Atrial Fibrillation

Atrial fibrillation: pathophysiology, genetic and epigenetic mechanisms

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Summary

Atrial fibrillation (AF) is the most common supraventricular arrhythmia affecting up to 1% of the general population. Its prevalence dramatically increases with age and could reach up to ~10% in the elderly. The management of AF is a complex issue that is object of extensive ongoing basic and clinical research, it depends on its genetic and epigenetic causes, and it varies considerably geographically and also according to the ethnicity. Mechanistically, over the last decade, Genome Wide Association Studies have uncovered over 100 genetic loci associated with AF, and have shown that European ancestry is associated with elevated risk of AF. These AF-associated loci revolve around different types of disturbances, including inflammation, electrical abnormalities, and structural remodeling. Moreover, the discovery of epigenetic regulatory mechanisms, involving non-coding RNAs, DNA methylation and histone modification, has allowed unravelling what modifications reshape the processes leading to arrhythmias. Our review provides a current state of the field regarding the identification and functional characterization of AF-related genetic and epigenetic regulatory networks, including ethnic differences. We discuss clear and emerging connections between genetic regulation and pathophysiological mechanisms of AF.

Keywords: Atrial fibrillation; Epigenetics; Genetics; Mechanisms

Introduction

Atrial fibrillation (AF) is a problem of global scope and increasing dimension.[1] AF is diagnosed by electrocardiographic recording. The typical finding is rapid (>300/min), irregular and often low-amplitude atrial activity, with an irregular ventricular response.

The risk of AF is significantly lower in non-White races and ethnicities (including Blacks, Hispanics and Asians) than Whites.[2–5] These differences occur despite a comparable or higher burden of risk factors (especially in Blacks) and point to a possible genetic basis.[6] Indeed, there is an decreased risk of AF in first-degree relatives of non-White (Black or Hispanic) early-onset AF-patients compared to first-degree relations of Whites, pointing to genetic factors.[7] In addition to genetic factors, epigenetics (factors controlling gene expression rather than gene sequence variations *per se*) have the capacity to influence racial/ethnic and regional occurrence and consequences of AF. Finally, emerging work points to an important role of inflammation, including inflammatory signaling in non-inflammatory cells, as playing an important role in AF occurrence.[8] This paper aims to review the rapidly evolving areas of genetic and epigenetic control of AF, with a view to considering their possible implication in the regional distribution and occurrence of AF as well as future developments in research and therapeutic innovation.

Genetic basis of AF and ethnic/racial variability

AF is increasingly recognized as having a strong genetic component.[9,10] Three genetic approaches have historically been applied to AF: (i) linkage analysis using families

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Key messages

- Atrial fibrillation (AF) is the most common supraventricular arrhythmia affecting up to 1% of the general population: its prevalence reaches up to ~10% in the elderly.
- AF insurgence depends on intertwined genetic and epigenetic causes.
- The risk of AF is significantly lower in non-White races and ethnicities (including Blacks, Hispanics and Asians) than Whites.
- Genome Wide Association Studies (GWAS) and Epigenome Wide Association Study (EWAS) in multiethnic studies of AF in underrepresented populations are powerful approaches to investigate the genetic basis of racial and ethnic variation in AF.
- GWAS and EWAS help in generating clinically potentially usable polygenic risk scores and epigenetic clocks for AF, with most studies conducted in individual of European ancestry.
- Integration of "omics" (genomics, transcriptomics, proteomics, and metabolomics) and artificial intelligence-driven network approaches are increasingly robust tools for detecting AF and for stratifying patients.
- A prototype case of a molecular link between epigenetic, genetic and pathophysiological mechanisms influencing AF and its racial and ethnic variations is PITX2, a homeobox transcription factor regulating cardiac conduction, modulation of ion channels and cardiac development.

with Mendelian forms of AF, (ii) Genome Wide Association Studies (GWAS), and (iii) coding variation from genome sequence data. The oldest approach, linkage analysis, was performed in families with a clear hereditary pattern, based on the tendency for a genetic marker near a disease-causing gene to be inherited together with the causal gene. AF-associated genes that were discovered through family based or gene-based studies include the ion channel subunit KCNQ1,[11] atrial natriuretic peptide ANP,[12] the transcription factor TBX5,[13] cytoskeletal/contractile proteins MYL4[14] and TTN[15] in different ethnicities (Table 1). Genetic linkage studies have been very informative but the percentage of even early-onset AF (<60 years-old) cases that reveal mutations is only ~10%, with mutations located predominantly in the TTN gene, among others.[33,34] GWAS were applied to AF for the first time in 2007 in people of European and Chinese descent and identified variants in chromosome 4q25, for which the closest gene is Pitx2, associated to AF.[19] Since then, GWAS studies have identified >100 AF candidate genes and risk loci, with protein-coding genes implicated in fibrosis and extracellular matrix remodeling, cardiomyogenesis, cell–cell coupling, ion-channel function or nuclear structure. In fact, most AF-associated GWAS variants are located in the noncoding genome, with a range of genes, such as Pitx2, ZFHX3, PRRX1, TBX5, NKX2–5, HAND2, MYL4, TTN and SYNPO2L that have been implicated in mediating effects.[35–39] A study based on Finnish biobanks from 12,859 AF cases and 73,341 controls, recently identified the loss-of-function splice site variant with a substantial effect size in the SYNPO2L gene, encoding a cytoskeletal/contractile protein involved in sarcomere organization, which was associated with a significantly increased risk of AF.[40] A larger GWAS, comparing a total of 60,620 cases and 970,216 controls of European ancestry from six contributing studies (including the UK Biobank and the AFGen Consortium), identified risk variants that fall near genes where more deleterious mutations have been reported to cause serious heart defects in humans (i.e., PITX2, TBX5), or near genes important for cardiac muscle function and integrity[20] (Table 1). Coding variants can be inherited, but also acquired, or somatic, which historically has been a focus of cancer genomics. A potential role of somatic mutations in connexin (40 and 43) genes has been shown in the pathogenesis of some AF cases.[41,42] However, other recent small scale studies deep sequencing DNA from AF patients found no evidence of somatic variants within the coding regions of LA posterior wall tissue, suggesting that atrial-specific mutations might be rare and unlikely to exert a prominent role in AF pathogenesis; or they might be technical artifacts linked to sample storage conditions.[43,44]

GWAS in multiethnic studies of AF genetics in underrepresented populations represent an invaluable approach to investigate the genetic basis of racial and ethnic variation in AF. Genome-wide admixture analysis of CHS (n = 4173), the ARIC (n = 12,341) study, and the Health ABC (n = 1015) showed that the protective allele of the AF SNP rs10824026 was more common among Black than White patients.[16] This SNP is located 5 kb upstream of the SYNPO2L gene.[16] Investigation of both common and rare variants in a large collection of individuals in the AFGen Consortium, by meta-analyses of 33 GWAS studies, including 22,346 individuals with AF and 132,086 referents, identified 12 new AF loci.[21] Among these, only variants in PITX2 genomic region significantly associated with AF in European, African and Japanese ancestries[21] (Table 1). The largest multi-ethnic meta-analysis of GWAS for AF to date has been performed by Roselli et al., and consisted of >500,000 individuals, including 65,446 with AF from the UK Biobank, AFGen Consortium, Broad AF Study and Biobank Japan.[28] The study identified 97 loci significantly associated with AF, including 67 that were novel, in a combined-ancestry analysis, and 3 that were novel in a European-specific analysis.[28] The latter 3 loci were located close to or within the genes CDK6 involved in cell cycle control, EPHA3 (a receptor involved in tyrosine kinase signaling), and GOSR2 (involved in intracellular vesicular transport) (Table 1). Importantly, single nucleotide variations identified by GWAS helped in generating clinically usable polygenic risk scores (PRS) for AF.[45–47] In more detail, PRS is a single value estimate of an individual's genetic liability to a disease. It is calculated by the sum of an individual's risk alleles, weighted by risk allele effect sizes derived from GWAS data. Several private companies nowadays use PRS to offer a comprehensive and integrated approach to enhance care management for CVD, including AF, as companion diagnostics. Genetic

