of galectin-3, a molecule that accelerates fibrosis by stimulating myofibroblast activation, have been associated with increased mortality and worse prognosis in heart failure. However, no associations were found between galectin-3 levels and CVF [69]. Circulating levels of miR-21, one of the regulators of fibroblast activity, have been shown to correlate with myocardial expression of type I collagen mRNA [70]. Its potential use as a blood biomarker for CVF and its cut-off values remain, however, questionable. Higher TGF-β levels have been reported in patients with heart failure compared with control [71]. However, its correlation with CVF remains to date unclear.

Cardiac magnetic resonance imaging (MRI) with T1 relaxometry can provide rapid information on cardiac edema, fibrosis, and deposition diseases, whereas replacement fibrosis can be evaluated using gadolinium MRI [72]. It should be noted, however, that this latter technique is not reliable in settings characterized by diffuse fibrosis in a homogeneous myocardium, although MRI-quantified fibrosis did correlate with cardiac function in patients with heart failure with preserved ejection fraction [73]. In the absence of edema or infiltrative disease, calculating the extracellular cardiac volume by T1 relaxometry after gadolinium injection allows evaluating even small amounts of fibrosis, and the results have been shown to correlate better with biopsy results than those obtained using other MRI-based techniques [72].

With its favorable safety profile and relative ease of use, ***echocardiography*** is often the first investigation used for assessing myocardial function and structure and for obtaining indirect data on cardiac fibrosis. Using speckle tracking echocardiography, one can quantify myocardial thickening, shortening, and rotation dynamics [74]. In hypertrophic cardiomyopathy, regional impairment of myocardial function assessed by speckle tracking echocardiography correlated with the presence of fibrosis detected by MRI [75]. Echocardiographic measurement of calibrated integrated backscatter is a technique developed to quantify the ultrasonic reflectivity of the myocardium in relation to the high reflectivity of the pericardium and the low reflectivity of blood [76]. In patients with dilated or hypertrophic cardiomyopathy and extensive fibrosis, the results have been shown to correlate significantly with the amount of myocardial fibrosis measured histologically [76]. The most important limitation of all echocardiographic methods remains, however, the need to obtain high-quality images.

4. Targeting Myocardial Fibrosis—A Magic Pill in Cardiovascular Medicine?

Immediately after any cardiac injury, a dynamic process of remodeling is initiated that is critical for stabilization of the cardiac wall. Expansion of non-contractile, collagen-rich tissue will lead, however, not only to scar tissue maturation but also to progressive adverse remodeling, which stands at the foundation of many CVDs. Targeting myocardial fibrosis could therefore provide tremendous benefits in many CVDs. However, because of the critical role that fibrosis plays in wound healing and tissue repair, concerns remain that fibrosis manipulation strategies may not completely innocuous. One of the major goals of novel fibrosis-oriented therapies is therefore not to withhold the process of fibrosis but rather to modify the properties of the scar tissue and to direct fibrotic pathways toward the formation of a functionally efficient fibrotic tissue.

4.1. Cardiac Antifibrotic Effects of Non-Antifibrotic Drugs

Clinical and experimental studies have shown that for numerous drugs created for various, non-antifibrotic purposes, the benefit could be at least partially linked to their antifibrotic effects (Table 2).

Table 2. Clinical and experimental studies of drugs studied for their antifibrotic effects.

Therapeutic Class	Drug	Study Type	Species	Duration	Underlying CVD	Results	References
RAAS inhibitors	Spironolactone (12.5–50.0 mg/day)	Placebo-controlled randomized trial	Human	6 months	HFrEF	Reduced PINP/PIIINP	[77]
	Lisinopril (5–20 mg/day)	Double-blind randomized trial	Human	6 months	Hypertensive heart disease	Reduced CVF and improved diastolic function	[78]
	Enalapril (5 mg/day)	Double-blind, randomized controlled clinical trial	Human	6 months	HFpEF-ESRF	Reduced PICP	[79]
	Losartan (50 mg/day)	Double-blind, randomized controlled clinical trial	Human	6 months	HFpEF-ESRF	Reduced CVF and improved diastolic function in severe fibrosis	[79]
Angiotensin receptor neprilysin inhibitor	Sacubitril-valsartan (200mg bid)	Double-blind, randomized controlled clinical trial	Human	9 months	HFpEF	No significant change in PIIINP/MMP2	[80]
Statins	Atorvastatin (40 mg/day)	Randomized open label study	Human	6 months	HFrEF	Reduction in PIIINP levels	[81]
	Rosuvastatin (40 mg/day)	Double-blind, randomized, placebo-controlled study	Human	6 months	HFrEF	No significant change in PINP/PIIINP	[82]
Pyridones	Pirfenidone	Double-blind, randomized, placebo-controlled study	Human	52 weeks	HFpEF	Ongoing	[83]
Mast cell degranulation inhibitor	Tranilast (400 mg/kg/day)	Experimental	Rat	12 weeks	2K1C renovascular hypertension	Decreased fibrotic area to total left ventricular area ratio	[84]
Endothelin receptor blocker	Bosentan (100 mg/kg/day)	Experimental	Rat	4 weeks	Myocardial hypertrophy	Decreased histological interstitial and perivascular fibrosis	[85]
Pacemaker current inhibitor	Ivabradine (5 mg bid)	Double-blind, randomized, placebo-controlled study	Human	8 months	HFrEF	Reversed LV volumes and increased LVEF	[86]
Phosphodiesterase type 5 inhibitors	Sildenafil (100 mg/day)	Double-blind, randomized, placebo-controlled study	Human	3 months	Type 2 diabetes	Improved LV contraction parameters and reduced TGF-β and MCP-1	[87]
Beta-blocker	Propranolol (40 mg/kg/day)	Preclinical	Rat	10 weeks	Left ventricular pressure overload, hypertrophy	No significant reduction in interstitial fibrosis	[88]
Calcium channel blockers	Mibefradil (10 mg/kg/d ay)	Preclinical	Rat	6 weeks	Myocardial infarction	Decreased infarct size and perivascular fibrosis	[89]

2K1C—two-kidney, one-clip; CVD—cardiovascular disease; CVF—collagen volume fraction; ESRF—end-stage renal disease; HFpEF—heart failure with preserved ejection fraction; HFrEF—heart failure with reduced ejection fraction; LV—left ventricle; LVEF—left ventricular ejection fraction; MCP-1—monocyte chemoattractant protein-1; MMP-2—matrix metalloproteinase-2; PICP—carboxy-terminal propeptide of procollagen type I; PINP—amino-terminal propeptide of procollagen type I; PIIINP—amino-terminal propeptide of procollagen type III; RAAS—renin–angiotensin–aldosterone system; TGF-β—transforming growth factor-*beta*.

Given the major role that ***RAAS*** plays in cardiac fibrosis pathogenesis, molecules that act at different RAAS levels have been investigated for their potential antifibrotic effects (Table 2). Aliskiren, a molecule that binds to renin and limits the initial step required for Ang II synthesis, has been shown to limit myocardial collagen deposition via Ang II-dependent and (pro)renin receptor-related pathways [90]. Already used as first-line therapy in a vast majority of CVDs, ACE inhibitors were also shown to reduce myocardial fibrosis in several animal models [33]. The decrease in myocardial collagen content induced by ACE inhibitors was related to a significant decrease in type I (but not type III) collagen as well as to an increase in gelatinase activity [91]. However, several clinical trials have failed to associate ACE inhibitors with a reduction in hospitalization and mortality in patients with various conditions characterized by extensive cardiac fibrosis, suggesting that ACE inhibition may be insufficient to effectively block the activity of multiple fibrosis pathways [92]. The blockade of Ang II AT1 receptors efficiently reduced fibrosis in both clinical and experimental settings [79,93]. Independently of their antihypertensive effects, AT1 receptor inhibitors have been associated with a more important reduction in type I collagen than ACE inhibitors [91]. Aldosterone inhibition reduced ECM, decreased fibrotic markers levels, significantly improved ventricular function in animal studies, and significantly reduced mortality in patients with heart failure and reduced ejection fraction [94]. Although in the Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist (TOPCAT) trial aldosterone inhibition failed to significantly improve the composite endpoint of cardiovascular death, aborted cardiac arrest, or hospitalization [95], a post hoc analysis of the trial indicated that this strategy may improve symptoms and hospitalization in certain patient subgroups [96].

Beta-blockers have been shown to prevent fibrosis and improve survival in animal models and to favorably affect prognosis in heart failure patients with preserved ejection fraction [97,98]. However, in humans, the antifibrotic effects of *beta*-blockers remain highly controversial (Table 2) [99]. This discordance could be at least partly related to the type of *beta*-blocker administered; there are studies suggesting that different *beta*-blockers may have opposite effects on the development of cardiac fibrosis [37]. ***Calcium channels blockers*** have also been shown to reduce cardiac fibrosis in different animal studies. The long-term administration of mibefradil, verapamil, and amlodipine reduced adverse cardiac remodeling and improved ventricular function in rats with ischemic heart failure [100]. In humans, the calcium channel blocker tetrandrine prevented myofibroblast activation and reduced cardiac fibrosis via a mechanism independent of calcium channel blockade and of the reduction in hemodynamic load [101]. Despite their anti-inflammatory effects, the ability of ***statins*** to alleviate cardiac fibrosis remains questionable. In rats with hypertensive heart disease, statin therapy reduced adverse cardiac remodeling, ventricular dysfunction, and progression to heart failure [102]. In patients with heart failure, statins reduced type III procollagen [81], the amount of myocardial fibrotic tissue and plasma markers of fibrosis [103]. However, in a 6-month randomized placebo-controlled study, the effect of statins on cardiac remodeling was neutral [104], whereas in another study, statin use was associated with an increase in serum collagen markers [82]. In experimental studies, ***endothelin inhibitors*** reduced fibrosis in multiple organs, attenuated cardiac remodeling, and significantly increased survival [105]. This did not seem to be the case, however, in patients with heart failure [106]. Adding endothelin antagonists to ACE inhibitors, *beta*-blockers, or aldosterone antagonists also does not seem to provide additional benefits in terms of cardiac remodeling in patients with heart failure [107].

4.2. Novel Targets for Cardiac Fibrosis Prevention and Therapy

Studies have identified a number of novel targets for cardiac fibrosis prevention and therapy (Table 3) [108].

Table 3. Novel targets for cardiac fibrosis prevention and therapy.

Therapeutic Target	Strategy
Cell transplantation	Direct remuscularization Stimulation of endogenous cardiovascular progenitor cells
TGF-β signaling	Suppression of TGF-β1 TGFβRII plasmid transfection ALK5 inhibition TGFβRII inhibition
Biomaterials	Hydrogel (alginate, polyester-VEGF, decellularized ECM, gelatin-HGF) Patch (alginate-neonatal rat cardiomyocytes, decellularized ECM) Glue (fibrin-fibroblast growth factor) Scaffold (fibrin–endothelial cells–smooth muscle cells)
Direct reprogramming	GMT (retrovirus/lentivirus) GMHT (retrovirus) miRNAs (miR-1, miR-133, miR-208, miR-499) Chemical/small molecule cocktails

ALK5—transforming growth factor-*beta* 1 type I receptor kinase; ECM—extracellular matrix; GMHT—Gata4/Mef2c/Hand2/Tbx5; GMT—Gata4/Mef2c/Tbx5; HGF—hepatocyte growth factor; TGF-β1—transforming growth factor-*beta* 1; TGFβRII—transforming growth factor-*beta* receptor II; VEGF—vascular endothelial growth factor.

Due to its major role in cardiac fibrosis, the ***TGF-β signaling pathway*** has become one of the most tempting targets in this setting. Anti-TGF-β antibodies were shown to efficiently decrease fibroblast activation and to improve diastolic function in rats with cardiac pressure overload, although they did not provide any improvement in myocyte hypertrophy or systolic function in those rats [109]. Moreover, in an experimental model of myocardial infarction, although anti-TGF-β*1* antibodies reduced fibrosis, their use was associated with increased mortality and dilatation of the left ventricle [110]. Alternative approaches, such as soluble TβRII, a competitive inhibitor of TGF-β, and inhibitors of the TGFβRI kinase (ALK5) have also been investigated. Both strategies reduced collagen synthesis and cardiac fibrosis but also manifested non-negligible side effects [111,112]. GW788388, a blocker of both ALK5 and TGFβRII that has improved pharmacokinetics and minimal toxic effects, reduced left ventricular remodeling in rats with myocardial infarction [113], but the full effects of this agent remain to be established. Inhibition of TAK1 and p38-MAPK has also been investigated, and both showed promising effects on cardiac fibrosis [114,115]. Pirfenidone and tranilast, two other TGF-β inhibitors, have also shown promising results in preclinical studies [116,117]. For tranilast, clinical data on cardiac fibrosis are still lacking. Meanwhile, in a recent phase II clinical trial, pirfenidone significantly reduced myocardial fibrosis in patients with heart failure and low ejection fraction without causing serious adverse cardiac events [83]. Pirfenidone's numerous extra-cardiac, including gastrointestinal, neurological, and dermatological side effects [118], remain, however, a serious concern. In mice, both Smad3 deficiency and Smad3 inhibition efficiently reduced the amount of cardiac fibrosis and prevented the decline in left ventricular ejection fraction [119], pointing Smad3 inhibition as a promising new antifibrotic approach. Increased levels of endogline, a co-receptor of TGF-β1 and TGF-β3, have been associated with both heart failure and acute myocardial infarction [120]. Meanwhile, decreased endoglin expression reduced the amount of cardiac fibrosis and improved survival in mice with heart failure [121]. The antifibrotic effect of blocking other TGF-β-related pathways, such as the RhoA–serum response factor-myocardin-related transcription factor [122] or the transient canonical potential receptor channels pathways [123] also remains to be evaluated in future studies.

In cardiac myocytes, the ***PDGF*** family includes PDGF-A and -C, while PDGFRα-positive cells have been described in the cardiac interstitium. Endothelial cells express PDGF-B and -D, while pericytes and smooth muscle cells are PDGFRβ-positive [124]. All PDGFs have been shown to play a role in cardiac fibrosis. In transgenic mice, the overexpression of PDGF-C and -D has been associated with cardiac fibrosis and hypertrophy [125].

Meanwhile, PDGF blockade reduced interstitial fibrosis in rats with myocardial infarction and decreased fibroblast activation in dogs [126], and neutralizing PDGF receptor-specific antibodies suppressed cell proliferation and collagen expression in cardiac fibroblasts [127].

Elevated levels of connective tissue growth factor (***CTGF***) have been detected in myocardial infarction and heart failure and have been shown to correlate with the degree of myocardial fibrosis [128]. The profibrotic effects of CTGF appear to emerge from its ability to stimulate fibroblasts' proliferation and transformation into myofibroblasts, although its potential to intrinsically induce fibrosis seems to be rather low [129]. In some experimental studies, the overexpression of CTGF had no significant profibrotic effects [130]. Other studies indicated, however, that CTGF can exert profibrotic effects [131], and that CTGF inhibition with monoclonal antibodies enhances cardiac repair, limits fibrosis, and ensures better preservation of left ventricular systolic function post-myocardial infarction [132].

Although the exact mechanisms remain insufficiently understood, the administration of ***angiotensin receptor–neprilysin inhibitors*** (ARNIs), a drug complex composed of a neprilysin inhibitor precursor and a non-peptide Ang II receptor blocker, has been shown to decrease the risk of death and hospitalizations in heart failure patients [133]. Preclinical studies have shown significant improvement in ventricular remodeling following neprilysin inhibition, and clinical trials later confirmed these results in patients with heart failure treated with ARNIs [134,135]. The benefit appears to emerge from the synergistic actions of the two components of ARNIs on multiple mechanisms involved in pathological cardiac remodeling. Neprilysin inhibition increases the concentrations of vasodilator peptides, such as the atrial and brain natriuretic peptides, and bradykinin, thereby improving myocardial perfusion in the infarcted area, but also increases concomitantly the concentrations of Ang II, whose effects are efficiently counteracted by the Ang II receptor blocker [135,136]. According to preclinical data, cardiac fibrosis and adverse remodeling are counteracted by ARNIs mainly via inhibition of the Wnt/β-catenin pathway [137]. In addition, ARNIs appear to attenuate cardiomyocyte growth and to increase the capillary/cardiomyocyte ratio at the level of the border area between the infarcted and the healthy myocardium [135], and even to reduce myocardial fibrosis, as reflected by the reduction in MMP-2, MMP-9, and N-terminal propeptide of type I procollagen, leading to a reduction in left atrial size and to significant improvement in left ventricular ejection in patients with heart failure [138–140]. Disappointingly, however, ARNIs failed to reduce hospitalizations and cardiovascular death in patients with heart failure and a left ventricular ejection fraction $\geq$45% (Table 2) [141].

4.3. Targeted Blockade—Aiming to Obtain a 'Better Scar'

Although the direct manipulation of mechanisms involved in fibroblast recruitment is not currently regarded as a primary target in the management of CVDs, this strategy carries a major potential to favorably influence scar formation and tissue remodeling.

Monocyte chemoattractant protein-1 (***MCP-1***) provides key signals for the migration and infiltration of inflammatory cells and activated fibroblasts. The overexpression of MCP-1 at the cardiac level promotes fibroblast accumulation, contributing to improved cardiac function and myocardial remodeling in transgenic myocardial infarction mice [142]. Meanwhile, MCP-1 deletion significantly reduced Ang-II-induced fibrosis by reducing the uptake and differentiation of CD45+ fibroblast precursors [143]. The manipulation of MCP-1 could thus emerge as a promising strategy to influence progenitor fibroblast cells and to prevent fibrosis and adverse cardiac remodeling.

Modulation of collagen accumulation and maturation in order to obtain a myocardial collagen network adapted to the local mechanical conditions could represent another potential target in fibrosis-related CVDs. In myocardial infarction, expansion of the infarcted area is associated with poor mechanics and increased risk of rupture of the injured wall. Approaches that stimulate compaction of the infarcted area by increasing collagen cross-linking inside the scar could thus provide an option to counteract maladaptive cardiac fibrosis. Modulation of lysyl oxidases, enzymes produced by activated fibroblasts

that stiffen the collagen network by boosting collagen fibers cross-linking, appears to be particularly promising in this regard [144].

Modulation of cardiomyocyte-fibroblast coupling inside the scar area, while keeping the outer area unchanged may also help to create a 'better scar'. In myocardial infarction, this would translate into increased trans-scar communication and transformation of the infarcted area into an 'electrically-transparent scar', with more homogeneous electrical activity and lower risk of re-entry [145]. This could be obtained by upregulating heterotypic connexin (Cx)-coupling with drugs such as rotigaptide, which significantly enhanced metabolic coupling in Cx43-coupled cells and attenuated gap junction closure under metabolic stress [146].

Fibroblast-derived microRNA-enriched exosomes, paracrine signaling mediators of cardiac hypertrophy and remodeling, are also regarded as highly promising. In vivo silencing of miR-21 reduced fibrosis in pressure-overload-induced disease and increased survival following myocardial infarction [70]. Other in vivo and in vitro studies suggested that miR-125b promotes profibrotic signaling in endothelial-to-mesenchymal transition and fibroblast activation [147]. miR-29 downregulation has also been associated with increased cardiac fibrosis, while miR-29 overexpression reduced collagen expression in myocardial infarction models [148]. In a mouse model of ATII-induced hypertension, mimetic miR-29 transfection also reduced the development of cardiac fibrosis via the TGF-β/Smad3 pathway [149]. More recently, miR-145, miR-30, and miR-133 have also been shown to modulate collagen deposition and to control structural ECM changes [150,151]. However, challenges in targeting microRNAs to prevent cardiac fibrosis remain, which are mainly related to their broad and non-specific effects. Nevertheless, ongoing efforts to identify the molecular targets of non-coding RNAs are promising for future clinical interventions.

Periostin targeting is also seen as a potential option in fibrosis-related CVDs. Periostin acts as a regulator of cardiac fibrosis by altering the deposition, diameter, and cross-linking of collagen fibers, by modifying the mechanical adhesion between fibroblasts and myocytes [152], and by recruiting activated fibroblasts via FAK-integrin signaling [153]. In heart failure patients, periostin distribution and expression has been associated with the amount of fibrotic tissue, suggesting that periostin may be a potential biomarker of cardiac remodeling in this setting [153]. In post-myocardial infarction mice, the genetic manipulation of periostin was shown to improve cardiac function. However, it also led to an overall increase in fibrosis [151]. Thus, the use of periostin as a potential target remains a sensitive issue.

4.4. Indirect Blockade of Fibrosis via Stimulation of Myocardial Regeneration/Repair

The targeted delivery of biomaterials composed of natural (e.g., naturally derived matrices) or synthetic (e.g., poly [N-isopropyl acrylamide]-based hydrogels) biomaterial +/− cells or growth factors has been investigated as a potential novel antifibrotic therapeutic strategy with promising results in rodent and large animal models [154]. Decellularized cardiac ECM alone can also be used as a biomaterial to control cardiac fibrosis and to provide support for the infarcted wall [154]. Transcatheter injection of processed decellularized cardiac ECM hydrogel has been shown to promote stem cells recruitment, proliferation, and differentiation into cardiac cells [155] and to be safe for administration in human patients [156]. Although the trial was not designed to assess efficacy, there was a decrease in heart failure symptoms, an increase in 6-min walk test distance, and an improvement in left ventricular remodeling in post-myocardial infarction patients [156]. Acellular patches that provide cells with tissue-specific biochemical cues important for cell migration and differentiation and tissue regeneration have also been investigated. The most used biomaterials include growth factors, ECM molecules, heparin, and thrombomodulin, which help to ensure a uniform surface coating of the polymeric cardiovascular scaffold [157]. The vascular endothelial growth factor, the insulin-like growth factor 1, the hepatocyte growth factor, the myeloid-derived growth factor, neuregulin 1, the epidermal growth

factor, and the fibroblast growth factor are the most widely employed to improve the bioresponsive properties of the scaffolds [157].

Cardiac patches that use collagen as a scaffold have also been studied in combination with a variety of cell types capable of exerting paracrine effects or to directly regenerate the injured myocardium [158], whereas fibrin cardiac patches improved cell delivery in a porcine model of post-infarction left ventricular remodeling [159]. Cells that promote adipose-derived stem cell regeneration embedded into platelet-rich fibrin and patched in myocardial infarction rats significantly decreased fibrotic mediators' levels and increased the expression of antifibrotic markers [160]. The implantation of pluripotent stem cells-derived cardiomyocytes placed on collagen scaffolds into dilated mouse hearts was also shown to decrease cardiac fibrosis and to increase the expression of osteopontin, which is an acidic phosphoglycoprotein that regulates the MMPs [161]. Multiple experimental studies provided highly promising results, and there are several ongoing clinical trials that test the localized delivery of biomaterials and antifibrotic agents.

Cell-sheet implantation has been shown to attenuate remodeling, restore the damaged myocardium, and improve cardiac function in several experimental models of myocardial infarction and dilated cardiomyopathy [162]. The method was tested in patients with myocardial infarction and dilated cardiomyopathy in a phase I clinical trial [163]. Although not adequately powered, the trial indicated a decrease in pulmonary pressure and resistance, as well as in the levels of BNP, an increase in walking distances on the 6-min walk test, and an improvement in the New York Heart Association classification in the treated patients, particularly in those with ischemic heart disease [163].

4.5. Modulation of Collagen Turnover

Procollagen processing by procollagen C-proteinase(s) is critical for the maturation of soluble collagen precursors into insoluble collagen and is potentiated by procollagen C-proteinase enhancers (PCPE-1 and -2) [164]. The expression levels of these later proteins have been shown to strongly correlate with the degree of fibrosis in different animal models [164]. The effect of PCPE-1 manipulation on cardiac fibrosis has not been evaluated to date. In the mouse liver, PCPE-1 deficiency decreased, however, the amount of fibrosis [165]. Meanwhile, PCPE-2 null hearts have been associated with a decrease in CVF and with lower myocardial stiffness in mice with aortic constriction [166].

Increased collagen production via Smad7 is amidst the many mechanisms by which miR-21 promotes cardiac fibrosis [167]. Elevated miR-21 expression has been shown to negatively affect collagen cross-linking and, implicitly, CVF [168], suggesting that miR-21 silencing could inhibit collagen synthesis and could thus exhibit antifibrotic effects.

Serelaxin is a recombinant form of human relaxin-2, which is a hormone that contributes, among others, to the degradation of the ECM. The antifibrotic effect of relaxin has been reported in both the kidney and the heart [169] and appears to rely on the prevention of cardiac fibroblast-to-myofibroblast transition via TGF-β/Smad3 pathway inhibition [170]. In addition, serelaxin has been shown to be safe in patients with acute heart failure [171], making this molecule particularly appealing for future clinical research.

Alterations in the balance between MMPs and their specific tissue inhibitors (i.e., TIMPs) have been incriminated as contributors to the abnormal production of ECM [172]. Cardiac expression of TIMP-1 and -2 was shown to be significantly increased and strongly correlated with the amount of cardiac fibrosis in patients with pressure overload [172]. Meanwhile, TIMP-3 has been shown to possess an increased affinity for ECM glycosaminoglycans and to alter the fibroblast phenotype [173]. The targeted administration of TIMP-3 could thus emerge as a promising collagen-decreasing strategy. In myocardial infarction pigs, the regional delivery of exogenous TIMP-3 showed positive effects on left ventricular ejection fraction and volume as well as on the extent of the infarcted area [173].

5. Gaps in Knowledge, Ongoing and Future Research

Cardiac fibrosis is a complex syndrome that affects not only the structure but also the function of the heart, suggesting that myocardial ECM homeostasis is essential for normal cardiac functioning. Identifying widely available, inexpensive, non-invasive, and highly accurate biomarkers for in vivo quantification of not only gross but also subtle cardiac fibrosis should continue to represent a major priority, as is the case in numerous other clinical settings [174,175]. Multiple strategies have been shown to efficiently counteract fibrosis. However, incomplete knowledge regarding the complex pathogenesis of fibrosis limits advancement in this field. Understanding the activation of cardiac fibroblasts and their role in cardiac fibrosis is necessary to improve our pharmaceutical arsenal. The development of safe and effective antifibrotic strategies also depends on a detailed decipherment of the pathways involved in the antifibrotic response. The window of therapeutic opportunity also remains unknown, at present, both spatially and temporally. Cardiac fibroblasts may respond differently to different therapeutic interventions, depending on the underlying profibrotic context [176]. In reparative fibrosis that follows myocardial infarction, the blockade of fibroblasts may have discordant effects at the periphery versus the center of the scar. Therapeutic approaches designed to block cardiac fibrosis should thus focus on preventing excessive ECM deposition at the periphery of the scar and should not affect the replacement of necrotic cardiomyocytes within the scar core. The most adequate moment for blocking the different profibrotic pathways remains another pending issue at this point. Blocking mediators at the wrong time could alter cellular responses that are critical for tissue repair. In reparative fibrosis that occurs after massive acute injury (e.g., after acute myocardial infarction), the beneficial effects of fibrotic tissue clearly outweigh its harmful effects. In such settings, early antifibrotic interventions could negatively affect the healing process and promote rupture of the heart wall, whereas delayed fibrosis inhibition may be ineffective if the fibrotic process is no longer reversible.

Numerous strategies aiming to prevent, block, and even reverse cardiac fibrosis have been extremely promising in animal models. However, their evaluation in human patients delays or, if they were tested in clinical settings, the results were rather disappointing. Interspecies discordances obviously mandate caution when trying to extrapolate data from animal studies to human medicine and can contribute to the discordant results obtained with different antifibrotic agents. Other factors may play, however, even greater roles. Drug doses used in animal studies are often much higher than those suitable for clinical use, and, with very few exceptions, currently used animal models have limited ability to adequately replicate human CVDs. Whereas in humans, CVDs are most often diseases of elderly individuals, with numerous concomitant cardiac and non-cardiac conditions, treated with different medications, including with a wide variety of cardioactive drugs, and in whom treatment adherence is often questionable, most animal data arise from young, healthy animals, fully compliant to all forms of therapy and who have no concomitant diseases and no concomitant therapy [177]. Using more clinically relevant animal CVDs models would certainly increase the translational value of data obtained in animal models. Meanwhile, clinical trials on innovative strategies have either been performed on a small number of patients or the follow-up period was much too short, considering the important interspecies differences regarding the time needed for the development of fibrosis, which seems to be much longer in humans [177]. Signaling pathways, profibrotic mechanisms, and even the type of fibrosis that develops have also been shown to vary greatly depending on the underling fibrose-promoting condition, suggesting that although most cardiac diseases involve a certain degree of fibrosis, a 'one size fits all' approach is unlikely to provide the solution in cardiac antifibrotic therapy. To date, with the exception of biomaterial-based approaches, which have been largely studied in post-myocardial infarction fibrosis, antifibrotic strategies have rarely been studied targeted on a specific type of fibrosis. Thorough understanding of the pathophysiological mechanisms underlying each type of myocardial fibrosis could provide the key for safe and efficient, targeted antifibrotic therapy.

