

# Molecular Basis of Atrial Fibrillation Initiation and Maintenance

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**Abstract:** Atrial fibrillation (AF) is the most common cardiac arrhythmia, largely associated to morbidity and mortality. Over the past decades, research in appearance and progression of this arrhythmia have turned into significant advances in its management. However, the incidence of AF continues to increase with the aging of the population and many important fundamental and translational underlying mechanisms remain elusive. Here, we review recent advances in molecular and cellular basis for AF initiation, maintenance and progression. We first provide an overview of the basic molecular and electrophysiological mechanisms that lead and characterize AF. Next, we discuss the upstream regulatory factors conducting the underlying mechanisms which drive electrical and structural AF-associated remodeling, including genetic factors (risk variants associated to AF as transcriptional regulators and genetic changes associated to AF), neurohormonal regulation (i.e., cAMP) and oxidative stress imbalance (cGMP and mitochondrial dysfunction). Finally, we discuss the potential therapeutic implications of those findings, the knowledge gaps and consider future approaches to improve clinical management.



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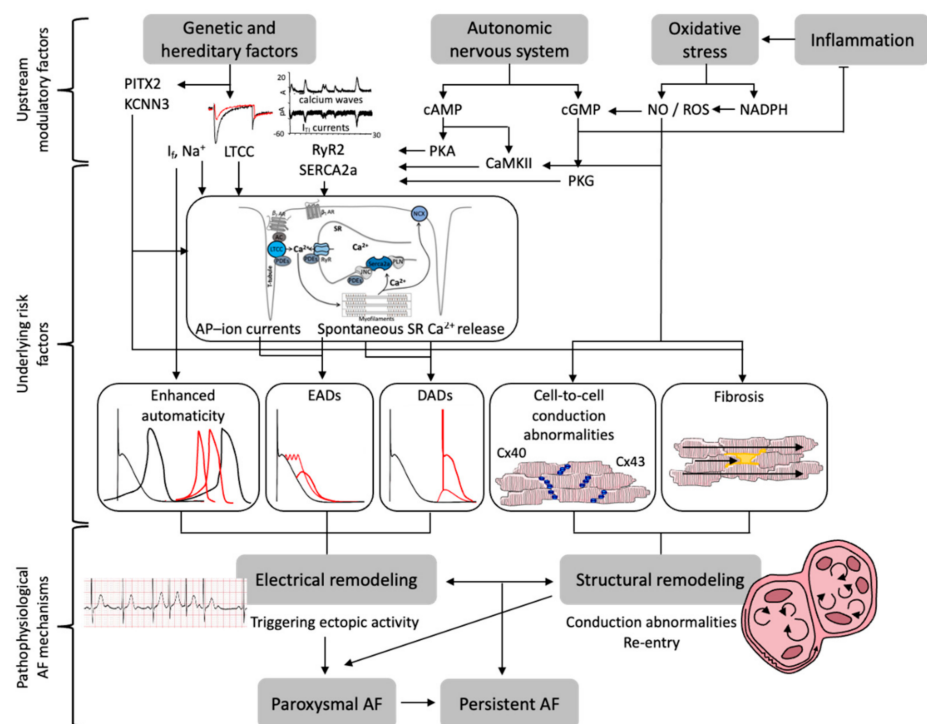
## 1. Introduction

Atrial fibrillation (AF) is a growing epidemic associated to increased morbidity and mortality [1]. Over the past decades, research in appearance and progression of this arrhythmia have turned into significant advances in its management [2]. Although the management of AF has drastically improved, it still has limited efficacy. Complete prevention, medium/long-term sinus rhythm maintenance after cardioversion or ablation of AF is not yet possible, and the progressive nature of AF is limiting the long-term success of current management therapies [3].

For AF to initiate and develop into a maintenance state, it requires a trigger and a vulnerable substrate [4]. Several mechanisms have been identified to serve as these triggers and substrates, including ectopic activity and conduction abnormalities, and several molecular upstream regulatory networks have been proposed to promote these mechanisms. This review highlights the recent advances in understanding the potential mediators and the molecular basis underlying initiation, maintenance and progression of AF, as well as highlighting resulting therapeutical implications and knowledge gaps.

## 2. Electrophysiological Mechanisms Associated with AF

A number of pathophysiological mechanisms have been proposed to underly initiation and maintenance of AF (Figure 1).



**Figure 1.** Pathophysiological mechanisms underlying initiation and maintenance of atrial fibrillation (AF). Summary of upstream modulatory factors, underlying risk factors and pathophysiological AF-associated mechanisms. Abbreviations: PITX2 = paired-like homeodomain transcription factor 2, KCNN3 = Potassium  $\text{Ca}^{2+}$ -Activated Channel,  $I_f$  = funny current, LTCC = L-type  $\text{Ca}^{2+}$  channel, RyR2 = ryanodine receptor 2, SERCA2a = SR  $\text{Ca}^{2+}$  ATPase, cAMP = 3',5'-cyclic adenosine monophosphate, PKA and PKG = protein kinase A and G, CaMKII =  $\text{Ca}^{2+}$ -calmodulin dependent protein-kinase type-II, NO = nitric oxide, ROS = reactive oxygen species, NADPH (Nicotinamide adenine dinucleotide phosphate), cGMP = cyclic guanosine monophosphate, AP = action potential, SR = sarcoplasmic reticulum, EADs/DADs = early/delayed afterdepolarizations, Cx40/43 = connexin 40/43, AF = atrial fibrillation.

