

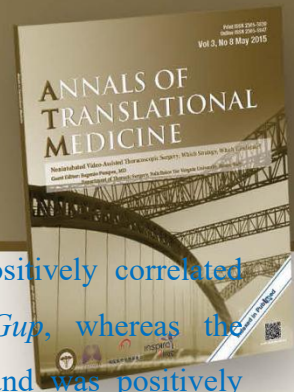
Peer Review File

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Reviewer A

Comment 1: The manuscript by Zhu et al. describes a population-based action potential modelling of atrial cardiomyocytes and furthermore studied the impact of Pitx2 in such a modelling, aiming to dissect the key determinant of AP variability in the AF context. The study is potentially interesting but there is a poor description of the biological rationale behind the distinct AP subgroups and their relation to Pitx2 impairment. The major caveat that I found in the manuscript is the fact that I do not see the correlation of the population AP modelling with Pitx2 levels. I fail to understand how the input of Pitx2 is related to the different models. The authors should provide a more clear and comprehensive description of it.

Reply 1: We would like to thank this reviewer for his or her positive endorsement, detailed suggestions and comments. We have modified our methods (Pages 9-10, Lines 12-23 and 1-3), results (Pages 13, Lines 1-8) and discussion (Pages 17-18, Lines 13-25 and 1-3) for providing a comprehensive description of the correlation of the population AP modelling with Pitx2 levels. Previous studies have shown that changes in Pitx2 levels lead to electrical remodelling linked to arrhythmogenesis (Chinchilla et al., 2011) and that Pitx2 regulates membrane effector genes associated with the electrical remodelling in a dose-dependent manner (Pérez-Hernández et al., 2016, Lozano-Velasco et al., 2016, Nadadur et al., 2016). Therefore, we assumed that changes in the Pitx2 level are translated to the extent of electrical remodelling (including GNa, GKs, GK1, GCaL, Grel and Gup) (Pérez-Hernández et al., 2016, Lozano-Velasco et al., 2016, Nadadur et al., 2016). The correlation of the Pitx2 levels with the different AF models developed based on Pitx2-induced electrical remodelling can be built. In the revised MS, we have used the AF models and the corresponding remodelled parameters to train the artificial neural network to determine the weights of remodelled parameters (listed in the new Table 3) for the classification of different AF subgroups (unshortened, shortened and triggered groups).



As shown in the new **Figure 8**, the incidence of shortened AP was positively correlated with *GKs* and *GK1* and was negatively correlated with *GNa*, *GCaL* and *Gup*, whereas the incidence of triggered AP was negatively correlated with *GKs* and *GK1* and was positively correlated with *GNa*, *GCaL* and *Gup*. According to experimental studies, *Pitx2* deficiency is associated with the increase in *GNa*, *Gup* (Nadadur et al., 2016) and *GCaL* (Dai et al., 2019) and the reduction in *GK1* (Syeda et al., 2016), whereas *Pitx2* overexpression is associated with upregulated *GKs* and downregulated *GCaL* (Pérez-Hernández et al., 2016). Therefore, we can conclude that the downregulation of *Pitx2* is associated with the triggered activity, whereas the upregulation of *Pitx2* contributes to AP shortening. These results indicated that the *Pitx2* level is positively correlated with the incidence of shortened AP and is negatively correlated with the occurrence of the triggered activity.

Chinchilla A, Daimi H, Lozano-Velasco E, et al. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circulation: Cardiovascular Genetics 2011; 4: 269-279.

Pérez-Hernández M, Matamoros M, Barana A, et al. Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing I_{Ks} and decreasing I_{Ca, L}. Cardiovascular research 2016; 109: 431-441.

Lozano-Velasco E, Hernandez-Torres F, Daimi H, et al. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovascular research 2016; 109: 55-66.

Nadadur R D, Broman M T, Boukens B, et al. Pitx2 modulates a Tbx5-dependent gene regulatory network to maintain atrial rhythm. Science translational medicine 2016; 8: 354ra115-354ra115.

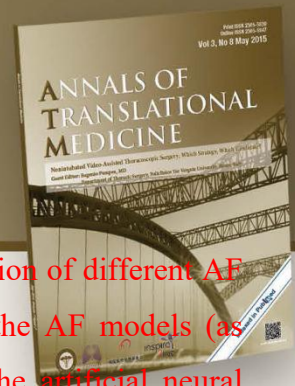
Syeda F, Holmes A P, Ting Y Y, et al. PITX2 modulates atrial membrane potential and the antiarrhythmic effects of sodium-channel blockers. Journal of the American College of Cardiology 2016; 68: 1881-1894.

Dai W, Laforest B, Tyan L, et al. A calcium transport mechanism for atrial fibrillation in Tbx5-mutant mice. Elife. 2019; 8:e41814.

Changes in text:

Pages 9-10, Lines 12-23 and 1-3

Previous studies have shown that changes in *Pitx2* levels lead to electrical remodelling linked to arrhythmogenesis (2) and that *Pitx2* regulates membrane effector genes associated with the electrical remodelling in a dose-dependent manner (3, 7, 8). Therefore, we assumed that changes in the *Pitx2* level are translated to the extent of electrical remodelling (including *GNa*, *GKs*, *GK1*, *GCaL*, *Grel* and *Gup*) (3, 7, 8). The correlation of the *Pitx2* levels with the different AF models developed based on *Pitx2*-induced electrical remodelling was built by evaluating the contribution of remodelled targets (including *GNa*, *GKs*, *GK1*, *GCaL*, *Grel* and *Gup*) to the changes in APs (including unshortened AP, shortened AP and triggered AP). The extent of electrical



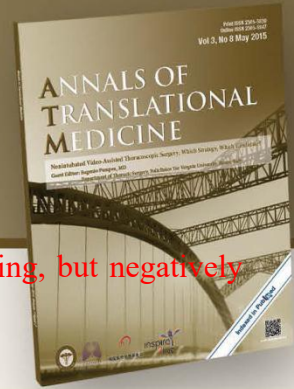
remodelling was quantified with weights of remodelled targets for classification of different AF subgroups (unshortened, shortened and triggered groups). Here, we used the AF models (as output) and the corresponding remodelled parameters (as input) to train the artificial neural network (28) to determine the weights of remodelled parameters. Based on the correlation of the Pitx2 levels with remodelled parameters observed in previous studies (3, 4, 7, 8, 29) and the correlation of remodelled parameters with changes in APs, the correlation of the Pitx2 levels with different AF models was built.

Pages 13, Lines 1-8

By using the artificial neural network, we got the weights of six remodelled parameters for classifying AF models (including shortened and triggered AP two categories) (Figure 8). For the shortened AP category, weights of GKs and GK1 are negative and weights of GNa, GCaL and Gup are positive, indicating the incidence of shortened AP is positively correlated with GKs and GK1 and is negatively correlated with GNa, GCaL and Gup. In contrast, for the triggered AP category, weights of GKs and GK1 are positive and weights of GNa, GCaL and Gup are negative. In addition, weights of Grel for shortened AP and triggered AP categories are positive. In detail, the values of weights of different remodelled targets can be found in Table 3.

Pages 17-18, Lines 13-25 and 1-3

Previous studies have shown that changes in Pitx2 levels lead to electrical remodelling linked to arrhythmogenesis (2) and that Pitx2 regulates membrane effector genes associated with the electrical remodelling in a dose-dependent manner (3, 7, 8). Therefore, based on the correlation of the Pitx2 levels with remodelled parameters observed in previous studies (3, 4, 7, 8, 29) and the correlation of remodelled parameters with changes in APs in the present study, the correlation of the Pitx2 levels with different AF models can be built. In the previous studies, Pitx2 deficiency is associated with the increase in G_{Na} , G_{up} (8) and G_{CaL} (29) and the reduction in G_{K1} (4). Interestingly, our simulated results indicated that the incidence of triggered AP was negatively correlated with G_{Ks} and G_{K1} and was positively correlated with G_{Na} , G_{CaL} and G_{up} . Therefore, the downregulation of Pitx2 may increase the incidence of triggered AP. In addition, the study of Pérez-Hernández et al.(3) showed that Pitx2 overexpression is associated with upregulated G_{Ks} and downregulated G_{CaL} . And our simulated results showed that the incidence of shortened AP was positively correlated with G_{Ks} and G_{K1} and was negatively correlated with G_{Na} , G_{CaL} and G_{up} . Therefore, the upregulation of Pitx2 may favor to AP shortening. Thus, the



Pitx2 level may be positively correlated with the occurrence of AP shortening, but negatively correlated with the incidence of triggered AP.