Data from clinical trials confirmed the safety of stem cells from different tissue sources, using different delivery routes, but their exact clinical benefit remains to be established. Large phase III clinical trials are in progress, and their results will be essential to determine the role of this novel, non-pharmacological approach. To fully understand the potential role of stem cell therapy in cardiac fibrosis, the mechanisms by which this therapy exerts its effects will also need to be clarified. Inhibition of the RAAS using anti-Ang II vaccines, administration of Ang (1–7), and ACE2 overexpression recently emerged as a promising new tool for myocardial fibrosis management in animal models and even in small clinical trials. Antifibrotic drugs used in different other settings would also be worth consideration. Pirfenidone, nintedanib, tranilast, bosentan, macitentan, ambrisentan, and thalidomide are drugs with excellent results in pulmonary fibrosis, and only a minority of them has been evaluated so far in CVDs. Hydralazine and ivabradine, already widely used in patients with CVDs, were shown to significantly attenuate renal fibrosis, but very few studies have assessed their cardiac antifibrotic effect (Table 2). Sildenafil was reported to exert antifibrotic effects not only in the genitals but also in the lungs and skin. A similar effect on cardiac fibrosis could thus contribute to the improvement in ventricular function associated with sildenafil usage. Bioengineering and cell transplant therapy have also demonstrated major potential in indirectly blocking fibrosis by stimulating myocardial regeneration/repair. Direct cell reprogramming and molecular targets, such as epigenetic modifiers and miRs, have also been proposed as novel promising pharmacological tools to prevent the development of cardiac scar tissue. Targeting cardiac fibrosis is still associated, however, with a number of major limitations, and the mechanisms that lead to excessive ECM formation remain incompletely understood. In the absence of myocardial regeneration, the degradation of large areas of fibrosis could result in catastrophic consequences. Future studies will need to fully elucidate the mechanisms involved in cardiac fibrosis, to identify safe and effective methods to counteract this harmful process, and to establish the most appropriate time to intervene.

6. Conclusions

Cardiac fibrosis is currently acknowledged as a central element in the vast majority of CVDs. Due to the complexity of the signaling pathways, to its dual, protective and deleterious impact, and to the numerous cell types involved in the fibrotic process, safe and effective therapies for cardiac fibrosis inhibition and/or reversal remain difficult to develop. Continuous research in this area will have to fully elucidate the mechanisms involved in cardiac fibrosis, to identify safe and effective antifibrotic methods, and to establish the most appropriate time to intervene.

Funding: This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS-UEFISCDI, project number PN-III-P1-1.1-TE-2019-0370, within PNCDI III.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 08 April 2021
doi: 10.3389/fphar.2021.596956

Long-Term Effects of Ivabradine on Cardiac Vagal Parasympathetic Function in Normal Rats

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Background: The complex interactions that exist between the pacemaker current, I_f, and the parasympathetic nervous system could significantly influence the course of patients undergoing chronic therapy with the I_f blocker ivabradine. We thus aimed to assess the effects of chronic ivabradine therapy on autonomic modulation and on the cardiovascular response to *in situ* and *in vitro* parasympathetic stimulation. The right atrial expression of HCN genes, encoding proteins for I_f, was also evaluated.

Methods: Sympathetic and parasympathetic heart rate variability parameters and right atrial *HCN*(1-4) RNA levels were analyzed in 6 Control and 10 ivabradine-treated male Wistar rats (IVA; 3 weeks, 10 mg/kg/day). The heart rate (HR) and systolic blood pressure (SBP) responses to *in situ* electrical stimulation of the vagus nerve (2–20 Hz) were assessed in 6 additional Control and 10 IVA rats. The spontaneous sinus node discharge rate (SNDR) response to *in vitro* cholinergic receptors stimulation using carbamylcholine (10^{-9}–10^{-6} mol/L) was also assessed in these later rats.

Results: Ivabradine significantly increased vagal modulation and shifted the sympatho-vagal balance toward vagal dominance. In Control, *in situ* vagus nerve stimulation induced progressive decrease in both the SBP ($p = 0.0001$) and the HR ($p < 0.0001$). Meanwhile, in IVA, vagal stimulation had no effect on the HR ($p = 0.16$) and induced a significantly lower drop in SBP ($p < 0.05$). IVA also displayed a significantly lower SNDR drop in response to carbamylcholine ($p < 0.01$) and significantly higher right atrial *HCN4* expression ($p = 0.02$).

Conclusion: Chronic ivabradine administration enhanced vagal modulation in healthy rats. In addition, ivabradine reduced the HR response to direct muscarinic receptors stimulation, canceled the cardioinhibitory response and blunted the hemodynamic response to *in situ* vagal stimulation. These data bring new insights into the

OPEN ACCESS

Edited by:
Heike Wulff,
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Reviewed by:
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Specialty section:
This article was submitted to Cardiovascular and Smooth Muscle Pharmacology, a section of the journal Frontiers in Pharmacology

Received: *20 August 2020*
Accepted: *26 February 2021*
Published: *08 April 2021*

Citation:
Scridon A, Halaţiu VB, Balan AI, Cozac DA, Moldovan V, Bănescu C, Perian M and Şerban RC (2021) Long-Term Effects of Ivabradine on Cardiac Vagal Parasympathetic Function in Normal Rats. Front. Pharmacol. 12:596956. doi: 10.3389/fphar.2021.596956

Abbreviations: ANS, autonomic nervous system; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; HF, high-frequency; HR, heart rate; HRV, heart rate variability; LF, low-frequency; NS, non-significant; pNN5, percentage of successive RR intervals that differ by > 5 ms; RMSSD, root mean square of the successive RR-interval differences; SBP, systolic blood pressure; SDNN, standard deviation of normal RR intervals; SNDR, sinus node discharge rate.

mechanisms of ivabradine-related atrial proarrhythmia and suggest that long-term I_f blockade may protect against excessive bradycardia induced by acute vagal activation.

Keywords: cardioinhibitory response, HCN4, I_f current, ivabradine, vagal tone

INTRODUCTION

The heart rate is the result of a highly coordinated sequence of electrical phenomena that normally take place in the pacemaker cells of the sinus node. The autonomic nervous system (ANS) ensures both long-term, tonic control and short-term, reflex adaptation of the HR to internal and external factors. The hyperpolarization-activated inward current (I_f) is critical in establishing the HR and one of the most relevant targets of HR modulation by the ANS (DiFrancesco, 2010).

Ivabradine is a specific I_f blocker devoid of dromotropic, inotropic, and lusitropic effects (Camm and Lau, 2003; Manz et al., 2003). In patients with heart failure, chronic ivabradine administration significantly improved clinical outcomes (Swedberg et al., 2010), while also causing a significant, although modest increase in atrial fibrillation, but not in ventricular arrhythmias occurrence (Martin et al., 2014; Cammarano et al., 2016). Studies have also shown that, by inhibiting I_f, ivabradine can significantly reduce the tachycardic response to acute sympathetic stimulation and can thus provide benefit in a wide range of settings associated with sympathetic hyperactivation. In patients with postural orthostatic tachycardia syndrome, ivabradine significantly improved the quality of life (Taub et al., 2021) and efficiently reduced the HR at rest and during tilting (Gee et al., 2018; Taub et al., 2021). In a small randomized controlled trial, ivabradine therapy significantly improved symptoms in patients with inappropriate sinus tachycardia (Cappato et al., 2012). Promising results have also been reported in patients with sinus tachycardia-mediated vaso-vagal syncope, in whom ivabradine was well tolerated and was associated with marked benefit or complete resolution of symptoms (Sutton et al., 2014).

The relationship between I_f blockade and parasympathetic activation appears to be, however, much more complex. In rats with post-myocardial infarction heart failure, long-term ivabradine therapy was shown to counteract the increase in the expression of genes encoding for hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, responsible for generating I_f, in the ventricular myocardium (Suffredini et al., 2012). Meanwhile, chronic ivabradine administration was associated with a significant increase in sinus node *HCN4* expression in mice (Leoni et al., 2006) If ivabradine exerts such an effect on sinus node cells, this could significantly alter the HR response to vagal stimulation. Acute (Mangin et al., 1998), but not chronic (Silva et al., 2016) intraperitoneal ivabradine administration has also been associated with marked increase in heart rate variability (HRV) parameters in rats. In addition, clinical and experimental data indicate that in the setting of heart failure, long-term ivabradine therapy shifts the sympatho-vagal balance toward vagal dominance (Milliez et al., 2009; Kurtoglu et al., 2014; Böhm et al., 2015). Accumulating data therefore suggest that chronic ivabradine therapy could alter both the vagal modulation and the HR response to vagal stimulation and could thus influence the risk of patients prone to cardiac arrhythmias and the clinical course of patients with vaso-vagal syncope. However, the effects of chronic I_f blockade on the HR response to acute parasympathetic stimulation have not been evaluated to date and its impact on vagal modulation in settings other than heart failure remains to date unknown.

We therefore aimed to help solving some of the existing controversies and to increase knowledge regarding the effects of the I_f blocker ivabradine. To achieve this goal, we designed an experimental study to assess the effects of chronic ivabradine therapy on the HR response to *in situ* and *in vitro* acute parasympathetic stimulation. The impact of chronic ivabradine administration on the sympatho-vagal modulation and on the right atrial expression level of *HCN* channels was also evaluated.

MATERIALS AND METHODS

Studied Animals

The flow chart of the study design is presented in **Figure 1**. Adult male Wistar rats (200–250 g) obtained from the local animal facility were initially randomized into two groups: Control (n = 12) and IVA (n = 20). All animals were housed individually in polycarbonate cages, in a temperature-controlled room (21 –24°C), with a 12/12 h light/dark cycle, and had free access to water and standard food. Rats in the IVA group received a daily dose of 10 mg/kg of body weight of ivabradine (ivabradine hydrochloride; Servier; Courbevoie, France) in their drinking water throughout the study, starting three weeks prior to any experimental procedure. To ensure that each rat consumed the adequate amount of ivabradine, the drug was dissolved in 15 ml of water; as soon as this amount was consumed, normal tap water was put at the rats' disposal for the rest of the day. Control rats received normal tap water throughout the study. The daily water intake was measured in all rats throughout the study. All protocols complied with the International Council for Laboratory Animal Science guidelines (Directive 2010/63/EU) and were approved by the local Ethics Committee and the National Sanitary Veterinary and Food Safety Authority. Partial blinding was applied in the present study: the researchers who performed the experimental procedures, (i.e., ECG device implantation, vagus nerve stimulation, atrial sampling, *in vitro* studies) were not blinded to the study group, but all parameters were measured and all statistical analyses were performed in a blinded manner.

Continuous 24 h ECG Monitoring and Heart Rate Variability Analysis

Three weeks after the beginning of the study, Control and IVA rats were re-randomized into two subgroups each. Six Control

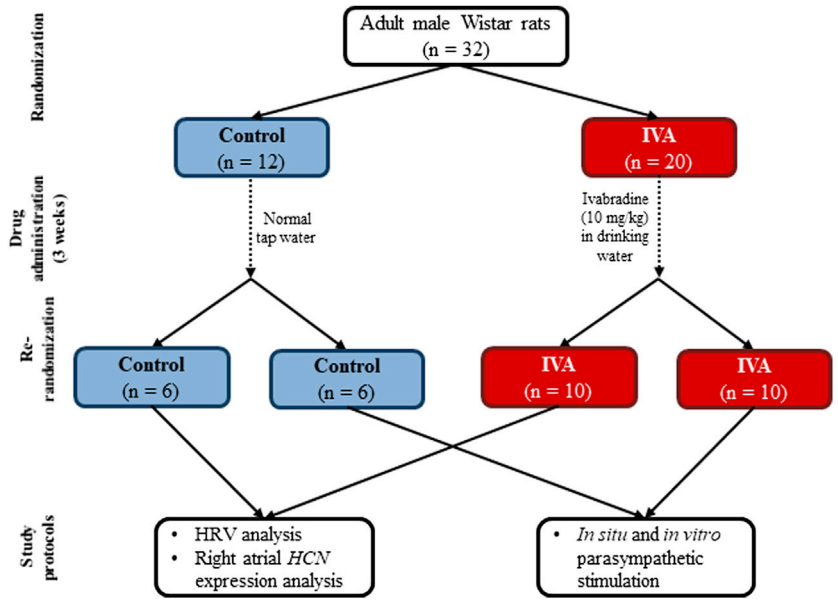

FIGURE 1 | The flow chart of the study design for the Control and the ivabradine-treated (IVA) groups. HRV – heart rate variability

and 10 IVA rats were implanted with radiotelemetry ECG devices (TA11 CA-F40; Data Sciences International, St. Paul, MN), as described previously (Doñate Puertas et al., 2017). After one week of post-implantation recovery, 24 h continuous ECG monitoring was performed in unrestrained, "conscious" rats, and the mean baseline HR was calculated using a program developed to automatically detect the R waves and measure the RR intervals (Gallet et al., 2013). Heart rate variability analysis was performed based on the 24 h ECG recordings by analyzing beat-to-beat variations in RR intervals in the time and frequency domains, as described previously (Scridon et al., 2012). For the time domain analysis, the standard deviation of normal RR intervals (SDNN), the root mean square of the successive RR-interval differences (RMSSD), and the percentage of successive RR intervals that differed by > 5 ms (pNN5) were assessed. For the frequency domain analysis, time series were resampled at 20 Hz using a cubic spline, then spectral power in the low-frequency (LF) band (0.3–0.6 Hz), the high-frequency (HF) band (0.6–2.5 Hz), and the LF/HF ratio were estimated on 2,048-point (102.4 s) segments windowed by the Hanning function and overlapping by 50% using a fast Fourier transform. All ECG tracings were assessed visually and all artifacts, arrhythmic events, and compensatory pauses were excluded prior to HRV analysis.

Right Atrial Expression Analysis of Genes Encoding for Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels

At the end of the ECG monitoring period, the 6 Control and 10 IVA rats were euthanized using an intraperitoneal injection of a terminal dose of sodium pentobarbital (>100 mg/kg). The thoracic cavity was opened and the heart was removed. The free wall of the right atrium was collected and rapidly immersed into RNA stabilization solution (RNAlater; Thermo Fisher Scientific, Waltham, MA). The RNA was isolated using iPrep PureLink Total RNA Kits and the iPrep Purification Instrument (Thermo Fisher Scientific). Reverse transcription was performed using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). The RNA expression levels of three target genes, (i.e. *HCN1*, *HCN2*, and *HCN4*) and one control gene, (i.e. glyceraldehyde 3-phosphate dehydrogenase [*GAPDH*]) were analyzed using a customized fast 96-well plate containing TaqMan Gene Expression Assays for the tested genes (Thermo Fisher Scientific). The expression of the neuron-specific *HCN3* isoform was also analyzed. All experiments were performed on a 7500 Fast Dx Real-Time PCR System (Applied Biosystems, Waltham, MA). The expression levels of *HCN1*, *HCN2*, *HCN3*, and *HCN4* were normalized with *GAPDH* housekeeping gene levels and compared between the two groups.

In situ and *in vitro* Parasympathetic Stimulation

Three weeks after the beginning of the study, the remaining 6 Control and 10 IVA rats were submitted to vagus nerve stimulation under ketamine/medetomidine anesthesia (i.p., 75.0/0.5 mg/kg). Briefly, the anterior cervical region was dissected and the sternohyoid and sternocleidomastoid muscles were separated and retracted laterally to allow visualization of the carotid artery. The right vagus nerve was carefully isolated from

the surrounding connective tissue. In order to avoid interferences from retrograde electrical vagus nerve stimulation, the nerve was secured using two surgical threads and was then cut. A stimulation electrode was placed beneath the distal end of the vagus nerve and was elevated to avoid contact with the surrounding tissues. The nerve was then stimulated electrically using rectangular impulses (pulse duration 0.5 ms; 20 V) at progressively higher frequencies, (i.e. 2, 5, 10, and 20 Hz). Each stimulation protocol was applied for 15 s, with 5 min intervals between stimulations. Surface ECG was continuously recorded during the entire duration of the protocol and the HR was calculated at baseline and during each stimulation protocol based on RR intervals duration. The ECG signal was captured using three electrodes placed on the two upper limbs and on the left lower limb, was amplified, and delivered to the acquisition board. The ECG signal was recorded using an acquisition program developed using the LabVIEW 8.20 software (National Instruments, Austin, TX). Systolic blood pressure (SBP) was measured non-invasively at baseline and during each stimulation protocol using a photoplethysmographic method, as described previously (Scridon et al., 2015). Briefly, a pneumatic tail cuff was placed proximally on the rat's tail and inflated/deflated using the PE-300 programmed electro-sphygmomanometer (Narco Bio-Systems Inc., Houston, TX). The photoplestimography sensor was placed on the tail distally to the pneumatic cuff, with the infrared beam at the level of the caudal artery. The cuff pressure and the phototransducer signals were routed to the signal acquisition board. The signals were recorded using an acquisition program developed using the LabVIEW 8.20 software (National Instruments).

At the end of the stimulation protocols, the anesthetized rats were euthanized by thoracotomy. The hearts were explanted and rapidly immersed into prewarmed (37°C) oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution containing NaCl (118.00 mM), KCl (4.70 mM), $NaHCO_3$ (25.00 mM), $MgSO_4$ (1.20 mM), $CaCl_2$ (1.25 mM), KH_2PO_4 (1.20 mM), and glucose (11.00 mM). The right atrium was isolated and transferred into the Steiert organ bath (Hugo Sachs Elektronik-Harvard Apparatus; March-Hugstetten, Germany) containing oxygenated Krebs-Henseleit solution at 37°C. The spontaneous sinus node discharge rate was measured at baseline and after direct cholinergic receptors stimulation using carbamylcholine solutions with progressively higher concentrations (10^{-9} mol/L to 10^{-6} mol/L, prewarmed at 37°C). Each solution was applied for 10 min. All samples were washed for 5 min with oxygenated Krebs-Henseleit solution between exposures to the successive carbamylcholine solutions.

Statistical Analysis

Statistical analyses were undertaken using MedCalc for Windows, version 12.4.3.0 (MedCalc Software; Ostend, Belgium). A two-tailed p-value < 0.05 was considered statistically significant. All data were tested for normality and are expressed as means ± standard error of the mean or median and interquartile range, as appropriate. Between-group comparisons were performed using the unpaired Student's t-test or the Mann–Whitney U-test, as appropriate. Differences within the same group were tested for significance using the paired Student's t-test or the Wilcoxon matched-pairs signed-rank test, as appropriate, and repeated-measures ANOVA. Changes in the spontaneous sinus node discharge rate in response to carbamylcholine administration were also analyzed by a nonparametric two-way ANOVA, factoring for the effects of ivabradine treatment status (ivabradine-treated vs. non-treated) and carbamylcholine concentrations (10^{-9} mol/L–10^{-6} mol/L). Due to the very limited amount of data available on this topic, the sample size could not be calculated prior to the study. However, based on previous studies in non-treated rats, Control rats were expected to present low interindividual variability and a sample size of 6 was therefore considered sufficient for these groups. To compensate for potentially higher interindividual differences in the ivabradine-treated animals, the two IVA groups were designed larger (n = 10) than the Control groups.

RESULTS

There was no significant difference in the daily water intake between the IVA and the Control rats (53.21 ± 2.49 ml/24 h vs. 57.50 ± 3.30 ml/24 h; p = 0.34).

Chronic Ivabradine Administration Increases Vagal Modulation and Shifts the Sympatho-Vagal Balance Toward Vagal Dominance in Healthy Rats

Figure 2 depicts typical telemetric ECG tracings recorded in Control and IVA rats. As expected, mean 24 h HR was significantly lower in the ivabradine-treated rats compared to their non-treated counterparts (p< 0.01; **Table 1**). Mean awake and mean asleep HR were both significantly lower in the ivabradine-treated compared to the non-treated rats (both p< 0.0001; **Table 1**). In the Control rats, there was a 22.6 ± 2.8 bpm (p< 0.001) awake/asleep HR difference, compared with an 18.7 ± 5.9 bpm (p = 0.01) awake/asleep HR difference in the ivabradine-treated rats (p = 0.63).

Similarly to what was previously reported in the setting of heart failure (Milliez et al., 2009; Kurtoglu et al., 2014; Böhm et al., 2015), HRV analysis revealed a significant increase in vagal modulation and a shift of the sympatho-vagal balance toward vagal dominance in ivabradine-treated healthy rats (**Table 1**). The RMSSD, pNN5, and the HF components of the HRV spectrum, reflecting vagal modulation, were all significantly higher in the IVA compared with the Control rats (awake, asleep, and over 24 h; all p < 0.05; **Table 1**). The LF/HF ratio, an index of sympathetic and parasympathetic interactions, was significantly lower in the IVA than in the Control rats (awake, asleep, and over 24 h; all p < 0.05; **Table 1**), demonstrating a shift of the sympatho-vagal balance toward vagal dominance in the ivabradine-treated rats. No significant change was observed in the LF components of the HRV spectrum (awake, asleep, and over 24 h; all p = NS; **Table 1**).

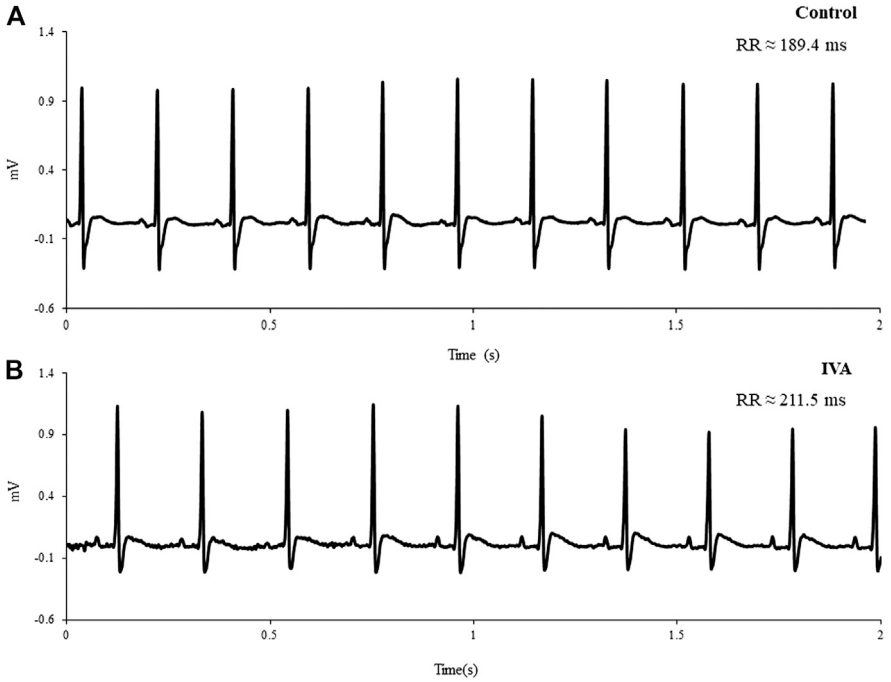

FIGURE 2 | Representative telemetric ECG tracings recorded in a Control **(A)** and an ivabradine-treated (IVA) rat **(B)**

Chronic Ivabradine Administration Up-Regulates the Right Atrial Expression of the Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel 4

Right atrial *HCN1* and *HCN2* expression levels were similar (both p = NS) between the IVA and the Control rats (**Figure 3**). There was also no significant difference in the right atrial expression of the neuron-specific *HCN3* isoform between the two groups (p = 0.22; **Figure 3**). However, the right atrial expression of *HCN4*, the most highly expressed *HCN* isoform in the sinus node (Sartiani et al., 2017), was significantly higher in the ivabradine-treated compared to the non-treated rats (p = 0.02; **Figure 3**).

Long-term Ivabradine Administration Cancels the Heart Rate Response and Reduces the Blood Pressure Response to Acute *in situ* Vagal Stimulation

As expected, baseline HR (derived from surface ECG recordings performed in anesthetized rats prior to vagus nerve sectioning) was significantly lower in the IVA compared with the Control rats (202.2 ± 6.6 bpm vs. 246.3 ± 13.6 bpm; p = 0.01), whereas SBP was similar between the two groups (143.5 ± 6.9 mmHg vs. 154.1 ± 9.8 mmHg; p = 0.58).

In the Control rats, electrical stimulation of the vagus nerve induced a significant, progressive decrease in both the SBP (p = 0.0001) and the HR (p < 0.0001) (**Figure 4**). In the IVA rats, electrical stimulation of the vagus nerve also induced a significant, progressive decrease in the SBP (p < 0.0001) (**Figure 5**). However, in the ivabradine-treated rats, vagus nerve stimulation had no effect on the HR (p = 0.16; **Figure 5**). In addition, the decrease in SBP in response to vagus nerve stimulation was also significantly lower in the IVA compared with the Control rats for all four stages of the stimulation protocol (all p < 0.05; **Figure 6**).

Ivabradine Reduces the Drop in the Sinus Node Discharge Rate in Response to *in vitro* Cholinergic Receptors Stimulation

When right atrial samples were assessed *in vitro* under progressively higher carbamylcholine concentrations, both IVA and Control rats displayed a significant, progressive decrease in the spontaneous discharge rate of the sinus node (both p< 0.0001; **Figure 7**). There was no significant difference in the sinus node discharge rate drop in response to similar carbamylcholine concentrations between the two groups (p = NS for all carbamylcholine concentrations; **Figure 7**). However, in absolute value, the drop in the sinus node discharge rate was ~30–40 bpm lower in the ivabradine-treated than in the non-treated rats for all carbamylcholine concentrations (**Figure 7**). Moreover, when the responses of the sinus node discharge rate to all carbamylcholine concentrations were combined, the decrease in the spontaneous discharge rate of the sinus node in response to carbamylcholine administration was significantly lower in the IVA compared with the Control rats (p < 0.01; **Figure 7**). In addition, two-way ANOVA factoring for the effects of ivabradine treatment status (ivabradine-treated vs. non-treated) and carbamylcholine concentrations (10^{-9} mol/L–10^{-6} mol/L) demonstrated that the response of the spontaneous discharge

TABLE 1 | Mean 24 h heart rate and heart rate variability parameters in ivabradine-treated (IVA) and non-treated (Control) rats.

Parameter	Control (n = 6)	IVA (n = 10)	p-value
Heart rate (HR)			
Mean 24 h HR (bpm)	341.5 ± 8.3	301.3 ± 7.5	<0.01
Mean HR awake (bpm)	356.6 ± 5.5	304.6 ± 6.9	<0.0001
Mean HR asleep (bpm)	334.0 ± 6.4	285.9 ± 4.9	<0.0001
24 h *Heart rate variability analysis*			
Time domain			
SDNN (ms)	23.3 ± 1.9	26.6 ± 1.7	0.23
RMSSD (ms)	3.9 ± 0.3	5.5 ± 0.2	<0.01
pNN5 (%)	16.9 ± 2.6	28.1 ± 1.7	<0.01
Frequency domain			
LF (ms^2)	1.9 ± 0.2	2.5 ± 0.2	0.11
HF (ms^2)	6.8 ± 1.5	11.6 ± 1.2	0.03
LF (n.u.)	6.1 ± 0.9	6.1 ± 0.4	1.00
HF (n.u.)	19.8 ± 3.4	32.1 ± 4.2	0.04
LF/HF	0.30 ± 0.02	0.23 ± 0.02	0.04
Heart rate variability analysis – awake			
Time domain			
SDNN (ms)	21.0 ± 1.4	23.8 ± 1.7	0.25
RMSSD (ms)	3.8 ± 0.3	5.3 ± 0.3	<0.01
pNN5 (%)	15.3 ± 2.9	26.1 ± 2.0	<0.001
Frequency domain			
LF (ms^2)	2.2 ± 0.2	2.7 ± 0.1	0.43
HF (ms^2)	6.2 ± 1.4	11.3 ± 1.3	<0.01
LF (n.u.)	6.4 ± 0.8	6.6 ± 0.4	0.46
HF (n.u.)	18.5 ± 3.7	31.9 ± 4.3	0.04
LF/HF	0.30 ± 0.03	0.21 ± 0.02	0.04
Heart rate variability analysis – asleep			
Time domain			
SDNN (ms)	22.7 ± 1.7	26.4 ± 1.4	0.14
RMSSD (ms)	4.2 ± 0.3	6.0 ± 0.3	<0.01
pNN5 (%)	18.7 ± 2.5	31.8 ± 2.3	<0.01
Frequency domain			
LF (ms^2)	1.6 ± 0.2	2.2 ± 0.2	0.42
HF (ms^2)	7.4 ± 1.6	13.5 ± 1.6	0.03
LF (n.u.)	5.9 ± 1.0	6.2 ± 0.4	0.79
HF (n.u.)	21.2 ± 4.2	34.6 ± 4.2	0.04
LF/HF	0.32 ± 0.03	0.23 ± 0.03	0.04

The values are expressed as means ± standard error of the mean; p-*values refer to between-group comparisons based on the unpaired Student's* t-*test.*
HF – high-frequency (0.6–2.5 Hz) signals; HR – heart rate; LF – low-frequency (0.3–0.6 Hz) signals; LF/HF – the ratio of low-to high-frequency components; n.u. – normalized units; pNN5 – percentage of successive RR intervals that differed by > 5 ms; RMSSD – root mean square of successive RR-interval differences; SDNN – standard deviation of normal RR intervals.

rate of the sinus node to parasympathetic stimulation was significantly affected by both carbamylcholine concentration (p = 0.04) and ivabradine administration (p < 0.01), and that there was no interaction between the two factors (p = 0.93).

DISCUSSION

The ANS is one of the most important contributors to HR regulation, and, although several subcellular components contribute to HR regulation by the ANS, including the acetylcholine-dependent potassium current, calcium currents, and the Na^+/Ca^{2+} exchanger, much of this regulation is achieved *via* I_f modulation (DiFrancesco, 2010). The ANS is, however, a double path (afferent and efferent) neural system and any significant change in cardiovascular parameters is naturally followed by adaptive changes in ANS functioning. The autonomic control of I_f and the molecular mechanisms underlying I_f modulation by the sympathetic and the parasympathetic nervous systems are well understood (DiFrancesco, 2010). Conversely, the effects of chronic I_f blockade on the ANS and on the HR response to acute autonomic changes are still largely unknown. Our study demonstrates that chronic ivabradine administration enhances vagal modulation and shifts the autonomic balance toward vagal dominance in healthy rats. Moreover, we show that chronic I_f blockade using ivabradine increases right atrial *HCN4* expression, reduces the HR response to direct muscarinic receptors stimulation, cancels the cardioinhibitory response and blunts the hemodynamic response to *in situ* vagal stimulation. These data bring new insights into the mechanisms of ivabradine-related atrial proarrhythmia and provide evidence that long-term I_f blockade may protect against excessive bradycardia induced by acute vagal activation.