### 2.1. Ectopic Activity

Abnormal pro-arrhythmic depolarizations, also called ectopic triggered activity, have been shown to originate mainly in the pulmonary vein (PV), where they have been linked to onset and maintenance of AF [4–7]. Another source of ectopic beats has been found in atrial muscle [6,8,9]. In both cases, anatomical variations and ion-channels properties predispose to ectopic triggered activity. Two types of abnormal depolarizations have been characterized: delayed (DADs) and early (EADs) afterdepolarizations [10]. EADs are favored by low heart rates, prolonged action potential durations (APD), partial inhibition of  $\text{K}^+$  channels, or by an increase in the inward  $\text{Ca}^{2+}$  currents during the plateau phase [11–13] or as a downstream effect in response to cellular stress [14]. DADs are favored by high heart rates and sarcoplasmic reticulum (SR)  $\text{Ca}^{2+}$  overloading and have been linked more directly to initiation of AF. Basically, a DAD occurs in response to a spontaneous SR  $\text{Ca}^{2+}$  release event, expanding as a  $\text{Ca}^{2+}$  wave through the cytosol and depolarizing the membrane, due to part of  $\text{Ca}^{2+}$  being expelled from the myocytes via  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) [15,16]. Repetitive ectopic activity initiates atrial arrhythmias in myocytes [17] and in whole hearts [18]. Thus, the most prominent channels causing ectopic activity are the ones responsible of the magnitude of the SR  $\text{Ca}^{2+}$  loading and release, the SR  $\text{Ca}^{2+}$  ATPase (SERCA2a) and ryanodine receptor 2 (RyR2) [14]. Phosphorylation of RyR2 has been shown to impair its interaction with one stabilizing agent, FKBP12.6, resulting in a “leaky” channel in canine ventricular myocytes, and RyR2 mutations linked to exercise induced arrhythmias have also been shown to decrease FKBP12.6 binding [19,20]. Additionally, reducing the

open duration of RyR2 in HEK293 cells and mice ventricular myocytes with carvedilol, effectively suppressed store-overload-induced  $\text{Ca}^{2+}$  release [21]. SERCA2a function is regulated by phospholamban (PLB) and in atrial cells additionally by sarcolipin (SLN) [14]. In myocytes of a canine heart failure model PLB's regulatory effect on SERCA2a was impaired due to an increased phosphorylation by type 1 phosphatase protein [22]. SLN mediates  $\beta$ -adrenergic response in the atrium and was significantly reduced in AF patients, resulting in an increased SR  $\text{Ca}^{2+}$  uptake through SERCA2a [23]. Rescuing SERCA2a function in failing rabbit myocytes by adenoviral gene transfer reversed the contractile dysfunctions, further highlighting the importance of SERCA2a function in preventing arrhythmias [24]. Overall,  $\text{Ca}^{2+}$  handling abnormalities reduce cellular  $\text{Ca}^{2+}$  stores and decrease contractile function, as well as causing beat-to-beat response irregularities [14,25]. Irregular beat-to-beat responses in human atrial myocytes (HAMs) are known to be caused by impaired L-type  $\text{Ca}^{2+}$  currents ( $I_{\text{Ca,L}}$ ) in combination with RyR2 upregulation. Large numbers of myocytes with irregular beat-to-beat patterns could hamper the synchronized intercellular propagation of  $\text{Ca}^{2+}$ -signaling between adjacent myocytes [25] and tissues.

## 2.2. Conduction Abnormalities

Fibrosis, resulting in a disorganized myocardial architecture, has been shown to support arrhythmia in multiple ways; however, mainly promoting conduction abnormalities or re-entry [26]. Myofibroblasts are able to effect electrical properties by heterocellular coupling to myocytes, shown in murine ventricular myocyte cultures, causing major conduction delays, partial cardiomyocyte depolarization or decreased contraction durations, all of which can increase the risk of fibrillation [27–31]. Importantly, the extended fibrotic extracellular matrix disrupts the unidirectional block which increase the likelihood of re-entry [32]. Although the role of atrial fibrosis in AF maintenance is well known, there are still sizeable knowledge gaps to enable optimal targeting of fibrotic areas during catheter ablation [26]. Cardiac stress, due to surgery or fibrillation, can activate quiescent fibroblasts [33], therefore fibrosis promoting arrhythmias can result in more fibrosis, making treatment improvements even more important. A recent study found calcitonin, a hormone product so far mainly linked to the thyroid gland, to also be produced by atrial myocytes and have protective effects on fibrosis. Patients with persistent AF, showed sixfold lower levels of calcitonin compared to controls with sinus rhythm and disruption of calcitonin signaling in mice triggered fibrogenesis and arrhythmias. Additionally, atrial specific overexpression of calcitonin in mice protected from fibrosis, highlighting a potential new treatment option for fibrosis [34]. Variations in connexin expression and function have been identified as another substrate causing conduction abnormalities. A study of patients with AF compared to those with sinus rhythm showed lateralization of connexin 40 (Cx40), Cx43 and N-cadherin, as well as significantly decreased levels of Cx43 in right atrial (RA) appendages and RA free wall only for AF patients [35]. A mutation in the Cx40 gene identified in AF patients lead to impaired gap junction formation and reduced intercellular electrical coupling, highlighting its role in coordinating atrial electrical coupling [36]. A review by Sanchez-Quintana et al. The authors of [4] highlighted one issue with the current state of knowledge linking connexin expression to AF, this being that decreased, increased and no change in expression levels of both Cx40 and Cx43 have been identified in AF patients, although these studies included a wide range of clinical sub-sets and would probably need to be investigated in more detail.