Figure 8 and Table 3

Table 3. Weights of remodelled parameters (including G_{Na} , G_{Ks} , G_{K1} , G_{CaL} , G_{Jrel} and G_{up}) for classifying unshortened, shortened and triggered AP three categories in AF models.

	G_{Na}	G_{Ks}	G_{K1}	G_{CaL}	G_{up}	G_{rel}
<i>Unshortened AP</i>	4.9065	-5.5615	7.8389	9.4655	-2.8048	-7.969
<i>Shortened AP</i>	-7.4592	14.6805	3.736	-21.6137	-4.0507	6.1308
<i>Triggered AP</i>	3.1941	-9.3017	-11.7621	12.4555	6.9198	1.4855

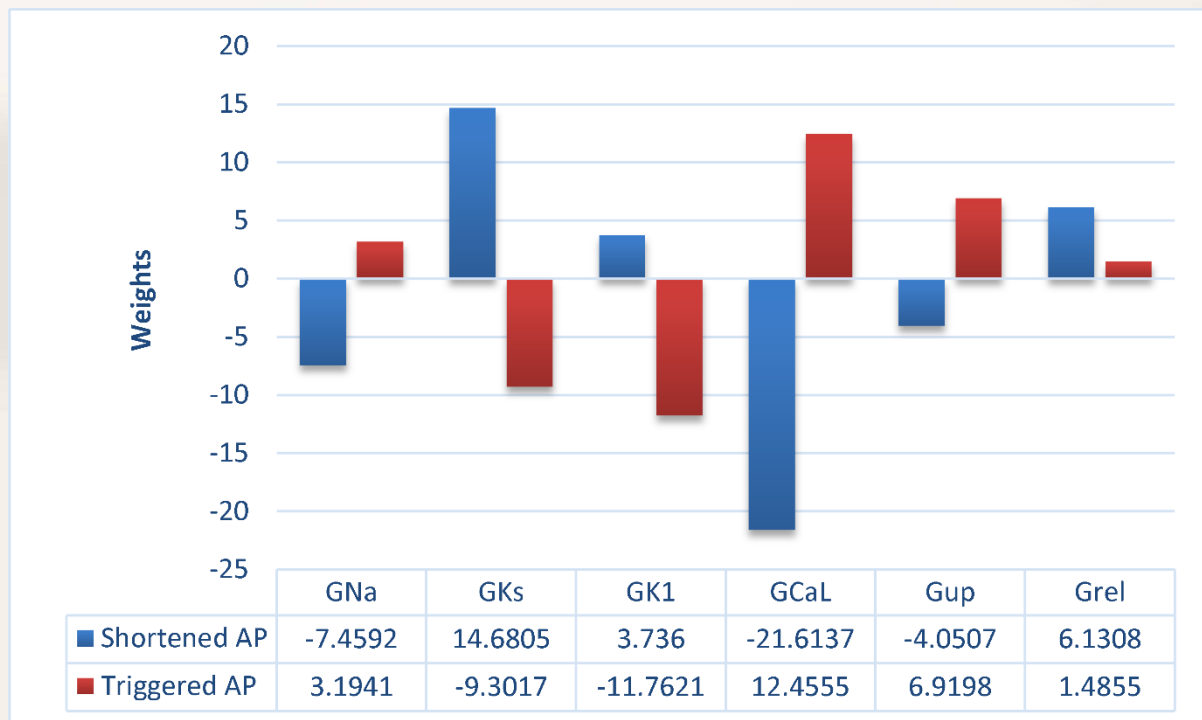
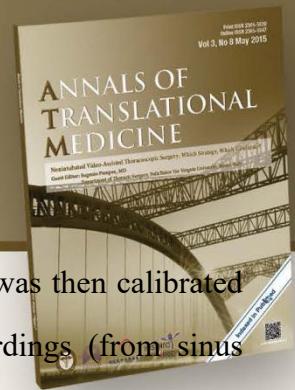


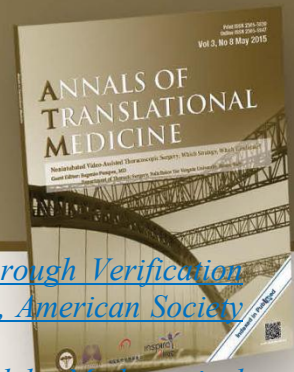
Figure 8. Weights of remodelled parameters (including G_{Na} , G_{Ks} , G_{K1} , G_{CaL} , G_{Jrel} and G_{up}) for classifying shortened and triggered AP three categories in AF models.



Comment 2: Lanes 14-16 The authors stated: “The TPA model population was then calibrated using a set of cellular biomarkers extracted from experimental AP recordings (from sinus rhythm (SR) and AF patients) (17) to capture key AP properties.”. It is unclear to me what the authors mean with calibrate. They should provide a more comprehensive description of the methods and criteria of such calibration.

Reply 2: We would like to thank this reviewer for his comments. According to the suggestions of this reviewer, we have modified our methods ([Page 7, Lines 16-25](#)) for providing a more comprehensive description of the methods and criteria of such calibration.

Physiological variability is difficult to investigate with experimental methods alone ([Carusi et al., 2012](#), [Sarkar et al., 2012](#)) due to the need to average data to control experimental error. Recently, a body of research ([Britton et al., 2013](#), [Groenendaal et al., 2015](#), [Sarkar et al., 2012](#)) has shown the power of computer models for investigations into the sources and modulators of biological variability. Specifically, populations of models – also referred to as ensembles of models – have proven useful in investigations of cardiac electrophysiological variability as reviewed by ([Sarkar et al., 2012](#)). Recent studies have furthered the methodology by explicitly incorporating experimental data into the construction of populations of models, thus yielding experimentally-calibrated populations of models ([Britton et al., 2014](#), [Britton et al., 2013](#), [Muszkiewicz et al., 2014](#), [Passini et al., 2015](#), [Sánchez et al., 2014](#), [Zhou et al., 2013](#)). In the present study, 30,000 candidate models were created by sampling parameters of ionic current conductances over a range from -100% to +200% of their original value with the Latin hypercube sampling (LHS) methodology ([Britton et al., 2013](#)). The population of candidate models generated in the previous step is simulated and calibrated, to select the models whose simulated electrophysiological properties are in range with the same properties in experimental data. This step yields the experimentally-calibrated population of models. It is crucial that the simulations mimic the experimental conditions and protocols as closely as possible, considering stimulation frequency (1Hz). Such calibration follows the ASME V&V40 Standard ([ASME V&V 40, 2018](#)), a new consensus Standard developed by the medical device community which outlines a framework for credibility assessment for models with medical device applications; reports advocating for formalized methods and education into credibility assessment ([National Research Council, 2012](#)).



[ASME V&V 40 \(2018\). Assessing Credibility of Computational Models Through Verification and Validation: Application to Medical Devices. Washington, DC: Standard, American Society of Mechanical Engineers.](#)

[National Research Council \(2012\). Assessing the Reliability of Complex Models: Mathematical and Statistical Foundations of Verification, Validation, and Uncertainty Quantification. Washington, DC: The National Academies Press.](#)

[Britton O J, Bueno-Orovio A, Van Ammel K, et al. Experimentally calibrated population of models predicts and explains intersubject variability in cardiac cellular electrophysiology. Proceedings of the National Academy of Sciences 2013; 110: E2098-E2105.](#)

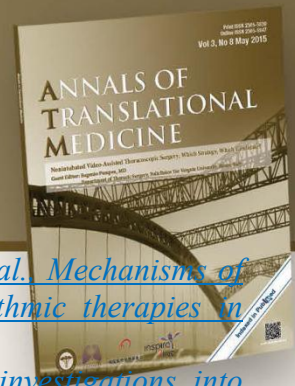
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Page 7, Lines 16-25

Following the ASME V&V40 Standard proposed by the Subcommittee of the American Society of Mechanical Engineers (ASME) on Verification and Validation (V&V) in Computational Modeling of Medical Devices (27) for developing a structured approach for establishing the credibility of computational models for a specific use, we used the candidate models to simulate human atrial AP by considering stimulation frequency (1Hz) under the experimental conditions (17) and calculate AP biomarkers of each candidate model. The candidate models generated in the previous step were selected to constitute SR and AF populations whose simulated electrophysiological properties are in range with the same properties in experimental data on AP biomarkers (including APA, $dVdt_{max}$, RMP, APD₂₀, APD₅₀ and APD₉₀) in **Table 2**. This step yields the experimentally-calibrated population of models.