Chronic Ivabradine Administration Increases Vagal Modulation and Shifts the Autonomic Balance Toward Vagal Dominance. Potential Implications for Ivabradine-Related Atrial Proarrhythmia

Autonomic imbalance with sympathetic hyperactivity is a common denominator of a wide variety of cardiovascular diseases (Brook and Julius, 2000; Hellstrom, 2007; Floras and Ponikowski, 2015). In settings such as heart failure, sympathetic hyperactivity develops as an adaptive mechanism aiming to preserve the cardiac output (Floras and Ponikowski, 2015). However, over the long term, this increased sympathetic activity significantly contributes to impaired prognosis and increased mortality rates (Floras, 2009; Jacobson et al., 2010; Fallavollita et al., 2014). Meanwhile, strategies aiming to decrease the sympathetic and/or increase the parasympathetic tone are believed to have great potential (van Bilsen et al., 2017), and *beta*-blockers, which decrease the cardiac effects of sympathetic activation and circulating catecholamine, have already been shown to increase survival and improve prognosis in heart failure (Hjalmarson et al., 1999; Lechat et al., 1999; Flather et al., 2005).

Accumulating data suggest that "pure" HR-lowering agents could provide similar benefits (Swedberg et al., 2010) and clinical and experimental studies suggest that this beneficial effect could be at least partly due to the ability of I_f blockers to improve HRV parameters and to induce sustained increase in vagal tone (Mangin et al., 1998; Milliez et al., 2009; Kurtoglu et al., 2014; Böhm et al., 2015; El-Naggar et al., 2018), although this was not the case in the study by Silva et al. (Silva et al., 2016). However, in this latter study, ivabradine was administered intraperitoneally, for only 7–8 days, and the magnitude of HR reduction induced by ivabradine was significantly greater (28%) than that achieved in the present study (13.3%) and in the large clinical trials (15–20%) (Swedberg et al., 2010). This increased drop in HR was also translated into a significant decrease in SBP in the ivabradine-

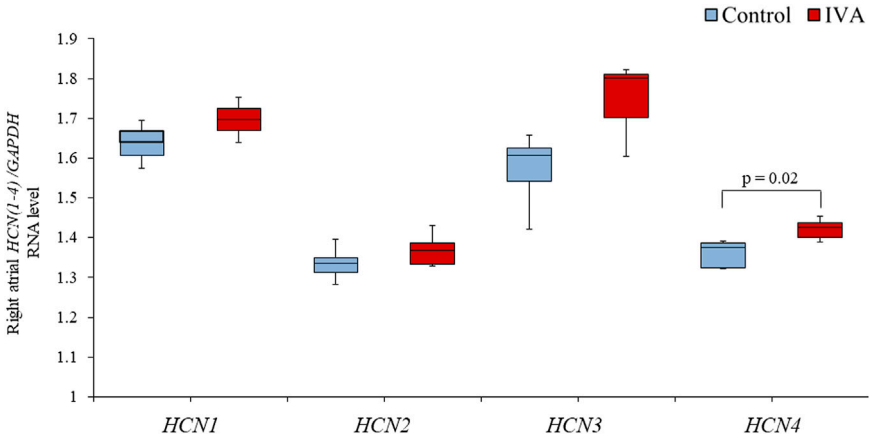

FIGURE 3 | Right atrial RNA expression of genes encoding for hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the ivabradine-treated (IVA; $n = 10$) and non-treated (Control; $n = 6$) rats. Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

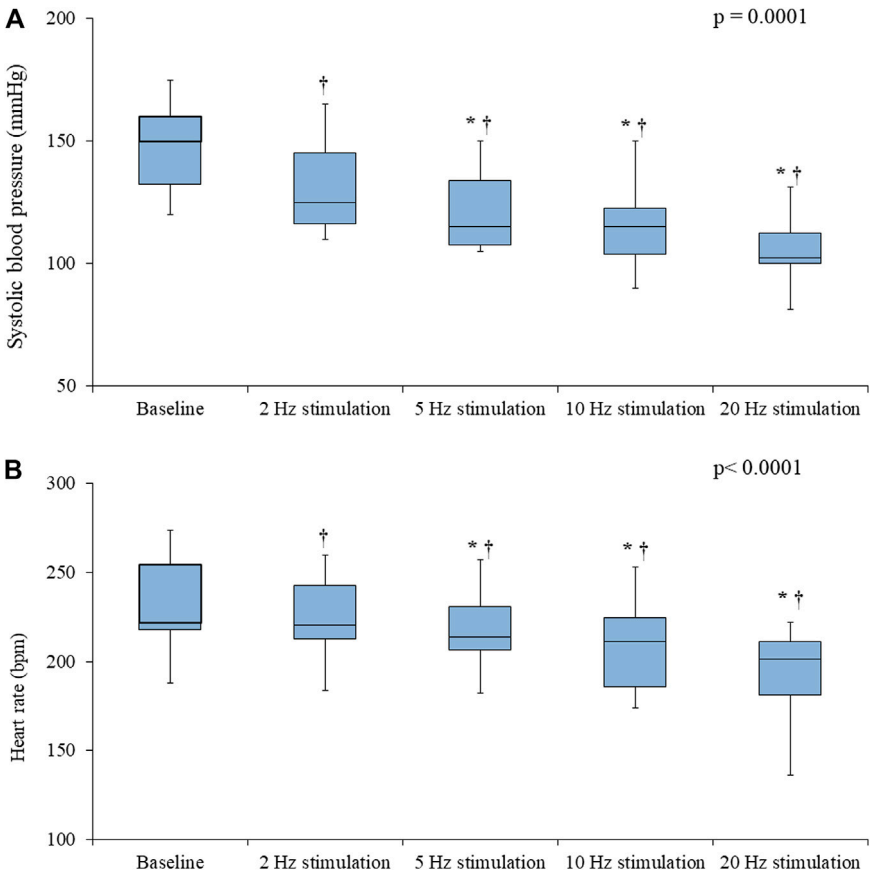

FIGURE 4 | Systolic blood pressure (**A**) and heart rate (**B**) values prior to (baseline) and during vagus nerve stimulation at 2, 5, 10, and 20 Hz in the Control rats ($n = 6$). Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile. *p*-values in the right upper corners were obtained using nonparametric repeated-measures ANOVA (Friedman test). *$p < 0.05$ vs. the value obtained during the previous stimulation using the paired Student's *t*-test or the Wilcoxon matched-pairs signed-rank test, as appropriate; †$p < 0.05$ vs. baseline using the paired Student's *t*-test or the Wilcoxon matched-pairs signed-rank test, as appropriate.

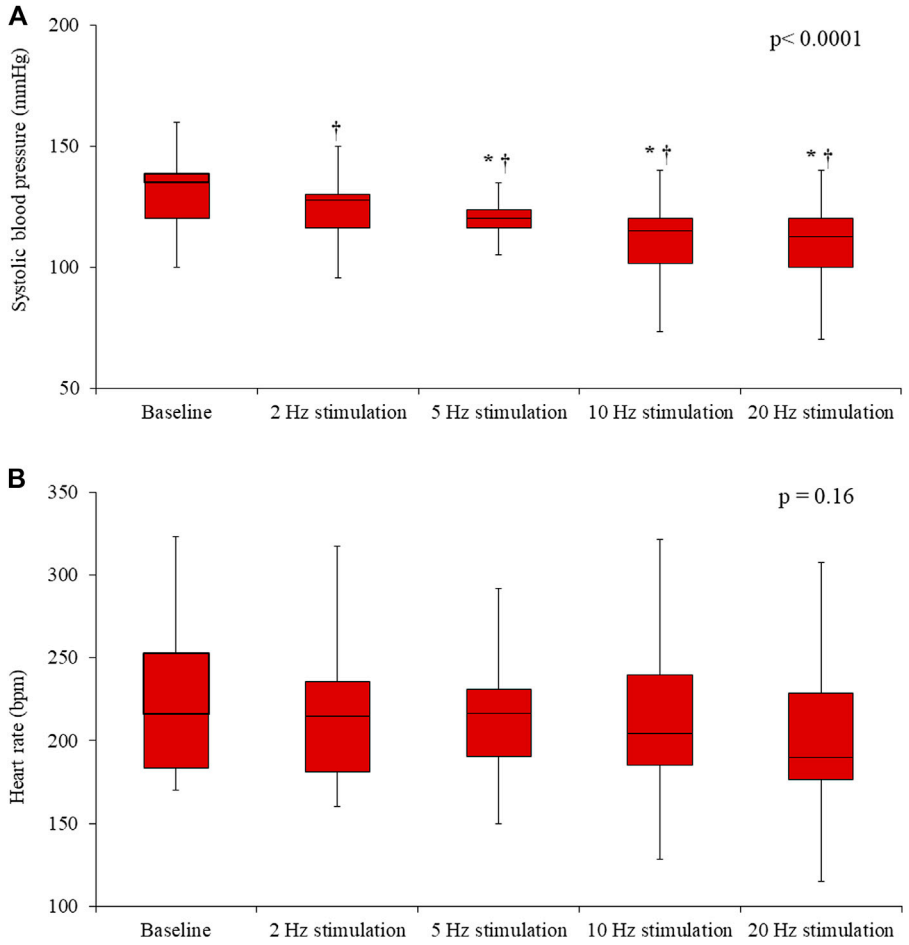

FIGURE 5 | Systolic blood pressure **(A)** and heart rate **(B)** values prior to (baseline) and during vagus nerve stimulation at 2, 5, 10, and 20 Hz in the ivabradine-treated rats (n = 10). Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile. p-values in the right upper corners were obtained using nonparametric repeated-measures ANOVA (Friedman test). *p < 0.05 vs. the value obtained during the previous stimulation using the paired Student's t-test or the Wilcoxon matched-pairs signed-rank test, as appropriate; †p < 0.05 vs. baseline using the paired Student's t-test or the Wilcoxon matched-pairs signed-rank test, as appropriate.

treated rats (Silva et al., 2016), effect that is not encountered at clinically relevant ivabradine doses (Swedberg et al., 2010). Although the exact mechanisms that underlie this effect remain to date unknown, the increase in vagal modulation induced by I_f blockade could be related to the lengthening of the diastolic filling time and to the consequent ventricular stretch, leading to mechanoreceptors stimulation and thus increasing the vagal and decreasing the sympathetic tone (Oren et al., 1993).

Given the well-known association between heart failure and sympathetic hyperactivity, this shift in autonomic balance toward vagal dominance was interpreted in the previous studies as an ivabradine-induced "improvement" in sympatho-vagal balance (Kurtoglu et al., 2014; Böhm et al., 2015). Although this may be true in settings associated with sympathetic hyperactivity, our data show that chronic ivabradine administration in clinically relevant doses exerts similar effects in healthy subjects. In the present study, chronic ivabradine therapy significantly reduced the HR in 24 h, awake, and asleep recordings, without causing significant dampening of circadian variations in HR. In addition, chronic ivabradine therapy significantly increased vagal modulation (as reflected by the higher parasympathetic indexes RMSSD and pNN5, and the increased HF components of the HRV spectrum), and shifted the sympatho-vagal balance toward vagal dominance (as reflected by the significantly lower LF/HF ratio) in healthy rats. These changes were present both when the animals were awake and while asleep. This demonstrates that the increase in vagal modulation induced by long-term I_f blockade is independent on the baseline status of the autonomic balance and is not restricted to settings associated with sympathetic hyperactivity. The autonomic changes induced by ivabradine cannot be therefore interpreted as "corrective"; rather, vagal hyperactivity appears to be a common ivabradine "side effect."

Clinical Implications

On the one hand, the fact that the increase in vagal modulation induced by ivabradine is not restricted to the heart failure setting

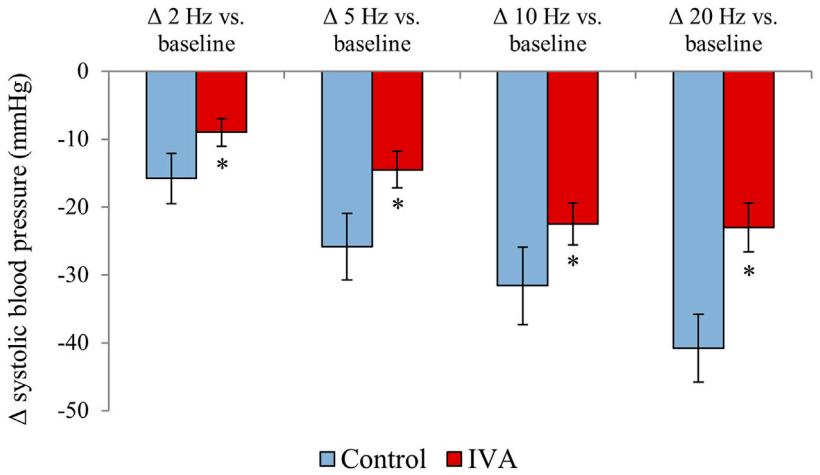

FIGURE 6 | Systolic blood pressure changes during vagus nerve stimulation at 2, 5, 10, and 20 Hz compared with the baseline values in the ivabradine-treated (IVA; $n = 10$) and non-treated (Control; $n = 6$) rats. Data are expressed as means ± standard error of the mean. *$p < 0.05$ for IVA vs. Control rats using the paired Student's *t*-test or the Mann-Whitney *U* test, as appropriate.

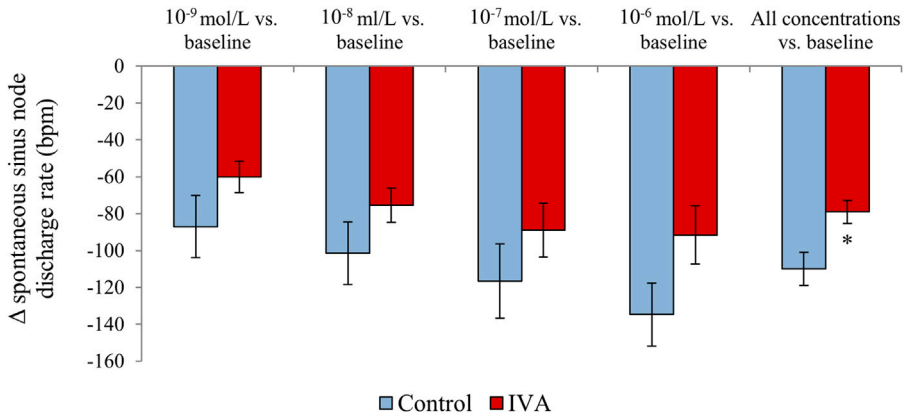

FIGURE 7 | Spontaneous sinus node discharge rate changes in response to *in vitro* carbamylcholine administration (10^{-9} to 10^{-6} mol/L) compared with the baseline values in the ivabradine-treated (IVA; $n = 10$) and non-treated (Control; $n = 6$) rats.

suggests that the beneficial effects of ivabradine could extend to other settings associated with increased sympathetic activity, in which the ivabradine-induced increase in vagal modulation could reduce myocardial oxygen demand and ischemia, diminish sympathetic stimulation and adrenoreceptor-mediated cytotoxicity, apoptosis, and hypertrophy, and reduce the likelihood of ventricular tachyarrhythmias and sudden death (Böhm et al., 2015). On the other hand, vagal hyperactivity has been shown to promote ectopic activity, reentry, and atrial fibrillation *via* multiple mechanisms (Scridon et al., 2018). Given the highly proarrhythmic effects of vagal activation at the atrial level, the increase in vagal modulation induced by ivabradine demonstrated in the present study could provide an explanation for the increased risk of atrial fibrillation associated with ivabradine use in clinical trials (Martin et al., 2014; Cammarano et al., 2016).

Chronic Ivabradine Administration Cancels the Cardioinhibitory Response and Blunts the Hemodynamic Response to Acute Vagal Stimulation. Potential Implications for Vaso-Vagal Syncope

According to estimates, 40% of individuals experience at least one episode of syncope during their lifetime (Colman et al., 2004), with vaso-vagal syncope representing up to 60% of all syncope cases (Brignole et al., 2006). Yet, the therapeutic management of vaso-vagal syncope remains highly challenging.

In the present study, *in situ* preganglionic vagus nerve stimulation elicited significant arterial hypotension and bradycardia in control rats. However, in the ivabradine-treated rats, vagal stimulation was not followed by the typical decrease in HR and the drop in SBP was significantly lower than that recorded in the control rats. Thus, the

present study demonstrates for the first time that chronic ivabradine administration in clinically relevant doses cancels the cardioinhibitory response and blunts the hemodynamic response to acute vagal stimulation in rats. Interestingly, reduced mean blood pressure response and almost complete elimination of HR response to vagus nerve stimulation was also reported following metoprolol administration (Gierthmuehlen and Plachta, 2016). Meanwhile, in apparent contradiction with our data, a higher drop in HR induced by vagus nerve stimulation was reported following ivabradine administration in two recent studies (Uemura et al., 2017; Kawada et al., 2019). Although the use of different anesthetic drugs may account for some of this discrepancy, in both those studies, acute ivabradine administration was used and the drug was administered in a single, intravenous dose. Chronic and acute ivabradine administration therefore appear to exhibit discordant effects on the HR response to vagal stimulation, suggesting that chronic ivabradine therapy could induce remodeling of one or several components of the parasympathetic nervous system-cholinergic receptors-I_f axis over the long term.

Head-to-head comparisons of sinus node discharge rate responses to direct cholinergic receptors stimulation for each carbamylcholine concentration did not show significant differences between the two groups. However, when the responses to all carbamylcholine concentrations were considered together, the decrease in the spontaneous discharge rate of the sinus node was significantly lower in the ivabradine-treated compared with the control rats. Two-factor ANOVA also demonstrated that the response of the spontaneous discharge rate of the sinus node to parasympathetic stimulation was significantly affected by ivabradine administration. Thus, the cancellation of the HR response to vagal stimulation induced by ivabradine appears to result from local, receptor and/or post-receptor ivabradine-parasympathetic nervous system interferences. In the same vein, our transcriptomic data showed that chronic ivabradine administration significantly up-regulates right atrial *HCN4* expression. In previous studies, ivabradine administration counteracted the increase in ventricular *HCN4* expression in post-myocardial infarction rats (Suffredini et al., 2012). Similarly, ivabradine has been shown to decrease *HCN2* and *HCN4* expression in a mixture of right and left atrial myocytes obtained from transgenic mice overexpressing the (pro) renin receptor (Wang et al., 2019). However, in the study by Leoni et al., 3 weeks ivabradine administration in mice led to a significant increase in sinus node *HCN4* expression (Leoni et al., 2006). The right atrial samples examined in our study contained both nodal and non-nodal tissue. However, since *HCN4* is highly expressed in the nodal pacemaker cells and only sparsely present in the remaining atrial myocardium (Chandler et al., 2009; Li et al., 2014), it is likely that the *HCN4* changes observed in our study reflect sinus node, rather than non-nodal alterations. Thus, in line with the data reported by Leoni et al. (Leoni et al., 2006), our data indicate that, contrary to its effects on non-pacemaker cells, long-term ivabradine therapy augments *HCN4* expression at the level of the sinus node. Eventually, *HCN4* and the consequent I_f up-regulation could render the sinus node less sensitive to acute vagal inputs and protect against excessive vagal-induced bradycardia. The recent finding of Kozasa et al. that *HCN4* overexpression attenuates the bradycardic response to vagus nerve stimulation (Kozasa et al., 2018) strongly supports this hypothesis.

Ivabradine does not pass the brain-blood barrier (Postea and Biel, 2011) and our data showed that long-term ivabradine therapy does not affect the expression of the neuronal specific *HCN*3 channel, at least at the level of the heart. However, *HCN* channels are also expressed in peripheral autonomic and somatosensory neurons, where they are responsible for generating the neuronal homologous of I_f – I_h, on which ivabradine has also been shown to exhibit significant inhibition (Savelieva and Camm, 2006; León-Hernández et al., 2016). An inhibiting effect of ivabradine on vagus nerve I_h activity could therefore also contribute to the lack of the HR response to *in situ* vagal stimulation observed in the ivabradine-treated rats.

Clinical Implications

The present study indicates for the first time that ivabradine is not only safe, but is also highly effective in preventing exaggerated vagal-induced bradycardia in rats. These data serve as a basis for future studies that will have to assess the validity of these findings in humans. If confirmed in clinical settings, this protective effect of ivabradine may place ivabradine as a drug that should not only not be avoided in patients with cardioinhibitory vaso-vagal syncope, but could even protect these patients from exaggerated vagal-induced cardioinhibitory response and reduce the risk of vaso-vagal syncope.

Strengths and Limitations

In the present study, the effects of long-term I_f blockade on autonomic function and on the cardiovascular response to parasympathetic stimulation were assessed by the use of clinically relevant ivabradine dose and route of administration. Both *in situ* and *in vitro* studies were performed, providing a comprehensive view on the impact of ivabradine therapy on the cardiovascular response to parasympathetic activation. The present study indicates ivabradine-induced sinus node *HCN4* up-regulation as potential mechanism for the blunted cardiovascular response to vagal stimulation seen in these rats. However, further studies are needed to explore other potential mechanisms responsible for this effect. *HCN* expression was not specifically assessed at the level of the sinus node; RNA levels were analyzed using a mixture of nodal and non-nodal right atrial tissue. *HCN4* changes observed in our study could therefore reflect not only sinus node, but also atrial myocardial changes. However, since HCN4 is highly expressed in the nodal pacemaker cells and only sparsely present in the atrial myocardium (Chandler et al., 2009; Li et al., 2014), it is likely that the *HCN4* changes reflect sinus node, rather than non-nodal *HCN4* alterations. *HCN* changes were only assessed by RNA quantification. Due to the small size of the rat right atrium, additional tissue analyses could not be performed; changes at the protein and I_f levels were not investigated. Although altered gene expression is expected to result in protein variations, post-transcriptional and post-translational regulatory mechanisms may also influence protein level and function. Diastolic blood pressure was not assessed in the present study. Although diastolic blood pressure changes are also reflected in the SBP, a potential direct impact of ivabradine on the vagal-induced vasodepressor response cannot be excluded. However, given that ivabradine is a pure HCN channels blocker, direct interference at the vascular level is highly unlikely. In the present study, vagal modulation of the HR was evaluated using HRV analysis. Vagal tone evaluation using muscarinic receptors

blockade, the Goldberger index, or vagal nerve activity recordings would have also been of interest. Finally, we acknowledge that by causing reduction in baseline HR, ketamine anesthesia could have diminished the impact of vagus nerve stimulation on the HR. However, whereas ketamine anesthesia manifested similar effects on the HR in the ivabradine-treated and non-treated rats, the HR response to vagus nerve stimulation was only abolished in the IVA rats. Thus, the lack of HR response to vagus nerve stimulation observed in the ivabradine-treated rats cannot be ascribed to the effects of ketamine anesthesia.

CONCLUSION

The present study demonstrates that long-term ivabradine therapy produces a significant increase in vagal modulation and shifts the sympatho-vagal balance toward vagal dominance. This increase in vagal modulation induced by ivabradine could contribute to the improved outcomes observed in patients with sympathetic hyperactivity, but could also provide an explanation for the increased risk of atrial fibrillation associated with ivabradine therapy in clinical trials. Ivabradine abolished the cardioinhibitory and blunted the hemodynamic response to acute vagal activation in rats, suggesting that I_f blockade may emerge as a promising therapy for patients with cardioinhibitory vaso-vagal syncope. Our data indicate sinus node *HCN4* up-regulation as a potential mechanism underlying the protective effect of ivabradine against excessive vagal-induced bradycardia in rats.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by the local Ethics Committee and the National Sanitary Veterinary and Food Safety Authority.

AUTHOR CONTRIBUTIONS

AS and RCS contributed to conception and design of the work. AS drafted the manuscript. AS, VBH, AIB, DAC, VM, CB, MP, and RCS contributed to acquisition, analysis or interpretation of data for the work. VBH, AIB, DAC, VM, CB, MP, and RCS performed critical revision of the manuscript for important intellectual content. All authors approved the final version of the manuscript.

FUNDING

This work was supported by the University of Medicine, Pharmacy, Science and Technology "George Emil Palade" of Târgu Mureş Research Grant number 615/2/17.01.2019.

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

Journal of Thrombosis and Haemostasis, **17**: 538–550 DOI: 10.1111/jth.14386

ORIGINAL ARTICLE

Vascular protease-activated receptor 4 upregulation, increased platelet aggregation, and coronary lipid deposits induced by long-term dabigatran administration – results from a diabetes animal model

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To cite this article: Scridon A, Mărginean A, Huțanu A, Chinezu L, Gheban D, Perian M, Vântu A, Gherțescu D, Fișcă PC, Șerban RC, Chevalier P, Dobreanu D. Vascular protease-activated receptor 4 upregulation, increased platelet aggregation, and coronary lipid deposits induced by long-term dabigatran administration – results from a diabetes animal model. *J Thromb Haemost* 2019; **17**: 538–50.

Essentials

- The impact of long-term thrombin inhibition outside the coagulation cascade is far from clear.
- We aimed to assess the impact of dabigatran etexilate (DE) in diabetic and control rats.
- In diabetic rats, DE increased platelet aggregation and lead to coronary lipid deposits.
- Long-term thrombin inhibition may increase atherosclerotic and atherothrombotic risk.

Summary. *Background:* Besides its role in the coagulation cascade, thrombin contributes to platelet aggregation and to a plethora of non-hemostatic functions. *Objectives:* To assess the impact of long-term thrombin inhibition with dabigatran etexilate (DE) on platelet aggregation and on extrahemostatic thrombin-related functions in diabetic and control rats. *Methods:* Markers of inflammation, endothelial dysfunction, oxidative stress, angiogenesis and cell adhesion molecules were quantified in control rats (Control; $n = 6$), DE-treated control rats (Control-Dabi; $n = 8$), diabetic rats (Diabetes; $n = 5$), and DE-treated diabetic rats (Diabetes-Dabi; $n = 8$). Agonist-induced platelet aggregation, aortic and coronary lipid deposits and aortic protease-activated receptor 4 (PAR4) expression were also assessed. *Results:* Control-Dabi rats showed significantly higher high-sensitivity C-reactive protein, von Willebrand factor (VWF), vascular endothelial growth factor (VEGF) and fibronectin levels, and significantly lower PAR4 agonist-induced aggregation, than Control rats. Control-Dabi rats also showed mild aortic lipid deposits, whereas no such changes were observed in Control rats. Diabetes-Dabi rats showed significantly higher VWF, VEGF and fibronectin levels than Diabetes rats, and similar PAR4 agonist-induced aggregation as Diabetes rats, and significantly higher ADP-induced aggregation than Diabetes rats. Coronary lipid deposits were observed in 75% of Diabetes-Dabi rats and in none of the Diabetes rats. PAR4 expression was 20.4% higher in Control-Dabi rats and 27.4% higher in Diabetes-Dabi rats than in their non-treated peers. *Conclusions:* This study indicates that long-term thrombin inhibition increases vascular PAR4 expression, promotes atherosclerosis-related mechanisms, and, in diabetic rats, increases platelet aggregation and favors the occurrence of coronary lipid deposits. These experimental data suggest that long-term thrombin inhibition may increase atherosclerotic and atherothrombotic risk, particularly in the presence of diabetes.

Keywords: atherosclerosis; dabigatran; platelet aggregation; thrombin; thrombin inhibition.

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Received: 7 November 2018,
Manuscript handled by: J. Morrissey
Final decision: P. H. Reitsma, 5 January 2019

Introduction

Thrombin is one of the major procoagulant enzymes, ensuring fibrin formation and cross-linking, but also the

amplification of the coagulation cascade via multiple feedback loops. Thrombin is also one of the strongest platelet activators, via protease-activated receptors (PARs) [1]. In addition to its role in hemostasis, thrombin contributes to a plethora of non-hemostatic functions on and within the vasculature. By interacting with PARs on leukocytes, endothelial cells, vascular smooth muscle cells, and fibroblasts, thrombin promotes inflammation, oxidative stress, endothelial dysfunction, angiogenesis, and cell proliferation [1,2].

Although the pharmacological effects of thrombin inhibition on coagulation assays have been extensively studied [3], the impact of long-term thrombin inhibition on platelet function and its effects outside the hemostatic system are far from being elucidated. In the Randomized Evaluation of Long Term Anticoagulant Therapy With Dabigatran Etexilate (RE-LY) trial, despite dabigatran's non-inferiority as compared with warfarin in terms of stroke reduction, higher rates of myocardial infarction were reported in the dabigatran arm [4]. In a meta-analysis of 14 randomized controlled trials in various patient populations, dabigatran was associated with a 34% higher risk of myocardial infarction (95% confidence interval 1.08–1.65; $P = 0.007$) than control agents [5]. However, postmarket studies do not seem to support this dabigatran–myocardial infarction association [6–8]. Moreover, experimental studies in apolipoprotein E-deficient mice suggested that thrombin inhibition may have beneficial effects on endothelial function and have antiatherosclerotic effects [9,10]. In transgenic mice fed a high-fat diet, dabigatran protected from obesity and reduced microvesicular liver steatosis [11], whereas, in a transgenic mouse model of Alzheimer's disease, long-term dabigatran administration reduced the expression of inflammatory proteins and reactive oxygen species [12]. Studies assessing the effect of thrombin inhibition on platelet function have also yielded conflicting results [13–15]. Thus, it remains unclear whether and how long-term thrombin inhibition impacts on thrombin functions outside the coagulation cascade. If present, such effects of long-term thrombin inhibition may be more prominent in settings already associated with impaired PAR expression and altered thrombin-related functions, such as diabetes mellitus [16]. Among PARs, PAR4, the predominant thrombin receptor in rodent platelets [17], has been shown more recently to play critical roles in diabetes-related cardiovascular complications. In mice with type 1 diabetes, increased susceptibility to arterial thrombosis was associated with enhanced platelet sensitivity to PAR4 [18]. Increased PAR4 expression was also reported in the aorta and carotid arteries of diabetic mice, which also developed a greater degree of neointima formation after coronary artery ligation than the non-diabetic mice, whereas such an effect was absent in PAR4-deficient diabetic mice [16]. More recently, increased PAR4 expression was reported in diabetic mouse cardiac fibroblasts, which also showed higher thrombin-induced migration, remodeling-associated gene expression, and oxidative stress [19].