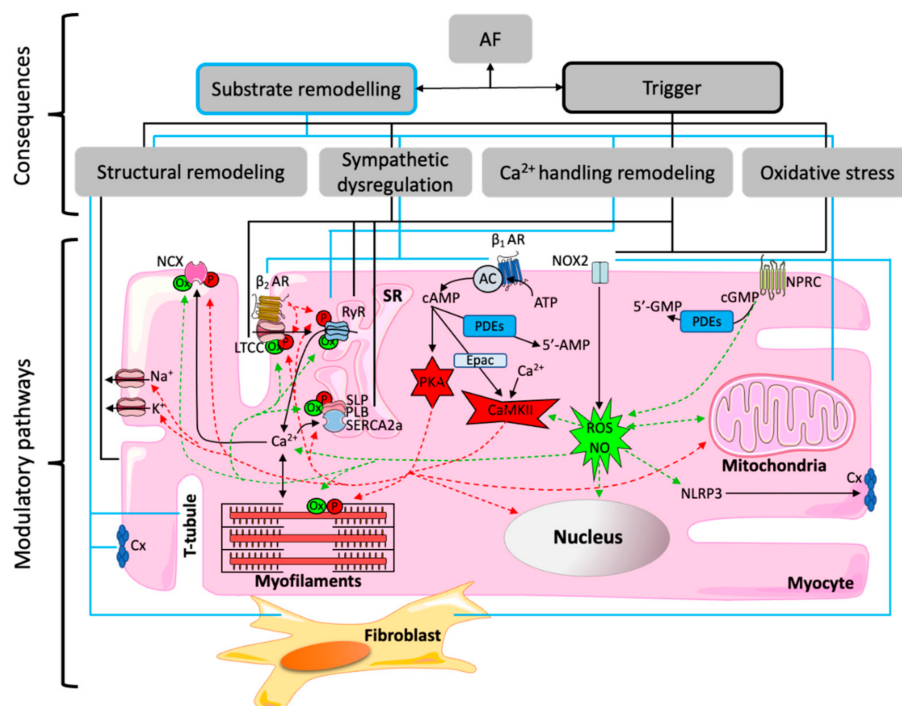
## 2.3. Enhanced Automaticity

The sinoatrial node (SAN) as primary pacemaker generates spontaneous APs (action potentials), the rate of which is controlled by a coupled-clock system involving the surface membrane ion channel M-clock and the SR  $\text{Ca}^{2+}$  clock [37]. A canine model of pacing induced atrial fibrillation with SAN dysfunction showed alterations in  $\text{Ca}^{2+}$  clock responsiveness to adrenergic stimulation, as well as a downregulation of RyR2 [38]. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, carrying the funny

current ( $I_f$ ), have also been linked to abnormal enhanced automaticity and atrial HCN shows increased expression in end-stage failing human hearts [39]. The degree to which enhanced automaticity causes AF is unclear and would require further investigation.

### 3. Molecular Regulatory Networks of Proarrhythmic Mechanisms in the Atria

Multiple potential mediators have been described to cause pathologic changes leading to the atrial substrate and electrical remodeling associated to AF (Figure 2), including hereditary genes, inflammation, fibrosis, oxidative stress, pressure and/or volume overload and autonomic changes.



**Figure 2.** Molecular regulatory networks: Many different microdomains can be modulated by oxidation (Ox) via cGMP, reactive oxygen species (ROS) and nitric oxide (NO), or by phosphorylation (P) via cAMP, protein kinase A (PKA) and  $\text{Ca}^{2+}$ -calmodulin dependent protein-kinase type-II (CaMKII). Regulated  $\text{Ca}^{2+}$ -handling microdomains include  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) and L-type  $\text{Ca}^{2+}$  channel (LTCC) at the cell membrane, ryanodine receptor (RyR) and sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA2a), which is inhibited by sarcolipin (SLP) and phospholamban (PLB), at the sarcoplasmic reticulum (SR) membrane, the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), and contractile proteins at the myofilaments. PKA/CaMKII are activated by  $\beta$ -adrenoreceptor ( $\beta$ AR) signaling via adenylyl cyclase (AC), phosphodiesterases (PDEs) and exchange protein directly activated by cAMP (Epac). ROS/NO is produced/increased by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) and natriuretic peptide clearance receptor (NPRC). Other modulations of atrial fibrillation-related remodeling are ion channels, such as  $\text{Na}^+$  and  $\text{K}^+$  channels, activation of fibroblasts or structural changes such as dilation or stretch affecting the tubular network, as well as connexins (Cx) function.

#### 3.1. Genetic Regulation

There is growing evidence for a genetic involvement in the development and maintenance of AF, with heritable mutations linked to 5% of all AF cases and approximately 15% of early-onset cases without concomitant disease [40]. Understanding of these genetic modifications and their effect on therapeutic interventions are crucial to increase the effectiveness of rhythm control therapies. Genome-wide association studies (GWASs) identified over 100 independent risk variants, many of which lying close to genes and transcrip-

tion factors linked to AF, for example the paired-like homeodomain transcription factor 2 (PITX2) and the T-Box Transcription Factor 5 (TBX5) [41–44]. Van Ouwerkerk et al. [45] described the networks of transcription factors linked to AF, but many other gene-regulatory networks are also known to promote AF.