Comment 3: Page 8, lane 1, the authors divided the AP into triggered and untriggered patterns. How do they divide them into triggered and untriggered? Which are the criteria to do so? In line with the previous comment, a more comprehensive description of the methods and criteria of such division should be provided.

Reply 3: We would like to thank this reviewer for his/her comments. In the revised MS, we have given the criteria for distinguishing whether each AP pattern is triggered. According to methods used in previous works, triggered patterns were identified when a positive derivative of the membrane potential ([Passini et al., 2016](#)) or a deflection of the membrane potential larger than 1 mV during the diastolic phase ([Trovato et al., 2020](#)) was observed. According to the suggestions of this reviewer, we have modified our methods (**Page 8, Lines 4-6**) for providing a more comprehensive description of the methods and criteria of such division.



[E. Passini, A. Mincholé, R. Coppini, E. Cerbai, B. Rodriguez, S. Severi, et al. Mechanisms of pro-arrhythmic abnormalities in ventricular repolarisation and antiarrhythmic therapies in human hypertrophic cardiomyopathy, J. Mol. Cell. Cardiol. 96 \(2016\) 72–81.](#)

[C Trovato et al., Human Purkinje in silico model enables mechanistic investigations into automaticity and pro-arrhythmic abnormalities, J. Mol. Cell. Cardiol. 142 \(2020\) 24–38.](#)

Changes in text:

Page 8, Lines 4-6

These models firstly were divided into triggered and untriggered AP groups to identify the main ion channel conductances involved in the genesis of triggered activity. Triggered patterns were identified when a positive derivative of the membrane potential (28) or a deflection of the membrane potential larger than 1 mV during the diastolic phase (29) was observed

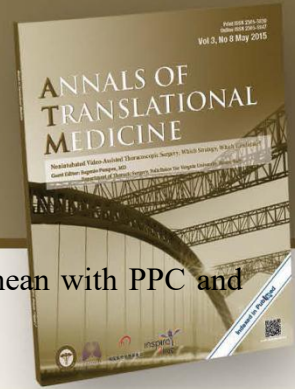
Comment 4: Similarly, the authors also distinguish between shortened and unshortened, but they failed to provide the criteria for such distinctions.

Reply 4: We would like to thank this reviewer for his/her comments. In the revised MS, we have given the criteria for distinguishing whether each AP pattern is shortened. According to the ranges of APD₉₀ for SR (190-440 ms) and AF (140-330 ms) (listed in **Table 2**), shortened AP patterns were identified when their APD₉₀ is within the range (140-330 ms) of AF but not within the range (190-440 ms) of SR. According to the suggestions of this reviewer, we have modified our methods (**Page 8, Lines 8-12**) for providing a more comprehensive description of the methods and criteria of such distinctions.

Changes in text:

Page 8, Lines 8-12

Then, the models in the untriggered AP group were divided into shortened and unshortened AP groups to examine the contribution of each remodelled target to the APD shortening (Figure 2). Shortened patterns were identified when APD₉₀ of the untriggered AP model in the AF population is smaller than that of the AP model in the SR population. In detail, shortened AP patterns were identified when their APD₉₀ is within the range (140-330 ms) of AF data but not within the range (190-440 ms) of SR (Table 2).



Comment 5: Finally, I personally do not understand what do the authors mean with PPC and what are their biological significance. I more detailed description is required.

Reply 5: We would like to thank this reviewer for his/her comments. We used a partial correlation to determine correlations between the properties of individual ionic currents and properties of the whole AP. We chose to use partial correlation over other correlation measures because partial correlation controls for the effects of one or more additional variables when looking for correlations between two quantities, which is important given that our models are generated by varying multiple parameters simultaneously. According to the suggestions of this reviewer, we have modified our methods (Page 9-10, Lines 19-24 and 1-6) for providing a more comprehensive description of the methods.

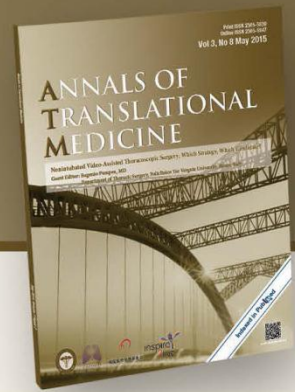
Changes in text:

Page 9-10, Lines 19-24 and 1-6

To quantify the relative importance of ionic conductances in determining changes in the biomarkers, partial correlation coefficients (PCCs) were used on the SR and AF populations to evaluate the role of each ionic current on properties of the whole AP (16). Partial correlation is a method to find correlations between two variables, after accounting for the linear effects of one or more additional variables (30). PCC between x and y , given the set of N additional variables z_i , is then defined as the correlation coefficient between the residuals $r_x = x - \hat{x}$ and $r_y = y - \hat{y}$ (16). \hat{x} and \hat{y} are the respective sample means or the following linear regression models:

$$\hat{x} = c_0 + \sum_{i=1}^N c_i z_i \quad \text{and} \quad \hat{y} = b_0 + \sum_{i=1}^N b_i z_i$$
$$PPC(x, y, z_i) = \frac{Cov(r_x, r_y)}{Var(r_x)Var(r_y)}$$

where $Cov(r_x, r_y)$ represents the covariance between r_x and r_y , while $Var(r_x)$ and $Var(r_y)$ are respectively the variance of r_x and variance of r_y .



Reviewer B

Comment 1: Zhu et al. present the generation of populations of electrophysiologic atrial cardiomyocyte models for describing current changes, resting membrane potential stability, action potential quality, and susceptibility to/incidence of ectopic electrical activity (E/DADs). The authors utilize these models to describe differences between normal sinus rhythm and Pitx2-induced electrical remodelling associated with atrial fibrillation. The manuscript is missing methodological detail, particularly relating to the clinical data obtained, but more importantly the claims related to Pitx2-induced remodelling associated with atrial fibrillation and the lack of a succinct unifying conclusion prevents this manuscript from reaching the necessary clarity for translational impact. Furthermore, it is unclear how this body of work relates to previously published manuscripts on Pitx2. While I am certainly interested in the genetic contributors to atrial fibrillation, both in familial and lifestyle cases, and do believe that this study has merit, I cannot endorse this manuscript in its current format for publication.

Reply 1: We sincerely thank the reviewer for taking his/her time to evaluate our study and provide extremely helpful criticisms and suggestions. We have hence made significant modifications and improvements to our MS. Please see our answers below for a detailed



explanation for each specific question. We believe that these changes have significantly improved our manuscript in terms of novelty, robustness, and clarity. We hope that our efforts will favor a positive decision for our manuscript. The description of experimental dataset (See reply number 8, **Pages 6-7, Lines 15-24 and 1-4**), the novelty of our MS (See reply number 5, **Pages 9-10, Lines 12-23 and 1-3, Pages 13, Lines 1-8, Pages 17-18, Lines 13-25 and 1-3**) and the relevance to previous studies (See reply number 2, **Pages 18-19, Lines 5-25 and 1-16, Pages 20-21, Lines 20-24 and 1-2**) have been added in the revised MS.

Changes in text:

Pages 6-7, Lines 15-24 and 1-4

Pages 9-10, Lines 12-23 and 1-3, Pages 13, Lines 1-8, Pages 17-18, Lines 13-25 and 1-3

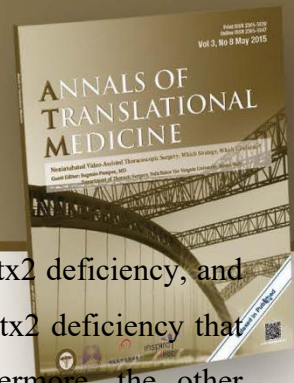
Pages 18-19, Lines 5-25 and 1-16, Pages 20-21, Lines 20-24 and 1-2

Details can be found from our answers for each specific question.