We therefore aimed to assess the effects of long-term thrombin inhibition on platelet aggregation and on extra-hemostatic thrombin-related functions in adult diabetic and control rats, and to investigate the mechanisms underlying these effects, with a focus on PAR4.

Materials and methods

Studied animals

Six-week-old male Wistar rats purchased from the Cantacuzino Experimental Station (Bucharest, Romania) were randomly assigned to four groups: control rats (Control; $n = 6$), control rats treated with dabigatran etexilate (DE) (Control-Dabi; $n = 8$), rats with diabetes mellitus (Diabetes; $n = 8$), and rats with diabetes mellitus treated with DE (Diabetes-Dabi; $n = 10$). Rats were housed individually in a climate-controlled room (21–24 °C), under a 12-h light–dark cycle, and had free access to food and water. All protocols complied with the International Council for Laboratory Animal Science guidelines (Directive 2010/63/EU), and were approved by the local Ethics Committee.

Diabetes was induced in Diabetes and Diabetes-Dabi rats at 11 weeks of age by intraperitoneal injection of a single dose of streptozotocin (60 mg kg^{-1} body weight), as described previously [20]. Control-Dabi and Diabetes-Dabi rats received DE (Boehringer Ingelheim, Ingelheim, Germany) in their chow (1 mg g^{-1} of chow for Control-Dabi rats; 0.3 mg g^{-1} of chow for Diabetes-Dabi rats) for 12 consecutive weeks, starting at the age of 27 weeks, as described previously [21]. The doses were calculated on the basis of previous studies showing lower body weight and higher food intake in diabetic rats than in age-matched control rats [20], and recommendations from Boehringer Ingelheim, and were designed to ensure a total dose of DE of 50 mg kg^{-1} body weight per day.

Blood sampling and analysis

At the end of the study (38 weeks of age), plasma glucose was measured in all rats under ketamine/xylazine anesthesia (intraperitoneal, 50:5 mg kg^{-1}) with a clinical glucometer (SensoCard; Elektronika, Budapest, Hungary) and commercially available test strips (SensoCard), as described previously [20]. The lipid profile (i.e. total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides) was evaluated by the use of enzymatic colorimetric methods with automatic analyzers (Cobas 6000 analyzer series [Roche Diagnostics, Basel, Switzerland] and Dimension RxL Max [Siemens Healthcare Global, Newark, DE, USA]). Diluted thrombin time (dTT) was determined at the Department of CardioMetabolic Disease Research of Boehringer Ingelheim with a CL4 coagulometer (Behnk Elektronik, Norderstedt, Germany), based on the clotting-based Hemoclot thrombin inhibitor

assay (Hyphen BioMed, Neuville sur-Oise, France), as described previously [21].

Several markers of inflammation, endothelial dysfunction, oxidative stress, angiogenesis and cell adhesion molecules were also evaluated. High-sensitivity C-reactive protein (hs-CRP), von Willebrand factor (VWF), vascular endothelial growth factor (VEGF), fibronectin, superoxide dismutase (SOD), glutathione peroxidase (GPx) and malondialdehyde (MDA) levels were determined with the ELISA technique on the Dynex DSX Automated ELISA System (Dynex Technologies, Chanilly, VA, USA).

Platelet aggregation was quantified with whole-blood impedance aggregometry (Multiplate Analyzer; Roche Diagnostics). Briefly, blood samples were diluted with NaCl (0.9%), and stirred at 37 °C for 3 min. Platelet aggregation was stimulated with ADP (9.7 μmol L^{-1}), PAR4 agonist (442 μmol L^{-1}), and arachidonic acid (AA) (60 μmol L^{-1}). Electrical impedance changes were recorded, and the area under the aggregation curve (AUC) was determined. Agonist concentrations were chosen to ensure the highest AUC [22].

Tissue sampling and analysis

The thoracic cavity was opened immediately after blood sampling, and the heart and large vessels were removed, as described previously [23]. The ascending aorta was separated from the heart, and an aortic ring was isolated, and placed in formalin. A sample of the left ventricular free wall exposing a visible epicardial coronary artery was also collected.

Lipid deposits within the myocardial tissue, the aortic wall and the coronary artery wall were evaluated by the use of Oil Red O staining. Briefly, slides containing 8–10-μm-thick tissue sections were rinsed in tap water (10 min) and in isopropyl alcohol (60%), and stained with fresh Oil Red O solution. Slides were then rinsed again in isopropyl alcohol (60%), counterstained with hematoxylin, rinsed in distilled water, and mounted in glycerin jelly. All samples were examined with a Leica DM750 microscope (Leica Microsystems, Mannheim, Germany) and photographed with a Leica ICC50 HD camera (Leica Microsystems). Lipid deposits within the three sites (i.e. myocardium, aortic wall, and coronary artery wall) were evaluated semiquantitatively by the use of a 0–3 scale: 0 indicated the absence of lipid deposits (no stain), 1 indicated the presence of mild, focal deposits (slightly stained), 2 indicated the presence of moderate lipid deposits (intensely stained), and 3 indicated the presence of severe lipid deposits (very intensely stained).

The density of PAR4 within aortic wall sections was determined with a manual immunohistochemistry technique. Tissue sections were dewaxed in xylene, and dehydrated through a graded series of ethanol. Antigen retrieval was performed with a water-bath at high temperature in citrate buffer (10 mmol L^{-1}; pH 6.0) for 20 min. After cooling of the sections, an endogenous peroxidase quenching step was performed with hydrogen peroxide 3% for 10 min. The slides were then incubated with the primary PAR4 antibody (rabbit polyclonal antibody #APR-034; Alomone Labs, Jerusalem, Israel) for 30 min at room temperature. The labeled streptavidin–biotin (LSAB+) system was used according to the manufacturer's instructions (Dako Real Detection System, K5001; Dako, Glostrup, Denmark) in order to reveal the presence of antigens in the aortic wall sections. The final reaction product was visualized with 3,3′-diaminobenzidine substrate-chromogen as a brown deposit. The nuclei were counterstained with hematoxylin. All images were analyzed with IMAGEJ. The results are expressed as percentage of positive immunolabeled area relative to the total analyzed area.

Statistical analysis

Data regarding the presence of lipid deposits within the three analyzed sites are expressed as absolute numbers and percentages, and were compared by the use of Fisher's exact test. All other data are expressed as means ± standard error of the mean or as median (interquartile range) and were compared by the use of Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. All comparisons involving the Diabetes group ($n = 5$) were performed with the Mann–Whitney *U*-test. For comparisons between the Control and Control-Dabi groups, data were tested for normality; if normally distributed, the data were analyzed with an unpaired Student's *t*-test; if not normally distributed, the data were analyzed with the Mann–Whitney *U*-test. Data regarding PAR4 density were also analyzed with a non-parametric (using a rank transform) two-way ANOVA, with factoring for the effects of both diabetes status (diabetic versus non-diabetic) and dabigatran treatment (treated versus non-treated). A *P*-value of < 0.05 was considered to be statistically significant. Statistical analyses were performed with MEDCALC for Windows, version 12.4.3.0 (MedCalc Software, Ostend, Belgium).

Results

Three Diabetes and two Diabetes-Dabi rats died during the study and were excluded from all analyses. Both Diabetes-Dabi rats died prior to DE administration. At the end of the study, measurement of dTT revealed relevant anticoagulation in both Control-Dabi and Diabetes-Dabi rats (Fig. 1), and there was no significant difference in dTT between Control-Dabi and Diabetes-Dabi rats ($P = 0.10$). Diabetes and Diabetes-Dabi rats showed significantly higher plasma glucose, total cholesterol and plasma triglyceride levels than their non-diabetic controls (all $P < 0.01$). DE administration had no effect on plasma glucose, or on lipid profile, in either control or diabetic rats (all $P > 0.05$).

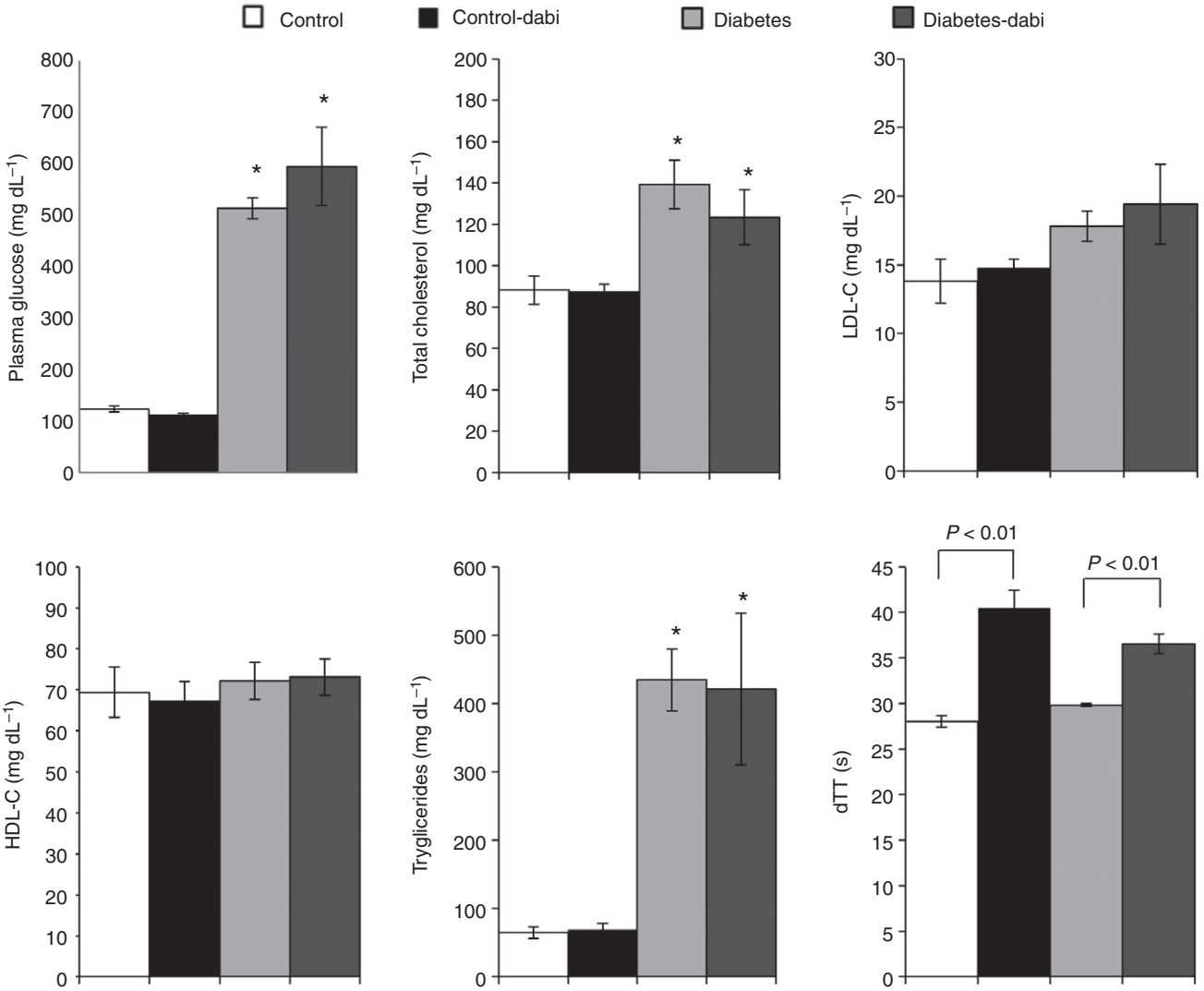

Fig. 1. Plasma glucose, lipid profile and diluted thrombin time (dTT) in the four study groups. dTT values show relevant anticoagulation in the dabigatran-treated control and diabetic rats. Dabigatran-treated and non-treated diabetic rats had significantly higher plasma glucose, total cholesterol and plasma triglyceride levels than their non-diabetic peers. Dabigatran had no effect on any of these parameters. *$P < 0.01$ as compared with non-diabetic controls based on an unpaired Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. Control, control ($n = 6$); Control-Dabi, dabigatran etexilate-treated control ($n = 8$); Diabetes, diabetic ($n = 5$); Diabetes-Dabi, dabigatran etexilate-treated diabetic ($n = 8$); HDL-C, HDL cholesterol; LDL-C, LDL cholesterol.

Impact of long-term thrombin inhibition in non-diabetic rats

In non-diabetic rats, DE administration was associated with significantly higher hs-CRP, VWF, VEGF and fibronectin levels, and with significantly lower GPx levels (all $P < 0.05$; Fig. 2). There was no significant change in SOD and MDA levels between Control and Control-Dabi rats (both $P > 0.05$). Control-Dabi rats showed significantly lower PAR4 agonist-induced aggregation than Control rats ($P = 0.03$), whereas ADP-induced and AA-induced platelet aggregation were similar between the two groups (both $P > 0.05$; Fig. 3). Myocardial fatty deposits were absent in Control rats, whereas four Control-Dabi rats (50%) showed mild (grade 1) myocardial fatty dystrophy ($P = 0.08$). No Control rats showed aortic lipid deposits, whereas six Control-Dabi rats (75%) showed mild (grade 1) aortic lipid deposits ($P < 0.01$). Coronary lipid deposits were not seen in any Control or Control-Dabi rats (Fig. 4). Immunohistochemical analysis of aortic wall sections (Fig. 5) revealed significantly higher PAR4 expression in Control-Dabi rats than in Control rats (24.7% ± 1.3% versus 20.5% ± 0.9%, $P = 0.02$).

Impact of long-term thrombin inhibition in diabetic rats

Similarly to Control-Dabi rats, Diabetes-Dabi rats showed significantly higher VWF, VEGF and fibronectin levels than Diabetes rats (all $P < 0.05$; Fig. 6). However, neither hs-CRP nor GPx levels were significantly different between Diabetes and Diabetes-Dabi rats (both

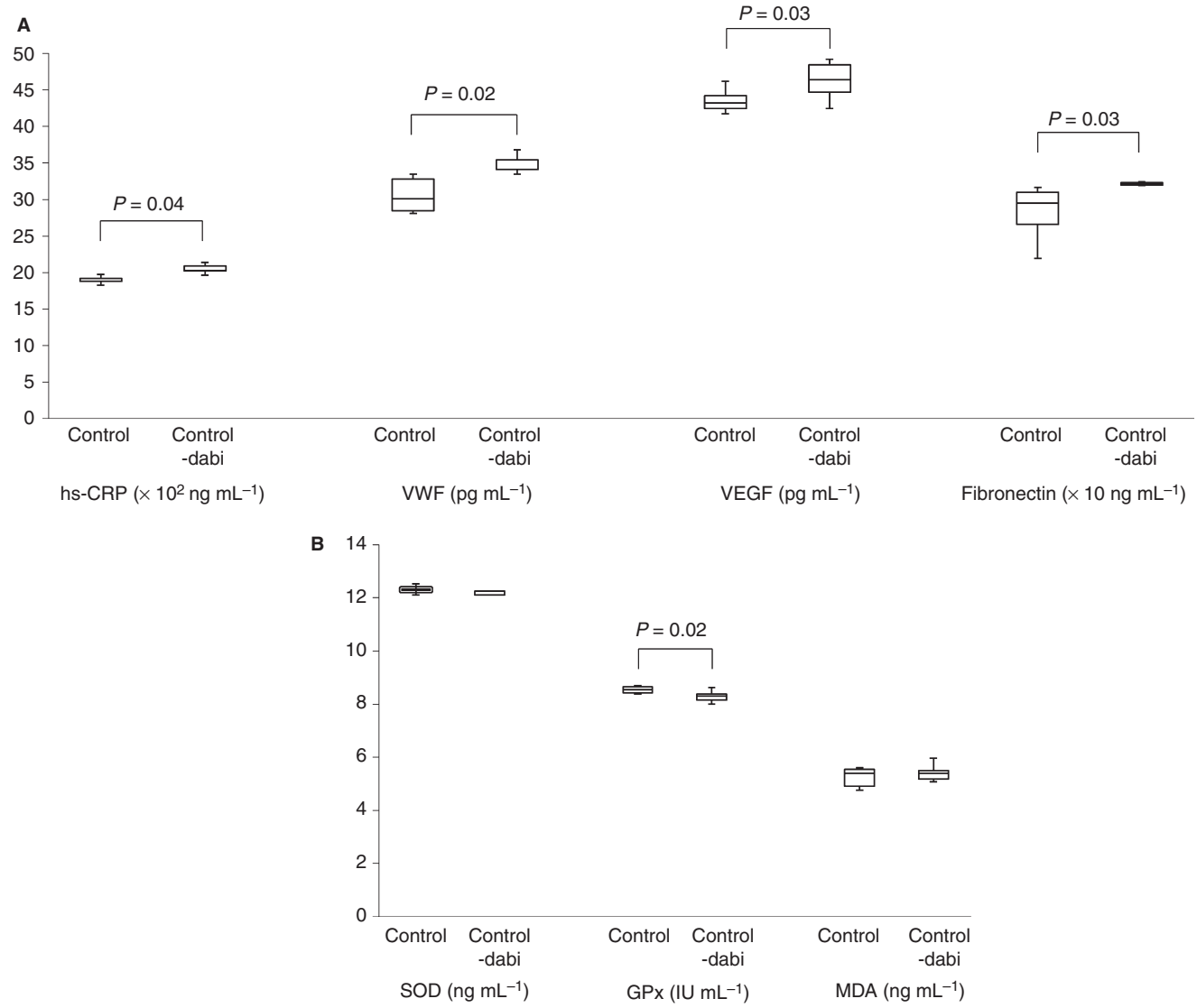

Fig. 2. Blood parameters in control rats treated and not treated with dabigatran. Plasma levels of markers of inflammation and endothelial dysfunction, angiogenic factors, and cell adhesion molecules (A), and oxidative stress (B), in non-diabetic rats treated and not treated with dabigatran. In non-diabetic rats, dabigatran administration was associated with significantly higher high-sensitivity C-reactive protein (hs-CRP), von Willebrand factor (VWF), vascular endothelial growth factor (VEGF) and fibronectin levels, and with significantly lower glutathione peroxidase (GPx) levels. Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile. *P*-values were obtained with an unpaired Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. Control, control ($n = 6$); Control-Dabi, dabigatran etexilate-treated control ($n = 8$); MDA, malondialdehyde; SOD, superoxide dismutase.

$P > 0.05$). Also, there was no significant change in SOD and MDA levels between Diabetes and the Diabetes-Dabi rats (both $P > 0.05$). As in non-diabetic rats, AA-induced platelet aggregation was also similar between Diabetes-Dabi and Diabetes rats ($P = 0.84$). However, in contrast to Control-Dabi rats, Diabetes-Dabi rats did not show lower PAR4 agonist-induced aggregation ($P = 0.52$) and showed significantly higher ADP-induced platelet aggregation ($P = 0.03$) than Diabetes rats (Fig. 7). Oil Red O staining (Fig. 4) demonstrated the presence of mild (grade 1) aortic lipid deposits in four (80%) Diabetes rats and in six (75%) Diabetes-Dabi rats ($P = 1.00$). No Diabetes rats showed coronary lipid deposits, whereas six (75%) Diabetes-Dabi rats showed coronary lipid deposits ($P = 0.02$), including grade 2 (moderate) deposits in two Diabetes-Dabi rats and grade 3 (gross) deposits in one Diabetes-Dabi rat. Myocardial fatty deposits were absent in Diabetes rats, whereas seven Diabetes-Dabi rats (87.5%) showed myocardial fatty dystrophy ($P < 0.01$). As in the non-diabetic rats, PAR4 expression (Fig. 5) was significantly higher in Diabetes-Dabi rats than in Diabetes rats ($37.2\% \pm 1.7\%$ versus $29.2\% \pm 1.6\%$, $P < 0.01$). Two-factor ANOVA demonstrated that PAR4 expression was

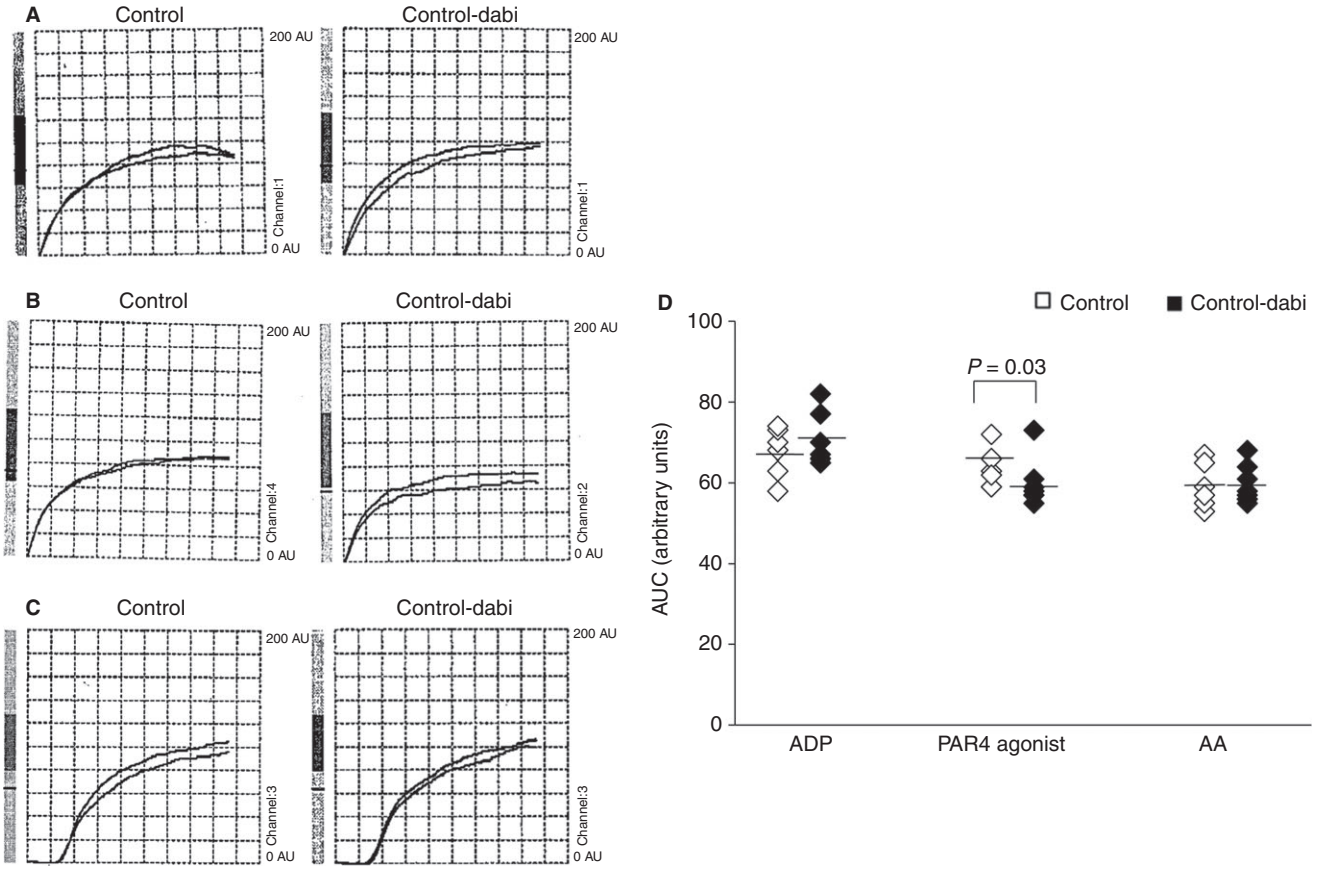

Fig. 3. Platelet aggregation in control rats treated and not treated with dabigatran. (A–C) Representative aggregometry tracings obtained in a non-treated control rat and in a dabigatran-treated control rat (measurements in duplicate), with (A) ADP, (B) protease-activated receptor 4 (PAR4) agonist and (C) arachidonic acid (AA) as agonists. (D) Platelet aggregation in non-diabetic rats treated and not treated with dabigatran. Dabigatran etexilate-treated control rats (Control-Dabi; $n = 8$) showed significantly lower PAR4 agonist-induced aggregation than their non-treated controls (Control; $n = 6$), whereas ADP-induced and AA-induced platelet aggregation were similar between the two groups. *P*-values were obtained with an unpaired Student's *t*-test or the Mann–Whitney *U*-test, as appropriate. AU, arbitrary units; AUC, area under the aggregation curve.

significantly affected by both DE administration ($P = 0.02$) and diabetes status ($P < 0.01$).

Discussion

The main findings of the present study were that: (i) in control rats, long-term dabigatran administration in relevant doses promoted inflammation, endothelial dysfunction, and oxidative stress, increased the expression of angiogenic and cell adhesion molecules, and favored the occurrence of aortic (but not coronary) lipid deposits; (ii) in diabetic rats, long-term dabigatran administration was associated with increased plasma levels of markers of endothelial dysfunction and angiogenesis, and cell adhesion molecules, as well as with the occurrence of coronary lipid deposits; (iii) in diabetic rats, these effects were also accompanied by increased agonist-induced platelet aggregation; and (iv) long-term thrombin inhibition was associated with increased vascular PAR4 expression in both diabetic and non-diabetic rats.

Clinically, there is still an ongoing controversy regarding the potential risks associated with the long-term use of thrombin inhibitors. Several post-approval cohort studies found no evidence of an increased risk, and even suggested a lower risk of myocardial infarction with dabigatran than with warfarin [6–8]. However, in the large multicenter RE-LY trial, there was a significant increase in myocardial infarction with dabigatran [4], at least before the inclusion of silent myocardial infarctions [24], and almost all randomized trials showed a numerical excess of myocardial infarction with dabigatran [25–28]. Nevertheless, none of these studies was designed to detect a difference in myocardial infarction between the different groups, and none of them had the power to allow conclusions to be drawn in this regard. Several meta-analyses combining these trials point, however, towards an increased risk of myocardial infarction in patients treated with dabigatran [5,8,29–31]. In contrast, several experimental studies showed improved endothelial function and reduced oxidative stress, and impaired formation, reduced size and enhanced stability of

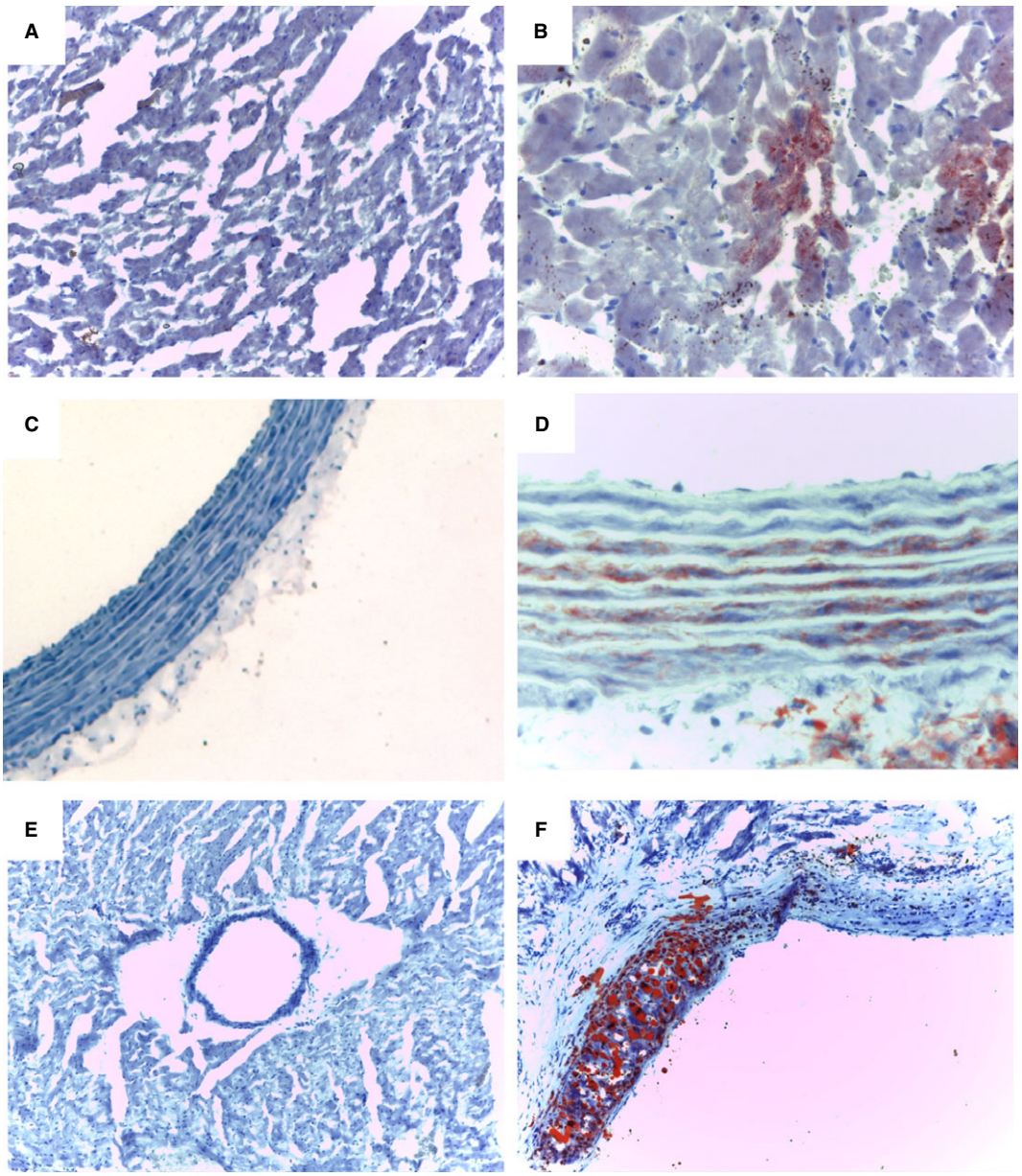

Fig. 4. Representative Oil Red O staining of myocardium, ascending aorta, and coronary artery wall. The images show: (A) normal myocardium in a control rat and (B) mild (grade 1) intramyocardial lipid accumulation in a dabigatran-treated diabetic rat; (C) normal aortic wall in a control rat and (D) mild (grade 1) lipid deposits in the aortic wall of a dabigatran-treated control rat; and (E) normal coronary artery wall in a dabigatran-treated control rat and (F) severe (grade 3) lipid deposits in the coronary artery wall of a dabigatran-treated diabetic rat. Lipid deposits are colored red.

atherosclerotic lesions, following administration of direct thrombin inhibitors [9,10,32–34]. However, all of these experimental studies were performed with a single model – the apolipoprotein E-deficient mouse. Although this model has a number of advantages, including early onset and increased severity of atherosclerotic plaques, its use in atherosclerosis research remains a matter of debate and the model has been criticized for its lack of clinical relevance. Atherosclerotic plaques in these mice are usually restricted to the aorta, whereas the coronary arteries, which are the sites of the major pathophysiological processes in human ischemic heart disease, are often lesion-free [35]. Also, acute plaque disruption and erosion are rarely observed in this model [9].