Right and left atrial samples from patients with sustained AF, displayed a significant decrease in *PITX2c* expression [46]. A reduced *Pitx2c* expression in mice shortened atrial APD and increased susceptibility to induced AF [47], and a double knockout (atrial-specific (NppaCre<sup>+</sup>Pitx2<sup>-/-</sup>) and ventricular (Mlc2vCre<sup>+</sup>Pitx2<sup>-/-</sup>)) *Pitx2c* mutant mice resulted in a significantly more polarized resting membrane potential (RMP) and a smaller AP amplitude [46,48]. It is known that in embryonic stage PITX2 is responsible for right-left differentiation and in adult hearts it is primarily confined to the left atrium (LA) [47,49]. Current studies suggest that, within the LA, PITX2 controls the expression and/or function of ion channels controlling RMP. A recent in silico study supports these results showing shortened APD, elevated RMP and a slow conduction in LA human cells with PITX2 down-regulation. They identified several targets of PITX2, including I<sub>KS</sub> (slow delayed rectifier potassium current), I<sub>Ca,L</sub>, RyR and SERCA2a. PITX2 deficiency led to an enhanced SERCA2a function resulting in increased SR Ca<sup>2+</sup> load, as well as increased I<sub>KS</sub> and decreased I<sub>Ca,L</sub> currents, both resulting in reduced APD [50]. Another study looking at partial or complete loss of function (LoF) models of *Pitx2* in mice found multiple calcium-handling genes (*Atp2a2*, *Casq2* and *Plb*) to be altered in a dose-dependent manner. They also found *Wnt8a* upregulation in the complete LoF model, correlating with spontaneous AF onset compared to only triggered AF in the partial model [51]. Additionally, PITX2 inhibits SAN formation through repression of *Shox2* and *Tbx3* and a loss of this function has been suggested to result in the ectopic formation of SAN like cells in LA [47]. However, other studies found a significantly higher *PITX2c* mRNA expression in human right atrial myocytes from patients with persistent AF compared to those with sinus rhythm [52,53]. As both increased and decreased levels of PITX2 have been observed in atrial fibrillation, it could be suggested that any change in expression promotes AF related remodeling or that the location of the expression change matters. A number of single nucleotide polymorphisms (SNPs) have been identified on chromosome 4q25 proximal to *PITX2* through GWAS, although they did not correlate with changes in *PITX2* expression [52,54], therefore the underlying mechanisms for increasing AF risk could be PITX2 independent. However, it would be interesting to repeat these measurements only in cardiomyocytes, or to measure PITX2 dependent biomolecules, or to study proarrhythmogenic effects of up- and down-regulation of PITX2 expression in human atrial myocytes (HAMS), in order to confirm or to disprove direct PITX2 and AF risk link. One of these SNPs (rs13143308T) clearly links AF to defective Ca<sup>2+</sup> homeostasis in HAMS. This risk variant has been associated with excessive Ca<sup>2+</sup> releases, spontaneous electrical activity linked to increased SERCA2a expression and RyR2 phosphorylation, which likely causes the high incidence of Ca<sup>2+</sup> induced I<sub>ti</sub> (transient inward currents) and spontaneous membrane polarizations seen in these patients. A single risk allele resulted in a five-fold increase in spontaneous DADs [54]. The exact mechanisms, by which this risk variant causes these modifications, have not been fully identified and require further investigation.

Recently, a mutation in the T-box region of TBX5 has been identified within a family of AF patients, reducing its transcriptional activity [55]. TBX5 has been shown to be crucial for cardiac development by in situ hybridization studies on human embryonic tissue [56], and it is therefore believed that this mutation increased AF susceptibility due to hypoplasia of the conduction system [55]. Adult-specific removal of *Tbx5* caused AF in mice and cardiomyocytes from this model revealed a multilevel gene regulatory network activated by TBX5 and repressed by PITX2 linking seven GWAS AF loci, including *Scn5a*, *Atp2a2* and RyR2 [57]. A similar interaction has been identified through mouse genetics between *Tbx5* and *Gata4*, with their interaction being crucial in atrial rhythm control via regulating the expression of *Atp2a2* and RyR2 [58].

A GWAS study based on lone AF patients with increased heritability of AF had identified another risk locus on chromosome 1q21, with its most significant SNP being intronic to *KCNN3*, a potassium channel involved in atrial repolarization [41]. A screen for genetic variation in the *KCNN3* gene of lone AF patients found no mutations but identified a synonymous SNP exonic to *KCNN3*, supporting the hypothesis of *KCNN3* being involved in AF pathogenesis [59]. Even though no mutations were identified, atrial *KCNN3* expression was reduced in AF with concomitant heart failure (HF) patients who also displayed a significant downregulation of histone deacetylase (HDAC) and atrial myocytes from a corresponding pig model revealed a direct regulation of *KCNN3* mRNA by HDAC [60].

In addition to heritable risk factors, expression changes in many non-heritable AF genes and their products, like RyR2, NCX or SERCA2a, have been identified; for a detailed review see Heijman et al. [61]. The above changes are mostly linked to  $\text{Ca}^{2+}$ -handling abnormalities, yet other pathways are also modulated by non-heritable expression changes. The Notch signaling pathway is dysregulated in an AF-dependent manner in end-stage HF patients, with over a quarter of its downstream transcripts being directly linked to atrial arrhythmias [62]. Several genes from the autonomic signaling cascade, including some phosphodiesterase (PDE) families, also showed pathology specific expression changes in atrial samples from AF patients [63]. Although many other genes have been linked to heritable and/or non-heritable predisposition to AF, mutations in the cardiac  $\text{Na}^+$  channel gene (*SCN5A*) deserve special mention because have not only been associated with AF, importantly they also increase susceptibility to ventricular arrhythmias, by promoting ectopic activity and conduction abnormalities [64–66].