Major Comments:

Comment 2: Author Jichao Zhao is an accomplished scientist with over 100 published manuscripts, many of which focused on atrial fibrillation. At least four of his manuscripts (1. Ionic and cellular mechanisms underlying TBX5/PITX2 insufficiency-induced atrial fibrillation: Insights from mathematical models of human atrial cells, 2. Proarrhythmia in the p.Met207Val PITX2c-Linked Familial Atrial Fibrillation-Insights From Modeling, 3. PITX2 upregulation increases the risk of chronic atrial fibrillation in a dose-dependent manner by modulating IKs and ICaL—insights from human atrial modelling, and 4. In silico investigation of the mechanisms underlying atrial fibrillation due to impaired Pitx2) relate directly to evaluating Pitx2 implication in familial atrial fibrillation and the last mentioned manuscript (In silico Investigation of the Mechanisms...) is pragmatically indecipherable from the present manuscript under review.

It is for this reason that I recommend rejection, unless the authors can clarify what the novel findings of this manuscript are, and highlight them side-by-side with the February 2020 PLOS Computational Biology publication which concludes elevated Ca²⁺ ATPase function with Pitx2 deficiency (unclear whether claim relates to enzymatic efficiency [turnover number] or



increased enzyme count), depolarization/repolarization heterogeneity with Pitx2 deficiency, and a multichannel mechanism to shortened atrial action potential duration in Pitx2 deficiency that exceeds sodium handling (flecainide antiarrhythmic drug) alone. Furthermore, the other previously published manuscripts describing Pitx2 effects should be addressed with greater clarity, so as to build on the previously described scientific findings and advance the computational cardiology field in a more concise manner.

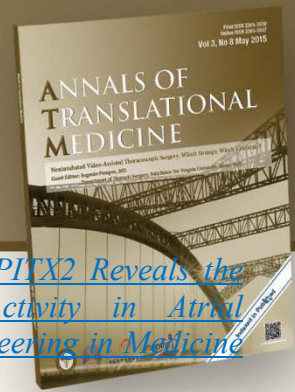
Reply 2: We thank the reviewer for this question and apologies for the confusion. As suggested, we have addressed previously published manuscripts with greater clarity (**Pages 18-19, Lines 5-25 and 1-16**) and summarized the novelty of this manuscript: 1) In spite of its potential importance, variability is often ignored in both experimental and theoretical studies (Bai et al., 2018, Bai et al., 2019, Bai et al., 2020a, Bai et al., 2020b, Bai et al., 2020c) of atrial electrophysiology properties of Pitx2-induced atrial fibrillation (AF), but we first considered the cell level variability and also identified vulnerable parameter regimes in the present study of Pitx2-induced AF. 2) Although previous studies have shown that upregulated Pitx2 is associated with shorten AP (Bai et al., 2019, Bai et al., 2020a, Bai et al., 2020b) and downregulated Pitx2 is associated with triggered AP (Bai et al., 2018, Bai et al., 2020b, Bai et al., 2020c), the correlation of the Pitx2 levels with the different models was not built. In the present study, the correlation of the Pitx2 levels with the different models (including shortened and triggered AP models) of action potential (AP) in the AF population was built by correlating the extent of Pitx2-induced electrical remodelling with the different AP models. The incidence of shortened AP was positively correlated with G_{Ks} and G_{KI} and was negatively correlated with G_{Na} , G_{CaL} and G_{up} , whereas the incidence of triggered AP was negatively correlated with G_{Ks} and G_{KI} and was positively correlated with G_{Na} , G_{CaL} and G_{up} . These simulated results suggested that the Pitx2 level was positively correlated with the incidence of shortened AP, and was negatively correlated with the incidence of triggered AP.

Bai J, Gladding P A, Stiles M K, et al. Ionic and cellular mechanisms underlying TBX5/PITX2 insufficiency-induced atrial fibrillation: Insights from mathematical models of human atrial cells. Scientific reports 2018; 8: 1-13.

Bai J, Lu Y, Lo A C, et al. Proarrhythmia in the p. Met207Val PITX2c-linked familial atrial fibrillation-insights from modelling. Frontiers in physiology 2019; 10: 1314.

Bai J, Lu Y, Lo A, et al. PITX2 upregulation increases the risk of chronic atrial fibrillation in a dose-dependent manner by modulating IKs and ICaL-insights from human atrial modelling. Annals of translational medicine 2020; 8: 191.

Bai J, Lo A, Gladding P A, et al. In silico investigation of the mechanisms underlying atrial fibrillation due to impaired Pitx2. PLoS computational biology 2020; 16: e1007678.



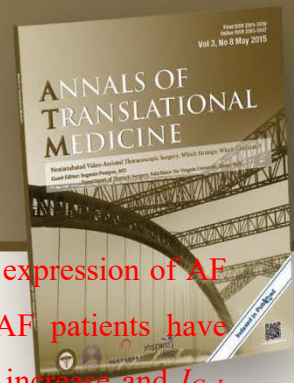
[Bai J, Zhu Y, Lo A, et al. In Silico Assessment of Genetic Variation in PITX2 Reveals the Molecular Mechanisms of Calcium-Mediated Cellular Triggered Activity in Atrial Fibrillation. 2020 42nd Annual International Conference of the IEEE Engineering in Medicine & Biology Society \(EMBC\), Montreal, QC, Canada, 2020, pp. 2353-2356.](#)

Changes in text:

Pages 18-19, Lines 5-25 and 1-16

Gain-of-function/loss-of-function of Pitx2 has been found to be associated with AF in humans (2-4, 9, 10, 19, 21). A number of previous studies have shown expression levels of Pitx2 are reduced in the left atrium in humans (2, 4, 55) and transgenic mice (2, 8, 9), or with loss-of-function mutations (56, 57). However, Pitx2 expression has been found to be increased in the left atrium (58) and in right atrial myocytes (3), from AF patients, or with the gain-of-function mutation p.Met207Val (59). The present study is consistent with this notion, indicating clearly a marked function impact of gain-of-function/loss-of-function of PITX2 on atrial cell electrophysiology that would promote susceptibility to AF. In previous simulated studies, we have shown that a shorten APD₉₀ arising from the Pitx2 overexpression (17) or the gain-of-function mutation p.Met207Val (19) increased atrial susceptibility to arrhythmias, and that abnormalities in intracellular calcium handling due to the Pitx2 downregulation (18, 20) or the loss-of-function mutation rs13143308T (31) increased the inducibility of triggered activity which can be suppressed by drugs such as dantrolene and flecainide. Our data are consistent with this. They indicate that Pitx2-induced electrical remodelling not only contributes to the APD₉₀ shortening in spatial vulnerability to arrhythmia, but also increases the susceptibility of the human atrial cell to the genesis of triggered activity. Therefore, our study substantiates a causative link between Pitx2-induced electrical remodelling and AF.

Accumulating evidence places particular emphasis on atrial arrhythmogenicity due to intersubject variability in Pitx2 levels (9), in producing the electrical remodelling in a dose-dependent manner (3, 7). The present study is both consistent with and extends this notion, indicating a relationship between the PITX2 alteration and arrhythmia onset. The general incidence of AF increases with age. The previous study of Scridon et al. have shown that PITX2 expressions showed a progressive, age-dependent change and were negatively correlated with both age and heart weight in hypertensive rats (5). It indicated a possible temporal relationship between the PITX2 alteration and arrhythmia onset, suggesting PITX2 alteration is an age-dependent process that starts before the occurrence of arrhythmias. And Lozano-Velasco et al. identified a complex regulatory network orchestrated by PITX2,

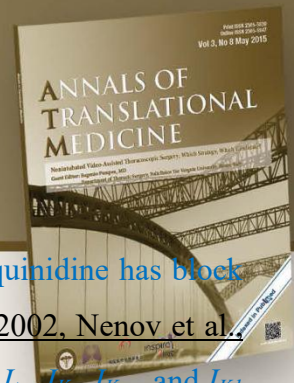


demonstrating a dose-dependent relation between PITX2 expression and the expression of AF susceptibility genes (7). Importantly, human right atrial myocytes from AF patients have shown PITX2 expression increases and this increase correlates with the I_{Ks} increase and I_{CaL} decrease that characterizes AF-induced electrical remodeling, demonstrating the substrate for arrhythmogenesis in these AF patients is dependent upon electrical remodeling modulated by the PITX2 expression in a dose-dependent manner (3). Our data are consistent with this. They indicated that the incidence of shortened AP was correlated with the extent of electrical remodelling due to Pitx2 upregulation and the incidence of triggered activity was correlated with the extent of electrical remodelling due to Pitx2 downregulation. Therefore, the correlation of the Pitx2 levels with arrhythmia onset with different mechanisms was built.