To the best of our knowledge, this is the first study to explore the effects of direct thrombin inhibition in control rats and in the setting of diabetes. In contrast to the antiatherosclerotic effects observed in the apolipoprotein E-deficient mice, in diabetic rats dabigatran administration was associated with increased plasma levels of markers of endothelial dysfunction (VWF) and angiogenesis (VEGF), and cell adhesion molecules (fibronectin), as

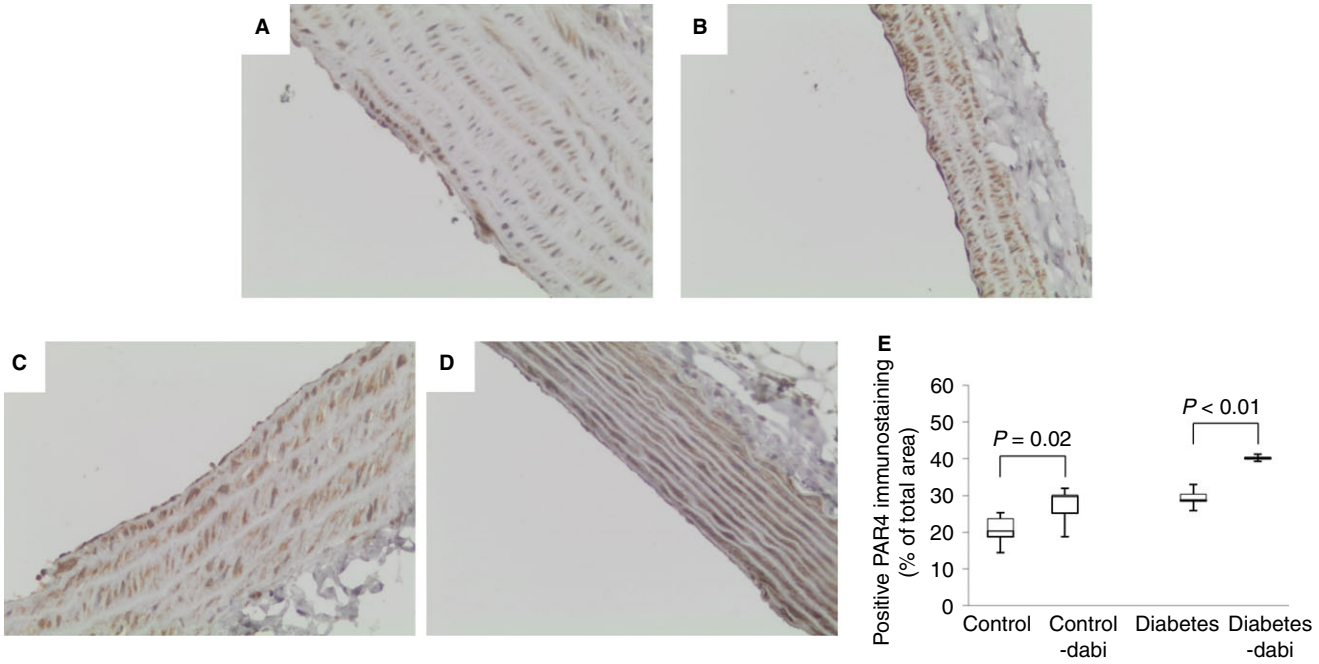

Fig. 5. Protease-activated receptor 4 (PAR4) expression in the aortic wall of the studied animals. (A–D) Representative immunohistochemical staining for PAR4 (colored in brown) within the aortic wall of (A) a control rat, (B) a dabigatran etexilate-treated control rat (Control-Dabi), (C) a diabetic rat (Diabetes), and (D) a dabigatran etexilate-treated diabetic rat. (E) Percentage of positive PAR4 immunostaining relative to the total analyzed area in the four study groups. Dabigatran-treated rats showed significantly higher aortic PAR4 expression than their non-treated peers. In (E), data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile. *P*-values were obtained with an unpaired Student's *t*-test and the Mann–Whitney *U*-test, respectively.

well as with the occurrence of coronary lipid deposits. Moreover, dabigatran administration was also associated with inflammation, endothelial dysfunction, and oxidative stress, with increased expression of angiogenic and cell adhesion molecules, and with the occurrence of aortic (but not coronary) lipid deposits in the non-diabetic rats. These discordant results may be attributable to interspecies differences, to the use of different experimental models, or to the younger age of the animals evaluated in the vast majority of previous studies. In the study by Preusch *et al*. [34], although 20 weeks of DE administration was associated with reductions in lesion areas in the aortic sinuses of 28-week-old mice, this was not the case in 40-week-old mice. Furthermore, anti-inflammatory and/or antiatherosclerotic effects of direct thrombin inhibitors have never been reported in clinical settings, whereas increased levels of markers of inflammation (interleukin-18 and C-reactive protein) have been observed in postmyocardial infarction patients treated with ximelagatran [36].

Although previous studies [18,37] have showed increased platelet reactivity in diabetics as compared with non-diabetics, this was not the case in our study. In the present study, diabetic rats showed similar PAR4 agonist-induced platelet aggregation as their non-diabetic peers ($P = 0.92$). However, a number of previous studies have reported similar findings, failing to show increased *in vitro* platelet aggregation in diabetics as compared with their non-diabetic controls [38–41]. To date, it remains unclear whether these discordances are attributable to interspecies differences, different diabetes models, and/or duration of diabetes, or to different laboratory techniques or different agonist concentrations used to assess platelet aggregation. Also, in agreement with previous clinical studies [13,42,43], dabigatran-treated control rats showed similar ADP-induced and AA-induced platelet aggregation to their non-treated peers, and lower PAR4 agonist-induced platelet aggregation. Increased ADP-induced platelet aggregation together with a relative increase in PAR4 agonist-induced aggregation (as compared with what was observed in the non-diabetic controls) was noticed in the dabigatran-treated as compared with the non-treated diabetic rats.

As most thrombin functions outside the coagulation cascade are mediated by PARs, we investigated whether a potential impact of long-term thrombin inhibition on PAR expression could provide an explanation for our findings. We therefore examined the density of PAR4 within the aortic walls of the studied animals, and demonstrated that prolonged exposure to dabigatran of control rats led to a 20.4% relative increase in the density of aortic PAR4. These data are in agreement with those from previous studies, which showed higher PAR1 and PAR4 expression in platelets of dabigatran-anticoagulated patients [43,44]. As already demonstrated in previous studies, vascular PAR4 expression was also significantly

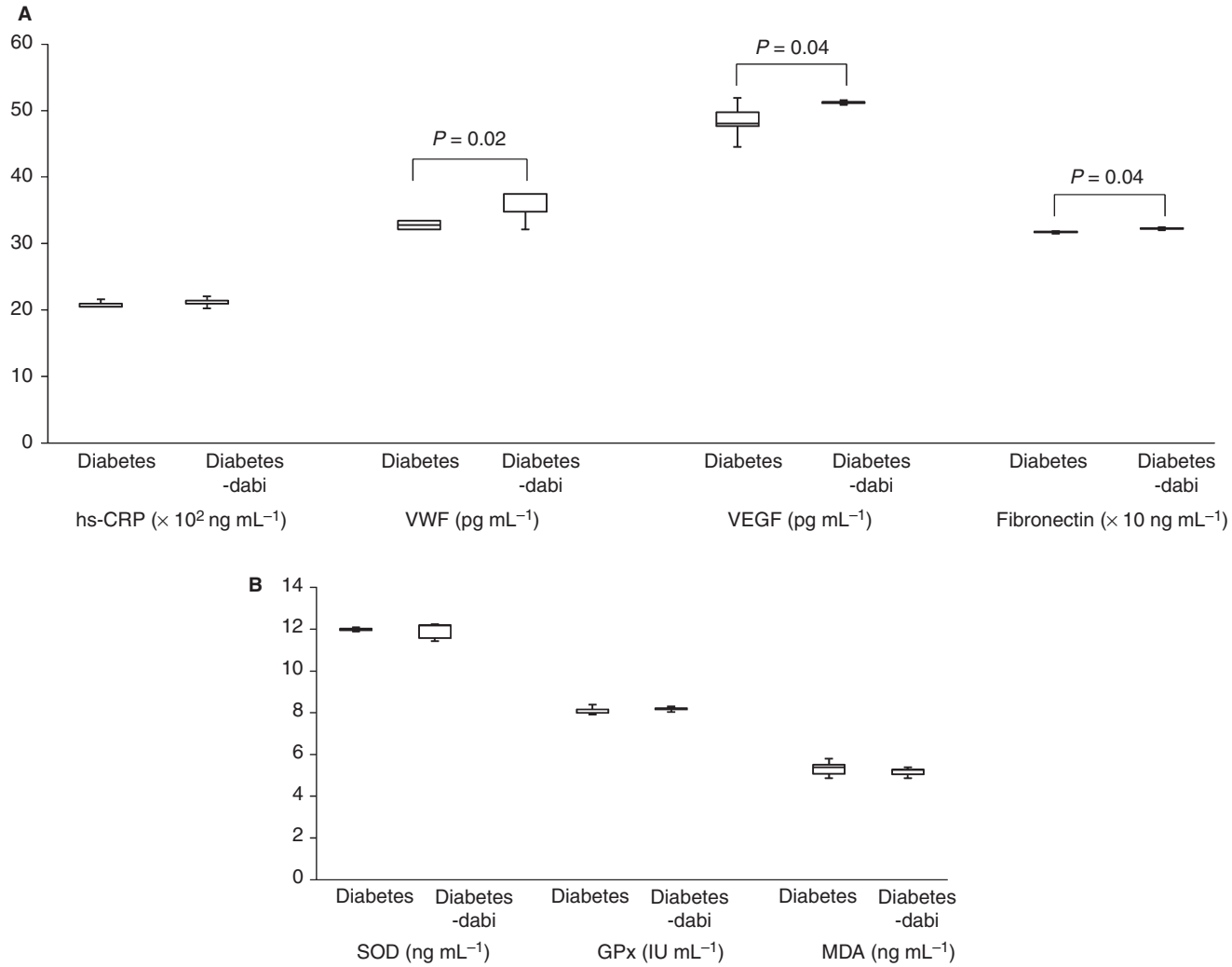

Fig. 6. Blood parameters in the diabetic rats treated and not treated with dabigatran. Plasma levels of markers of inflammation and endothelial dysfunction, angiogenic factors, and cell adhesion molecules (A), and oxidative stress (B), in diabetic rats treated and not treated with dabigatran. In diabetic rats, dabigatran administration was associated with significantly higher von Willebrand factor (VWF), vascular endothelial growth factor (VEGF) and fibronectin levels. Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile. *P*-values were obtained with the Mann–Whitney *U*-test. Diabetes, diabetic rats ($n = 5$); Diabetes-Dabi, dabigatran etexilate-treated diabetic rats ($n = 8$); GPx, glutathione peroxidase; hs-CRP, high-sensitivity C-reactive protein; MDA, malondialdehyde; SOD, superoxide dismutase.

affected by the diabetes status [16]. However, in the present study, dabigatran administration was associated with increased PAR4 density independently of diabetes status. Moreover, dabigatran-induced PAR4 overexpression was higher in the diabetic than in the non-diabetic rats (27.4% relative increase in the diabetic rats versus 20.4% in the non-diabetic rats), and this may explain the more prominent effects of dabigatran administration on coronary lipid deposits as well as on platelet aggregation observed in this group.

Given the indisputable role of PARs in atherosclerosis-related processes [1,2], the coexistence of increased PAR4 expression and increased levels of markers of endothelial dysfunction, inflammation, and angiogenesis, and cell adhesion molecules, as well as with the occurrence of vascular lipid deposits is not surprising. Also, this is not the first time that long-term dabigatran administration has been associated with increased platelet function. In the phase II Stroke Prevention in Patients With AF by Treatment With Dabigatran, With and Without Aspirin, Compared to Warfarin (PETRO) trial, the levels of thromboxane, which is a marker of platelet activation, were significantly increased in dabigatran-treated patients [45], although this was not the case in a substudy from the RE-LY trial [46]. In the study by Olivier *et al.* [14], dabigatran administration was associated with an increase in thrombin receptor-activating peptide (TRAP)-induced platelet aggregation, and this effect was more pronounced in patients taking higher dabigatran doses and for a longer time. Similarly, in the study by Achilles *et al.* [44], the authors

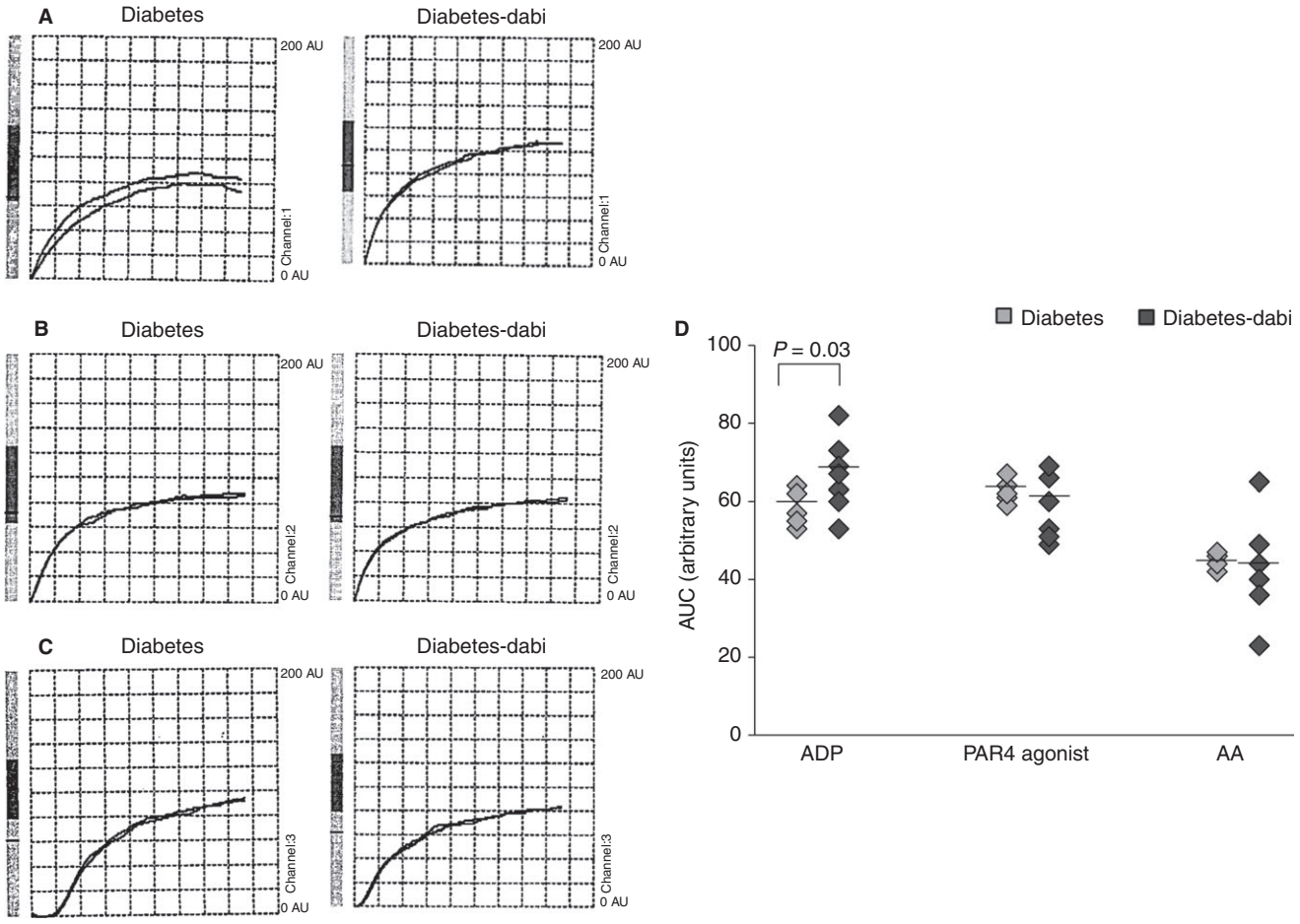

Fig. 7. Platelet aggregation in the diabetic rats treated and not treated with dabigatran. (A–C) Representative aggregometry tracings obtained in a non-treated diabetic rat and in a dabigatran-treated diabetic rat (measurements in duplicate) with (A) ADP, (B) protease-activated receptor 4 (PAR4) agonist and (C) arachidonic acid (AA) as agonists. (D) Platelet aggregation in diabetic rats treated and not treated with dabigatran. Dabigatran etexilate-treated diabetic rats (Diabetes-Dabi; $n = 8$) showed significantly higher ADP-induced aggregation than their non-treated controls (Diabetes; $n = 5$), whereas PAR4 agonist-induced and AA-induced platelet aggregation were similar between the two groups. *P*-values were obtained with the Mann–Whitney *U*-test. AU, arbitrary units; AUC, area under the aggregation curve.

demonstrated enhanced TRAP-induced platelet aggregation coupled with increased platelet expression of PAR1 and PAR4 in atrial fibrillation patients following dabigatran treatment. Other studies, however, reported no change or even decreased TRAP-induced platelet aggregation in dabigatran-treated patients [13,42,43].

Increased ADP-induced platelet aggregation following thrombin inhibition has not been reported to date, suggesting that this effect may be restricted to the diabetes model used in this study. However, there have been antithrombotic agents that induced a prothrombotic state via pathways other than those primarily targeted [47]. Furthermore, in the study by Christersson *et al.* [48], although thrombin inhibition diminished the formation of procoagulant microparticles and tissue factor expression within the platelet–granulocyte aggregates, there was a tendency for there to be increased formation of platelet–monocyte aggregates by the thrombin inhibitors upon ADP stimulation. In addition, there have been studies showing increased ADP-induced platelet aggregation following phenprocoumon administration [49] and studies showing no difference in ADP-induced platelet aggregation in patients treated with dabigatran as compared with those taking phenprocoumon [50].

Clinical implications

Pleiotropic effects of direct thrombin inhibitors on atherosclerosis and atherothrombosis have been suggested by both clinical and experimental studies. However, it remains unclear whether these effects are beneficial or deleterious [51]. The present study indicates that, in diabetic rats, long-term dabigatran administration promotes inflammation, endothelial dysfunction, and cell adhesion, favors the occurrence of coronary lipid deposits, and increases platelet aggregation. It remains to be established whether these effects play a role in the increased risk of myocardial infarction associated with dabigatran administration in certain clinical studies. Given the expanding use

of dabigatran, particularly in patients exposed to anticoagulation for decades, such as those with atrial fibrillation, the potential impact of long-term thrombin inhibition on atherosclerosis initiation and/or progression deserves special attention. The clinical trials performed so far might have been too short to reliably capture atherosclerotic events associated with direct oral anticoagulant administration. Studies using sensitive methods of intravascular imaging may provide an answer. Increased PAR expression induced by long-term dabigatran administration may also place these patients at increased risk of thrombotic events following abrupt drug discontinuation, as already shown for the direct activated factor X inhibitors rivaroxaban and apixaban [52,53]. In addition, if confirmed in human patients, increased ADP-induced platelet aggregation associated with long-term dabigatran treatment may interfere with the antiplatelet activity of $P2Y_{12}$ inhibitors, as integrated results from previous studies appear to suggest [49,50].

Strengths and limitations

In the present study, the effects of long-term thrombin inhibition were assessed by the use of relevant dabigatran doses. Multiple thrombin-related pathways were studied, providing a comprehensive view on the impact of thrombin inhibition on and within the vasculature, not only in a disease model, but also in control rats. Our study also has a number of limitations. In the present study, PAR4 expression was assessed only in the vessel wall; PAR4 expression analysis in platelets was not performed. However, previous studies have already demonstrated increased PAR1 and PAR4 expression in platelets of dabigatran-anticoagulated patients [43,44]. Evaluating vascular PAR1 expression and exploring PAR1 and PAR4 function in endothelial cells would also have been of interest. Future studies will also have to address the mechanisms underlying the ADP hypersensitivity observed in the diabetic rats after long-term dabigatran exposure. Although the diabetes model used in this study has been shown to replicate the most common features of human diabetes [20], extrapolation of these results to humans should be performed with caution. The overall changes observed in this study, although statistically significant, are rather subtle. Future studies will have to fully establish the biological significance of these changes and assess their relevance in clinical settings.

Conclusions

This study provides evidence that long-term thrombin inhibition increases the expression of vascular PAR4 receptors, promotes atherosclerosis-related mechanisms, and, in diabetic rats, favors the occurrence of coronary lipid deposits and increases agonist-induced platelet aggregation. Pending confirmation in human patients, our data suggest that long-term thrombin inhibition may have unwanted effects in terms of atherosclerotic and atherothrombotic risk, particularly in the presence of diabetes. Future studies are needed to fully understand the potential benefits and drawbacks of long-term thrombin inhibition.

Addendum

A. Scridon contributed to study conception and design, and acquisition, analysis and interpretation of data, and drafted the manuscript. A. Mărginean, A. Huțanu, L. Chinezu, D. Gheban, M. Perian, A. Vântu, D. Gherțescu, and P. C. Fișcă contributed to the acquisition, analysis and interpretation of data. R. C. Șerban, P. Chevalier, and D. Dobreanu contributed to study conception and design, and interpretation of data, and performed the critical revision of the manuscript.

Acknowledgements

This work was supported by the University of Medicine and Pharmacy of Tîrgu Mureș Research Grant number 16/11.12.2013 and Boehringer Ingelheim GmbH. Boehringer Ingelheim GmbH provided the chow for the animal study and performed diluted thrombin time analysis. The sponsors had no other roles in data collection or interpretation, or in the writing of the manuscript.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Journal of Diabetes •• (2017), ••–••

ORIGINAL ARTICLE

Plasma lipids affect dabigatran etexilate anticoagulation in rats with unbalanced diabetes mellitus

Highlights
- Diabetic rats exhibit significantly more intense dabigatran-induced anticoagulation that does not seem to be solely related to altered kidney function.
- Plasma cholesterol significantly affects dabigatran-induced anticoagulation in this setting.
- These findings could explain the similar benefits of dabigatran in reducing ischemic stroke compared with warfarin in patients with and without diabetes mellitus, despite the significantly higher intrinsic thrombotic risk in the former, as well as the lack of benefit of reducing major bleeding in diabetic patients.

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Received 30 December 2016; revised 12 May 2017; accepted 9 June 2017.

doi: 10.1111/1753-0407.12580

Abstract

Background: Dabigatran etexilate (DE) has similar stroke prevention efficacy in patients with and without diabetes mellitus (DM). However, the benefit of reducing major bleeding was not seen in diabetics. Thus, this study investigated anticoagulant responses to DE and the biological predictors of this response in a DM model.

Methods: Experiments were performed in six control (C), eight DE-treated control (CD), five diabetic (D), and eight DE-treated diabetic (DD) rats. Dabigatran etexilate (50 mg/kg/day) was administered in chow for 12 weeks. At the end of the study, plasma glucose, triglycerides, total cholesterol (TC), high-density lipoprotein cholesterol, low-density lipoprotein cholesterol (LDL-C), and plasma creatinine were measured. Correlations were ascertained with the diluted thrombin time (dTT).

Results: When corrected for similar DE intake, dTT was significantly higher in DD than CD rats ($P < 0.001$). There was a significant negative correlation between creatinine clearance (CCr) and dTT ($r = -0.91$, $P < 0.01$) in DD rats. In addition, dTT was positively correlated with TC ($r = 0.96$, $P < 0.01$), LDL-C ($r = 0.75$, $P = 0.04$), and glucose ($r = 0.83$, $P = 0.02$). In multiple regression analysis, CCr ($r = -0.81$, $P = 0.01$), TC ($r = 0.93$, $P < 0.001$), and LDL-C ($r = 0.74$, $P < 0.01$) remained the only independent predictors of dTT.

Conclusions: The results show a significantly more intense DE-induced anticoagulation in diabetic rats that does not seem to be solely related to altered kidney function, and demonstrate that plasma cholesterol can significantly affect DE anticoagulation in this setting.

Keywords: animal model, dabigatran, diabetes mellitus, hypercholesterolemia, oral anticoagulants.

Introduction

Vitamin K antagonists (VKAs) have represented the gold standard for thromboembolism prophylaxis in atrial fibrillation (AF) patients for more than 50 years. By decreasing the risk of stroke by more than 60%, VKAs are among the most efficient preventive tools medicine has ever known. However, due to unpredictable anticoagulant responses and difficulties in maintaining adequate International Normalized Ratio (INR) values, VKA use is overtly suboptimal. Over recent years, two new classes of oral anticoagulants (NOACs), direct thrombin inhibitors and direct Factor Xa inhibitors, have emerged as an appealing alternative. Large clinical trials have demonstrated that these new agents are at least as effective as the VKAs in preventing strokes in AF patients[1–4] and, due to fixed-dose regimens, they are easier to administer. Moreover, the more stable pharmacokinetics and pharmacodynamics of NOACs, their relatively low drug and food interactions, and wider therapeutic windows ensure a much more predictable anticoagulant response, allowing them to be administered without the need for routine laboratory monitoring.[5]

However, in pharmacokinetic studies, significant interindividual variability has been reported, even among apparently healthy individuals.[6–8] Recent studies indicate that drug and food interactions, kidney function, and even genetic variability can significantly affect the anticoagulant response to NOACs.[9,10] Furthermore, a degree of interindividual variability remains inexplicable to date.[9]

Adequate oral anticoagulation is even more challenging in the setting of diabetes mellitus (DM). Whereas DM significantly contributes to unstable anticoagulation in VKA-treated patients,[11] in subgroup analyses of NOAC trials, despite similar efficacy in stroke prevention in diabetic and non-diabetic subjects,[12,13] the benefit of reducing major bleeding with these new agents compared with VKAs was not seen in diabetic subjects.[14–16]

Accordingly, the aim of the present study was to investigate anticoagulant responses to the direct thrombin inhibitor dabigatran etexilate (DE) and to identify biological predictors of this response using an experimental model of DM in rats.

Methods

Animals and housing

Thirty-two 6-week-old male Wistar rats (mean [± SEM] weight 178 ± 4 g) were purchased from the Cantacuzino Experimental Station (Bucharest, Romania). The rats were randomly assigned to one of four groups, namely a control (C; $n = 6$) group, a control group treated with DE (CD; $n = 8$), a diabetic group (D; $n = 8$), and a diabetic group treated with DE (DD; $n = 10$). Rats were housed individually in polycarbonate cages in a climate-controlled room (21–24°C) under a 12-h light–dark cycle and had free access to food and water. For each rat, food was weighed twice a week, at 24-h intervals, and 24-h food intake was calculated. The body weight of all rats was measured weekly throughout the study. All experiments complied with the International Council for Laboratory Animal Science guidelines (Directive 2010/63/EU; http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:EN:PDF, accessed 10 July 2017) and were approved by the local ethics committee of the University of Medicine and Pharmacy of Tîrgu Mureş.

Induction of DM

At 11 weeks of age, DM was induced in D and DD rats using a single intraperitoneal injection of streptozotocin (STZ; 60 mg/kg, i.p.), as described previously.[17,18] One rat in the D group died during STZ administration and was excluded from any further analysis. Insulin was not administered. The D and DD rats were considered diabetic if fasting plasma glucose measured 1 week after STZ administration exceeded 250 mg/dL.[17] The C and CD rats were injected with an equivalent volume of vehicle (citrate buffer), without STZ. Three rats in the D group and two in the DD group died during the study. Both DD rats died before initiation of DE administration.

Administration of DE

Starting at 27 weeks of age, CD and DD rats received DE (Boehringer Ingelheim, Ingelheim, Germany) in their chow (1 mg/g chow for CD rats; 0.3 mg/g chow for DD rats) for 12 weeks. This dose was designed to ensure a total DE dose of 50 mg/kg bodyweight per day, based on recommendations from Boehringer Ingelheim and previous studies showing lower body weight and higher food intake in diabetic compared with age-matched control rats.[17]

Blood sampling and analysis

At 38 weeks of age, non-fasting plasma glucose was measured in all rats, as described previously;[17,18] blood samples were collected in two vacutainers, one containing EDTA and the other containing sodium citrate.

A complete blood count was performed using the direct current detection method (Sysmex XP-300 Automated Hematology Analyzer; Sysmex Corporation, Kobe, Japan). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride levels were measured by enzymatic colorimetric methods using automatic analyzers (Cobas 6000 analyzer series [Roche Diagnostics, Basel, Switzerland] and Dimension RxL Max [Siemens Healthcare Global, Newark, DE, USA]). Plasma creatinine was measured using a buffered kinetic Jaffe reaction without deproteinization and creatinine clearance (CCr) was calculated using an equation validated for rats as follows:[17]

$$\mathrm{CCr\,(mL/min)} = [220\,\mu\mathrm{mol/min\ per\ kg} \times \mathrm{bodyweight\,(kg)}]/\mathrm{plasma\ creatinine\,(\mu mol/L)}$$

The INR was determined using the fully automated blood coagulation analyzer Sysmex CA-1500 (Sysmex Corporation). Alanine and aspartate aminotransferases (ALT and AST, respectively) were measured using a reduced nicotinamide adenine dinucleotide method, according to the International Federation of Clinical Chemistry guidelines,[19,20] without pyridoxal-5′-phosphate, using an automatic biochemistry analyzer (ARCHITECT c4000 Clinical Chemistry Analyzer; Abbott Diagnostics, Abbott Park, IL, USA).