### 3.2. Autonomic Remodeling and Upregulation of Sympathetic Signaling

Sympathetic neurotransmission results from the excitation of sympathetic nerves promoting the release of a neurotransmitter at the presynaptic terminal, by the membrane depolarization of the neurons, nearby G protein-coupled receptors (GPCRs). Neurotransmitters bind to GPCRs promoting the G protein dissociation activating the corresponding second messengers, mostly via adenylyl cyclase (AC), and a signaling cascade, which modulates enzymes that in turn control the phosphorylation state of important proteins for cardiomyocyte function. Thus, adrenaline-binding to  $\beta$ -adrenoreceptors ( $\beta$ AR) leads to AC activation, increasing 3',5'-cyclic adenosine monophosphate (cAMP) which activates protein-kinase A (PKA). PKA phosphorylates a plethora of proteins, including  $\text{Ca}^{2+}$ -handling proteins and ion channels. L-type  $\text{Ca}^{2+}$  channel (LTCC) phosphorylation increases  $I_{\text{Ca,L}}$ , RyR2 phosphorylation increases SR  $\text{Ca}^{2+}$ -release and phosphorylated PLB loses inhibitory effects on SERCA2a. At the same time, the increase of  $\text{Ca}^{2+}$  due to cAMP-PKA-dependent phosphorylation of LTCC and/or RyR2 activates  $\text{Ca}^{2+}$ -calmodulin dependent protein-kinase type-II (CaMKII), which may amplify phosphorylation effects of PKA on  $\text{Ca}^{2+}$ -handling proteins. Furthermore, cAMP also activates CaMKII via the exchange protein directly activated by cAMP (Epac). Importantly, cAMP acts within discrete subcellular microdomains to fine-tune ion channel and transporter activities controlling excitation-contraction coupling due to specific isoforms of PDEs. These degrade cAMP, terminating and spatially restricting cAMP signals, thus also providing special and temporal control of the cAMP effectors, like kinases, that regulate phosphorylation of key  $\text{Ca}^{2+}$ -handling proteins.

Several neurohormonal factors have been studied for their involvement in the initiation and perpetuation of AF. The main focus was always placed on the adrenergic (sympathetic) nervous system (ANS). Even though in AF the  $\beta$ AR transcription is unchanged [63,67], *PKACA*, *IPP2*, *PDE4B*, *PDE3A*, *AKAP9*, *PKIA* and *AC9* are downregulated [63]. By contrast, *PIK3G* is upregulated in left human atria and *PDE8A*, *PP2CA* and *EPAC2* are upregulated in human RA [63]. Regarding the functional and structural remodeling, increased atrial sympathetic innervation has been associated with AF in both animal models and humans [68–70]. Generalized sympathetic hyperactivation plays an important role in AF initiation and maintenance of the arrhythmia, as well as in its related

comorbidities. In line, cAMP seem to be increased in AF [71]. Both the sympathetic and parasympathetic activation have been shown to promote downstream relevant effects in the onset of AF by increasing  $\text{Ca}^{2+}$  transient and diastolic  $\text{Ca}^{2+}$ , promoting ectopic depolarizations due to spontaneous release of  $\text{Ca}^{2+}$  from SR, thereby facilitating spontaneous ectopic firing and increasing heart rate variability [72,73]. Indeed, common mechanisms proposed to underlie  $\text{Ca}^{2+}$ -handling remodeling, including increased phosphorylation of the RyR2 at ser2808 via PKA [17,71,74,75] and ser2814 via CaMKII [71,76], increased PLB phosphorylation at ser16 via PKA [77] and reduced phosphorylation state of the LTCC via phosphatases [78] and PDEs [79,80]. In human atria, cAMP hydrolytic activity is mainly controlled by PDE3 [79,81]. However, reduced PDE4 activity in AF was suggested to be, at least in part, responsible for the enhanced frequency of spontaneous SR  $\text{Ca}^{2+}$  release in AF [74,79]. PDE4 inhibition increases the incidence of arrhythmias in human atrial strips during  $\beta$ -adrenergic stimulation [79]. On the other hand, although the knock-in mouse model with constitutively phosphorylated RyR2 at ser2814 showed a higher incidence of  $\text{Ca}^{2+}$  sparks and increased susceptibility to pacing-induced AF [71], and although CaMKII inhibitors were found to reduce  $\text{Ca}^{2+}$  sparks frequency as well as to increase RyR2 open probability in HAMs [71,82], it has never been shown that CaMKII inhibition reduces  $\text{Ca}^{2+}$  waves or arrhythmias in AF human atrial myocytes or tissues. Thereby, it is not clear whether the role of CaMKII-dependent RyR2 phosphorylation is protective maintaining SR  $\text{Ca}^{2+}$  load by allowing small releases of  $\text{Ca}^{2+}$  from the SR without promoting membrane spontaneous depolarizations and contractions [21], or proarrhythmogenic as suggested during the last years. Several spatio-temporal specific signaling alterations have been linked to the development and maintenance of AF. One of them is a time-dependent switch in  $\beta_1$ -AR signaling from PKA to CaMKII which leads to myocyte apoptosis and maladaptive remodeling, as well as activation of LTCC increasing intracellular  $\text{Ca}^{2+}$  loading [83]. This mechanism has been linked to the induction of ectopic activity in rat PVs [84]. Additionally, a switch in the associated  $G_s$  subunit to  $G_i$  in response to prolonged agonist stimulation has been identified in  $\beta_2$ -AR signaling in HEK293 cells and HAMs [85,86], as having a cardioprotective effect via PI3K/Akt, MEK/ERK and PDE4 signaling cascades [83,87,88]. Additionally, PDE subtype specific inhibitors have been shown to have different effects on electrical activity in isolated rabbit PV and SAN tissue. The increase in SAN and PV spontaneous electrical activity upon PDE3 inhibition was abolished by protein kinase G (PKG) and PKA inhibition, suggesting that PDE3 links to PKG and PKA signaling. PDE4 inhibition led to an increase in intracellular cAMP levels and increased  $I_{\text{CaL}}$  but had no significant effect on spontaneous activity [89]. CaMKII has also been shown to autophosphorylate independently of Epac2 in atrial appendage samples of AF patients. Thus, CaMKII autophosphorylation increased  $\text{Ca}^{2+}$  sensitivity of apamin sensitive small-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current ( $I_{\text{KAS}}$ ) in AF [90].