Technological advance	European descent (targets)	African descent (targets)	Asian descent (targets)	Other descents (targets)
Genetics				
Familial AF and linkage analysis	ANP[12]; TBX5[13]; MYL4[14]			
Genome Wide Association Studies (GWAS)	MYOZ1[16]; PLEC[17]; MYL4[18]; PITX2[19–23]; TTN[15,24]; GATA4[20]; MYH6[20,25]; NKX2–5[20]; CFL2[20]; MYH7[20]; PKP2[20]; RBM20[20]; SGCG[20]; SSPN[20]; ZFHX3[26]; KCCN3[21,27]; CEP68[21]; KCNN2[21]; SOX5[21]; SH3PXD2A[21]; METTL11B[21]; CEP68[21]; KLHL3–WNT8A–FAM13B[21]; ASAH1[21]; KCNJ5[21]; SCN[22]; CDK6[28]; EPHA3[28]; GOSR2[28]; UBE4B[28]; CASZ1[28]; CASQ2[28]; GJA5[28]; NUCKS1[28]; KIF3C[28]; XP01[28]; REEP1[28]; KDM3A[28]; WIPF1[28]; CHRNA[28]; SPATS2L[28]; LRIG1[28]; PHLDB2[28]; GNB4[28]; WDR1[28]; SLC9B1[28]; CAMK2D[28]; ARHGAP10[28]; ARHGAP26[28]; NR3C1[28]; NKX2–5[28]; ATXN1[28]; KDM1B[28]; CDKN1A[28]; UST[28]; DGKB[28]; CREB5[28]; GTF2I[28]; COG5[28]; KCNH2[28]; XP07[28]; FBX032[28]; PTK2[28]; SLC24A2[28]; MLLT3[28]; ZNF462[28]; PSMB7[28]; REEP3[28]; NAV2[28]; SSPN[28]; PKP2[28]; NACA[28]; BEST3[28]; KRR1[28]; PHLDA1[28]; TBX5-AS1[28]; TBX3 intergenic[28]; DNAH10[28]; MYH7[28]; AKAP6[28]; SNX6[28]; CFL2[28]; LRRC 74[28]; IRF2BPL[28]; USP3[28]; TLE3[28]; UACA[28]; IGF1R[28]; POLR2A,[28] TNFSF1 2[28]; MYOCD[28]; MAPT[28]; KCNJ2,[28]CASC17[28]; SMAD7[28] CASC20[28]; BMP2,[28] PRRX1[23]; CAV1[23]; SYNE3[23]; FBP1/2[23]; HCN4[23]; SYNPO2L[23]; WNT8A[23]; SCN10A[29]; TUBA8[28]; ZFHX3[23]	MYOZ1[16]; PITX1[21]; UBE4B[21]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1[21]; KIF3C[21]; XP01[21]; REEP1[21]; KDM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5[21]; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032[21]; PTK2[21]; SLC24A2[21]; MLLT3[21]; ZNF462[21]; PSMB7[21]; REEP3[21]; NAV2[21]; SSPN[21]; PKP2[21]; NACA[21]; BEST3[21]; KRR1[21]; PHLDA1[21]; TBX5-AS1,[21] TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21]; TLE3[21]; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21]; KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21]; BMP2[21]; TUBA8[21]	KCNQ1[11]; CAV1[23,30]; PITX2[19–23]; PRRX1[23]; SH3PXD2A[21]; UBE4B[21]; ZFHX3[23]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1[21]; KIF3C[21]; XP01[21]; REEP1[21]; DM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5[21]; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032[21]; PTK2[21]; SLC24A2[21]; MLLT3[21]; ZNF462; PSMB7[21] REEP3[21]; NAV2[21]; SSPN[21] PKP2[21]; NACA[21]; BEST3[21]; KRR1[21]; PHLDA1[21]; TBX5-AS1[21]; TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21]; TLE3[21]; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21]; KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21]; BMP2[21]; TUBA8[21]	PITX2[31]; DTNA[32]; UBE4B[21]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1[21]; KIF3C[21]; XP01[21]; REEP1[21]; KDM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032;[21] PTK2[21]; SLC24A2,[21] MLLT3[21]; ZNF462[21]; PSMB7[21]; REEP3[21]; NAV2[21]; SSPN[21] PKP2[21]; NACA[21]; BEST3[21]; KRR1[21]; PHLDA1[21]; TBX5-AS1[21]; TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21]; TLE3[21]; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21]; KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21],[21]; TUBA8[21]

Table 1: **Genetic components of AF pathogenesis, identified by technological advance and by geography of ancestry.**

mutations revealed by genetic linkage studies, GWAS, and coding variation studies may also help to elucidate the sex bias in AF towards men[48] and the increased prevalence/risk of AF due to habitual alcohol consumption in East Asians compared to Europeans.[49,50] In addition, they have shown a substantial overlap between genetic variants implicated in AF and atrial cardiomyopathy.[37] In this respect, pathway and functional transcriptomic enrichment analyses suggested that putative AF genes act via cardiac structural remodeling, as observed in atrial cardiomyopathy.[20,51] It emerges from the studies discussed in this section that the majority of genetic analyses for AF have been performed in individuals of European descent. European ancestry *per se* is an independent risk factor for incident AF,[52] and the heritability of AF based on common genetic variants in individuals of European ancestry has been estimated to be ~22%.[53] However, the tendency to focus on European ancestry has led to a non-representative distribution of ancestries in genetic studies compared with the real-world diversity, which may negatively impact the applications of scores to other ethnicities.

Epigenetic basis of AF

Epigenetics includes three main regulatory systems that determine chromatin remodelling and gene transcription regulation: DNA methylation, regulation of transcription and translation by non-coding RNAs, and chromatin remodelling (histone modifications). The functional crosstalk among these epigenetic processes contributes strongly to determining cell phenotype in AF (Fig. 1). All epigenetic processes have been implicated in AF pathogenesis (Table 2). During DNA methylation, a methyl group is catalyzed by DNA methyltransferases (DNMTs) to shift from the S-adenosyl- L-methionine to the 5′ carbon of cytosine, which are mostly located in cytosine-phosphate-guanine (CpG) islands. Gene-promoter hypermethylation causes transcriptional silencing, whereas hypomethylation leads to increased expression of the gene. AF might be associated with global DNA hypermethylation.[60] The concept of Epigenome Wide Associated Studies (EWAS) was first introduced in 2011,[71] and led to spurring of many correlative studies. In 2016, a report on whole genome methylation in the Offspring Cohort of the Framingham Heart Study, including 183 participants of European descent with prevalent AF and 220 with incident AF with up to 9 year follow up revealed that a CpG site locus in CUX2, a transcription factor, was the most significant SNP associated with AF.[54] The promoter of the PITX2 gene, which consistently emerges from most AF-related GWAS, was found hypermethylated in the regions

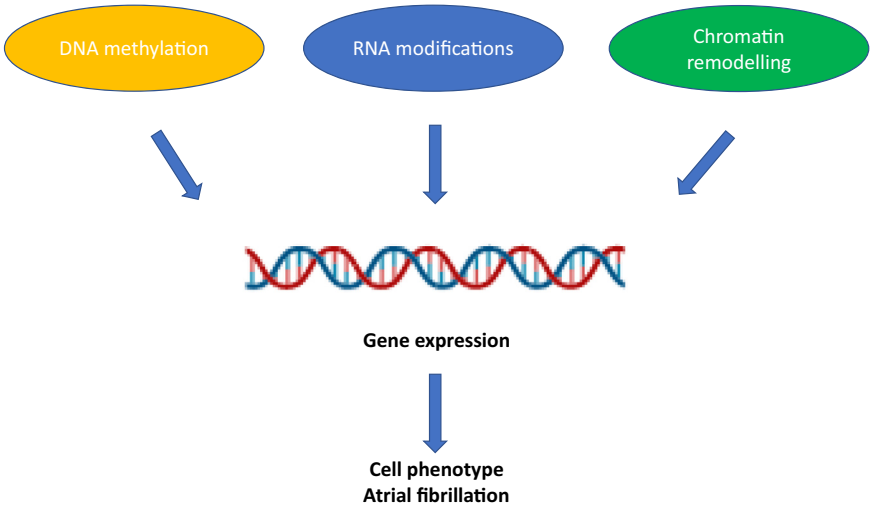

Fig. 1: Major epigenetic mechanisms regulating gene expression and cell phenotypes in AF.

encompassing LA or pulmonary veins-LA junctions of affected individuals of European descent in a small sample size study.[55] The promoter of KIF15, a cytoskeletal protein is hypermethylated in a small size Chinese AF cohort, and predictive tools computed a concomitant regulation of PSMC3, TINAG, and NUDT6 genes.[61] New-onset AF is a known postoperative complication after cardiac surgery: Fischer et al. identified two loci associated with AF in this setting, namely EYS and LINC01683,[56] and developed a DNA methylation biomarker-based prediction model for the future occurrence of postoperative AF associated with cardiac surgery in a European cohort.[72] The latter prospective study was conducted on a medium size cohort (n = ~200), with ROC curves displaying AUC values in the range of 0.7–0.8, thus demonstrating the potential of precision medicine to develop models combining epigenomic and clinical data to predict AF. Epigenetic clocks feed fine-tuned algorithms to calculate biological age based on a read-out of the extent to which dozens of CpG sites across an individual's DNA are methylated.[73] A multi-ethnic study involving 5600 participants with 905 incident AF cases during a mean follow-up of 12.9 years revealed that some epigenetic clocks were associated with a statistically significant higher incidence of AF after adjusting for chronological age, race, sex, and smoking variables, demonstrating that biological (epigenetically measured) aging might play a role in AF development independent of chronological age.[73,74] However, a potential causal relationship between epigenetic clocks (or DNA methylome), aging and AF remains unexplored. A recurrent feature of DNA methylation studies is that profiles were measured from blood, which could vary from the ones found in atria or specific tissues/cell types. However, invasive specimen collection is often not feasible in human studies, and blood is frequently used and accepted as a proxy tissue to assess DNA methylation.