Platelet-poor plasma was obtained from citrated blood by centrifuging blood samples at 3800 *g* for 20 min at 4°C. Plasma was divided into 2-mL aliquots and stored at −80°C until analysis. The diluted thrombin time (dTT) was determined using the clotting-based Hemoclot thrombin inhibitor assay (Hyphen BioMed, Neuville sur-Oise, France), as described previously,[21] at the Department of CardioMetabolic Disease Research of Boehringer Ingelheim. Clotting was measured using a CL4 coagulometer (Behnk Elektronik, Norderstedt, Germany), according to the manufacturer's instructions. Briefly, frozen (−80°C) plasma samples (~100 μL) were thawed for 5 min at 37°C, then stored on ice. All samples were prediluted 1:8 in 0.15 mol/L physiological saline (20 μL poor-platelet plasma + 140 μL NaCl). A 0.05-mL aliquot of the diluted plasma sample was pipetted into a test tube prewarmed at 37°C. Reagent-1 (0.1 mL normal pooled citrated plasma; Hyphen BioMed, Andresy, France) was added and the samples were incubated for 1 min at 37°C. Reagent-2 (0.1 mL human calcium thrombin at 37°C) was then added to initiate coagulation. The time that elapsed from the addition of Reagent-2 to the onset of plasma clotting, representing the dTT, was determined.

Separate calibration curves were generated using platelet-poor plasma pools from the C and D rat groups. Diluted thrombin time values corresponding to dabigatran concentrations of 0, 25, 50, 125, 250, 500, 1000, and 2000 ng/mL were determined. The curves constructed using C and D platelet-poor plasma were then used to determine the concentration of dabigatran in CD and DD plasma samples, respectively, based on dTT measurements.

Statistical analysis

Data are expressed as the mean ± SEM, and were compared using the Mann–Whitney *U*-test. Non-parametric analysis of variance (ANOVA Kruskal–Wallis test) was used for multiple comparisons. Correlations were investigated using Spearman's correlation method. Stepwise multiple regression analysis was used to identify independent predictors of dTT. All tests were two-sided and $P < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Dabigatran plasma concentrations and coagulation tests

Diluted thrombin time measurement using platelet-poor plasma pools from C and D rats and pre-established dabigatran concentrations generated diverging curves for the two groups (Fig. 1). Differences were already apparent at the lowest dabigatran concentration (25 ng/mL), when the dTT of the diabetic plasma was more than 10% higher than that of the control plasma (35.8 vs 32.5 s respectively), and became progressively greater at higher dabigatran concentrations. At the highest dabigatran concentration (2000 ng/mL), the dTT of the diabetic plasma was more than 50% higher than that of the control plasma (293.3 vs 195.0 s respectively).

At the end of the study, assessment of body weight and food consumption revealed a DE intake of 50.74 ± 1.98 mg/kg in CD rats, compared with only 33.79 ± 1.68 mg/kg in DD rats ($P < 0.001$). Even so, dTT was significantly prolonged in both CD and DD rats compared with their respective untreated controls (40.51 ± 1.90 vs 28.02 ± 0.65 s in CD vs C rats, respectively [$P < 0.001$]; 36.54 ± 1.09 vs 29.82 ± 0.17 s in DD vs D rats, respectively [$P < 0.01$]). Moreover, there was no significant difference in dTT between CD and DD rats ($P = 0.10$). Diluted thrombin time-derived dabigatran concentrations were significantly lower in DD than CD rats (34.44 ± 5.90 vs 88.03 ± 13.99 ng/mL, respectively; $P < 0.01$). To enable comparisons

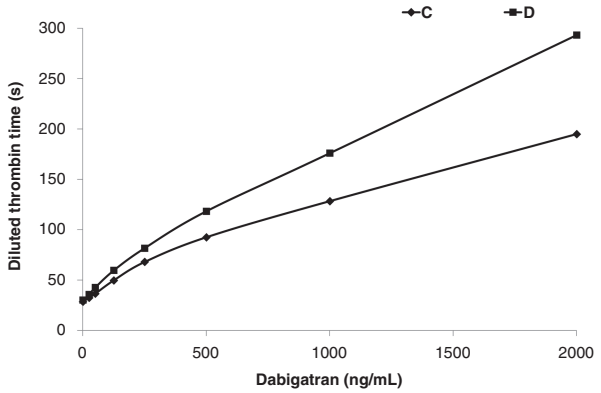

Figure 1 Calibration curves used for determining plasma dabigatran concentrations in dabigatran etexilate-treated control and diabetic rats using the clotting-based Hemoclot (Hyphen BioMed, Neuville sur-Oise, France) thrombin inhibitor assay. Plasma to construct the curves was obtained by pooling plasma from six control (C) and five diabetic (D) rats. The diluted thrombin time was determined after adding purified dabigatran in concentrations of 0, 25, 50, 125, 250, 500, 1000, and 2000 ng/mL.

between CD and DD rats, the dTT values obtained in DD rats were corrected for a DE intake of 50 mg/kg bodyweight per 24 h. Corrections were made considering linear changes in dTT between two values, given the a priori similar oral bioavailability of DE among the diabetic rats and the linear relationship between dabigatran concentration and dTT reported in humans[22] and confirmed in the in vitro studies described herein (see also Fig. 1), which also demonstrated a linear relationship between dabigatran concentrations and dTT ($R^2 = 0.99$, $P < 0.0001$) for the dTT values obtained in DD rats. Corrected dTT-derived dabigatran plasma concentrations were then determined based on the calibration curves obtained using diabetic plasma samples. When corrected for a similar DE intake of 50 mg/kg bodyweight per 24 h, dTT (54.65 ± 2.07 and 40.51 ± 1.90 s; $P < 0.001$) and dTT-derived dabigatran plasma concentrations (132 ± 11 and 88.03 ± 13.99 ng/mL; $P = 0.03$) were significantly higher in DD than CD rats.

Coagulation tests also revealed significantly higher INR values in both CD and DD rats compared with their respective controls (0.84 ± 0.01 vs 0.72 ± 0.01 in CD vs C rats, respectively [$P < 0.001$]; 0.78 ± 0.01 vs 0.71 ± 0.01 in DD vs D rats, respectively [$P < 0.01$]), whereas there was no significant difference in either dTT or INR values between C and D rats ($P > 0.05$ for both).

No hemorrhagic event was recorded in any of the rats studied.

Biochemical parameters

Glycemic and lipid profiles and CCr in the four study groups are presented in Table 1.

As expected, plasma glucose was significantly higher in D and DD rats compared with C and CD rats, respectively ($P < 0.001$ for both), whereas there was no significant difference between the C and CD rats ($P = 0.10$), or between the D and DD rats ($P = 0.43$).

Similarly, both D and DD rats had significantly higher TC and triglyceride levels compared with C and CD rats, respectively ($P \leq 0.02$ for all), whereas there were no significant differences for any of these parameters between C and CD rats ($P \geq 0.94$ for all), or between D and DD rats ($P \geq 0.42$ for all). Neither

Table 1 Biochemical and hematological parameters in the four study groups

	C ($n = 6$)	CD ($n = 8$)	D ($n = 5$)	DD ($n = 8$)	*P*-value
Biochemical parameters					
Glycemia (mg/dL)	124 ± 6	113 ± 3	513 ± 20	595 ± 76	<0.001
TC (mg/dL)	88.2 ± 6.9	87.6 ± 3.4	139 ± 12	123 ± 13	<0.01
LDL-C (mg/dL)	13.8 ± 1.6	14.8 ± 0.6	17.8 ± 1.1	19.4 ± 2.9	0.12
HDL-C (mg/dL)	69.4 ± 6.1	67.5 ± 4.5	72.2 ± 4.5	73.1 ± 4.4	0.79
Triglycerides (mg/dL)	64.6 ± 8.6	69.5 ± 9.0	434 ± 46	421 ± 111	<0.01
Creatinine (μmol/L)	43.6 ± 3.6	40.3 ± 1.5	47.0 ± 1.7	44.3 ± 3.2	0.19
CCr (mL/min)	2.6 ± 0.2	2.9 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	<0.001
AST (IU/L)	57.5 ± 9.6	52.6 ± 4.3	75.5 ± 8.4	62.8 ± 5.3	0.24
ALT (IU/L)	65.5 ± 22.7	54.7 ± 4.9	164 ± 15	173 ± 21	<0.01
Hematological parameters					
WBC ($\times 10^3/mm^3$)	2.9 ± 0.4	3.2 ± 0.5	5.2 ± 0.8	3.7 ± 0.7	0.16
RBC ($\times 10^6/mm^3$)	7.9 ± 0.1	8.0 ± 0.2	8.5 ± 0.2	7.8 ± 0.4	0.15
Platelets ($\times 10^3/mm^3$)	853 ± 29	949 ± 21	761 ± 26	774 ± 64	<0.01

Data are the mean ± SEM. *P*-values refer to between-group comparisons using non-parametric ANOVA (Kruskal–Wallis test).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control group; CD, control group treated with dabigatran etexilate; D, diabetic group; DD, diabetic group treated with dabigatran etexilate; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RBC, red blood cells; WBC, white blood cells.

HDL-C nor LDL-C levels were significantly different between D or DD rats and their non-diabetic controls ($P \geq 0.08$ for all). As expected, HDL-C was significantly higher than LDL-C in all groups ($P < 0.01$ for all).

Although there were no significant differences in plasma creatinine between D and DD rats and their non-diabetic controls ($P \geq 0.28$ for both), CCr was significantly lower in both D and DD rats compared with the C and CD rats, respectively ($P \leq 0.001$ for both).

Aminotransferase assessment revealed comparable AST levels in all groups. However, ALT levels were significantly higher in the D and DD rats compared with the C and CD rats, respectively ($P \leq 0.01$ for both), whereas there were no significant differences in ALT levels between C and CD rats ($P = 0.57$), or between D and DD rats ($P = 0.93$).

Hematological parameters

The white blood cell count was significantly higher in D than C rats ($P = 0.03$; Table 1); however, no such difference was observed between DD and CD rats ($P = 0.56$). There were no significant differences in the red blood cell count among the different groups ($P \geq 0.08$ for all). A significantly higher platelet count was observed in CD than C rats ($P = 0.02$), but not in DD compared with D rats ($P = 1.00$).

Predictors of dabigatran anticoagulant effect

No significant correlation was found between dTT and any of the parameters tested among the C or D rats ($P > 0.05$ for all). In CD rats, a significant positive correlation was observed between TC and dTT ($r = 0.73$, $P = 0.04$).

As expected, there was a significant negative correlation between CCr and dTT ($r = -0.91$, $P < 0.01$) in DD rats. In addition, dTT was significantly positively correlated with TC ($r = 0.96$, $P < 0.01$), LDL-C ($r = 0.75$, $P = 0.04$), and plasma glucose ($r = 0.83$, $P = 0.02$) (Fig. 2). However, in multiple regression analysis, CCr ($r = -0.81$, $P = 0.01$), TC ($r = 0.93$, $P < 0.001$), and LDL-C ($r = 0.74$, $P < 0.01$) remained the only independent predictors of dTT. None of the tested hematological parameters was correlated with dTT ($P > 0.05$ for all).

Discussion

The present study indicates a significantly more intense DE-induced anticoagulation in rats with unbalanced DM that does not seem to be solely related to altered kidney function, and demonstrates that plasma cholesterol can significantly affect DE anticoagulation in this setting.

In the vast majority of cases, AF is attributable to cardiovascular risk factors such as hypertension, valvular disease, heart failure, or coronary artery disease.[23,24] More recently, other disorders have been added to this list, including lung disease, sleep apnea, obesity, and DM.[25] Diabetes mellitus is extremely common in AF patients, affecting up to 25% of this population, and cohort studies have identified DM as one of the strongest independent predictors of AF,[26–28] promoting atrial proarrhythmic structural, electrical, and autonomic remodeling, as well as systemic and local atrial inflammation.[29–31] In AF patients, DM is a strong independent predictor for stroke.[32] Furthermore, strokes in diabetic AF patients are often more disabling and are associated with higher mortality than in non-diabetic subjects.[33,34] Therefore, with or without other comorbidities, the simple presence of DM imposes initiation of anticoagulation in AF patients for stroke prophylaxis.

For more than half a century, VKAs have represented the mainstay in stroke prevention in AF patients. However, VKA anticoagulation has proven difficult to control, making VKAs the most common drugs associated with adverse drug reaction-related hospital admissions.[35] In diabetic subjects, maintaining adequate INR is even more difficult than in the general AF population.[11]

With their shorter half-lives, more stable pharmacokinetics and pharmacodynamics, relatively few food and drug interactions, and wider therapeutic windows, NOACs have appeared as a promising alternative to VKAs. In the vast majority of subgroup analyses of NOAC trials, both direct thrombin inhibitors and direct Factor Xa inhibitors exhibited similar benefits in reducing stroke compared with warfarin in AF patients with and without DM.[12,14] However, the benefit of reducing major bleeding with DE and apixaban compared with warfarin, as reported in the general AF population,[1,3] was not seen in diabetic patients, and differences between diabetics and non-diabetics remained significant even after adjustment for known bleeding risk factors.[12,14] An explanation for this finding has not been proposed so far.

The present study demonstrates for the first time that the anticoagulant response to the direct thrombin inhibitor DE exhibits a significantly different pattern in diabetic than non-diabetic rats. Directly related to the lower DE intake in DD compared with CD rats, DD rats had lower dabigatran plasma concentrations than the CD rats. However, when corrected for similar DE

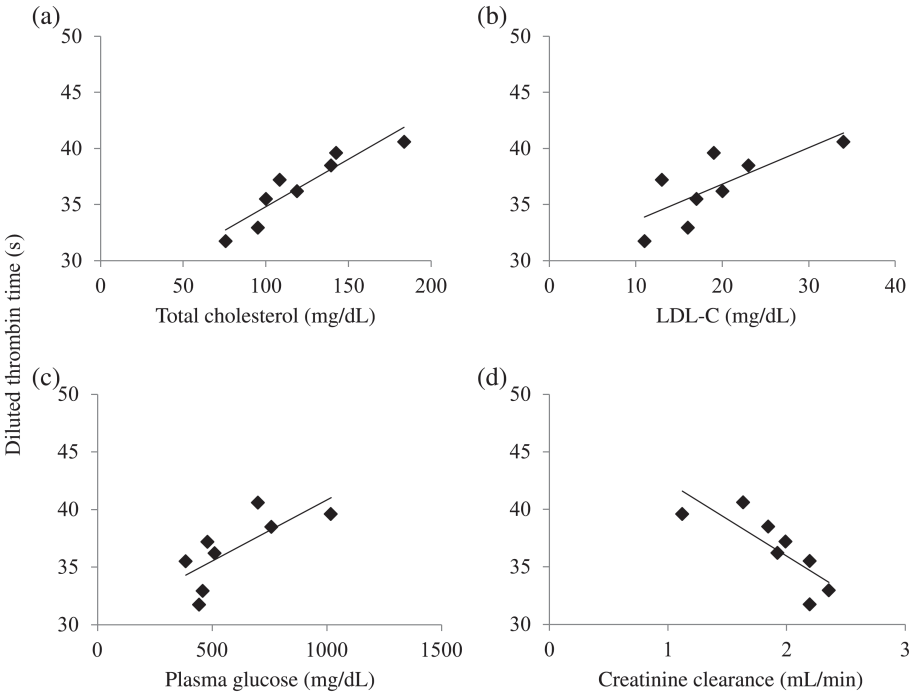

Figure 2 Correlation analysis between the diluted thrombin time and (a) total cholesterol, (b) low density lipoprotein cholesterol (LDL-C), (c) plasma glucose, and (d) creatinine clearance in dabigatran etexilate-treated diabetic rats.

intake, dTT and dTT-derived dabigatran plasma concentrations were significantly higher in diabetic rats. This is not surprising, given that 80% of the dabigatran dose is eliminated via the urine, and diabetic rats had significant kidney dysfunction, as demonstrated by their lower CCr values.

Interestingly, however, despite the significantly lower dabigatran concentrations in DD rats at the administered dose, dTT values were similar to those measured in DE-treated non-diabetic rats. The explanation for this relies on the results of the in vitro study, which demonstrated considerably higher dTT values in diabetic compared with non-diabetic plasma for similar dabigatran concentrations. Together, these data suggest that, beyond the inherent changes in dabigatran plasma concentrations related to kidney dysfunction, diabetic plasma could be more responsive than non-diabetic plasma to similar dabigatran concentrations.

Due to a potential fibrinogen deficit related to STZ-induced liver toxicity, this finding could be seen as a feature of the DM model used in the present study and not of DM per se. Indeed, STZ has been shown to induce liver toxicity,[36] and altered coagulation tests (decreased plasminogen, α_2-plasmin inhibitor, antithrombin III) have been reported in previous studies, although hepatocytes from STZ-treated rats appear to retain their coagulation activity.[37] In agreement with the results of those studies, ALT measurements in the present study confirmed a degree of liver dysfunction in the diabetic rats. Of note, in line with previous studies performed in DE-treated patients,[38] DE administration did not affect liver function in either CD or DD rats, in contrast to what has been observed with the direct thrombin inhibitor ximelagatran.[39] However, the INR, which is also affected by plasma levels of several coagulation factors synthetized by the liver, was not significantly different between diabetic rats and their controls in the present study. In addition, there was no correlation between dTT and the severity of liver dysfunction among DD rats. Moreover, despite similar liver function impairment in D and DD rats, dTT was not affected in the former group, suggesting that the effect of liver dysfunction on the dabigatran concentration–dTT functional relationship in DD rats was negligible, if present.

Although DM has been associated with altered coagulation function, dTT was prolonged only in DE-treated, and not in untreated, diabetic rats compared with their respective controls, indicating that the more intense DE-anticoagulation observed in the diabetic rats is due to the effect of DM-related factors on DE-induced anticoagulation and not to the effect of DM on coagulation per se. In addition to CCr, only glycemia, TC, and LDL-C showed significant correlations with dTT in the DD rats. However, in multiple regression analysis, lipid profile (TC and LDL-C), but not plasma glycemia, remained an independent predictor of dTT, suggesting that the DM-related hypercholesterolemia and not DM per se may affect DE anticoagulation in this model. Indeed, similar data were presented in a

study in apolipoprotein E-deficient mice with severe hypercholesterolemia.[40] Although the authors did not discuss this finding, calibration curves obtained in their in vitro studies exhibited major similarities with those seen in our diabetic rats, showing considerably higher dTT values in mice with hypercholesterolemia than in wild-type mice for similar dabigatran concentrations.

Because high plasma lipids have been shown to alter numerous laboratory parameters, one could suspect that hyperlipemic plasma could have affected dTT measurements in diabetic rats. However, despite similarly severe dyslipidemia in DE-treated and untreated diabetic rats, dTT was not affected in the latter group. In addition, there was no correlation between dTT and plasma lipids among the untreated diabetic rats. Together, these data indicate a direct functional relationship between hypercholesterolemia and DE-induced anticoagulation, and exclude a potential effect of increased plasma lipids on dTT itself.

Indeed, because DE absorption is strongly dependent on the p-glycoprotein transporting system, hypercholesterolemia could affect dabigatran concentrations by increasing p-glycoprotein activity.[41] However, in addition to potential pharmacokinetic interactions, our data suggest that hypercholesterolemia could affect DE pharmacodynamics, possibly via hypercholesterolemia-induced allosteric changes in thrombin receptor activity, as has already been demonstrated for numerous other G-protein-coupled receptors,[42] including those for oxytocin, serotonin (5-HT_{1A}), or neurotensin. In fact, in a recent study performed in DE-treated patients, the only parameter that differed significantly between patients with prolonged activated partial thromboplastin time (aPTT) and those with normal aPTT was HDL-C plasma concentrations.[39] In turn, dabigatran appears to also affect lipid metabolism, as indicated by the lower apolipoprotein B levels seen in AF patients after 3 months of DE administration, regardless of other lipid-lowering therapies.[43] Together, these data indicate complex thrombin inhibition–lipid metabolism interactions that deserve to be further investigated.

Clinical implications

The more intense DE-induced anticoagulation observed in the setting of DM could explain the similar benefits of DE in reducing ischemic stroke compared with warfarin in AF patients with and without DM,[12] despite the significantly higher intrinsic thrombotic risk of the former, as well as the lack of benefit of reducing major bleeding in AF diabetic patients.[12] Given the high prevalence of DM in the AF population, as well as the inter- and even intra-individual variability in plasma cholesterol in these patients, dedicated studies will have to address the issue of DE anticoagulation in diabetic patients and study the effect of plasma cholesterol on DE anticoagulation in clinical settings. Furthermore, because different factors appear to affect DE pharmacokinetics and pharmacodynamics, the HAS-BLED score[44] may not be the best tool to describe the hemorrhagic risk associated with the use of this new agent. New models designed to predict the hemorrhagic risk in this setting, potentially including hypercholesterolemia, may be required. Similar studies focused on direct Factor Xa inhibitors would also be of interest.

Potential limitations

In the present study, dabigatran plasma concentrations were only determined indirectly, based on dTT measurements. Direct chromatographic measurements were not performed. Assessment of dabigatran plasma concentrations based on other assays, as well as aPTT measurement, would be of interest. In addition, the present study assessed the anticoagulant effects of DE in rats with unbalanced DM. Further studies will have to confirm the relevance of these findings in clinical settings.

Conclusions

The present study indicates a significantly more intense anticoagulant response to DE administration in rats with unbalanced DM that does not seem to be solely related to altered kidney function, and demonstrates that plasma cholesterol can significantly affect DE anticoagulation in this setting. Specific calibration curves may be required for reliable determination of dabigatran plasma concentrations in specific population subgroups.

Acknowledgements

This work was supported by the University of Medicine and Pharmacy of Tîrgu Mureş (Research Grant no. 16/11.12.2013) and Boehringer Ingelheim GmbH (Biberach, Germany). The authors thank Johanna Schurer (Department of CardioMetabolic Disease Research, Boehringer Ingelheim) for performing the coagulation tests.

Disclosure

PC has received research funding from Boehringer Ingelheim GmbH. DD has received honoraria from

Pfizer Inc. and Bayer HealthCare. The remaining authors report no conflicts of interest.

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Mechanisms of Ageing and Development 166 (2017) 48–54

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Original Article

Age-dependent ventricular arrhythmias risk, structural and molecular remodeling in systemic arterial hypertension

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ARTICLE INFO

Keywords:
Aging
Left ventricular hypertrophy
Molecular remodeling
Systemic arterial hypertension
Ventricular arrhythmias

ABSTRACT

Introduction: The left ventricular hypertrophy (LVH)-ventricular arrhythmias relationship associated with arterial hypertension and aging remains controversial. We aimed to assess the age-dependency of ventricular arrhythmias in spontaneously hypertensive rats (SHRs) and the corresponding ventricular structural and molecular remodeling.

Materials and methods: Ventricular arrhythmias were quantified using 24-h radiotelemetry ECG monitoring in eight SHRs and four Wistar-Kyoto (WKY) rats at 14 (young), 24 (adult), and 48 (aging) weeks of age. Left ventricular histology and mRNA expressions of 89 proarrhythmogenic genes were assessed in six additional groups (n = 4 each) of young, adult, and aging SHRs and WKYs.

Results: Regardless of their age, SHRs presented more premature ventricular contractions (PVCs) than age-matched WKYs ($p < 0.01$). The arrhythmogenicity peak occurred in adult SHRs; ventricular tachycardias only occurred in adult SHRs. Among the SHRs, LV thickness, interstitial fibrosis, and the number of deregulated genes increased with age. *Kcnj11* expression was deregulated in adult, but not in young or aging SHRs.

Discussion: This study confirms the presence of higher ventricular ectopy in SHRs than in age-matched WKYs. LVH appeared to be an adaptive, antiarrhythmic process. Myocardial energetic changes with advancing age, as reflected by *Kcnj11* expression changes, could underlie this age-dependency of ventricular arrhythmias.

1. Introduction

Left ventricular hypertrophy (LVH) in the setting of persistent arterial hypertension develops as an adaptive process aiming to attenuate the deleterious effects of chronic hemodynamic overload. The relationship between LVH and ventricular arrhythmias remains controversial and poorly understood. Several studies have reported increased incidence of ventricular arrhythmias in the presence of LVH, and a linear relationship between arrhythmia burden and LV mass has been observed in this setting (Ghali et al., 1991). On the contrary, James et al. reported more frequent premature ventricular contractions (PVCs) in untreated hypertensive patients with normal LV mass than in the presence of LVH (James and Jones, 1989). Similarly, other data also suggest that hypertensive patients with LVH might actually experience fewer arrhythmias (James and Jones, 1990).

We aimed to assess the age-dependency of ventricular arrhythmia occurrence in spontaneously hypertensive rats (SHRs). We also evaluated the associated LV structural remodeling, as well as the corresponding LV molecular remodeling by analyzing mRNA expression of 89 genes encoding for proteins involved in Ca^{2+}-handling, ion channels, extracellular matrix, and cell–cell communication, and recognized for their involvement in cardiac arrhythmogenicity (Gaborit et al.,

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http://dx.doi.org/10.1016/j.mad.2017.07.002
Received 25 January 2017; Received in revised form 1 June 2017; Accepted 8 July 2017
Available online 10 July 2017
0047-6374/

2009; Scridon et al., 2014).

2. Materials and methods

2.1. Animals

Thirty-six male SHRs and Wistar-Kyoto (WKY) rats were purchased from Elevage Janvier (Le Genest Saint Isle, France). All animals were housed in a climate-controlled room (21–22 °C), with a 12-h light/dark cycle, in an accredited animal facility. Rats undergoing ECG recordings (eight SHRs and four WKY rats) were housed individually in polycarbonate cages. All other animals − three groups of SHRs of different ages (14 weeks, i.e. young; 24 weeks, i.e. adult; and 48 weeks, i.e. aging; n = 4 each) and three groups of age-matched WKY rats (n = 4 each) − were housed in groups of two to three rats per cage under the same controlled conditions. All rats were fed standard rat pellets and had free access to tap water. All experiments were performed in compliance with the French Ministry of Agriculture guidelines and were approved by the local Animal Ethics Committee.

2.2. ECG recording and analysis

Radiotelemetry ECG transmitters (TA11 CA-F40; Data Sciences International, St. Paul, MN) were implanted in eight SHRs and four WKY rats as previously described (Scridon et al., 2012). For baseline quantification of ventricular arrhythmic events, 24-h continuous ECG monitoring was performed on unrestrained, conscious rats. ECG recordings were performed in SHRs and WKY rats at 14 (young), 24 (adult), and 48 (aging) weeks of age, respectively. ECG signal capture was accomplished with receivers (RPC-1; Data Sciences International) placed under each experimental cage. ECG data were recorded and analyzed as described previously (Scridon et al., 2012). All ECG tracings were visually assessed and artefactual periods were discarded prior to analysis.

Premature ventricular contractions were defined as enlarged QRS complexes with morphology that was significantly different from sinus rhythm beats. Ventricular couplets were defined as a pair of consecutive ventricular premature activations. The regular succession of a normal sinus beat and a ventricular premature activation was defined as ventricular bigeminism. The succession of three PVCs was defined as a salvo of PVCs. Ventricular tachycardia (VT) was defined as rapid ventricular rhythm (enlarged and distorted QRS complexes) of at least four beats. Ventricular fibrillation (VF) was defined as a wandering, irregular baseline with no clearly identifiable QRS complexes or P waves. The total numbers of VT and VF episodes, PVCs, ventricular couplets and bigeminisms, and salvos of PVCs were quantified on the 24-h ECG recordings.

2.3. Heart sampling

Young, adult, and aging SHRs (n = 4 each) and age-matched WKY rats (n = 4 each) were weighted and euthanized using an intraperitoneal injection of a terminal dose of sodium pentobarbital (> 100 mg/kg). The hearts were excised and weighted. Left ventricular samples originating from the LV lateral wall were isolated, placed in sterile dry tubes, snap frozen in liquid nitrogen, and stored at −80 °C until processing. Additional samples from the LV lateral wall of each rat were immediately fixed in 10% buffered formalin and were processed for paraffin histology.

2.4. Gene expression analysis

Total RNA was isolated from frozen biopsies of LV lateral wall using TRIzol Reagent (Life Technologies; Villebon sur Yvette, France) according to the manufacturer's instructions. RNA concentration was measured using Nanodrop ND1000 (Thermo Scientific; Illkirch, France) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies; Massy, France). The expressions of 89 genes known for their involvement in cardiac arrhythmogenicity (Gaborit et al., 2009; Scridon et al., 2014) were investigated using TaqMan Low-Density Array (TLDA) analysis. The TLDA card was configured into four identical 89 genes sets. For each set of genes, six endogenous control genes: *Ppia*, *beta*-actin, *Gapdh*, *Hprt1*, *Tbp*, and 18 s RNA, were used. Briefly, 1 μg of total RNA was reverse-transcribed to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) according to the manufacturer's instructions. The cDNA (2 μl) was mixed with 48 μl of H_2O and 50 μl of 2 × TaqMan Universal PCR Mix (Applied Biosystems). Each sample (100 μl) was loaded into a port of the micro-fluid card and run on an ABI 7900HT System (Applied Biosystems) for 2 min at 50 °C, then 10 min at 94.5 °C, followed by 40 cycles of 30 s at 97 °C and 1 min at 59.7 °C.