Serotonin (5-hydroxytryptamine, 5-HT) receptor mRNA transcripts are reduced by 36% in AF patients [67]. The maximum inotropic responses to 5-HT are also reduced in trabeculae of patients with AF [91] due to diminished cytosolic cAMP responses upon 5-HT in these patients regulated via PDE3 and to a lesser extend PDE4 [92]. Nevertheless, PDE3 or PDE4 regulation of 5HT-dependent cAMP levels seems to be highly compartmentalized. Thus, contrary to what happens with contraction, the PDE3 or PDE4 control on propensity of 5-HT-evoked arrhythmias on human atrial trabeculae from patients in sinus rhythm or with paroxysmal AF, is lost in persistent AF [93]. 5-HT stimulation has been shown to increase  $I_f$  [94] and  $I_{\text{CaL}}$  [91,94] in HAMs, but less than  $\beta$ -adrenergic stimulation [91,95] and without regulation of PDE3 or PDE4 [90].

Muscarinic receptor ( $M_2R$ ) activation by acetylcholine induces negative chronotropy via G-protein-activated inwardly rectifying  $\text{K}^+$  current ( $I_{\text{K,ACh}}$ ) [96,97]. Elevated  $I_{\text{K,ACh}}$  promotes proarrhythmic effects by hyperpolarizing RMP and shortening APD and refractory period. However, muscarinic receptor-mediated  $I_{\text{K,ACh}}$  activation is reduced in AF [98]. Conversely, agonist-independent constitutive  $I_{\text{K,ACh}}$  activation has been implicated in AF [99,100] via adenosine  $A_1$  receptors [101].

Adenosine exerts dose-dependent antiarrhythmic and proarrhythmic actions.  $A_1$  and  $A_3$  are  $G_i$ -coupled receptors predominantly expressed in atria and sinus node [102,103].  $A_1$  receptors promote anti-adrenergic effects by reducing isoproterenol-induced cAMP-PKA-dependent phosphorylation of  $Ca^{2+}$ ,  $K^+$  channels and contractile proteins within others [104].  $A_1$  receptors show higher expression in the RA versus LA [8].  $A_3$ , like  $A_2B$  receptors, need high concentrations of adenosine to be activated due to their low affinity.  $A_2A$  and  $A_2B$  are  $G_s$ -coupled receptors also expressed in human atria.  $A_2A$  receptor activation increases the incidence of spontaneous SR  $Ca^{2+}$  releases via RyR2 phosphorylation [105], promotes beat-to-beat irregularities [106] and activates  $K^+$  channels [107]. Importantly,  $A_2A$  receptors are upregulated in AF [108]. Anti-adrenergic actions of  $A_1A$  are counteracted by  $A_2A$  receptors [109]. However,  $A_1$  and  $A_3$  stimulation would neutralize  $A_2A$  receptors signaling [101] during ischemia or hypoxia, where  $A_1$  and  $A_3$  receptors are also activated by high levels of accumulated inosine [110].  $A_3$  activates phospholipase C (PLC) stimulating antioxidant cardio protection [111].  $A_2B$  activation inhibits endothelin-1 (ET-1) induced fibroblast proliferation, as well as angiotensin II (AngII)-mediated fibrosis via cAMP, Epac, PI3K-dependent pathways in neonatal and adult rat cardiac fibroblasts [112–114]. In contrast,  $A_2B$  knockdown or using a selective  $A_2B$  antagonist attenuated the effects of myocardial infarction on fibrosis and ventricular fibrillation in mice and rat [115,116]. The failing myocardium from chronic heart failure patients showed decreased gene expressions for  $A_2A$ ,  $A_2B$  and  $A_3$  receptors, as well as for adenosine deaminase (ADA). The decreased receptor expression likely impaired the cardioprotective signaling effects of adenosine. However, this could have been attenuated by the increased cardiac adenosine levels due to the reduced ADA expression and resulting decreased activity [117].

Mineralocorticoid receptors mRNA expression is increased in AF [118]. Aldosterone binding and subsequent cardiac fibrosis formation has been associated with AF [119]. Furthermore, aldosterone could contribute to increased intracellular  $Ca^{2+}$ , activating phospholipase C (PLC) and protein kinase C (PKC). Thus, inhibition of the renin-angiotensin-aldosterone system (RAAS) with angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARB) has been proposed for the treatment of AF to prevent oxidative stress and structural and electrical remodeling [120].