Within the structure of the nuclear chromatin, the N-terminal histone tails of all histones and the C-terminal tails of H2A histones stem from the nucleosome core structure. These tails are preferentially accessible and target sites for many post-translational modifications (PTMs).[75] PTMs such as phosphorylation, acetylation, and methylation can strongly modify chromatin conformation and affect gene expression. Each PTM type is regulated by *ad hoc* enzymes. For instance, histone deacetylase (HDACs)-related epigenetic mechanisms have been implicated in AF regulation, by ventricular electrical remodeling (reviewed in[76,77]). Few examples related to enzymes regulating specific PTM in the context of AF are illustrated below.

Both rare and common variants in the gene encoding KCNN3, a calcium-activated K^+ channel, have been associated with AF.[21,27] KCNN3 expression is reducedin AFvia HDAC2-dependent histone PTM in atrial cardiomyocytes.[78–80] The expression of EZH2, encoding the histone methyltransferase responsible for methylation of histone H3 lysine 27, a hallmark of epigenetic gene silencing is upregulated in permanent AF patients with atrial fibrosis, and its repression/activation correlated with anti-fibrotic/pro-fibrotic mechanisms, respectively.[57] In this respect, chromatin immunoprecipitation (ChIP) is a powerful tool to analyze protein-DNA interactions in vivo. ChIP-seq, which combines ChIP with next generation sequencing (NGS) technology, can efficiently detect genome-wide DNA segments that interact with histones or other epigenetic players. A ChIP assay showed increased specific binding of EZH2 to the α-SMA promoter, providing evidence that EZH2 causes atrial fibroblast activation through this binding.[57]

Technological advance	European descent (targets)	African descent (targets)	Asian descent (targets)	Other descents (targets)
Epigenetics				
Epigenome Wide Association Study (EWAS)	CUX2[54]; PITX2[55]; EYS,[56] LINC01683.[56]			
Chromatin Immunoprecipitation sequencing (ChIP-Seq)	αSMA[57]; PITX2[58]	MYOZ1[16]; PITX1[21]; UBE4B[21]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1[21]; KIF3C[21]; XP01[21]; REEP1[21]; KDM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5[21]; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032[21]; PTK2[21]; SLC24A2[21]; MLLT3[21]; ZNF462[21]; PSMB7[21]; REEP3[21]; NAV2[21]; SSPN[21]; PKP2[21]; NACA[21]; BEST3[21]; KRR1[21]; PHLDA1[21]; TBX5-AS1,[21] TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21]; TLE3[21]; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21]; KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21]; BMP2[21]; TUBA8[21]	KCNQ1[11]; CAV1[23,30]; PITX2[19–23]; PRRX1[23]; SH3PXD2A[21]; UBE4B[21]; ZFHX3[23]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1 ([21]; KIF3C[21]; XP01[21]; REEP1[21]; DM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5[21]; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032[21]; PTK2[21]; SLC24A2[21]; MLLT3[21]; ZNF462; PSMB7[21] REEP3[21]; NAV2[21]; SSPN[21]; PKP2[21]; NACA[21]; BEST3 ([21]; KRR1[21]; PHLDA1[21]; TBX5-AS1[21]; TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21] TLE3 2221; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21] KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21]; BMP2[21]; TUBA8[21]	PITX2[31]; DTNA[32]; UBE4B[21]; CASZ1[21]; CASQ2[21]; GJA5[21]; NUCKS1[21]; KIF3C[21]; XP01[21]; REEP1[21]; KDM3A[21]; WIPF1[21]; CHRNA[21]; SPATS2L[21]; LRIG1[21]; PHLDB2[21]; GNB4[21]; WDR1[21]; SLC9B1[21]; CAMK2D[21]; ARHGAP10[21]; ARHGAP26[21]; NR3C1[21]; NKX2–5[21]; ATXN1[21]; KDM1B[21]; CDKN1A[21]; UST[21]; DGKB[21]; CREB5; GTF2I[21]; COG5[21]; KCNH2[21]; XP07[21]; FBX032,[21] PTK2[21]; SLC24A2,[21] MLLT3[21]; ZNF462[21]; PSMB7[21]; REEP3[21]; NAV2[21]; SSPN[21] PKP2[21]; NACA[21]; BEST3[21]; KRR1[21]; PHLDA1[21]; TBX5-AS1[21]; TBX3 intergenic[21]; DNAH10[21]; MYH7[21]; AKAP6[21]; SNX6[21]; CFL2[21]; LRRC 74[21]; IRF2BPL[21]; USP3[21]; TLE3[21]; UACA[21]; IGF1R[21]; POLR2A[21]; TNFSF1 2[21]; MYOCD[21]; MAPT[21]; KCNJ2[21]; CASC17[21]; SMAD7[21]; CASC20[21],[21]; TUBA8[21]
Integrated omics approaches				
ChIP-Seq, transcriptomics	NKX2-5[59]; TBX3[59]; ZFHX3[59]; SYNPO2L[59]; MYH7[59]		NPRA[60]; KIF15[61]	
ChIP-Seq, mass spectrometry	ATP5L[62]; CES1D[62]; MYL7[62]; NDUFA12[62]; NDUFA8[62]; NDUFS7[62]; PDHA1[62]; TNNI3[62]; UQCR10[62]			
GWAS, CHiP-Seq, ATAC-Seq, transcriptomics	NKX2.5[63]			
GWAS, ChIP-Seq, transcriptomics, mass spectrometry	NKX2.5, TNNT2, CYB5R3, NDUFB3, HIBADH NDUFA9, DLAT, PPIF, MYL4, CKM, NKX2-5, MYL7[64]; PGAM2[64]; TNNC1[64]; CYC1[64]; ETFB[64]; PRDX5[64]; AK1[64]; ALDOA[64]; TCAP[64]; TOM1L2[64]		UBADC1,[65] Quinic acid,[65]Diosmetin,[65] IGK,[65] alpha-Ergocryptine,[65] Fludrocortisone acetate,[65] 2,6-Di-tert-butylbenzoquinone,[65] NBL1,[65] NPC2,[65] SNCA[65]	
GWAS, EWAS, transcriptomics	ADORA1[66]; ATP1A3[66]; ATP1B2[66]; CACNA1D[66]; KCNQ4[66]; NR3C2[66]; THRA[66]			
ATAC-Seq, transcriptomics	BMP10[35]; SMYD2[35]; PITX2[35]; MYOT, TBX5[35]; GJA1[35]; CAV1[35]; HCN4[35]; SPATS2L[35]; PLN[35]; KCNH2[67]			
ATAC-Seq, Cut&Run, transcriptomics	TBX5-p.G125R variant[68]			
Network/artificial intelligence				
CNN GWAS	PITX2[69]; SCN5A[70]; TTN[70]	PITX2[69]		PITX2[69]

Table 2: **Epigenetic components of AF pathogenesis, identified by technological advance and by geography of ancestry.**

However, it remains unexplained how deposition of H3K27me3 would reconcile with increased with pro-fibrogenic α-SMA gene expression in atrial fibroblasts. Decreased expression of targets of the CREB/CREM/ATF transcription factor family was previously linked to AF susceptibility in humans, and the CREM repressor isoform CREM-IbΔC-X is upregulated in human AF.[62] Heart-specific transgenic CREM-IbΔC-X mice

represent a model of spontaneous onset AF. Prolonged treatment with valproic acid (VPA, an anticonvulsant drug and a potent HDAC inhibitor) treatment increased H4-acetylation (a readout of HDAC activity) in atria of CREM-IbΔC-X mice.[62] A ChIP-PCR assay directed against the CREM and its targets in CREM-IbΔC-X versus wild type mice, revealed a counter-regulatory effect of VPA in this AF mouse model, supporting the VPA-dependent functional delay of AF onset.[62]

This approach identified nine VPA-upregulated genes/proteins (Atp5l, Ces1d, Myl7, Ndufa12, Ndufa8, Ndufs7, Pdha1, Tnni3, Uqcr10), which were downregulated in the atria of a transgenic mouse model of AF (CREM-IbΔC-X)[62] (Table 2). ChIP-Seq analysis revealed that Pitx2 directly binds to conserved chromatin upstream of two specific microRNAs (miRs): miR-17-92 and miR-106b-25[58] (see also section "*Genetic regulators operating via pathophysiological mechanisms that remain to be clarified- the prototype case of PITX2*"). In turn miRs are a class of non-coding RNAs [ncRNAs, which also include long ncRNAs (lncRNAs) and circular RNAs (circRNAs)], epigenetic modulators of gene expression recognized as major regulatory gatekeepers of protein-coding genes in the human genome. The role of post-transcriptional ncRNA regulators in specific events related with AF has started to be unveiled in the last years and has been extensively reviewed.[76,77,81,82] There is evidence that miRs are involved in both electrical and structural remodeling in AF. MiR-26 is an example of the former: its increased expression in AF resulted in augmented expression of KCNJ2 encoding a potassium channel, causing an enhanced vulnerability to AF in mice.[83] Conversely, miR-21 is an example for the involvement of miRs in structural remodeling. Upregulation of miR-21 in AF indirectly promotes fibrosis via downregulation of SPRY1, a negative regulator of profibrotic cellular signaling pathways.[84,85] MiRs therapeutics for AF appear promising but they are still in their infancy. One of the primary concerns is miRs' ability to target multiple pathways in multiple tissues. Further research must be carried out to confirm their safety and therapeutic potential for AF. Overall, a better understanding of the mechanisms of epigenetic gene regulation may provide improved tools to define the links between genetic variation and atrial function in AF across different ethnicities.