The TLDA data were analyzed using SDS 2.3 software (Applied Biosystems). Threshold cycle data for all target and control genes were transformed into molecule numbers (attomoles) considering a PCR efficiency of 100%. Gene expression levels were normalized using TATA box binding protein (TBP). A complete list of the 89 studied genes is provided in the Supplementary Table 1.

2.5. Histological examination

Left ventricular samples were dehydrated, embedded in paraffin and cut with a LeicaRM2245 microtome (Leica Microsystems, Germany). Four-μm sections were then stained with Masson's trichrome. After digital acquisition of slides (MIRAX MIDI slide scanner, Carl Zeiss, Oberkochen, Germany), the thickness of the LV myocardial layer and interstitial ventricular fibrosis were quantified using the Panoramic Viewer 3 1.15.3 software (3DHISTECH Ltd., Budapest, Hungary).

2.6. Statistical analyses

Data are expressed as means ± SEM or median and range, as appropriate. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test. Within-group differences were tested for significance with the Wilcoxon signed-rank test. Nonparametric repeated-measures ANOVA (Friedman test) was used for repeatability data. Correlations were ascertained with Spearman's rank correlation method. A p-value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism software (GraphPad Software; San Diego, CA).

3. Results

3.1. Ventricular arrhythmic activity at baseline

Fig. 1 depicts typical ECG tracings recorded in adult SHRs. At all ages, SHRs presented significantly more frequent PVCs (Fig. 2A) compared to age-matched WKY rats ($p < 0.01$). Similar differences were observed in the numbers of ventricular couplets (Fig. 2B), bigeminisms (Fig. 2C), and salvos of PVCs (Fig. 2D). None of the WKY rats presented ventricular couplets or salvos of PVCs, and only one young WKY rat presented a single episode of ventricular bigeminism.

Among the SHRs, the highest number of PVCs was recorded in adults (median 99, range 58–309 PVCs/24-h), with significantly smaller numbers in young (median 57, range 23–88 PVCs/24-h, $p < 0.01$) and aging SHRs (median 43, range 23–69 PVCs/24-h, $p < 0.01$); no significant difference was observed between young and aging SHRs ($p = 0.46$). Similarly, the numbers of ventricular couplets (both $p < 0.02$), bigeminisms (both $p < 0.05$), and salvos of PVCs (both $p < 0.03$) were higher in adult SHRs compared to both aging and young SHRs. In multiple comparisons, the differences between SHRs of different ages remained significant ($p = 0.01$ for PVCs;

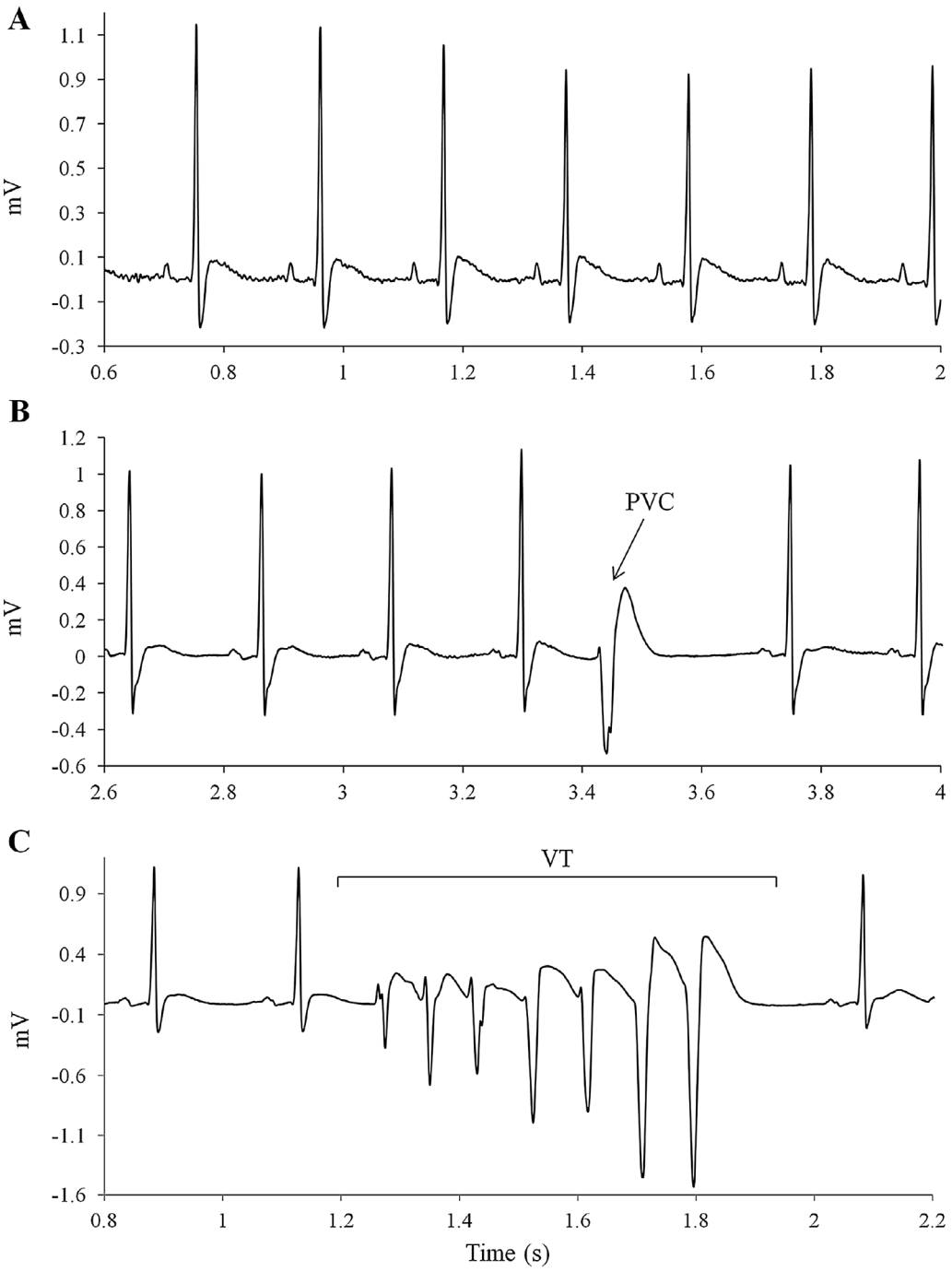

Fig. 1. ECG tracings in adult SHRs showing normal sinus rhythm (A), premature ventricular contraction (PVC) (B), and unsustained ventricular tachycardia (VT) (C).

p = 0.02 for ventricular couplets; p = 0.04 for ventricular bigeminisms; and p = 0.02 for salvos of PVCs).

During the 24-h recording periods, one adult SHR presented three episodes of unsustained VT, while two adult SHRs presented each a single episode of unsustained VT. No episode of VT was recorded in young or aging SHRs, or in WKY rats, regardless of their age. No episode of VF was recorded in any of the SHR or WKY rats.

3.2. Left ventricular hypertrophy indexes and structural remodeling

Heart weight-to-body weight ratios were calculated to assess the presence and severity of LVH in SHRs. In the young rats, heart weight-to-body weight ratios were not significantly different between SHRs and WKY rats (p = 0.11). Heart weight-to-body weight ratios of adult SHRs were 30 ± 2% higher compared to those of age-matched WKY rats (p = 0.02). Aging SHRs showed heart weight-to-body weight ratios that were 56 ± 3% higher than those measured in age-matched WKY rats (p = 0.01).

The thickness of the LV myocardial layer (Fig. 3A and C) was also similar in young SHRs and WKY rats (p = 0.11), whereas both adult and aging SHRs presented higher LV thickness compared to age-matched WKY rats (3,889.9 ± 271.7 μm in adult SHRs *versus* 2,763.7 ± 234.3 μm in adult WKY rats, p = 0.04; 4,524.4 ± 158.6 μm in aging SHRs *versus* 2,643.3 ± 195.3 μm in aging WKY rats, p = 0.02). Whereas among the SHRs LV thickness was significantly positively correlated with age (Spearman r = 0.89, p < 0.001), this was not the case in the WKY rats (p = 0.82).

The extent of interstitial fibrosis (Fig. 3B and D) was not significantly different between young SHRs and age-matched WKY rats (p = 0.69), nor between adult SHRs and WKY rats (p = 0.11).

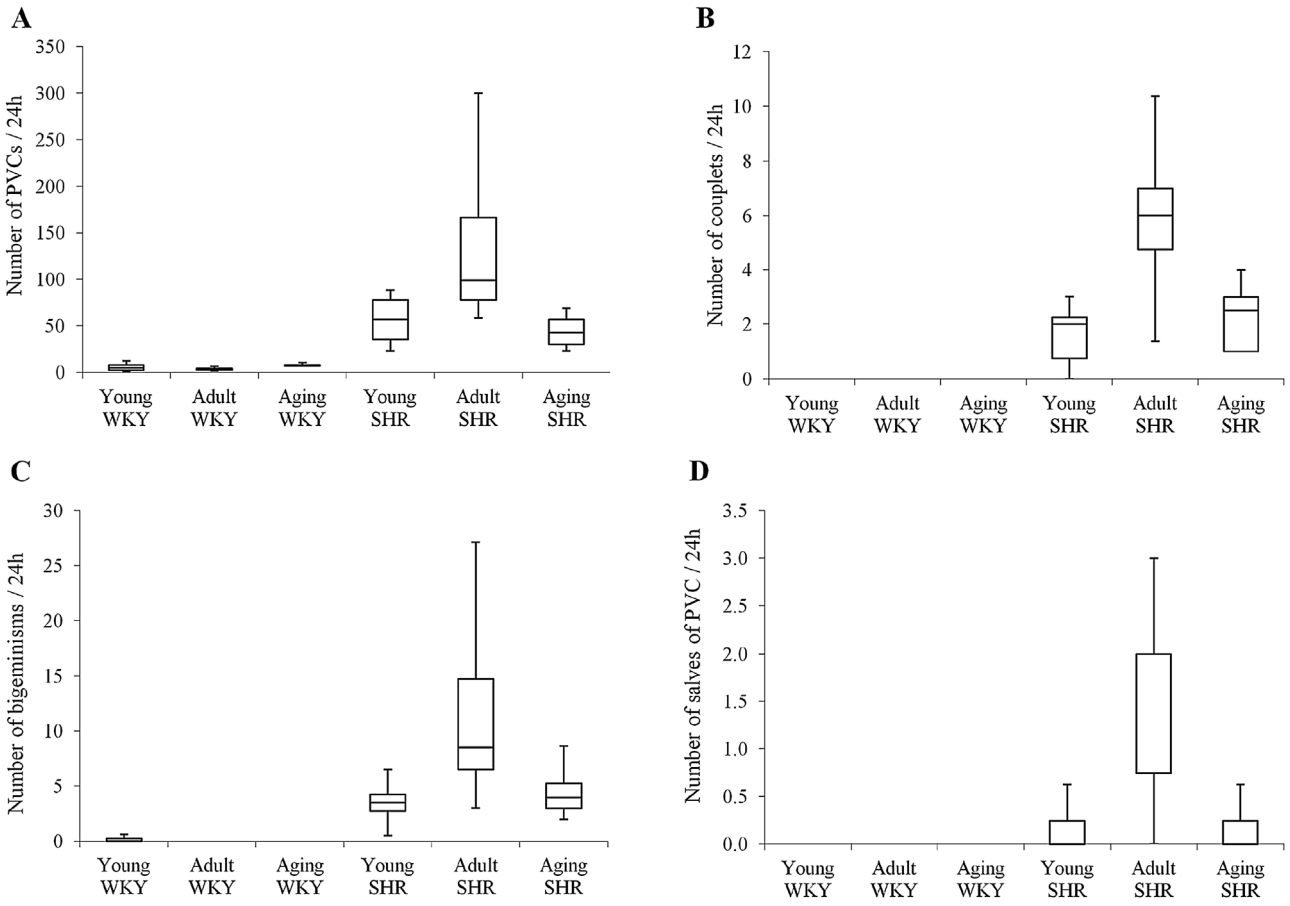

Fig. 2. Number of ventricular arrhythmic events − premature ventricular contractions (PVCs) (A), ventricular couplets (B), ventricular bigeminisms (C), and salvos of PVCs (D) − during 24-h ECG recordings in young, adult, and aging SHRs (n = 8 each) and age-matched WKY rats (n = 4 each). Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile, and the highest value within 1.5 IQR of the third quartile.

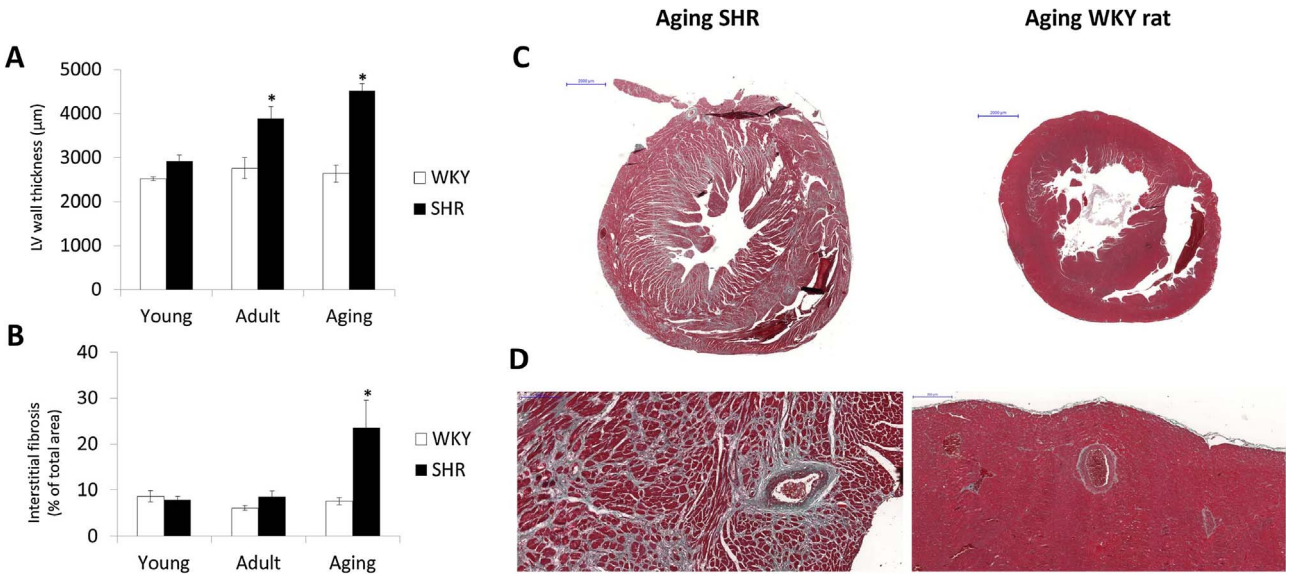

Fig. 3. (A) Left ventricular (LV) wall thickness and (B) interstitial fibrosis in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) controls. Representative Masson's trichrome-stained left ventricular samples for an aging hypertensive rat and an age-matched WKY rat showing (C) increased left ventricular wall thickness and (D) increased interstitial and perivascular fibrosis (stained in blue) in the aging SHR. *- $p < 0.05$ compared to age-matched WKY rats.

However, interstitial fibrosis was significantly more important in aging SHRs compared to age-matched WKY rats ($p = 0.04$). The extent of interstitial fibrosis was also significantly positively correlated with age among the SHRs ($r = 0.71$, $p = 0.02$), but not among the WKY rats ($p = 0.60$).

3.3. Left ventricular molecular profile in hypertensive versus normotensive rats

Significantly altered transcripts were selected by applying a fold-change cutoff of 1.5 and a p-value cutoff of 0.05. In young rats, nine of

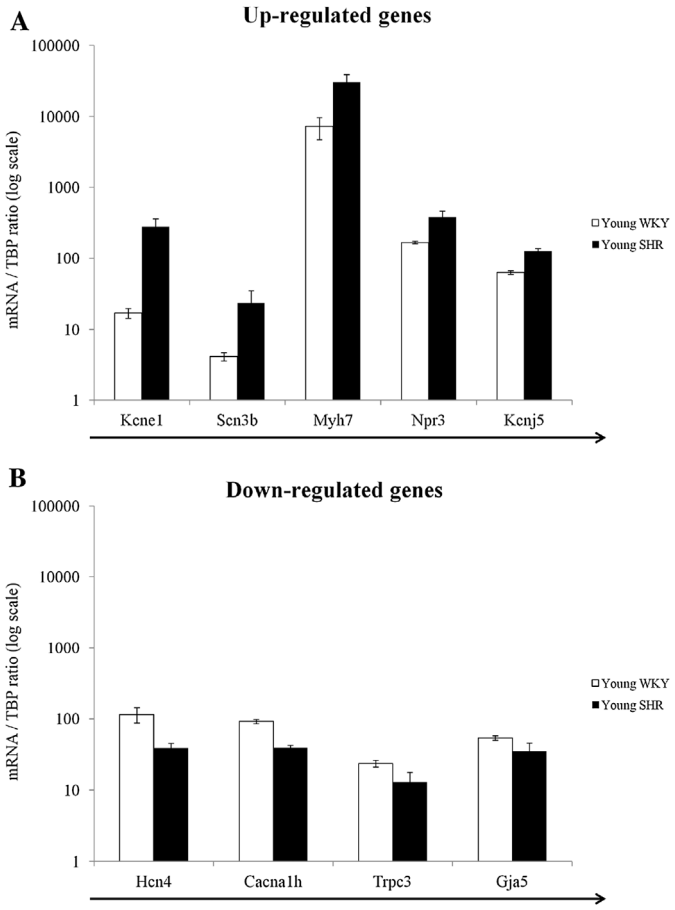

Fig. 4. mRNA/TBP ratios for genes differentially expressed between young SHRs and age-matched WKY rats, including upregulated (A) and downregulated (B) genes. Data are expressed as means ± SEM. $p < 0.05$ for young SHRs *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney *U* test. Arrows are directed from the most deregulated to the least deregulated gene.

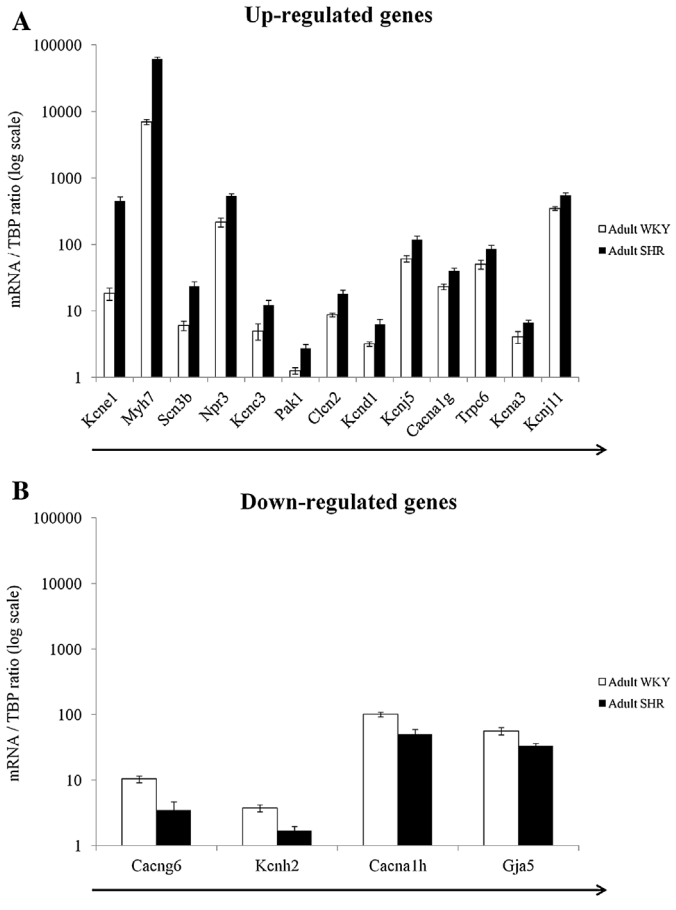

Fig. 5. mRNA/TBP ratios for genes differentially expressed between adult SHRs and age-matched WKY rats, including upregulated (A) and downregulated (B) genes. Data are expressed as means ± SEM. $p < 0.05$ for adult SHRs *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney *U* test. Arrows are directed from the most deregulated to the least deregulated gene.

the 89 studied genes were differentially expressed between SHRs and WKY rats, five of which were upregulated and four downregulated (Fig. 4A and B). In this group, the total fold-change value of overexpressed genes was higher than that of underexpressed genes (total fold-change: 30.6 for overexpressed genes *versus* 8.7 for underexpressed genes).

In adult rats, 17 of the 89 studied genes were differentially expressed between SHRs and WKY rats, 13 of which were upregulated and four downregulated (Fig. 5A and B). Again, the total fold-change value of overexpressed genes was higher than that of underexpressed genes (total fold-change: 57.3 for overexpressed genes *versus* 8.9 for underexpressed genes). Seven of the nine genes that were deregulated in young SHRs presented the same pattern in adult SHRs: *Kcne1*, *Scn3b*, *Myh7*, *Npr3*, *Kcnj5*, *Cacna1 h*, and *Gja5*. *Kcnj11* expression was deregulated in adult, but not in young or aging SHRs.

In aging rats, 22 of the 89 studied genes were differentially expressed between SHRs and WKY rats, 16 of which were upregulated and six downregulated (Fig. 6A and B). Twelve of the 17 genes that were deregulated in adult SHRs presented the same pattern in aging SHRs: *Myh7*, *Kcne1*, *Kcnd1*, *Kcnc3*, *Kcnj5*, *Scn3b*, *Cacna1g*, *Cacng6*, *Cacna1h*, *Clcn2*, *Pak1*, and *Npr3*.

Six genes appeared deregulated in all SHR groups compared to age-matched WKY rats: *Kcne1*, *Scn3b*, *Myh7*, *Npr3*, *Kcnj5*, and *Cacna1h*. All but *Cacna1h* were significantly upregulated in all SHRs compared to age-matched WKY rats.

In SHRs, *Kcne1* ($r = 0.56$, $p = 0.04$), *Scn3b* ($r = 0.62$, $p = 0.03$), *Npr3* ($r = 0.92$, $p < 0.001$), and *Cacna1h* ($r = 0.68$, $p = 0.02$) expressions were significantly positively correlated with age. In WKY rats, *Myh7* ($r = 0.71$, $p = 0.01$) and *Npr3* ($r = 0.68$, $p = 0.02$) expressions were significantly positively correlated with age.

4. Discussion

The present study demonstrates that, regardless of their age, hypertensive rats present more frequent ventricular arrhythmic events compared to age-matched normotensive controls. However, despite the more severe LV hypertrophy and more important interstitial fibrosis in the aging SHRs, the arrhythmogenicity peak was noted in the adult SHRs. In line with the age-dependent LV structural remodeling, transcriptomic analysis of the 89 target genes demonstrated increasing number of deregulated genes in SHRs with advancing age. Six genes were significantly deregulated in SHRs of all ages, and the expressions of four of these genes were significantly correlated with age. *Kcnj11* expression was only deregulated in adult, but not in young or aging SHRs.

4.1. Ventricular arrhythmogenicity in early-stage versus evolved left ventricular hypertrophy

In line with previous studies (Rodenbaugh et al., 2003), our results confirm the association between higher ventricular ectopy and persistent arterial hypertension. However, the number of both isolated (PVCs) and more complex (ventricular couplets, bigeminisms, and salvos of PVCs) ventricular arrhythmic events was still quite small, with a median of less than 100 PVCs/24-h in all SHRs, regardless of their age

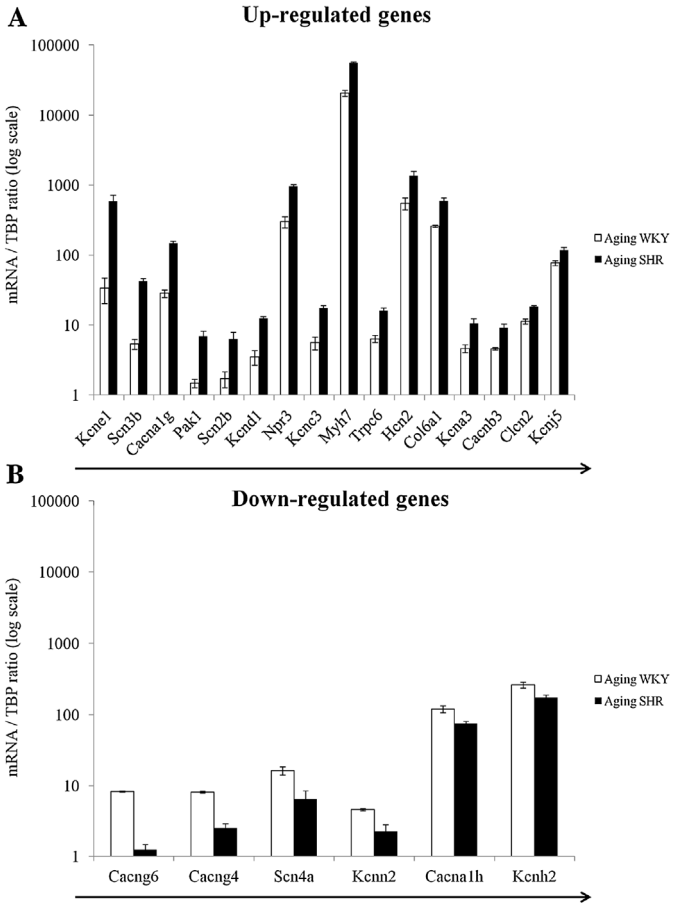

Fig. 6. mRNA/TBP ratios for genes differentially expressed between aging SHRs and age-matched WKY rats, including upregulated (A) and downregulated (B) genes. Data are expressed as means ± SEM. $p < 0.05$ for aging SHRs *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney *U* test. Arrows are directed from the most deregulated to the least deregulated gene.

and of the severity of LV structural remodeling.

As expected, heart weight-to-body weight ratios and LV wall thickness measurement showed that LVH was not yet present in young SHRs, mild early-stage LVH was present in adult SHRs, and much more severe LVH was observed in aging SHRs. A similar progression was observed in LV interstitial fibrosis among the SHRs. However, despite the more severe LV hypertrophy and more important interstitial fibrosis in the aging SHRs, the arrhythmogenicity peak was noted in the adult and not the aging SHRs. Episodes of unsustained VT were also noted exclusively in adult SHRs, while no such arrhythmic events were recorded in young or aging SHRs. These findings suggest that the development of myocardial hypertrophy may have antiarrhythmic effects and that factors other than the severity of LV structural remodeling may play important roles in ventricular arrhythmogenicity in this setting. Indeed, comparable results have already been reported in clinical studies that did not find a correlation between LVH severity and complex ventricular arrhythmia burden (Gatzoulis et al., 2000). In a rat model of LVH, James et al. tested the effects of sudden blood pressure changes on normal and hypertrophied isolated hearts; the authors induced ventricular arrhythmias in the normal, but not in the hypertrophied hearts (James and Jones, 1990). Repeating the experiments in cation-depleted environment showed that hypertrophied hearts were particularly sensitive to sudden blood pressure changes and presented significantly more frequent and more severe ventricular arrhythmias than the normal hearts. The authors concluded that the occurrence of ventricular arrhythmias in patients with LVH might be explained not by ventricular hypertrophy itself, but more likely by the combination of hypertrophy and other conditions that are rather common in hypertensive patients, such as cation depletion induced by diuretic therapy (James and Jones, 1990). Evans et al. demonstrated that low magnesium combined with low potassium perfusion increased the complexity of ventricular arrhythmias in hypertrophied hearts (Evans et al., 1996). Similarly, Mayet et al. demonstrated significantly more frequent ventricular ectopy in hypertensive patients who had received long-term antihypertensive therapy compared to untreated hypertensive patients (Mayet et al., 1995). Indeed, although antihypertensive drugs are highly efficient in reducing the risk of fatal and non-fatal myocardial infarction, it has been suggested that they do not reduce the incidence of sudden cardiac death (Taverny et al., 2016). Our results emphasize the question of timing as a critical point for antihypertensive drugs efficiency.

4.2. Age-dependent arterial hypertension-related proarrhythmic left ventricular molecular remodeling

In SHRs, the number of deregulated genes increased with advancing age, from nine genes deregulated in the young SHRs, to 17 in the adult SHRs, and finally to 22 in the aging SHRs. *Myh7* gene expression was significantly upregulated in all SHR groups compared to normotensive controls. This was not surprising, since *Myh7* encodes for the cardiac *beta*-myosin heavy chain, a well-established marker of cardiac hypertrophy (Pandya et al., 2009).

The six genes that were deregulated in all SHRs, regardless of their age, exhibited the same directional change (either upregulation or downregulation) in all SHR groups. The expression levels of four of these six genes were significantly correlated with age, suggesting a potential implication of these genes in the adaptive mechanisms related to arterial hypertension and LVH development with advancing age. Of these four genes, *Npr3* expression was also significantly correlated with age in WKY rats; thus, both aging and hypertension seem to influence this gene's level of expression. In line with the more important interstitial fibrosis observed in the aging SHRs, these rats also showed significantly higher expression levels of *Col6a1*, encoding for type VI collagen, compared to age-matched WKY rats.