Comment 3: Zhu et al. claim that all major cations implicated in the cardiac action potential (sodium, calcium, and potassium) are implicated in the “Pitx2-related remodeling”. While this may very well be the case, it is unclear how this information is 1) novel (see major comment number 2 regarding multichannel mechanism), or 2) pragmatically useful as any clinical electrophysiologist or cardiologist would seek a single current to target for therapeutic conversion of atrial fibrillation to normal sinus rhythm.

Reply 3: Thank you for this important question. As the reviewer suggests, we agree that Pitx2-induced AF may be a multichannel mechanism (The incidence of shortened AP was positively correlated with G_{Ks} and G_{KI} and was negatively correlated with G_{Na} , G_{CaL} and G_{up} , whereas the incidence of triggered AP was negatively correlated with G_{Ks} and G_{KI} and was positively correlated with G_{Na} , G_{CaL} and G_{up}).

In the revised MS, the novel findings of this manuscript (See reply number 2 regarding the novel findings of MS and **Pages 20-21, Lines 20-24 and 1-2**) were added. Based on these findings and AP models in the Pitx2-induced AF population, in further studies, we can predict the effects of antiarrhythmic drugs used in clinical practice on Pitx2-induced APs and then screen potential drugs for the therapeutic conversion of Pitx2-induced AF to normal sinus rhythm. Although electrophysiologist or cardiologist would seek a single current to target, most antiarrhythmic drugs have block effects on multiple ion channels. For example, experimental studies on Class I ADDs showed flecainide has block effects on I_{Na} , I_{Kr} and RyR (Watanabe et al., 2009, Belardinelli et al., 2013), disopyramide has block effects on I_{Na} , I_{CaL} , I_{to} , I_{Ks} , I_{Kr} and I_{Kur} (Kramer



et al., 2013, Hanada et al., 2003, Satoh et al., 2000, Aréchiga et al., 2008), [quinidine has block effects on \$I_{Na}\$, \$I_{CaL}\$, \$I_{to}\$, \$I_{Ks}\$, \$I_{Kr}\$, \$I_{Kur}\$ and \$I_{K1}\$ \(Kramer et al., 2013, Zhang et al., 2002, Nenov et al., 1998, Kang et al., 2001\) and propafenone has block effects on \$I_{Na}\$, \$I_{CaL}\$, \$I_{to}\$, \$I_{Kr}\$, \$I_{Kur}\$ and \$I_{K1}\$ \(Zhang et al., 2015, Katchman et al., 2006, Hancox et al., 1997, Gross et al., 1998, Franqueza et al., 1998, Amorós et al., 2013\).](#)

[Watanabe H, Chopra N, Laver D, Hwang HS, Davies SS, Roach DE, et al. Flecainide prevents catecholaminergic polymorphic ventricular tachycardia in mice and humans. *Nature medicine*. 2009;15\(4\):380. pmid:19330009](#)

[Belardinelli L, Liu G, Smith-Maxwell C, Wang W-Q, El-Bizri N, Hirakawa R, et al. A novel, potent, and selective inhibitor of cardiac late sodium current suppresses experimental arrhythmias. *Journal of Pharmacology and Experimental Therapeutics*. 2013;344\(1\):23–32. pmid:23010360](#)

[Kramer J, Obejero-Paz CA, Myatt G, et al. MICE models: superior to the HERG model in predicting Torsade de Pointes. *Scientific reports* 2013;3\(1\):1-7.](#)

[Hanada E, Ohtani H, Hirota M, et al. Inhibitory effect of erythromycin on potassium currents in rat ventricular myocytes in comparison with disopyramide. *Journal of pharmacy and pharmacology* 2003;55\(7\):995-1002.](#)

[Satoh H. Comparative actions of cibenzoline and disopyramide on \$I_{Kr}\$ and \$I_{Ks}\$ currents in rat sino-atrial nodal cells. *European journal of pharmacology* 2000;407\(1-2\):123-29.](#)

[Aréchiga IA, Barrio-Echavarria GF, Rodríguez-Menchaca AA, et al. \$Kv1.5\$ open channel block by the antiarrhythmic drug disopyramide: molecular determinants of block. *Journal of pharmacological sciences* 2008;108\(1\):49-55.](#)

[Zhang YH, Hancox JC. Mode-dependent inhibition by quinidine of \$Na^+-Ca^{2+}\$ exchanger current from guinea-pig isolated ventricular myocytes. *Clinical and experimental pharmacology and physiology* 2002;29\(9\):777-81.](#)

[Nenov NI, Crumb Jr WJ, Pigott JD, Harrison Jr LH, Clarkson CW. Quinidine interactions with human atrial potassium channels: developmental aspects. *Circulation research* 1998;83\(12\):1224-31.](#)

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[Zhang H, Zou B, Du F, et al. Reporting sodium channel activity using calcium flux: pharmacological promiscuity of cardiac \$Nav1.5\$. *Molecular pharmacology*. 2015, 87\(2\): 207-217.](#)

[Katchman AN, Koerner J, Tosaka T, Woosley RL, Ebert SN. Comparative evaluation of HERG currents and QT intervals following challenge with suspected torsadogenic and nontorsadogenic drugs. *J Pharmacol Exp Ther*. 2006 Mar;316\(3\):1098-106. doi: 10.1124/jpet.105.093393. Epub 2005 Nov 8. PMID: 16278312.](#)

[Hancox JC, Mitcheson JS. Inhibition of L-type calcium current by propafenone in single myocytes isolated from the rabbit atrioventricular node. *British journal of pharmacology* 1997;121\(1\):7-14.](#)

[Gross GJ, Castle NA. Propafenone inhibition of human atrial myocyte repolarizing currents. *Journal of molecular and cellular cardiology* 1998;30\(4\):783-93.](#)

[Franqueza L, Valenzuela C, Delpón E, et al. Effects of propafenone and 5-hydroxy-propafenone on \$hKv1.5\$ channels. *British journal of pharmacology* 1998;125\(5\):969.](#)



[Amorós I, Dolz-Gaitón P, Gómez R, et al. Propafenone blocks human cardiac Kir2. x channels by decreasing the negative electrostatic charge in the cytoplasmic pore. Biochemical Pharmacology 2013;86\(2\):267-78.](#)

Changes in text:

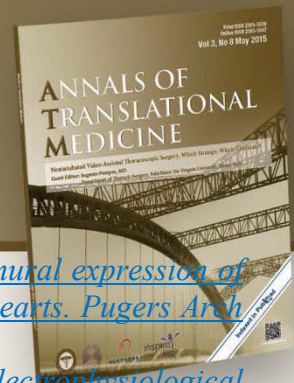
Pages 18-19, Lines 5-25 and 1-16 (See reply number 2) and **Pages 20-21, Lines 20-24 and 1-2**

By using this computational framework, our results suggest that the incidence of shortened AP was positively correlated with IKs and IK1 and was negatively correlated with INa, ICaL and SERCA, whereas the incidence of triggered AP was negatively correlated with IKs and IK1 and was positively correlated with INa, ICaL and SERCA. Based on the correlation of Pitx2-induced remodelling with different AF models and the correlation of Pitx2 levels with the electrical remodelling in previous studies, we can conclude that the Pitx2 level may be positively correlated with the occurrence of AP shortening, but negatively correlated with the incidence of triggered AP.

Comment 4: Zhu et al. claim that “the ionic and cellular mechanisms may differ among patients because the underlying genetics of each patient determines the Pitx2-induced remodelling process and its effects on ion channel currents.” This claim is not satisfactory as it would suggest that there is not a conserved effect between all humans expressing both wild type Pitx2 and abnormal Pitx2. It seems highly improbable to me that the altered transcription and/or translation of Pitx2 yields no reproducible effect on cardiomyocytes with respect to membrane or action potential homeostasis. Hox genes are highly conserved with respect to structure and one would anticipate that knockout or knockdown of Pitx2 would also be somewhat consistent in effect. Furthermore, if the mechanisms of Pitx2 effects differ among patients, what is the utility in devoting computational resources to this endlessly heterogeneous population? A citation is needed for this claim.