Integrated and artificial intelligence-driven analysis in AF

Technologies such as NGS and mass spectrometry will help enormously in understanding molecular pathophysiology, and relating the results obtained in animal models to those obtained in man, to ultimately translate basic information into for clinical application. "Integrated omics" combine multiple omics datasets from sources like genomics, transcriptomics, proteomics, and metabolomics. By integrating three types of omics data (i.e., GWAS, EWAS and transcriptome-wide association study), many more relevant AF-related genes were identified than using GWAS alone from the AFGen consortium.[66,86] Most integrated omics studies have focused on individuals of European ancestry. Benaglio et al. analyzed transcriptomic and epigenomic data from induced pluripotent stem cell-derived cardiomyocytes from seven related individuals and identified ~2000 single-nucleotide variants associated with NKX2-5 binding—as assessed by ChIP-Seq, showing that differential binding at numerous regulatory variants across the genome might contribute to ECG phenotypes in AF.[63] The same study experimentally and functionally validated two NKX2-5 binding sites in these variants (rs3807989, within the locus of CAV1; and rs590041, within the locus of SSBP3), showing that they underlie ECG GWAS signals.[63]

Van Ouwerkerk et al. unraveled the genetic predisposition for AF by probing the non-coding regions of the genome. Using available and newly generated data, they prioritized candidate genes that may be affected by 104 SNP previously associated to AF,[28] by cross-referencing transcriptomic, epigenomic, and chromatin conformation datasets, and studied the impact of SNP-containing regulatory elements (RE) on putative target gene expression.[35] RE activity is limited to topologically associating domain, which in turn is a tissue independent feature. The authors assessed which gene promoters are contacted by the putative RE by integrating available chromatin conformation data (measured with Hi-C, a method to study the three-dimensional architecture of genomes) and the levels of genes expressed in the human left atrial posterior wall that play a role in cardiac conduction, force of contraction, and heart development.[35] Finally, they also identified new potential cardiac regulatory elements by Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq), a method for genome-wide mapping of chromatin accessibility at the regions associated with AF, generating a dataset for transcription factor motif enrichment, and deployed an enhancer prediction tool (EMERGE). This integrative study ultimately identified BMP10, SMYD2, PITX2, MYOT, TBX5, GJA1, CAV1, HCN4, SPATS2L, and PLN genes, among others, to be likely affected by AF associated variants and proposed a role for new regulatory region variants in modulating the expression of the potential AF genes.[35] This sophisticated approach is able to prioritize potentially AF-related functional variants and, interestingly, it was validated for some AF-associated regions (i.e., GJA1) in the mouse using genome editing. Nevertheless, the involvement of such variant REs in AF-relevant gene regulation and pathophysiology was based on a number of assumptions and prompts further studies. Weber Hall et al. recently created a model of left atrium (LA)

chromatin states (profiling 7 histone post-translational modifications by ChIP-Seq: H3K4me3, H3K4me2, H3K4me1, H3K27ac, H3K36me3, H3K27me3, H3K9me3), integrated with transcriptomics (RNA-Seq), DNA methylation, long range chromatin interaction data from primary human left atrium and ventricle derived from Hi-C, GWAS data and electrocardiographic traits in AF patients.[59] In more detail, the authors first profiled histone modifications in 5 healthy human LA tissues and produced a model of potential chromatin states. This model was then integrated with gene expression profiles, and DNA methylation to validate the functionality of chromatin states. They then identified active enhancers (RE) defined by H3K27ac and H3K4me1 colocalization and by bioinformatics tools. They identified gene-enhancer connections using HiC performed in LA, analyzed these genes for enriched pathways, and prioritized CVD effector genes found at GWAS. Adopting this workflow, they have defined a robust gene regulatory network underlying AF susceptibility, composed of known players such as NKX2-5, TBX3, ZFHX3, SYNPO2L and MYH7.[59] Although not leading to the identification of new AF-targets, the latter integrated genetic/epigenetic/transcriptomic pipeline could be applied to large AF cohorts and to study inter-ethnic/racial differences. Technological advances also led to combine single cell ATAC-Seq with RNA-Seq, allowing in parallel chromatin accessibility and transcriptome, in adult human hearts. This approach led to the identification and characterization of AF variants associated to the locus of KCNH2, a potassium channel[67]; and of the pathogenic missense variant p. G125R in TBX5 causing Holt–Oram syndrome and early onset of atrial fibrillation.[68] A landmark study by Assum et al. performed a multi-omics analysis integrating genomics, transcriptomics and proteomics in human atrial tissue in a AF case control cohort (n = 118).[64] In genetics, cis and trans regulatory elements differ from their ability to regulate nearby and distant genes, respectively. Using published data or data newly obtained with Affymetrix GeneChip Arrays, Assum et al. first integrated cis-expression quantitative trait loci (cis-QTL) and cis-protein quantitative trait loci (pQTL) analysis to allow the distinction between functional regulatory mechanisms with consequences for mRNA and protein levels. Second, they extended the cis-QTL analysis by combining it with a PRS for AF, a concept previously applied for blood-derived gene expression and termed eQTS.[87] Integration of partially divergent eQTS and pQTS, trans-associations with AF GWAS SNPs and gene ontology analysis led to identify core gene pathways for AF. Third, they focused on the key AF gene NKX2-5, largely based on the above-mentioned functional study of Benaglio et al.,[63] and they observed a strong correlation between the AF disease variant SNP rs9481842 and the NKX2-5 transcript. Finally, the authors showed that in addition to cis-regulatory elements, trans-acting mechanisms are important for the NKX2-5-mediated link between the genetic variant rs9481842 and AF. Through integration of NKX2-5 ChIP-Seq, HiC and the genetic variant rs9481842 data, NKX2-5 specific targets were identified and proposed as AF priority putative core genes, for further experimental research: TNNT2, CYB5R3, NDUFB3, HIBADH NDUFA9, DLAT, PPIF, MYL4, CKM, MYL7, PGAM2, TNNC1, CYC1, ETFB, PRDX5, AK1, ALDOA, TCAP, TOM1L2.[64]

Artificial intelligence (AI), and in particular machine learning (ML, the application of AI into a system or machine, which helps it to self-learn and improve continually), research has become increasingly common and popular for AF diagnosis and patient stratification.[64,88] Neural networks are a branch of ML models that are built using principles of neuronal organization constituting animal brains. In this context, neural network (CNN) analysis of large-scale genetic data such as GWAS and EWAS is expected to be faster, more efficient and accurate than other types of neural networks, such as artificial neural network (ANN) and recurrent neural network (RNN). Application of a CNN analysis to a multi-ethnic AF network GWAS for early-onset AF, including 6358 subjects from four independent cohorts (Korean, Japanese, European, multi-ethnic), led to moderate-to-high predictive power and confirmed the known target PITX2 by assigning it a high saliency score among the AF associated SNPs.[69] Libiseller-Egger et al. used a previously published CNN[89] to predict the cardiovascular age of 36,349 participants of the UK biobank from their ECGs, and performed a GWAS on the difference between predicted and chronological age (delta age). The analysis identified eight loci associated with delta age including genes linked to AF, such as SCN5A and TTN, indicating a potentially increased disease susceptibility.[70] AI-driven network approaches are increasingly robust and useful tools for detecting polygenic diseases such as AF, by capturing the cumulative effects and interactions between omics.