Since *Kcnj11* encodes for an ATP-sensitive potassium channels (K-ATP) subunit, expression changes in this gene can be seen as a marker of LV energetic status, reflecting the dynamics of myocardial energetic balance with advancing age (Brown and O'Rourke, 2010). These age-dependent changes in myocardial energetics in hypertensive rats may provide an explanation for the age-dependency of ventricular arrhythmias seen in this model. Indeed, alterations in substrate metabolism and energy utilization, recognized for their involvement in cardiac arrhythmias (Brown and O'Rourke, 2010), have been reported in both human patients with LVH (Smith et al., 2006) and in experimental LVH models (Ye et al., 2001). Altered LV energetic status due to profound supression of fatty acids oxidation pathway and reduced ATP production, already demonstrated in adult SHR hearts (Christe and Rodgers, 1994), and reflected by *Kncj11* upregulation in our adult SHRs, may play a key role in the increased ventricular arrhythmogenicity observed in this group. Indeed, K-ATP channels, inhibited at physiological intracellular ATP concentrations and stimulated by ATP deficit, are among the densest (Terzic et al., 1995) and the most active ion channels in the myocardium, opening of even a small proportion of these channels leading to significant action potential shortening (Faivre and Findlay, 1990), increased action potential heterogeneity, and cardiac arrhythmias (Billman, 2008). Meanwhile, with advancing age, a significant elevation in glucose oxidation appears to occur, causing a 4–5-fold increase in the ratio of glucose/fatty acids oxidation rates in the SHR hearts (Christe and Rodgers, 1994).

4.3. Potential limitations

Gene expression changes were only assessed by mRNA

quantification, with no investigation of changes at the protein or ion current levels. Although altered gene expression would probably result in abnormal protein variations, other posttranscriptional or post-translational regulatory mechanisms are known to influence protein level and function. The small sample size of the groups may have lowered the study's statistical power and may have led to exclusion of certain potentially relevant genes. However, measures have been taken to avoid false positive results by applying a fold-change cutoff of 1.5 in gene expression and a p-value cutoff of 0.05. These cutoff values, which have been validated in previous studies (Peart et al., 2005; Raouf et al., 2008), allowed us to identify the genes that vary the most widely between the different groups, are likely to have major biological impact, and are therefore valuable for understanding cardiac remodeling related to aging and arterial hypertension. The validity of our results is also supported by the fact that a number of genes known to be associated with LVH were also differentially expressed in our SHRs. Consistent with previous studies (Pandya et al., 2009), *Myh7* expression was significantly upregulated in our hypertensive rats, regardless of their age, compared to normotensive controls. Also in accordance with previous studies that have demonstrated increased transient outward potassium (I_{to}) current densities in hypertrophied hearts (Ten Eick et al., 1993), SHRs displayed significantly higher expressions of the *Kcnc3*, *Kcnd1*, and *Kcnd2* genes, encoding for the I_{to} current.

5. Conclusions

This study confirms the presence of higher ventricular ectopy in SHRs of all ages, compared to age-matched WKY rats. However, in the present model, evolved and stable LVH appeared to have antiarrhythmic effects. Myocardial energetic changes with advancing age, as reflected by the *Kcnj11* expression changes, could explain this age-dependent ventricular arrhythmia behavior. Early up-stream therapy of hypertension with drugs that optimize myocardial energy efficiency should be encouraged.

Funding

This work was partially supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS – UEFISCDI, project number PN-II-RU-TE-2014-4-1544.

Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mad.2017.07.002.

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Europace Advance Access published June 7, 2014

Europace
doi:10.1093/europace/euu139

BASIC SCIENCE

Long-standing arterial hypertension is associated with *Pitx2* down-regulation in a rat model of spontaneous atrial tachyarrhythmias

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Received 20 October 2013; accepted after revision 7 May 2014

Aims The timecourse of left atrial *Pitx2* down-regulation in the setting of atrial tachyarrhythmias remains unknown. Accordingly, we aimed to assess the age dependency of left atrial *Pitx2* expression in an experimental model of spontaneous atrial tachyarrhythmias in rats.

Methods and results Atrial sampling was performed in three groups ($n = 4$ each) of young (14-week-old), adult (24-week-old), and ageing (48-week-old) spontaneously hypertensive rats (SHRs), in which we previously demonstrated the age dependency of spontaneous atrial tachyarrhythmias, and three groups ($n = 4$ each) of age-matched normotensive Wistar-Kyoto (WKY) rats. mRNA expression of *Pitx2* was studied using real-time polymerase chain reaction. Ageing SHRs presented significantly lower left atrial *Pitx2* expressions compared with age-matched WKY rats ($P = 0.02$), while no significant difference was observed between young or adult SHRs and age-matched WKY rats (both $P > 0.05$). Among SHRs, *Pitx2* expressions showed a progressive, age-dependent decrease (34.9 ± 6.7 in young SHRs, 17.1 ± 3.6 in adult SHRs, and 10.7 ± 1.7 in ageing SHRs, $P = 0.04$) and were significantly negatively correlated with both age (Spearman $r = -0.86$, $P < 0.01$) and heart weight (Spearman $r = -0.76$, $P < 0.01$).

Conclusion The present study suggests the presence of age-dependent left atrial *Pitx2* down-regulation in SHRs. The strong negative correlation between left atrial *Pitx2* expression and heart weight among SHRs may indicate a link between long-standing arterial hypertension and *Pitx2*-related atrial arrhythmogenicity.

Keywords Atrial tachyarrhythmia • Experimental model • Transcriptomics • *Pitx2* • Hypertension

Introduction

Although atrial fibrillation (AF) is the most common sustained cardiac arrhythmia, the molecular substrate of this arrhythmia remains elusive. Recent genome-wide association studies in AF patients identified strong associations between several risk variants on chromosome 4q25 and the occurrence of AF.[1–3] Furthermore, a common polymorphism on chromosome 4q25 independently predicted AF recurrence after cardioversion.[4] The closest gene in this region is *Pitx2*, a member of the pituitary homeobox family of transcription factors that plays an important role in morphogenesis and early left/right determination during embryonic development.[5,6] Chinchilla *et al.*[7] recently demonstrated a significant decrease in *Pitx2c* expression in AF patients, providing a link between *Pitx2* loss-of-function and AF. In transgenic mice with heterozygous deletion of the *Pitx2* gene, reduction of *Pitx2* expression to ~60% of normal was associated with increased susceptibility to AF,[8] suggesting that *Pitx2* down-regulation is more likely a causative element in AF occurrence rather than a consequence of the arrhythmia. However, while studies on transgenic mice with *Pitx2* deletion have provided valuable insights into our understanding concerning the pro-arrhythmic consequences of *Pitx2* deficiency, *Pitx2* expression has never been assessed in a clinically relevant experimental model of atrial arrhythmias or in the

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What's new?

- Similarly to what has been observed in humans, there is an association between left atrial *Pitx2* down-regulation and increased atrial arrhythmogenicity in this experimental model of spontaneous atrial arrhythmias.
- The results of the present study provide evidence for an association between *Pitx2* down-regulation and long-standing arterial hypertension and indicate a possible temporal relationship between *Pitx2* down-regulation and arrhythmia onset, suggesting that in hypertensive rats *Pitx2* down-regulation is an age-dependent process that starts before the occurrence of the arrhythmias.
- The strong negative correlation between left atrial *Pitx2* expression and heart weight among hypertensive rats indicates that *Pitx2* down-regulation may be involved in long-standing arterial hypertension-related atrial arrhythmogenicity.

presence of arrhythmogenesis-promoting factors such as arterial hypertension. Clinical and experimental data demonstrated that the association between ageing and arterial hypertension significantly increases atrial arrhythmogenicity,[9–11] but *Pitx2* expression changes have never been assessed in this setting.

Accordingly, we sought to assess the presence and the timecourse of *Pitx2* expression changes in a spontaneously hypertensive rat (SHR) model of unprovoked atrial tachyarrhythmias (AT). The age dependency of atrial arrhythmias in this model has already been documented.[9] For this study, atrial mRNA expression of *Pitx2* was investigated in SHRs in comparison with age-matched Wistar-Kyoto (WKY) controls.

Methods

Animals

Three groups of male SHRs 14 weeks (further mentioned as young; $n = 6$), 24 weeks (further mentioned as adult; $n = 4$), and 48 weeks (further mentioned as ageing; $n = 2$) of age, and three groups of age-matched WKY controls ($n = 4$ each) were purchased from Elevage Janvier.

All animals were housed in a climate-controlled room (21–24°C), with a 12 h/12 h light/dark cycle, in an accredited animal facility, and had free access to standard rat pellets and tap water. Rats undergoing electrocardiogram (ECG) recordings were housed individually in polycarbonate cages. All other animals were housed in groups of two to three rats per cage under the same controlled conditions.

All experiments conform to the Guide for the Care and Use of Laboratory Animals were performed in compliance with the French Ministry of Agriculture guidelines for animal experimentation, and were approved by the local Animal Ethics Committee.

Electrocardiogram recording and analysis

To confirm the arrhythmic status of SHRs and the age dependency of AT episodes, radiotelemetry ECG transmitters (TA11 CA-F40; Data Sciences International) were implanted under isoflurane anaesthesia in two of the six young SHRs as previously described.[9] The animals underwent 24 h continuous ECG monitoring every 4 weeks until the age of 46 weeks, when they joined the ageing SHR group. To test for day-to-day reproducibility, 46-week-old SHRs underwent 48 h continuous ECG monitoring, instead of the standard 24 h recording. Data used to assess the age dependency of arrhythmic events corresponded to the first 24 h period. The total number and duration of AT events were quantified based on 24 h ECG recordings as previously described.[9]

Heart sampling

Four of the young SHRs, the four adult SHRs, the two ageing SHRs, and all WKY rats were euthanized upon purchase with an intraperitoneal injection of a terminal dose of sodium pentobarbital (>100 mg/kg). The two remaining young SHRs were only euthanized upon completion of the ECG monitoring period, when they reached the age of ageing SHRs. The hearts of rats belonging to all six groups (i.e. young, adult, and ageing SHRs and age-matched WKY rats; $n = 4$ each) were excised and weighted; the left and right atria were isolated, placed in sterile dry tubes, snap frozen in liquid nitrogen, and stored at −80°C until processing. Heart weights of SHRs compared with those of WKY rats were used as indirect markers of arterial hypertension. The hypertensive status of rats of this strain was also documented in a previous study using intra-arterial blood pressure measurement in conscious, unrestrained rats.[9]

Gene expression analysis

Ribonucleic acid extraction

Total RNA was isolated from frozen biopsies using TRIzol® Reagent (Life Technologies) following the manufacturer's instructions. RNA concentration was measured using Nanodrop ND1000 (Thermo scientific) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies).

Pitx2 expression analysis

One microgram of total RNA was reverse-transcribed to complementary DNA by using 100 U Superscript II (Life Technologies) and a mixture of random hexamers and oligo(dT) primers (Promega). Real-time polymerase chain reaction assays were performed as previously described,[12] using a Rotor-Gene 6000 (QIAGEN). Values were normalized using TATA box binding protein (TBP). Primers used to detect *Pitx2* were GTG-TGG-ACC-AAC-CTT-ACG-G (sense primer) and AGT-TGA-AGA-AGG-GGA-AGC-TC (antisense primer).

To allow comparison with previous studies, expression levels of two additional genes, *Myh7*, encoding for β-myosin heavy chain 7, and *Gja1*, encoding for connexin-43, were studied in left atrial samples from ageing SHRs and age-matched WKY rats using TaqMan Low Density Array analysis, as previously described.[13]

Statistics

Data are expressed as the mean ± standard error (SE). Non-parametric analysis of variance (Kruskal–Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann–Whitney *U* test. Correlations were ascertained with Spearman's rank correlation method. Stepwise multiple regression analysis was performed to identify independent predictors of left atrial *Pitx2* expression.

A *P* value of <0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism® software (GraphPad Software).

Results

Atrial arrhythmic activity in hypertensive rats

Figure 1 depicts typical ECG tracings recorded in the two SHRs at 46 weeks of age, showing spontaneous AT episodes.

No tachyarrhythmic episodes were recorded at the age of 14, 18, or 22 weeks, in neither of the two SHRs. In one SHR, the onset of AT episodes occurred at the age of 26 weeks, with a number of 286 AT episodes/24 h, while in the other SHR, AT episodes were present starting from the age of 34 weeks, with a number of 325 AT episodes/24 h. Both SHRs presented AT episodes on all 24 h ECG recordings, with 376 and 504 AT episodes/24 h, respectively, at the age of 38 weeks, and 363 and 191 AT episodes/24 h, respectively, at the age of 42 weeks (*Figure 2*). At the age of 46 weeks, the two SHRs presented 299 and 543 AT episodes, respectively, on the first 24 h interval and 354 and 246 AT episodes, respectively, on the second 24 h interval. At 46 weeks of age, the median duration of tachyarrhythmic episodes in the two SHRs was 0.64 s. Episode duration ranged from 0.33 to 12.78 s.

When all ECG recordings were taken into account, there was a significant correlation between age and the number of tachyarrhythmic episodes (Spearman $r = 0.89$, $P < 0.01$); however, when only recordings obtained after the onset of tachyarrhythmic episodes were considered (starting from the age of 34 weeks), statistical significance of this correlation was no longer present ($P = 0.46$).

Myocardial hypertrophy indices

Heart weights were measured to assess the presence and the severity of myocardial hypertrophy in SHRs. In young rats, heart weights were not significantly different between SHRs and WKY rats ($P = 0.49$). However, heart weights of adult SHRs were 16 $\pm$ 3% higher compared with those of age-matched WKY rats (1.80 $\pm$ 0.32 g in adult SHRs vs. 1.55 $\pm$ 0.33 g in adult WKY rats, $P = 0.03$), while heart weights of ageing SHRs were 72 $\pm$ 2% higher compared with those of age-matched WKY rats (2.67 $\pm$ 0.12 g in ageing SHRs vs. 1.55 $\pm$ 0.07 g in ageing WKY rats, $P = 0.02$).

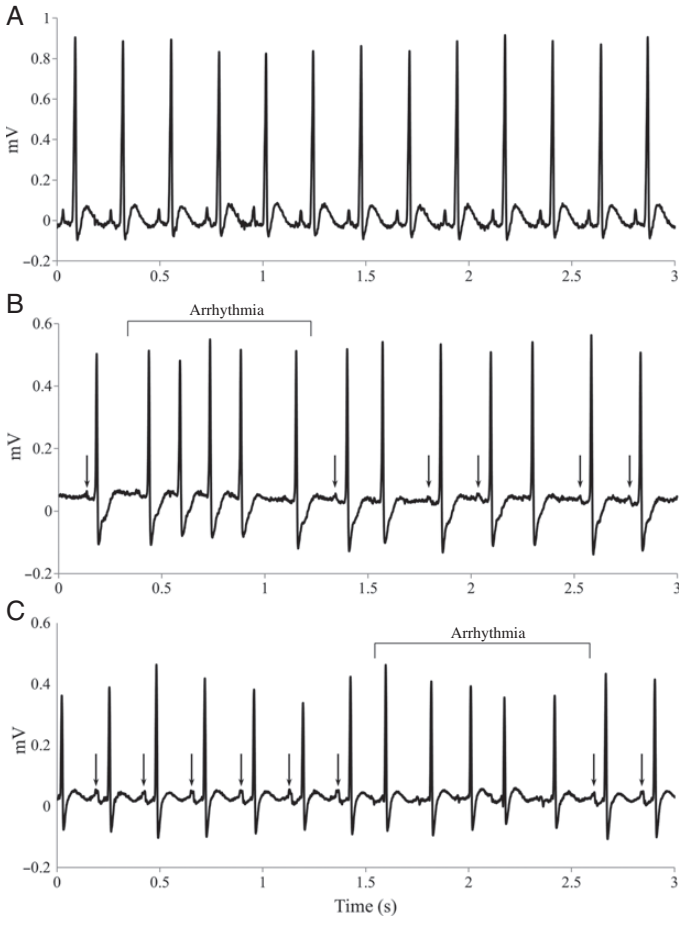

Figure 1 Electrocardiogram tracings in 46-week-old SHRs showing sinus rhythm (*A*); AT episode suggesting a diagnosis of AF (rapid, irregular rhythm, with persistence of narrow QRS complexes, in the absence of visible P-waves) (*B*); and AT episode suggesting a diagnosis of multifocal AT (rapid, irregular rhythm, with persistence of narrow QRS complexes and wide, inverted atrial waves, with continually changing morphology) (*C*). *B* and *C*, arrows: sinus rhythm beats.

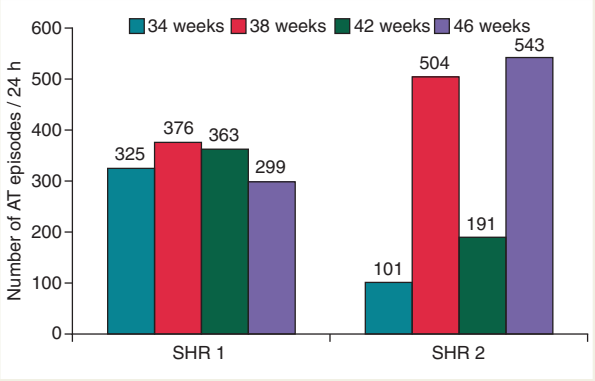

Figure 2 Total number of AT episodes/24 h in two individual SHRs at 34, 38, 42, and 46 weeks of age, respectively.

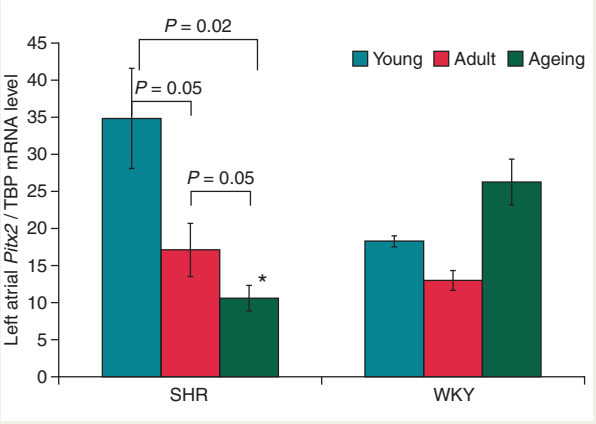

Figure 3 Left atrial *Pitx2*/TBP mRNA expression in young, adult, and ageing SHRs ($n = 4$ each) and age-matched WKY rats ($n = 4$ each). Data are expressed as the mean ± SE. *$P = 0.02$ for ageing SHRs vs. ageing WKY rats using the Mann–Whitney *U* test. *P*-values on the figure refer to comparisons between SHR groups using the Mann–Whitney *U* test.

Among SHRs, heart weights were highly dependent on age (Spearman $r = 0.87$, $P < 0.01$), while no correlation was present between heart weights and age among normotensive rats ($P = 0.80$).

Pitx2 expression in the left atria of hypertensive rats

When right atrial expression of *Pitx2* was compared between hypertensive and normotensive rats, no significant difference was observed between groups, regardless of the age of animals (all $P > 0.05$). Similarly, there was no significant difference between right atrial levels of *Pitx2* among young, adult, and ageing SHRs ($P = 0.11$), nor among young, adult, and ageing WKY rats ($P = 0.63$).

With the exception of ageing SHRs, all other groups (i.e. young and adult SHRs, and young, adult, and ageing WKY rats) presented significantly higher expressions of *Pitx2* in the left than in the right atria (all $P < 0.05$). However, in ageing SHRs, there was no significant difference between left and right atrial levels of *Pitx2* ($P = 0.45$).

No significant difference was found between left atrial levels of *Pitx2* in young SHRs compared with age-matched WKY rats ($P = 0.09$), nor in adult SHRs compared with age-matched WKY rats ($P = 0.49$). On the other hand, ageing SHRs had significantly lower left atrial *Pitx2* expression levels compared with age-matched WKY rats ($P = 0.02$; *Figure 3*).

Among hypertensive rats, left atrial *Pitx2* expression showed a gradual, age-dependent decrease (34.9 ± 6.7 in young SHRs vs. 17.1 ± 3.6 in adult SHRs vs. 10.7 ± 1.7 in ageing SHRs; $P = 0.04$) (*Figure 3*). When comparisons were ascertained among various SHR groups (*Figure 3*), adult SHRs tended to have lower left atrial *Pitx2* expression levels than young SHRs and higher left atrial *Pitx2* expression levels than ageing SHRs (both $P = 0.05$), while ageing SHRs had significantly lower left atrial *Pitx2* levels compared with young SHRs ($P = 0.02$). On the other hand, there was no significant difference in left atrial *Pitx2* expression levels between neither of the WKY rats groups (all $P > 0.05$).

In addition, left atrial *Pitx2* expression levels were significantly negatively correlated with age (Spearman $r = -0.86$, $P < 0.01$) among SHRs. A significant negative correlation was also found among SHRs between left atrial *Pitx2* expression levels and heart weights (Spearman $r = -0.76$, $P < 0.01$). In multivariate analysis, both age ($R^2 = 0.66$; $P < 0.01$) and heart weight ($R^2 = 0.49$; $P = 0.01$) independently predicted decreased left atrial *Pitx2* expression levels. The combination of age and heart weight was a significant predictor of left atrial *Pitx2* expression levels in SHRs ($R^2 = 0.69$; $P < 0.01$), while no significant correlation was found between left atrial *Pitx2* expression levels and neither age or heart weight among WKY rats (both $P > 0.05$).

Left atrial expression levels of *Myh7* were 1.9 times higher in ageing SHRs compared with age-matched WKY rats (4519.1 ± 83.6 in ageing SHRs vs. 2377.4 ± 52.4 in ageing WKY rats; $P = 0.02$). Contrarily, *Gja1* expression was 2.8 times lower in ageing SHRs compared with age-matched WKY rats (949.7 ± 44.7 in ageing SHRs vs. 2611.7 ± 61.1 in ageing WKY rats; $P = 0.04$).

Discussion

The main findings of the present study were: (i) in ageing SHRs with spontaneous AT left atrial *Pitx2* expression was significantly downregulated compared with age-matched WKY rats; (ii) among SHRs, *Pitx2* expression was significantly negatively correlated with both age and heart weight.

Pitx2 insufficiency and atrial arrhythmias in spontaneously hypertensive rats

Cardiovascular pathologies offer numerous examples of cardiac conditions characterized by re-expression or reactivation of a battery of genes that are normally only expressed in foetal hearts. Recent genome-wide association studies identified several risk variants on chromosomes 4q25, 16q22, and 1q21 strongly associated with AF.[1,14–16] The single-nucleotide polymorphisms most significantly associated with AF are located on chromosome 4q25, in a large, intergenic region, without any known genes. The closest gene in this region is *Pitx2*, a member of the pituitary homeobox family of

transcription factors that plays important roles in early embryonic development of the heart.[5,6]

Chinchilla *et al.*[7] were the first to demonstrate that *Pitx2c* expression is significantly decreased in AF patients, providing a link between *Pitx2* loss-of-function and AF. Moreover, several experimental studies demonstrated increased susceptibility to atrial arrhythmias in transgenic mice with heterozygous deletion of the *Pitx2* gene, indicating a direct relationship between *Pitx2* deficiency and atrial arrhythmias.[8,17] On the other hand, Tessari *et al.*[18] demonstrated that PITX2$^{-/-}$ knockout mice display severe heart defects and die before birth. Taken together, these observations suggest that *Pitx2* down-regulation is more likely related to a dysfunction that occurs later in life, and not in the developmental period.

The present study suggests that, similarly to what has been observed in humans, there is an association between left atrial *Pitx2* down-regulation and increased atrial arrhythmogenicity in this clinically relevant experimental model of atrial arrhythmias. Electrocardiogram monitoring in SHRs confirmed the presence and the age dependency of spontaneous AT in this group, which paralleled the decrease in left atrial *Pitx2* expression levels. While left atrial *Pitx2* expression showed no significant difference between non-arrhythmic young or adult SHRs and age-matched normotensive controls, *Pitx2* expression levels were significantly decreased in ageing SHRs with atrial arrhythmias compared with age-matched non-arrhythmic normotensive controls. Furthermore, among SHRs, left atrial *Pitx2* expression presented a gradual, age-dependent decrease. These results further suggest that, at least in the presence of underlying cardiac conditions such as arterial hypertension, *Pitx2* down-regulation is more likely to occur during adulthood, than in the developmental period.

Expression changes in *Pitx2*, a transcription factor that regulates the activity of various other genes, could then lead to further pro-arrhythmic molecular remodelling,[7,8] contributing to the high propensity to atrial arrhythmias observed in this setting. *Pitx2* down-regulation has been related to re-expression of *Shox2*, a transcription factor responsible for a sinoatrial nodal gene programme in the developing heart.[17] *Pitx2* misexpression has also been shown to impair connexin-40 expression,[7,19] providing a putative link to AF, since mutations in the connexin-40 gene have been associated with AF. In addition, *Pitx2* could modulate ion channel expression, leading to overexpression of HCN4, encoding for the pacemaker current I_f, impaired expression of various genes encoding for K^+ channels, including KCNQ1 (encoding for the α-subunit of I_{Ks}) and KCNK2 (encoding for a stretch-activated K^+ channel).[17] In the study of Kirchhof *et al.*,[8] *Pitx2*$^{-/+}$ hearts displayed shorter atrial action potential duration and increased susceptibility to AF elicited by programmed stimulation with a single atrial premature beat. Gene expression patterns and functional analyses in *Pitx2*-deficient atria also demonstrated altered Ca^{2+} handling, cell–cell communication, and melanocyte function, providing a mechanistic link between *Pitx2* down-regulation and arrhythmia occurrence. It is likely that the interaction between various molecular pathways, some related and others unrelated to *Pitx2* down-regulation, which are not mutually exclusive and are likely to coexist, determines the onset and the severity of the arrhythmia.

The presence of AF has also been associated with re-expression of various embryonic genes, including calcineurin, troponin I, α-smooth muscle actin, or β-myosin heavy chain.[20–24] Consistent with the results of these previous studies, arrhythmic ageing SHRs presented significantly higher left atrial expression levels of *Myh7*, encoding for β-myosin heavy chain 7, and significantly lower expression levels of *Gja1*, encoding for connexin-43.[20,24]

Clinical implications

The results of the present study provide evidence for an association between *Pitx2* down-regulation and long-standing arterial hypertension and indicate a possible temporal relationship between *Pitx2* down-regulation and AT onset in hypertensive rats, suggesting that in SHRs *Pitx2* down-regulation is an age-dependent process that starts before the occurrence of the arrhythmias. This finding may indicate that a timely appropriate therapy targeting this process in hypertensive patients may prevent AF onset.

In addition, the strong negative correlation between left atrial *Pitx2* expression and heart weight among SHRs indicates that *Pitx2* down-regulation may be involved in long-standing arterial hypertension-related atrial arrhythmogenicity. However, the mechanisms responsible for *Pitx2* insufficiency in hypertension-related AT are yet to be established. Similarly to other cardiac disorders-related molecular remodelling, *Pitx2* down-regulation could represent an adaptive process in response to various insults, such as haemodynamic afterload imposed by long-standing arterial hypertension. This hypothesis is supported by the fact that among SHRs left atrial *Pitx2* expression levels were significantly negatively correlated with both age and heart weight. In multivariate analysis, both age and heart weight independently predicted decreased left atrial *Pitx2* expression levels. The combination of age and heart weight was a significant predictor of left atrial *Pitx2* expression levels in hypertensive rats, while no significant correlation was found between left atrial *Pitx2* expression levels and neither age or heart weight among normotensive rats. On the other hand, there was no significant difference in right atrial *Pitx2* expression levels between hypertensive and normotensive rats, regardless of the age of animals, neither among hypertensive or normotensive rats of different ages. Consequently, *Pitx2* down-regulation seems confined to the left atrium of hypertensive rats and this could be due to long-standing left atrial haemodynamic overload due to persistent arterial hypertension. Additional markers, such as atrial or brain natriuretic peptides, could have brought additional evidence regarding the potential role of haemodynamic changes in left atrial *Pitx2* down-regulation.

However, while this study has demonstrated an association between long-standing arterial hypertension and *Pitx2* down-regulation, at this point it is difficult to support a causal relationship between the two processes. This is further complicated by the fact that *Pitx2* down-regulation itself seems to induce molecular changes leading to left atrial electrical and structural remodelling that could further impair atrial haemodynamics.[8]

In this light, it is likely that identification and controlling of the mechanisms leading to *Pitx2* down-regulation in this setting would represent a valuable new approach for atrial arrhythmias treatment and/or prophylaxis. Given the clinical relevance of the model used in this study, this experimental model could represent a valuable tool in this regard.

Potential limitations

Given the small size of rats' atria, concomitant histological examination of the samples could not be performed. However, structural abnormalities within the left atria of ageing SHRs and WKY rats have already been described.[25]

Since ECG monitoring was only performed in two SHRs, direct correlations between AT burden and *Pitx2* expression levels could not be ascertained. Indeed, on the last ECG recording, performed just before euthanasia and heart sampling, SHR1 presented a number of 299 AT episodes/24 h, while SHR2 presented 543 AT episodes/24 h (*Figure 2*), in concordance with *Pitx2* expression levels, which were lower in SHR2 compared with SHR1 (7.50 in SHR2 vs. 12.92 in SHR1). Nevertheless, continuous ECG monitoring in SHRs demonstrated a high variability in the number of AT episodes among various recordings. Therefore, despite the high arrhythmogenicity present in these animals, the selection of ECG recordings for statistical analysis would be highly arbitrary and it seems unlikely for *Pitx2* expression levels to be significantly correlated with atrial arrhythmia burden. Also, the small sample size of the cohorts may have lowered the study's statistical power.

The results of the present study indicate a possible link between long-standing arterial hypertension and *Pitx2*-related atrial arrhythmogenicity. The relationship between these factors and AT, which may not necessarily be causal, deserves to be further studied.

Conclusion

The present study suggests the presence of age-dependent left atrial *Pitx2* down-regulation in SHRs. The strong negative correlation found between left atrial *Pitx2* expression and heart weight among hypertensive rats indicates a possible link between long-standing arterial hypertension and *Pitx2*-related atrial arrhythmogenicity.

Acknowledgements

The authors thank Mrs Valérie Oréa (Institut de Biologie et Chimie des Protéines, FRE 3310 CNRS/Université Claude Bernard Lyon1, Lyon, France), Drs Maximilien Giovannetti, Stéphane Wroblewski, and Daniel Grinberg for performing heart sampling.

Conflict of interest: none declared.

Funding

This work was partially supported by a University of Medicine and Pharmacy of Tîrgu Mureş Research Grant number 10/30.01.2013.

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