### 3.3. Oxidative Stress and Inflammation

Inflammatory states have been frequently associated with AF due to inducing oxidative stress, which plays an important role in atrial structural remodeling [121]. Inflammation can readily promote AF through oxidative stress and  $Ca^{2+}$ -dependent triggered activity. Long-term inflammation processes may promote not only electrical but also structural remodeling [122,123]. Steroidal anti-inflammatory agents can suppress atrial tachycardia induced electrophysiological changes in dogs through significantly reducing C-reactive protein (CRP) and attenuating the tachycardia induced activation of endothelial nitric oxide synthases (eNOS) [124]. Patients developing AF after coronary bypass surgery have been shown to have significantly higher preoperative CRP levels [125]. Additionally, increased CRP baseline levels have been linked to an increased risk of developing AF in the general population in a Korean health screening program spanning 11 years and a stepwise CRP elevation was seen with an increasing AF burden [126,127].

The NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome, which can be activated by oxidative stress, can in turn activate cardiac fibroblasts [128], displaying a link between inflammation and cardiac fibrosis. Importantly, increased NLRP3 inflammasome activity has been shown in patients with paroxysmal AF and chronic AF [129], and suppressing NLRP3 inflammasome activity in a mouse model of left ventricular hypertrophy attenuated cardiac fibrosis [130]. Neutrophil derived inflammation has also been shown to create an AF substrate, via myeloperoxidase (MPO) activity resulting in conduction abnormalities in a canine model [131]. RA samples of AF patients also displayed higher MPO plasma levels and larger MPO burden compared to sinus rhythm [131]. Additionally, MPO-deficient mice were protected from AF and atrial fibrosis [132]. Cell-to-

cell conduction abnormalities, like those caused by MPO activity, are often due to stress induced atrial remodeling modulating Cx40 and Cx43, interfering with gap junctions and intercellular transmission among atrial myocytes [133]. In a sheep coronary infarct model, Cx43 was redistributed from the intercalated discs to the lateral surface, increasing fibroblast-cardiomyocyte coupling and resulting in an increased associated AF risk, likely due to impaired signal transduction between myocytes [134,135]. Another study showed Cx43 mediates the induction and maintenance of AF in a canine model [136]. Connexins can be regulated through phosphorylation by multiple kinases like CaMKII, PKA or PKC [137–139]. CaMKII can be activated by oxidative stress [140], additionally resulting in phosphorylation of RyR2 and Nav1.5 [141,142], as well as causing CaMKII independent oxidation of RyR2 leading to calstabin dissociation and a  $\text{Ca}^{2+}$  leak [131,143]. Phosphorylation of Nav1.5 leads to an increase in late sodium current ( $I_{\text{NaL}}$ ) and APD, as well as increased DADs expression levels in mice atrial myocytes, and increased Nav1.5 phosphorylation levels in atrial samples from AF patients [133].

In response to oxidative stress, NOS function can become “uncoupled”, shifting the electron transfer to molecular oxygen, which causes superoxide anion formation, a reactive oxygen species (ROS) [144]. During AF, but not sinus rhythm, NOS contribute significantly to atrial ROS production. This is one possible mechanism leading to atrial oxidative injury and electrophysiological remodeling observed in AF patients [145]. In addition to uncoupled NOS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX2) has been identified as main source of ROS production during AF and sinus rhythm [145]. These two different pathways of ROS production during AF have been shown to change overtime, highlighting important implications for the prevention and management of AF. In postoperative AF patients, NOX2 and inducible NOS (iNOS) upregulation was responsible for ROS production, and statins inhibiting NOX2 and iNOS were effective at preventing AF onset. In contrast, statins were not effective in managing long-term AF, which correlated with a switch to uncoupled NOS and myocardial oxidases causing the majority of ROS production [146]. A recent study, looking at independent effects of NOX2 on AF, suggested NOX2 to mostly be a biomarker for AF-induced remodeling. This work showed that NOX2 overexpression only led to a modest increase in AF susceptibility, which was associated with a modest RyR2 dysregulation, but had no impact on AF duration [147,148].

ROS can increase the open probability of mammalian RyR2 by increasing channel sensitivity to cytosolic  $\text{Ca}^{2+}$  and ATP, due to an altered redox state of sulfhydryl groups [149]. Additionally, they can trigger depolarization of the mitochondrial inner membrane potential in guinea pig ventricular myocytes, which can cause oscillations in APD and lead to arrhythmias [150]. There is also evidence that ROS are able to activate TGF- $\beta$  signaling, which has been linked to a protective effect via attenuation of oxidative stress and reducing pro-inflammatory cytokine release [151,152].

NOS-derived nitric oxide (NO) can regulate myocardial  $\text{Ca}^{2+}$  flux in multiple ways, depending on NOS type. In mice left ventricular (LV) myocytes, neuronal NOS (nNOS)-derived NO inhibits  $\text{Ca}^{2+}$  influx via LTCC, increases SR  $\text{Ca}^{2+}$  reuptake by increasing phospholamban phosphorylation and regulates  $\text{Ca}^{2+}$  release from SR by changes to RyR2 S-nitrosylation [153,154]. Whereas eNOS-derived NO reduces myofilament  $\text{Ca}^{2+}$  sensitivity via PKG modulating cardiac relaxation in rat ventricular myocytes/guinea pig myocytes [155], reduces  $I_{\text{Ca,L}}$  in mice ventricular myocytes [156], and inhibits myocardial oxygen consumption in mice left ventricular myocytes [157]. Activation of eNOS due to mechanical stress has been shown to increase the open probability of RyR2 in porcine left atrial myocytes [158]. On the other hand, a link was observed between decrease in eNOS expression and NO bioavailability and AF, resulting from the loss of a protective effect against lesion and thrombus formation [159]. Another study found no change in eNOS expression in human left atrial appendages and also showed that NO inhibits  $I_{\text{Kv4.3}}$ , a main current of the  $\text{Ca}^{2+}$  independent component of the transient outward  $\text{K}^{+}$  current in humans, which could prolong the plateau phase of the chronic AF-remodeled AP [160]. These results suggest that restoring a physiological level of NO in human atria could play

a part in preventing and/or terminating AF, by affecting the above mechanisms as well as possibly preventing the uncoupling of NOS function.