Connecting genetic regulation to pathophysiological mechanisms

Genetic regulators operating via clear pathophysiological mechanisms

Many of the identified genetic and epigenetic factors controlling AF risk operate through well-identified pathophysiological mechanisms (Fig. 2). The details of the mechanisms shown in the figure go beyond the scope of the present manuscript-the interested reader is referred to some detailed reviews.[90,91] In brief, 2 main arrhythmogenic mechanisms lead to AF: atrial reentry and ectopic atrial activity. These principal mechanisms and their underlying promoters are color-coded brown and green, respectively, in Fig. 2.

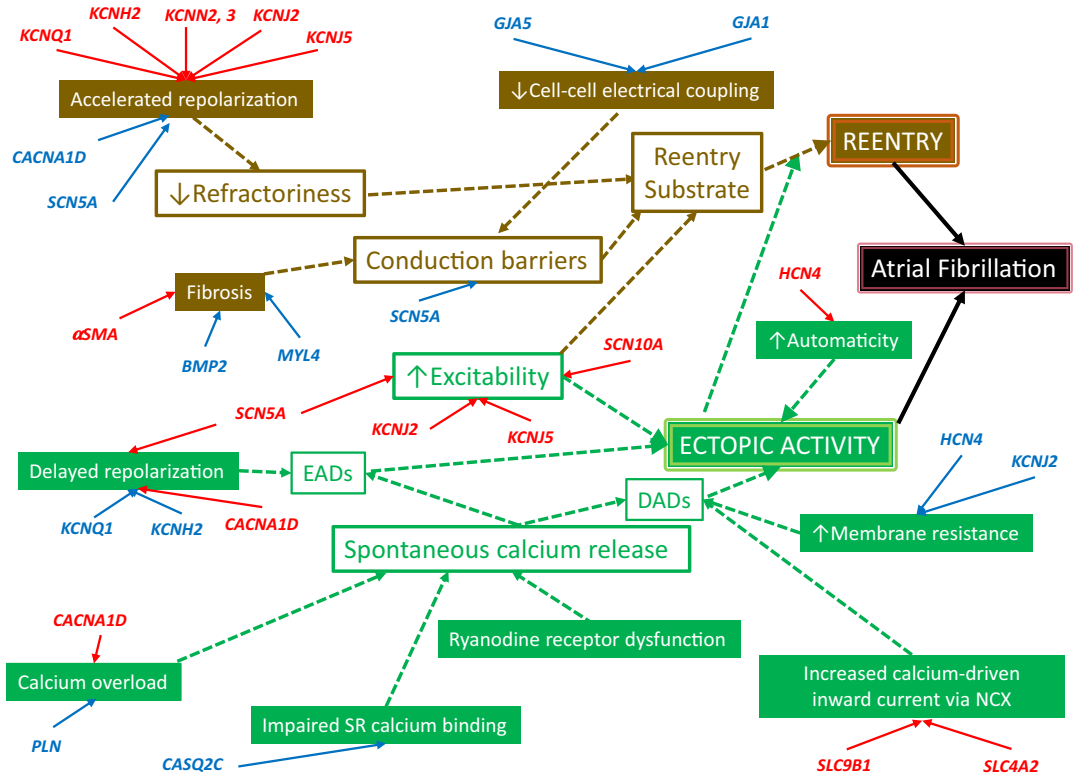

Fig. 2: Pathophysiological mechanisms linking functional changes caused by specific AF-associated gene-variants to AF. Gain-of-function is indicated by red, Loss-of-function by blue. Green boxes are associated with AF-promotion by enhanced spontaneous ectopic activity, brown boxes are associated with AF-promotion by forming a vulnerable substrate for reentry. Open boxes represent final common proximal changes in key electrical properties, whereas filled boxes are underlying changes that lead to final common proximal changes. The upward and downward arrows indicate the direction of change in a specific property; the connecting arrows indicate a causal relationship.

Reentry occurs when ectopic activity encounters a substrate that can support reentry and continues if the reentrant impulse continues to propagate without being stopped by an inexcitable barrier. Several proximal drivers create a favorable substrate for reentry: 1) Abbreviated refractoriness, generally due to accelerated repolarization, makes it easier for the reentering impulse to encounter excitable tissue throughout the circuit; 2) The presence of conduction barriers favors the initiation of reentry by promoting unidirectional block and makes it easier for reentry to be maintained by stabilizing reentry around a barrier; 3) Increased excitability stabilizes reentry by increasing the energy of the propagating reentrant wave. Conduction barriers are established by structural obstacles like tissue fibrosis, by impaired cell-to-cell coupling and by reduced strength of the Na^+ current (I_{Na}) responsible for impulse propagation. Increased excitability occurs when I_{Na} is increased (leading to the paradoxical reality that both gain or loss of I_{Na} function can promote reentry, via different mechanisms) or when the resting membrane potential is made more negative by enhanced background potassium current (inward rectifier I_{K1} or acetylcholine-dependent current I_{KACh}).

Ectopic activity leads to AF primarily by acting as a trigger to initiate reentry, but sustained repeated rapid activity can also maintain AF even in the absence of reentry. Ectopic activity typically results from spontaneous after depolarisations, either early after depolarisations (EADs), occurring before, or delayed after depolarisations (DADs), occurring after full repolarization. DADs are likely the most common precipitant of AF-related ectopic activity. DADs typically result from spontaneous diastolic Ca^{2+}-release events from the sarcoplasmic reticulum (SR), with the released Ca^{2+} being exchanged for extracellular Na^+ via the Na^+/Ca^{2+}-exchanger (NCX), causing during diastole a depolarizing inward current carried by Na^+. SR Ca^{2+} release is controlled by the cardiac ryanodine receptor (RyR2) gating mechanism, which is Ca^{2+} sensitive. Increased cell Ca^{2+} can trigger abnormal RyR2 Ca^{2+} releases, while impaired SR Ca^{2+} binding or abnormalities of RyR2 (e.g., phosphorylation) can make it hypersensitive to Ca^{2+}. Other changes that can enhance the risk of ectopic

activity are increases in NCX function, which enhance the inward current generated by any given quantity of released Ca^{2+}. Furthermore, increased membrane resistance increases the degree of depolarization for any given depolarizing current carried by NCX.

The genes identified to be associated with AF risk (Tables 1 and 3) that have known or strongly suspected pathophysiological mechanisms are indicated in red (for consequences of gain of function) or blue (for loss of function) in Fig. 2. Gain-of-function mutations in several K^+-channel genes accelerates repolarization, abbreviating refractoriness and leading to a reentrant substrate, as does loss of Ca^+- or Na^+ channel function variants. The opposite effects lead to delayed repolarization and risk of EAD-related ectopic activity. Gain-of-function in background K^+-channels enhance excitability by hyperpolarizing the membrane and removing Na^+-channel inactivation, as does gain-of-function in Na^+-channels. Conduction barriers are produced by impaired intercellular coupling due to loss-of-function variants in connexin genes *GJA5* and *GJA1*, as well as through tissue fibrosis caused by mutations in fibroblast genes (like αSMA gain of function or BMP2 loss of function) or atrial-selective contractile genes (loss of MYL4 function[110]). Spontaneous SR Ca^{2+}-release is favoured by Ca^{2+}-loading due to gain of Ca^{2+}-channel function or loss-of-function of phospholamban (PLN), which inhibits SR Ca^{2+}-uptake via the SR Ca^{2+}-ATPase. Impaired SR Ca^{2+}-binding by dysfunctional or down-regulated calsequestrin (CASQ) increases free SR Ca^{2+}-activity, functionally mimicking Ca^{2+}-loading. Gain-of-function mutations in NCX (encoded by *SLC* genes) increase the current for any given amount of Ca^{2+} released and loss-of-function channel mutations that reduce diastolic current flow and increase membrane resistance, increase the degree of voltage change caused by a given depolarizing NCX current, causing larger DADs for any given amount of Ca^{2+}-release induced current.

Genetic regulators operating via pathophysiological mechanisms that remain to be clarified-the prototype case of PITX2

For some of the genetic control mechanisms, their role is much less clear, despite a great deal of accumulated data. Because of space limitations, we will discuss in detail here only one genetic mechanism to illustrate the challenges in linking identified genetic mechanisms with clear pathophysiological mediators that mediate their influence on AF. The specific element to be discussed is the genetically controlled variation in the expression of PITX2, selected because it is the gene closest to the strongest AF-linked region, on chromosome 4q25 and because a large number of studies have addressed potential underlying mechanisms.