Reply 4: We apologize for any confusion in our manuscript presentation. We have changed the sentence to “the ionic and cellular mechanisms may be different because mRNA expression levels might vary with circadian rhythm ([Bray et al., 2008](#), [Jeyaraj et al., 2012](#)), gender differences ([Gaborit et al., 2010](#), [Ambrosi et al., 2013](#), [James et al., 2007](#)) and spatial location within the heart ([Soltysinska et al., 2009](#))” and given citations (**Page 4, Lines 10-14**).

[Gaborit N, Varro' A, Le Boulter S, Szuts V, Escande D, et al. \(2010\) Gender related differences in ion-channel and transporter subunit expression in nondiseased human hearts. J Mol Cell Cardiol 49: 639–646.](#)



[Soltysinska E, Olesen S, Christ T, Wettwer E, Varro' A, et al. \(2009\) Transmural expression of ion channels and transporters in human nondiseased and end-stage failing hearts. Pagers Arch 459: 11–23.](#)

[Ambrosi C, Yamada K, Nerbonne J, Efimov I \(2013\) Gender differences in electrophysiological gene expression in failing and nonfailing human hearts. PLOS ONE 8\(1\): e54635.](#)

[Nattel S, Frelin Y, Gaborit N, Loualt C, Demolombe S \(2010\) Ion-channel mRNA-expression profiling: Insights into cardiac remodeling and arrhythmic substrates. J Mol Cell Cardiol 48: 96–105.](#)

[Bray M, Shaw C, Moore M, Garcia R, Zanutta M, et al. \(2008\) Disruption of the circadian clock within the cardiomyocyte influences myocardial contractile function, metabolism, and gene expression. Am J Physiol Heart Circ Physiol 294: H1036–H1047.](#)

[Jeyaraj D, Haldar S, Wan X, McCauley M, Ripperger J, et al. \(2012\) Circadian rhythms govern cardiac repolarization and arrhythmogenesis. Nature 483:](#)

[James A, Choisy S, Hancox J \(2007\) Recent advances in understanding sex differences in cardiac repolarization. Prog Biophys Mol Bio 94: 265–319.](#)

Changes in text:

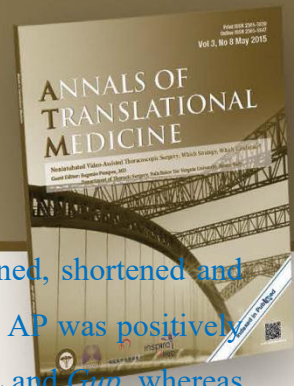
Page 4, Lines 10-14

Although AP duration (APD) shortening (9), depolarised resting membrane potential (RMP) (4) and triggered activity (8, 10) were observed, the ionic and cellular mechanisms may be different because mRNA expression levels might vary with circadian rhythm (11, 12), gender differences (13-15) and spatial location within the heart (16).

Comment 5: It was not easy to deduce why the authors believe that either a down- or upregulation of Pitx2 could yield the same physiologic effect.

Reply 5: We thank the reviewer for this question and apologies for the confusion. We agree with this reviewer that a down- or upregulation of Pitx2 leads to different physiologic effects. According to the comments of this reviewer, we have modified our methods (Pages 9-10, Lines 12-23 and 1-3), results (Pages 13, Lines 1-8) and discussion (Pages 17-18, Lines 13-25 and 1-3) for providing a comprehensive description of the different physiologic effect due to a down- or upregulation of Pitx2.

In our MS, we assumed that changes in the Pitx2 level are translated to the extent of electrical remodelling (including GNa, GKs, GK1, GCaL, Grel and Gup) (Pérez-Hernández et al., 2016, Lozano-Velasco et al., 2016, Nadadur et al., 2016). The correlation of the Pitx2 levels with the different AF models developed based on Pitx2-induced electrical remodelling can be built. In the revised MS, we have used the AF models and the corresponding remodelled parameters to train the artificial neural network to determine the weights of remodelled parameters (listed in



the new **Table 3**) for the classification of different AF subgroups (unshortened, shortened and triggered groups). As shown in the new **Figure 8**, the incidence of shortened AP was positively correlated with GKs and GKI and was negatively correlated with GNa , $GCaL$ and Gup , whereas the incidence of triggered AP was negatively correlated with GKs and GKI and was positively correlated with GNa , $GCaL$ and Gup . According to experimental studies, *Pitx2* deficiency is associated with the increase in GNa , Gup (Nadadur et al., 2016) and $GCaL$ (Dai et al., 2019) and the reduction in GKI (Syeda et al., 2016), whereas *Pitx2* overexpression is associated with upregulated GKs and downregulated $GCaL$ (Pérez-Hernández et al., 2016). Therefore, we can conclude that the downregulation of *Pitx2* is associated with the triggered activity, whereas the upregulation of *Pitx2* contributes to AP shortening.

Chinchilla A, Daimi H, Lozano-Velasco E, et al. PITX2 insufficiency leads to atrial electrical and structural remodeling linked to arrhythmogenesis. Circulation: Cardiovascular Genetics 2011; 4: 269-279.

Pérez-Hernández M, Matamoros M, Barana A, et al. Pitx2c increases in atrial myocytes from chronic atrial fibrillation patients enhancing I Ks and decreasing I Ca. L. Cardiovascular research 2016; 109: 431-441.

Lozano-Velasco E, Hernandez-Torres F, Daimi H, et al. Pitx2 impairs calcium handling in a dose-dependent manner by modulating Wnt signalling. Cardiovascular research 2016; 109: 55-66.

Nadadur R D, Broman M T, Boukens B, et al. Pitx2 modulates a Tbx5-dependent gene regulatory network to maintain atrial rhythm. Science translational medicine 2016; 8: 354ra115-354ra115.

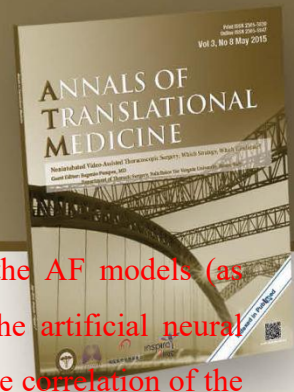
Syeda F, Holmes A P, Ting Y Y, et al. PITX2 modulates atrial membrane potential and the antiarrhythmic effects of sodium-channel blockers. Journal of the American College of Cardiology 2016; 68: 1881-1894.

Dai W, Laforest B, Tyan L, et al. A calcium transport mechanism for atrial fibrillation in Tbx5-mutant mice. Elife. 2019; 8:e41814.

Changes in text:

Pages 9-10, Lines 12-23 and 1-3

Previous studies have shown that changes in *Pitx2* levels lead to electrical remodelling linked to arrhythmogenesis (2) and that *Pitx2* regulates membrane effector genes associated with the electrical remodelling in a dose-dependent manner (3, 7, 8). Therefore, we assumed that changes in the *Pitx2* level are translated to the extent of electrical remodelling (including GNa , GKs , GKI , $GCaL$, $Grel$ and Gup) (3, 7, 8). The correlation of the *Pitx2* levels with the different AF models developed based on *Pitx2*-induced electrical remodelling was built by evaluating the contribution of remodelled targets (including GNa , GKs , GKI , $GCaL$, $Grel$ and Gup) to the changes in APs (including unshortened AP, shortened AP and triggered AP). The extent of electrical remodelling was quantified with weights of remodelled targets for classification of different AF



subgroups (unshortened, shortened and triggered groups). Here, we used the AF models (as output) and the corresponding remodelled parameters (as input) to train the artificial neural network (28) to determine the weights of remodelled parameters. Based on the correlation of the Pitx2 levels with remodelled parameters observed in previous studies (3, 4, 7, 8, 29) and the correlation of remodelled parameters with changes in APs, the correlation of the Pitx2 levels with different AF models was built.

Pages 13, Lines 1-8

By using the artificial neural network, we got the weights of six remodelled parameters for classifying AF models (including shortened and triggered AP two categories) (Figure 8). For the shortened AP category, weights of GKs and GK1 are negative and weights of GNa, GCaL and Gup are positive, indicating the incidence of shortened AP is positively correlated with GKs and GK1 and is negatively correlated with GNa, GCaL and Gup. In contrast, for the triggered AP category, weights of GKs and GK1 are positive and weights of GNa, GCaL and Gup are negative. In addition, weights of Grel for shortened AP and triggered AP categories are positive. In detail, the values of weights of different remodelled targets can be found in Table 3.