Other ways in which NO signaling can cause aberrant  $\text{Ca}^{2+}$  signals and abnormal excitation-contraction coupling have been described. NO activates guanylate cyclase (GC), which increased intracellular 3',5'-cyclic guanosine monophosphate (cGMP), in turn inhibiting  $\text{Ca}^{2+}$  signals. In ventricular myocytes NO has a biphasic effect on sarcolemmal  $\text{Na}^+/\text{H}^+$  exchanger (NHE1). Active NHE1 results in  $\text{Na}^+$  driven cellular  $\text{Ca}^{2+}$  loading (*via* NCX) and increases the possibility of spontaneous SR  $\text{Ca}^{2+}$  signals. NO modulates NHE1 through GC/cGMP, which activates NHE1 via PKG. High levels of cGMP then inhibit PDE3, resulting in an increase of cAMP and NHE1 inactivation by PKA, causing the biphasic effect of NO and major implications for targeting NHE1 as treatment for fibrillations [161]. The impact of cGMP/cAMP crosstalk via NO signaling has also been observed on the  $\text{Ca}^{2+}$  dynamics in HAMs [83,162].  $\text{I}_{\text{Ca,L}}$  density increased with increasing concentrations of NO-donor SNAP and PDE3 inhibition enhanced  $\text{I}_{\text{Ca,L}}$  to similar extent as SNAP. Conversely, SNAP reduced  $\text{I}_{\text{Ca,L}}$  when applied on top of PDE3 inhibition or  $\beta\text{AR}$  stimulation, when cAMP was previously elevated, pointing to cGMP-cAMP cross-regulation. However, in atrial myocytes from patient with AF, basal stimulatory effects of SNAP were lost, while its inhibitory effect when cAMP was previously elevated by PDE3 inhibition or  $\beta\text{AR}$  stimulation was preserved [77]. Importantly, cGMP levels were found to be significantly higher in patients with AF [163,164].

Besides the dual substrate PDEs (PDE2 and PDE3) leading to a crosstalk between cGMP and cAMP, one important effector target of cGMP is PKG. NO activates GC, which increase intracellular cGMP that stimulates PKG. PKG in turn phosphorylates the muscarinic receptor-activated  $\text{I}_{\text{K,ACh}}$  channel in sinus rhythm patients, but does not seem to be involved in the muscarinic-dependent activation of the constitutively active channel in cAF patients. This constitutive activation is due to abnormal phosphorylation by PKC, the expression of which is upregulated in right atrial appendage samples of chronic AF patients [100].

Natriuretic peptides (NP) can also activate the cGMP pathway. The atrial natriuretic peptide, which is secreted by atrial myocytes, promotes epicardial progenitor cells differentiation in adipocytes, favoring fibrosis substrate from the epicardial adipose tissue and promoting AF [165]. B-type natriuretic peptide antagonizes the pro-fibrotic effects of TGF- $\beta_1$ , AngII and ET-1 [166]. In accordance with this, a naturally 17-fold higher expression of natriuretic peptide clearance receptor (NPR-C) in the atria compared to ventricles of wildtype mice, has been linked to the increased atrial susceptibility to TGF- $\beta_1$ -mediated fibrosis [167,168].

#### 4. Therapeutic Implications and Future Directions

Despite the significant advances in AF management, especially regarding stroke prevention with antithrombotics and maintaining sinus rhythm with invasive strategies, current treatment options still have significant limitations. Catheter ablation has limited efficacy, particularly in patients with long standing persistent AF [169]. Early rhythm control therapy may lower the risk of AF-associated complications and ameliorate outcomes [170]. On the other hand, the complex nature of AF pathophysiology hinders drug-based AF-management of both rate and rhythm control. Although recent advances in our understanding of the underlying mechanisms promoting and maintaining AF have led to implementing novel antiarrhythmic drug therapies, the current available treatments has limited efficacy, extracardiac toxicity and ventricular proarrhythmogenic effects [171,172]. These weaknesses emphasize the need of (1) better understanding of all pathomechanisms underlying each type of AF, and (2) major conceptual changes in our approaches looking for pharmacological targets. Thus, multiple regulatory networks have been identified to promote and/or maintain AF (i.e., inflammation, oxidative stress, fibrosis, genes and autonomic changes), but not many studies give information about the role of specific targets on the different steps and progression of AF. Furthermore, early identification of

abnormalities may lead to early interventions to prevent AF, or AF progression. Next, the biggest dare would be to develop new therapeutic approaches to treat AF without side effects. Full understanding of AF mechanisms for defining patient-specific treatments depending on the progression stage of the arrhythmia and the specific driven mechanisms, achieving atrial selectivity on the new molecular therapeutic targets, and identifying new circulating biomarkers to detect and classify AF, may be the next potential major challenges in AF research.

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