Although many SNPs on chromosome 4q25 associate with a higher risk for AF, causality is difficult to demonstrate in the case of PITX2. Because PITX2 is the closest gene to the discovered AF-associated SNPs, it is assumed that these SNPs could causally promote AF by regulating the atrial expression levels of PITX2. This idea is further supported by data showing that PITX2 is a suppressor of sinoatrial node-specific gene expression exclusively located in the left atrium (LA), with PITX2 deficiency increasing the mRNA levels of HCN4, SHOX2 and TBX3.[96] However, published work did not find a significant correlation between the presence of risk SNPs on chromosome 4q25 and the atrial expression levels of PITX2.[98,111] Although at the mRNA level the expression of PITX2 is 100-fold higher in human LA than in right atrium (RA),[98] the PITX2 protein is only 2-fold higher in LA than in RA,[92] suggesting a much smaller LA-RA PITX2 protein gradient in the human atrium. In addition, despite some evidence at the mRNA level that patients with AF may have lower levels of PITX2 in LA compared to sinus rhythm controls,[55,97] PITX2 mRNA is increased in RA cardiomyocytes from AF patients.[108] In addition, PITX2 mRNA levels vary substantially in LA appendages of AF patients requiring rhythm control therapy with ablation,[101] there is no confirmation of reduced PITX2 in AF patients at the protein level, and no studies employed immunostaining to show and quantify the protein abundance of PITX2 in atrial cardiomyocytes or in other cell types of the human atrium. Rats with heart failure (HF)[93] or hypertension[94] reveal reduced PITX2 mRNA in LA, pointing to a potential influence of the AF-accompanying co-morbidities on PITX2 expression. Rapid atrial pacing also causes a decrease of PITX2 mRNA in pig LA,[95] suggesting that AF itself could modify atrial PITX2 expression. These findings are consistent with the hypermethylation of the PITX2 promoter in LA of AF vs sinus rhytm patients[55] and in LA of HF rats.[93] However, whether the protein levels of Pitx2 are reduced in the atria or in atrial cardiomyocytes of patients with AF remain to be demonstrated.

The consequences of PITX2 deficiency for atrial function and AF susceptibility were studied in many mouse lines with global or cardiac-restricted genetic knockdown of PITX2 (summarized in Table 3). There is a universal finding that PITX2 deficiency in mice is associated with either ECG changes resembling AF or with a higher susceptibility to pacing-induced AF (Table 3). Genetic knockdown of PITX2 or its major cardiac isoform PITX2c in mice and zebrafish or in isolated atrial cardiomyocytes and fibroblasts provided inconsistent and sometimes contradictory results about the underlying proarrhythmogenic substrate. For instance, action potential (AP) duration (APD) was abbreviated in some studies,[98,100,101] but unaltered in one study employing PITX2 deficient mice.[97] PITX2 deficiency in human IPSCs resulted in APD abbreviation.[107] AP upstroke velocity was unchanged in PITX2 deficient mice,[97] but was faster in human IPSCs with genetic

Study first author surname and year	Pubmed ID	Details and quality of evidence			Summary of key findings		
		Species	Model system	Key inclusion & exclusion criteria	Relevant outcome(s) to atrial remodeling	Key findings and important biases	Conclusion(s)
Key studies determining atrial PITX2 expression in patients and in experimental models and systems							
Kahr et al., 2011	[92]	Human	Right and left atrial appendages (RAA, LAA)	Patients in sinus rhythm undergoing heart surgery for CABG or valve replacement		Protein levels of PITX2 two-fold higher in LA vs RA	Systematic differences between LA and RA gene expression exist and support a potential role of PITX2 in shaping LA
Donate Puertas et al., 2017	[55]	Human	LAA and pulmonary veins-LA junction	Patients with or without AF undergoing heart surgery for valve replacement	Increased LA surface in AF is inversely correlated with PITX2 mRNA levels	- mRNA levels of both PITX2 and PITX2c about two-fold lower in LA of AF vs SR patients - PITX2 promoter is hypermethylated in AF vs SR patients - It is possible that besides AF co-morbidities also affect PITX2 mRNA - PITX2 may not be changed at the protein level in human AF	AF is associated with epigenetic LA changes including PITX2 promoter hypermethylation
Kao et al., 2013	[93]	Rat Mouse	Rat LA Mouse atrial HL-1 cells	Rats with isoprenaline-induced heart failure (HF) vs control animals	HF and Ang-II decrease atrial PITX2c protein levels	- PITX2c promoter is hypermethylated in LA of HF rats - Ang-II causes PITX2c promoter hypermethylation, along with a decrease in PITX2c and Kir2.1 protein levels	Heart failure and Ang-II promote atrial PITX2c promoter hypermethylation
Scridon et al., 2015	[94]	Rat	Rat LA	Young (14 weeks-old), adult (24 weeks-old) and aged (48 weeks-old) spontaneously hypertensive rats (SHRs)	Hypertension decreases atrial PITX2c mRNA levels	- mRNA levels of PITX2 were lower in LA of SHR vs age-matched control rats - Protein levels of PITX2 were not assessed	In SHRs PITX2 down-regulation is an age-dependent process that starts before the occurrence of atrial arrhythmias
Torrado et al., 2015	[95]	Pig Mouse	Pig LA Mouse atrial HL-1 cells	Pigs with AF induced by rapid atrial pacing	AF itself decreases LA PITX2 (and TBX5) protein levels	- Rapid atrial pacing reduces the protein levels (by 70%), but not the mRNA levels of PITX2c (and TBX5) in LA of pigs, along with an increase in miR-21 - Overexpression of miR-21 in HL-1 cells causes downregulation of PITX2c protein levels	Rapid atrial pacing mimicking AF decreases atrial PITX2c and TBX5 protein levels by upregulating miR-21
Key studies assessing the consequences of genetic manipulation of PITX2 expression levels for atrial function and AF susceptibility							
Wang et al., 2010	[96]	Mouse	Whole hearts and LA	Mice with global PITX2 deficiency (PITX2null ± mice)	PITX2 deficiency creates an atrial arrhythmogenic substrate	- PITX2null ± mice have a higher susceptibility to inducible atrial arrhythmias - PITX2c is a suppressor of sinoatrial node-specific gene expression in LA: PITX2c deficiency enhances the mRNA levels of HCN4, NPPA, KCNQ1, SHOX2, and TBX3	PITX2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification
Chinchilla et al., 2011	[97]	Human Mouse	Human RAA and LAA Mouse RA and LA Mouse atrial HL-1 cells	Patients with or without AF undergoing heart surgery for valve replacement Conditional atrial-specific PITX2 deficient mice (NppaCre + PITX2−/−)	Atrial-specific PITX2 deficiency causes atrial electrical and structural changes	- mRNA levels of PITX2c strongly decreased (80–90%) in both LA and RA of AF vs SR patients - PITX2c may not be changed at the protein level in human AF - On ECG P wave was missing in 85% of NppaCre + PITX2−/− mice, pointing to AF presence; Sinoatrial node function normal - NppaCre + PITX2−/− show atrial and ventricular enlargement and increased ventricular, but not atrial fibrosis; mRNA of Col1a1 and Col3a1 increased in the ventricle but reduced in RA and LA of mutant mice - Multicellular AP recordings: more depolarized RMP with smaller AP amplitude, but no change in Vmax or APD in LA only of NppaCre + PITX2−/−, along with mRNA reduction of SCN5A, SCN1B, KCNJ2, KCNJ12, and KCNJ4. Kir2.1 and Nav1.5 protein levels reduced in mutant mice, along with miR-1 increases. - Overexpression of PITX2c in HL-1 cells decreases miR-1, whereas miR-1 overexpression in HL-1 cells decreases GJA1 and KCNJ2, but not SCN5A and SCN1B transcripts - Ventricular dysfunction is a confounder of the atrial phenotype of NppaCre + PITX2−/−	PITX2 could be an upstream transcriptional regulator of atrial function left-sided pacemaker specification

(Table 3 continues on next page)