Pages 17-18, Lines 13-25 and 1-3

Previous studies have shown that changes in Pitx2 levels lead to electrical remodelling linked to arrhythmogenesis (2) and that Pitx2 regulates membrane effector genes associated with the electrical remodelling in a dose-dependent manner (3, 7, 8). Therefore, based on the correlation of the Pitx2 levels with remodelled parameters observed in previous studies (3, 4, 7, 8, 29) and the correlation of remodelled parameters with changes in APs in the present study, the correlation of the Pitx2 levels with different AF models can be built. In the previous studies, Pitx2 deficiency is associated with the increase in G_{Na} , G_{up} (8) and G_{CaL} (29) and the reduction in G_{K1} (4). Interestingly, our simulated results indicated that the incidence of triggered AP was negatively correlated with G_{Ks} and G_{K1} and was positively correlated with G_{Na} , G_{CaL} and G_{up} . Therefore, the downregulation of Pitx2 may increase the incidence of triggered AP. In addition, the study of Pérez-Hernández et al.(3) showed that Pitx2 overexpression is associated with upregulated G_{Ks} and downregulated G_{CaL} . And our simulated results showed that the incidence of shortened AP was positively correlated with G_{Ks} and G_{K1} and was negatively correlated with G_{Na} , G_{CaL} and G_{up} . Therefore, the upregulation of Pitx2 may favor to AP shortening. Thus, the Pitx2 level may be positively correlated with the occurrence of AP shortening, but negatively correlated with the incidence of triggered AP.

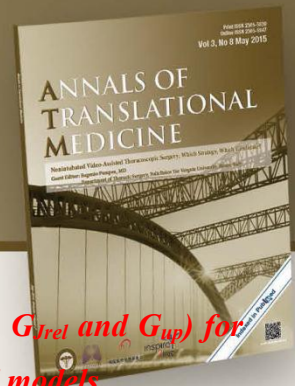


Figure 8 and Table 3

Table 3. Weights of remodelled parameters (including G_{Na} , G_{Ks} , G_{K1} , G_{CaL} , G_{Jrel} and G_{up}) for classifying unshortened, shortened and triggered AP three categories in AF models.

	G_{Na}	G_{Ks}	G_{K1}	G_{CaL}	G_{up}	G_{rel}
<i>Unshortened AP</i>	4.9065	-5.5615	7.8389	9.4655	-2.8048	-7.969
<i>Shortened AP</i>	-7.4592	14.6805	3.736	-21.6137	-4.0507	6.1308
<i>Triggered AP</i>	3.1941	-9.3017	-11.7621	12.4555	6.9198	1.4855

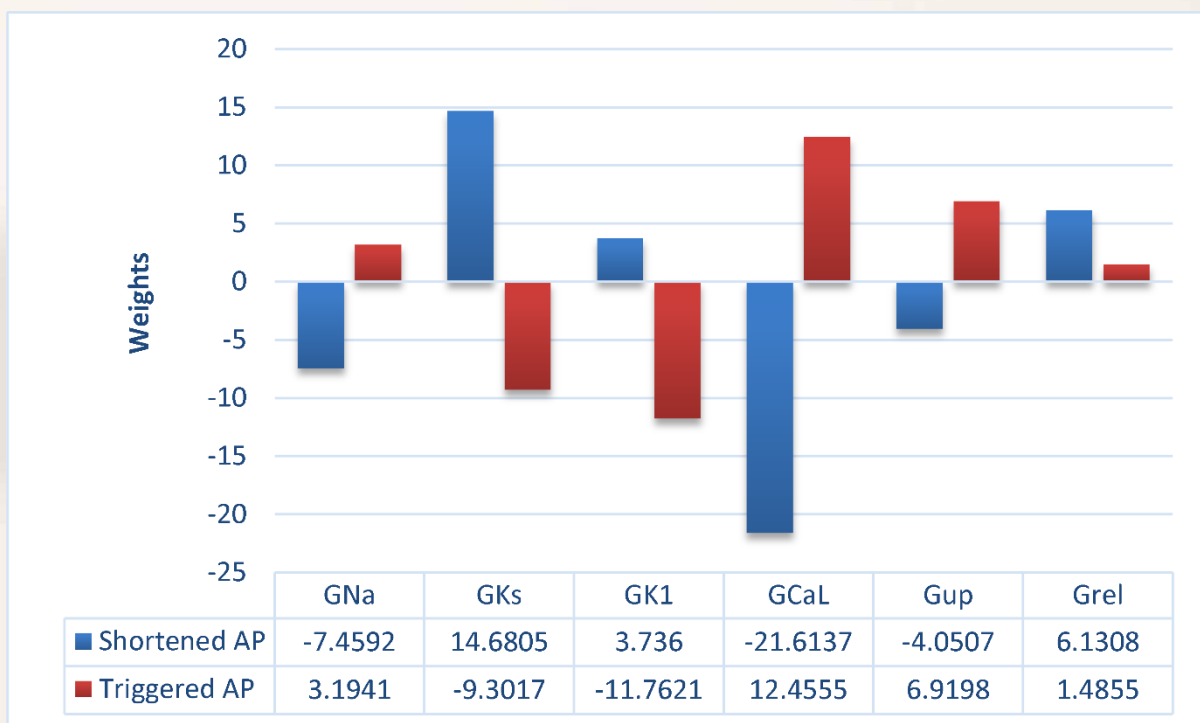
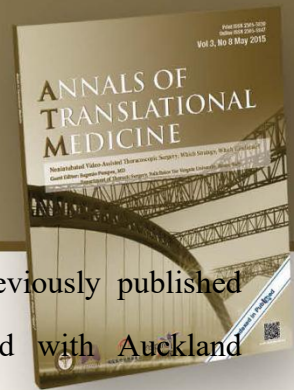


Figure 8. Weights of remodelled parameters (including G_{Na} , G_{Ks} , G_{K1} , G_{CaL} , G_{Jrel} and G_{up}) for classifying shortened and triggered AP three categories in AF models.

Minor Comments:



Comment 6: Author Jieyun Bai is listed on Google Scholar and in previously published manuscripts (please see major comment number one) to be affiliated with Auckland Bioengineering Institute, however in the present manuscript the affiliation for J Bai is Jinan University. Please clarify this discrepancy and rectify if need be.

Reply 6: We thank the reviewer for this question and apologies for the confusion. Between 2018 and 2019, I worked as a postdoctoral fellow with Jichao Zhao for postdoc training at Auckland Bioengineering Institute. From March 2019, I join Jinan University. However, online profiles were not updated, and I will update this information as soon as possible to avoid misleading.

Changes in text:

Page 1, Lines 5-8

YiJie Zhu¹, Jieyun Bai^{1*}, Andy Lo², Yaosheng Lu¹, Jichao Zhao²

¹Department of Electronic Engineering, College of Information Science and Technology, Jinan University, Guangzhou, 510632, China

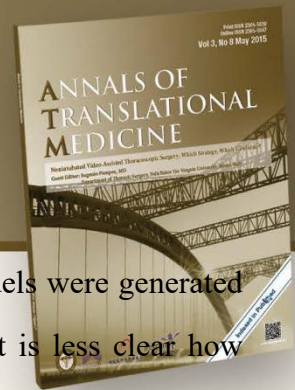
Comment 7: Abstract – Background (line 3): “substrate” refers to the necessary precursor for a given reaction/phenomenon to occur. Based on Zhu et al.’s use of the word “substrate” in this sentence, it would seem that the substrate in Pitx2-regulated atria is already present. Is that what the authors are attempting to conclude? Or perhaps that the arrhythmogenic substrate for atrial fibrillation is created via pathophysiologic processes such as hypertension, coronary artery disease, or type two diabetes mellitus, and may be accentuated by Pitx2 down- or upregulation? In summary, this background sentence produces more confusion than clarification in me.

Reply 7: We apologize for this confusion in our manuscript presentation. As suggested, we have changed the sentence to “Electrical remodelling as a result of the homeodomain transcription factor 2 (Pitx2)-dependent gene regulation induces atrial fibrillation (AF) with different mechanisms”

Changes in text

Page 2, Lines 2-4

Background: Electrical remodelling as a result of the homeodomain transcription factor 2 (Pitx2)-dependent gene regulation induces atrial fibrillation (AF) with different mechanisms.