Study first author surname and year	Pubmed ID	Details and quality of evidence			Summary of key findings		
		Species	Model system	Key inclusion & exclusion criteria	Relevant outcome(s) to atrial remodeling	Key findings and important biases	Conclusion(s)
(Continued from previous page)							
Kirchhof et al., 2011	[98]	Human Mouse	Human RAA and LAA Mouse RA and LA	Patients in sinus rhythm with or without AF undergoing heart surgery for CABG or valve replacement PITX2c deficient mice (PITX2c+/−)	PITX2c deficiency causes atrial electrical disturbances (action potential shortening) but no structural changes	- mRNA levels of PITX2c about 100-fold higher in human LA vs RA - PITX2c mRNA levels do not correlate to rs2200733 AF risk allele on chromosome 4q25 - PITX2c ± mice show action potential shortening and a higher susceptibility to inducible AF; Sinoatrial node function normal - On optical mapping, no difference in conduction time and activation patterns between mouse groups - Cardiac dimensions, structure and contractile function preserved in PITX2c ± mice (echocardiography); no evidence of fibrosis - PITX2c ± have normal ventricular function	Reduction of atrial PITX2c expression promotes AF inducibility by causing action potential shortening and is associated with complex changes in gene expression in the atria
Tao et al., 2014	[99]	Mouse	Whole atria	Conditional PITX2 deficient mice (PITX2 CKO)	Conditional PITX2 deficiency causes abnormal cardiac conduction, sinoatrial node dysfunction, and alterations in cardiomyocyte ultrastructure	- PITX2 CKO mice have sinoatrial node dysfunction and abnormal cardiac conduction - PITX2 CKO mice show disrupted intercalated discs and swollen and vacuolated mitochondria in LA cardiomyocytes - PITX2 CKO exhibit alterations in ion channel and calcium handling genes, and in genes that stabilize the intercalated discs	PITX2 directly regulates ion transport, calcium handling and intercalated dics genes
Wang et al., 2014	[58]	Mouse	Whole atria	Different genetically modified mouse lines	PITX2 deficiency upregulates miR-17-92 and miR-106b-25 and upregulation of these miRs promote pacing-induced AF in mice	- PITX2 positively regulates both miR-17-92 and miR-106b-25 expression - Mice with cardiac-specific miR-17-92 or miR-106b-25 both show higher susceptibility to inducible AF	PITX2 suppresses expression of miR-17-92 and miR-106b-25 and inhibits predisposition to AF
Nadadur et al., 2016	[100]	Mouse	Whole LA Isolated atrial myocytes	TBX5 and PITX2 deficient and double mutant mouse lines	PITX2 deficiency causes atrial APD abbreviation, but does not induce cellular triggered activity	- Atrial myocytes of PITX2 ± mice show shortened APD90 and APD50 and an increased AP amplitude, but no cellular triggered activity - PITX2 ± mice have a higher susceptibility to pacing-induced AF - Cross-breeding of PITX2 ± with TBX5 ± mice rescues the proarrhythmic phenotype (slowed atrial conduction, Ca2+ dependent triggered activity, APD50 and APD90 prolongation) of the latter	The TBX5 deficiency associated susceptibility to inducible AF was rescued by PITX2 haploinsufficiency in mice
Syeda et al., 2016	[101]	Human Mouse	Human LAA Mouse whole hearts Mouse LA Mouse LA myocytes	Patients undergoing bilateral thoracoscopic AF ablation PITX2c deficient mice (PITX2c+/−)	Pitx2 deficiency causes APD shortening and a more depolarized RMP	- PITX2 mRNA levels vary substantially in LAA of AF patients requiring rhythm control therapy with ablation - PITX2 mRNA levels do not correlate to rs2200733, rs6838973 or rs14448818 AF risk alleles on chromosome 4q25 - Flecainide suppresses atrial arrhythmias more efficiently in PITX2c ± mice - RMP is more depolarized in PITX2c+/−, along with decreased protein levels of TASK-2 (but not Kv1.6 or Nav1.5) - IK1 similar in PITX2c ± and WT mice - Conduction velocity similar in PITX2c ± and WT mice - LA optical APD is shorter in PITX2c ± mice	PITX2 deficiency results in more depolarized RMP, which causes greater sodium channel inactivation, thereby potentiating the antiarrhythmic effects of flecainide
Kao et al., 2019	[102]	Human	Cultured human atrial fibroblasts	PITX2c knockdown in human atrial fibroblasts	PITX2c deficiency increases activity of atrial fibroblasts, promoting their transition to collagen-secreting myofibroblasts	- PITX2c KO increase fibroblast migration, with no change in proliferation; CaMKII inhibition normalizes migration - PITX2c KO enhances Ca2+ influx and increases protein levels of CaMKII-P, αSMA, and MMP2, but not of Col1 or MMP9 - Effect of culture-driven transdifferentiation is a bias	PITX2c deficiency increases human atrial fibroblast activity potentially promoting atrial fibrosis

(Table 3 continues on next page)

Study first author surname and year	Pubmed ID	Details and quality of evidence			Summary of key findings		
		Species	Model system	Key inclusion & exclusion criteria	Relevant outcome(s) to atrial remodeling	Key findings and important biases	Conclusion(s)
(Continued from previous page)							
Collins et al., 2019	[103]	Zebra fish	Whole heart	PITX2c deficient zebra fish (PITX2c ± and PITX2c−/−)	PITX2c deficiency causes atrial conduction defects and alterations in cardiomyocyte metabolism and ultrastructure	- PITX2c deficiency causes atrial conduction defects, atrial enlargement and fibrosis - PTX2c deficiency induces sarcomere disassembly, alters mitochondrial morphology and cardiac metabolism, and increases ROS production, all of which precede the onset of cardiac arrhythmias - Incidence and severity of PITX2c deficiency related arrhythmias are reduced by antioxidant treatment with the ROS scavenger N-acetyl-cysteine, pointing to oxidative stress as a driver of the arrhythmia - Ventricular function is normal in PITX2c deficient zebra fish	PITX2c deficiency in zebra fish induces sarcomere and metabolic defects that precede the development of atrial conduction disturbances and arrhythmias
Holmes et al., 2021	[104]	Human Mouse	Human IPSCs HEK293 cells Mouse LA	HEK293 cells expressing Scn5a + Scn1b Human IPSC-derived myocytes PITX2c deficient mice (PITX2c+/−)	PITX2c deficiency causes a more positive RMP	- PITX2c ± mice have more positive RMP - Dronedarone causes stronger action potential prolongation in PITX2c+/− - Human IPSC-derived myocytes are not atrial-like	The more depolarized RMP due to PITX2c deficiency increases the efficacy of dronedarone to prolong atrial action potential
Tarifa et al., 2023	[105]	Mouse	Pooled LA and RA myocytes	Atrial-specific PITX2 deficient mice (NppaCre + PITX2+/−; NppaCre + PITX2−/−)	Atrial-specific PITX2 deficiency causes atrial electrical and Ca2+ handling alterations that may promote atrial ectopy	- PITX2 deficiency increases cell capacitance of RA and LA myocytes, pointing to cell hypertrophy - PITX2 deficiency causes a reduction in ICa,L in RA and LA - PITX2 deficiency increases frequency of Ca2+ sparks and waves and of transient inward current ITI in LA and RA myocytes - PITX2 deficiency enhances caffeine releasable SR Ca2+ load in RA and LA - PITX2 deficiency promotes DADs and triggered action potentials in RA myocytes	Atrial-specific PITX2 deficiency reduces ICa,L that may cause re-entry promoting action potential shortening and induces cellular Ca2+ handling abnormalities that may cause atrial ectopy and triggered activity
Kim et al., 2023	[106]	Mouse	Atrial myocytes	PITX2 deficient mice (PITX2+/−)	PITX2 deficiency causes Ca2+-dependent cellular triggered activity	- PITX2−/− mice show higher susceptibility to inducible AF, which is suppressed by the selective RyR2 inhibitor ent-verticulide - Normal sinoatrial node function - Atrial myocytes from PITX2 ± mice show increased frequency, amplitude and mass of Ca2+ sparks, along with more spontaneous Ca2+ waves - No changes in diastolic Ca2+, SR Ca2+ load or CaT decay in PITX2 ± mice - Ent-verticulide suppresses spontaneous Ca2+ waves in PITX2−/−	The higher susceptibility to AF of PITX2 ± mice is suppressed by selective RyR2 inhibition suggesting that ectopic (triggered) activity is one potential arrhythmogenic mechanism of PITX2 deficiency
Schulz et al., 2023	[107]	Human	Humans IPSCs	CRISPER/Cas9-mediated PITX2 deletion in healthy human atrial-like IPSCs Atrial engineered heart tissue	Atrial-specific PITX2 deficiency causes atrial electrical changes	- PITX2−/− reduces cell capacitance - PITX2 deficiency causes a reduction in Ito and ICa,L - Lack of Pitx2 decreases force of contraction and slows force relaxation, with beating rate being slightly lower in PITX2−/− - RMP more negative in PITX2−/−, but not due to a higher IK1 - APD20 is longer, APD90 is shorter; AP amplitude is higher with faster Vmax in PITX2−/− - PITX2−/− reduces mRNA of Cav.1.2, SERCA2a, RyR2, NCX1; no change in KCNJ2, KCNJ4, KCNJ12 - Atrial-like IPSCs differ from native atrial myocytes and there is no LA myocyte specification	PITX2 knockout induces key findings of electrical (shorter triangular AP with decreased Ito and ICa,L) and contractile (reduced force of contraction) remodeling typical of persistent AF

(Table 3 continues on next page)

Study first author surname and year	Pubmed ID	Details and quality of evidence			Summary of key findings		
		Species	Model system	Key inclusion & exclusion criteria	Relevant outcome(s) to atrial remodeling	Key findings and important biases	Conclusion(s)
(Continued from previous page)							
Perez-Hernandez et al., 2016	[108]	Human Mouse	Human RAA Mouse atrial HL-1 cells	Patients in sinus rhythm with or without AF undergoing heart surgery for CABG or valve replacement Mouse atrial HL-1 cells	Increased PITX2c is associated with reduced ICa,L and increased IKs, both potentially abbreviating action potential duration	- PITX2c mRNA is increased in RA myocytes from AF patients - The higher PITX2c mRNA in RA correlates with reduced ICa,L and increased IKs in RA cardiomyocytes of AF patients - Overexpression of PITX2 in HL-1 cells reduces ICa,L and increases IKs by direct modulation of KCNQ1 promoter activity - These findings might not apply to human LA cardiomyocytes of AF patients	Human chronic AF is associated with increased PITX2c expression and a reduction of ICa,L and an increase in IKs in RA cardiomyocytes that may contribute to re-entry promoting AP shortening
Key study linking 4q25 rs13143308T allele to atrial cardiomyocyte function of AF patients							
Herraiz-Martinez et al., 2019	[109]	Human	Human RAA	Patients in sinus rhythm with or without AF undergoing heart surgery for CABG or valve replacement	The risk variant rs13143308T associates with DAD-dependent triggered activity, but not with ICa,L function	- Rs13143308T increases SR Ca2+ load and frequency of Ca2+ sparks, transient inward current ITI, and DADs in RA myocytes - Rs13143308T does not associate with ICa,L changes - Ser2808-RyR2 increased, Ser2014-RyR2 unchanged - Protein levels of SERCA2a increased, those of NCX1 and CSQ unaltered - Putative association of the risk allele with atrial PITX2 expression levels not assessed	The 4q25 variant rs13143308T increase the risk for AF by causing ectopic (triggered) activity

Table 3: **Key studies determining atrial PITX2 expression in patients and in experimental models and systems (upper part); key studies linking 4q25 rs13143308T allele to atrial cardiomyocyte function of AF patients (lower part).**

PITX2 knockdown.[97] Similarly, resting membrane potential (RMP) was more depolarized[97,101] in PITX2 deficient mice, but hyperpolarized in human IPSCs with genetic PITX2 knockdown.[101] Surprisingly, the basal inward rectifier K^+ current I_{K1} was unchanged in both PITX2 deficient mice[101] and human IPSCs with genetic PITX2 knockdown.[101] Likely because of the more depolarized RMP, the antiarrhythmic drugs dronedarone and flecainide caused a stronger APD prolongation in PITX2c deficient mice.[101,104] Ca^{2+} influx appears disturbed, because PITX2 deficiency caused a reduction of L-type Ca^{2+} current ($I_{Ca,L}$) in mouse atrial cardiomyocytes[105] and in human IPSCs.[107] In contrast, the presence of the Rs13143308T risk allele did not associate with changes in $I_{Ca,L}$.[109] Atrial conduction defects were detected in PITX2 deficient zebrafish[112] and in one study using PITX2 deficient mice,[99] but conduction was unaltered in other PITX2 deficient mice studies.[98,101]

Atrial structure appears differentially affected in the individual studies using PITX2 deficient mouse lines. There is evidence for atrial enlargement[97] and alterations in cellular ultrastructure with disrupted intercalated discs and swollen and vacuolated mitochondria in LA cardiomyocytes[113] in PITX2 deficient mice, which are consistent with data from PITX2 knockout zebrafish, whereby atrial enlargement and fibrosis, sarcomere disassembly, altered cardiac metabolism, and increased ROS production occurred before the onset of cardiac arrhythmias.[103] Incidence and severity of PITX2c deficiency-related arrhythmias in zebrafish were reduced by antioxidant treatment with the ROS scavenger N-acetyl-cysteine, pointing to oxidative stress as a driver of the arrhythmia in this model.[103] PITX2 knockout in mice could also cause cardiomyocyte hypertrophy.[105] However, another study could not find differences in cardiac dimensions and structure or contractile function.[98] In addition, it is important to note that some mouse lines with PITX2 deficiency developed sinoatrial node[99] or ventricular dysfunction,[97] which might have confounded the results at the atrial level, potentially contributing to the inconsistent results between studies.

One study in cultured human atrial fibroblasts showed that PITX2 knockdown resulted in increased fibroblast migration, with no change in proliferation, which was associated with enhanced Ca^{2+} influx and increased protein levels of autophosphorylated (activated) CaMKII, α-smooth muscle actin, and MMP2, but not MMP9 or collagen-1.[102] Inhibition of CaMKII normalized fibroblast migration and rescued the phenotype, suggesting that PITX2 deficiency promotes the transition of fibroblasts to collagen-secreting myofibroblasts by activation of CaMKII, potentially causing structural remodeling.[102]

There is evidence for cellular triggered activity in PITX2 deficient mice.[102,106] PITX2 deficiency increased SR Ca^{2+} load, the frequency of RyR2-mediated Ca^{2+} sparks and waves and the resulting NCX-mediated transient inward current I_{TI} in LA and RA cardiomyocytes, thereby causing DAD-mediated triggered

Search strategy and selection criteria

We identified data for this Series paper by searching MEDLINE, Current Contents, PubMed, and references from relevant articles using the search terms "atrial fibrillation, genetics", "atrial fibrillation, epigenetics", "atrial fibrillation, artificial intelligence", "atrial fibrillation black", "atrial fibrillation asian", "atrial fibrillation racial differences", and "expression of PITX2 and atrial fibrillation". We considered data published in English between Jan 1, 1990, and June 16th, 2023.

activity.[102] As already mentioned, PITX2 suppresses expression of miR-17-92 and miR-106b-25 thereby inhibiting the predisposition to AF,[58] whereas loss of miR-106b-25 cluster promotes triggered activity and AF by enhancing RyR2 expression and SR Ca^{2+} release,[114] consistent with data from patients with paroxysmal AF.[115] Other miRs involved in the pathophysiology of PITX2-dependent AF include miR-29a, miR-200, miR-203, miR-21, miR-208ab, miR-1 and miR-26b.[95,116] In particular,miR-21, miR-106a, miR-203 and miR-208ab were down-regulated, whereas miR-1, miR-26b, miR-29a, miR-106b and miR-200 were up-regulated by the absence of PITX2. A miR-dependent gene regulatory network has been described leading to PITX2-dependent AF.[117] However, another study could not detect DAD-mediated cellular triggered activity in PITX2 deficient mice.[100] The selective RyR2 inhibitor *ent*-verticulide suppressed spontaneous Ca^{2+} waves and the related cellular triggered activity in PITX2 deficient mice, pointing to a critical role of RyR2 dysfunction.[106] Another study performed in human RA cardiomyocytes showed that the presence of the Rs13143308T risk allele was associated with increased SR Ca^{2+} load, frequency of Ca^{2+} sparks and transient inward current I_{TI}, and DADs, but no changes in $I_{Ca,L}$.[109] Although the putative association of the risk rs13143308T allele with human atrial PITX2 expression levels was not assessed, these data support the possibility that Ca^{2+}-dependent cellular triggered activity, as observed in AF patients,[115] could represent a potential arrhythmogenic mechanism associated with PITX2 deficiency.

In summary, the studies assessing the putative role of PITX2 in AF pathophysiology clearly illustrate the big challenges of defining and establishing causal mechanisms of PITX2 in atrial arrhythmogenesis. Although PITX2 deficiency could cause reentry-promoting APD abbreviation, the findings related to atrial electrical and structural remodeling are rather inconsistent among the individual studies (Table 3). This may also be due to species-specific differences and differences in the genetic background (e.g., in determinants of APD, calcium handling etc.), which may further hinder the comparison between experimental models (mice, pigs, zebrafish, iPSC). Whether PITX2 deficiency related DAD-mediated triggered activity could be translated to clinical AF requires direct demonstration. There are still many important gaps in our knowledge about the direction of change of PITX2 and the underlying arrhythmogenic mechanisms in humans with AF that will require extensive additional work to foster translation to the clinical management of AF.

Conclusions

A review of AF mechanisms in 2002 remarked on the paucity of genetic information relating to AF pathophysiology and commented on an expected rapid growth in the future.[104] The present review substantiates this prediction by documenting the explosion of information in this area over the past 20 years. Nevertheless, it also underlines the challenges in exploiting this vast body of information to understand the mechanistic link between many clear and important genetic factors and AF occurrence. While for many gene variants affecting cardiac functions associated with known AF mechanisms the pathophysiological links are relatively clear, this is not the case for most gene variants implicated in the arrhythmia. Clearly, major additional work needs to be done. Particularly important will be the development of robust methods through which to understand how gene variants in non-coding regions, genes encoding proteins with no known cardiac function and "gene deserts" lead to AF, which is at present not known in virtually all cases. An additional area needing major attention is the mechanistic basis for ethnic and racial variability in AF-risk, known to be important. Differences in genetic factors are already emerging as candidates to explain some of these observations, but again much additional work needs to be completed before we can truly understand why AF risk differs according to region, ethnic origin and racial factors.

Contributors

MV, DD, SN: literature search, figures, writing.

Declaration of interests

We declare no competing interests.

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