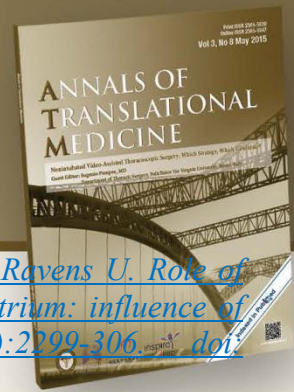
Comment 8: While it is redundantly clarified that 30,000 mathematical models were generated (however no justification for this number is provided to my knowledge), it is less clear how many normal sinus rhythm patients and atrial fibrillation patients were included in the study, nor what clinical equipment was utilized to collect the in vivo atrial action potentials.

Reply 8: Thank you for this important question. As the reviewer suggests we agree that the justification for the number of models generated ([Page 7, Lines 13-16](#)), and data on in vivo atrial action potentials of patients should be provided ([Pages 6-7, Lines 15-24 and 1-4](#)) in the MS.

A previous study ([Britton et al., 2013](#)) showed that 10,000 variants were sufficient for convergence of the sensitivity coefficients for both SR and AF model populations, because a larger number of virtual cells had to be excluded upon calibration and subsequent analyses, we tripled the initial size of the model population to 30,000 variants. For our experimental dataset, AP recordings from $n=254$ right atrial appendages of $N=214$ SR patients and from $n=215$ right atrial appendages of $N=149$ AF patients, were published in the previous study ([Sánchez et al., 2014](#)). In the previous experimental study, right atrial appendages were obtained when patients were undergoing cardiac surgery for coronary artery bypass grafting or mitral/aortic valve replacement. Antiarrhythmic drugs were discontinued before the study. Human myocytes were isolated enzymatically from atrial appendages as previously described ([Dobrev et al., 2000](#)) and APs were recorded with standard intracellular microelectrodes in atrial trabeculae ([Wettwer et al., 2004](#), [Wettwer et al., 2013](#)). Bath solution contained (in mM): NaCl 127, KCl 4.5, MgCl₂ 1.5, CaCl₂ 1.8, glucose 10, NaHCO₃ 22, NaH₂PO₄ 0.42, equilibrated with O₂-CO₂ [95:5] at $36.5\pm 0.58^{\circ}\text{C}$, pH 7.4. Preparations were regularly stimulated at 1 Hz for at least 1 h before data acquisition ([Wettwer et al., 2004](#), [Wettwer et al., 2013](#)). The following parameters were quantified to characterize inter-subject variability in human atrial AP: action potential amplitude (APA), maximum upstroke velocity (dV/dt_{max}), RMP, and APD at 20% (APD₂₀), 50% (APD₅₀) and 90% (APD₉₀). Minimum, maximum and mean \pm standard deviation values for these biomarkers are presented in **Table 2**.

[Britton O J, Bueno-Orovio A, Van Ammel K, et al. Experimentally calibrated population of models predicts and explains intersubject variability in cardiac cellular electrophysiology. Proceedings of the National Academy of Sciences 2013; 110: E2098-E2105.](#)

[Sánchez C, Bueno-Orovio A, Wettwer E, et al. Inter-subject variability in human atrial action potential in sinus rhythm versus chronic atrial fibrillation. PloS one 2014; 9: e105897.](#)



[Wettwer E, Hála O, Christ T, Heubach JF, Dobrev D, Knaut M, Varró A, Ravens U. Role of IKur in controlling action potential shape and contractility in the human atrium: influence of chronic atrial fibrillation. Circulation. 2004 Oct 19;110\(16\):2299-306. doi: 10.1161/01.CIR.0000145155.60288.71. Epub 2004 Oct 11. PMID: 15477405.](#)

[Wettwer E, Christ T, Endig S, Rozmaritsa N, Matschke K, Lynch JJ, Pourrier M, Gibson JK, Fedida D, Knaut M, Ravens U. The new antiarrhythmic drug vernakalant: ex vivo study of human atrial tissue from sinus rhythm and chronic atrial fibrillation. Cardiovasc Res. 2013 Apr 1;98\(1\):145-54. doi: 10.1093/cvr/cvt006. Epub 2013 Jan 22. PMID: 23341576.](#)

[Dobrev D, Wettwer E, Himmel HM, Kortner A, Kuhlisch E, Schüler S, Siffert W, Ravens U. G-Protein beta\(3\)-subunit 825T allele is associated with enhanced human atrial inward rectifier potassium currents. Circulation. 2000 Aug 8;102\(6\):692-7. doi: 10.1161/01.cir.102.6.692. PMID: 10931811.](#)

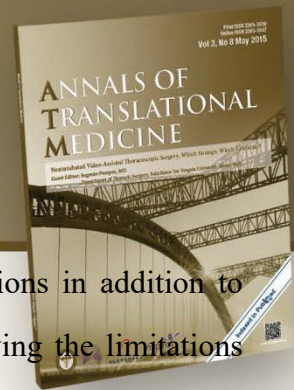
Changes in text:

Pages 6-7, Lines 15-24 and 1-4

For our experimental dataset, AP recordings from $n=254$ right atrial appendages of $N = 214$ SR patients and from $n=215$ right atrial appendages of $N = 149$ AF patients, were published in the previous study (23). In the previous experimental study, right atrial appendages were obtained when patients were undergoing cardiac surgery for coronary artery bypass grafting or mitral/aortic valve replacement. Antiarrhythmic drugs were discontinued before the study. Human myocytes were isolated enzymatically from atrial appendages as previously described (32) and APs were recorded with standard intracellular microelectrodes in atrial trabeculae (33, 34). Bath solution contained (in mM): NaCl 127, KCl 4.5, MgCl₂ 1.5, CaCl₂ 1.8, glucose 10, NaHCO₃ 22, NaH₂PO₄ 0.42, equilibrated with O₂-CO₂ [95:5] at $36.5 \pm 0.58^\circ\text{C}$, pH 7.4. Preparations were regularly stimulated at 1 Hz for at least 1 h before data acquisition (33, 34). The following parameters were quantified to characterize inter-subject variability in human atrial AP: action potential amplitude (APA), maximum upstroke velocity (dV/dt_{\max}), RMP, and APD at 20% (APD₂₀), 50% (APD₅₀) and 90% (APD₉₀) (**Figure 1B**). Minimum, maximum and mean \pm standard deviation values for these biomarkers are presented in **Table 2**. More information (including ethics approval, informed consent and basic information of participants) regarding the experimental conditions under which the data were collected is available in the study of Sánchez et al.(23).

Page 7, Lines 13-16

These parameters were sampled over a range from -100% to +200% of their original value with the Latin hypercube sampling (LHS) methodology (22) to create 30,000 candidate models that were sufficient for convergence of the sensitivity coefficients for both SR and AF model populations (22).



Comment 9: It would seem that the limitations section contains clarifications in addition to limitations. This information would be better placed in the discussion, leaving the limitations section for easily decipherable shortcomings in study design or limitations in currently available technology.

Reply 9: Thank you for this valid question. As the reviewer suggests we have moved clarifications (**Pages 14-15, Lines 23-25 and 1-13**) to the subsection (“AF population of models”) of the discussion, leaving shortcomings in study design.

Changes in text:

Pages 14-15, Lines 23-25 and 1-13

In the present study, the TPA model was used as the basal model to investigate the mechanisms underlying Pitx2-induced AF. It had been validated and fully assessed by comparing with existing human atrial models (including Lugo et al. model (42), Maleckar et al. model (43), Nygren et al. model (44), Koivumaki et al. model (45), Courtemanche et al. model (46), Grandi et al. model (47), CRN_TP model (18, 20) and Voigt et al. model (48)). This model was chosen as it is able to reproduce human AP morphology, APD rate dependence and triggered activity, i.e., early afterdepolarizations (EADs), delayed afterdepolarizations (DADs) and spontaneous depolarizations. It also was used to investigate the pro-arrhythmic effects of Pitx2-induced remodelling and it can reproduce phenomena (including triggered activity and AP shortening) observed in the experiments. In our previous study, simulations based on similar models, such as the CRN_TP model and Grandi et al. model, had been conducted to explain model dependence of results by reproducing Pitx2-induced AP shortening and triggered activity (18). Simulations with an alternative human atrial cell model should not alter the main findings of this study, although the inclusion of the intersubject variability approximates *in silico* experiments to more realistic clinical scenarios.