# Ionic Atrial Remodelling in Atrial Fibrillation and its effect in the cardiac action potential

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## Abstract

*Atrial fibrillation (AF), most common sustained arrhythmia in humans, especially in the elderly, is characterized by rapid ineffective atrial activity with irregular ventricular contractions. The increasing elderly population justifies the growing interest in the underlying mechanisms and the investigation of prophylactic treatment of this disease.*

*This project aims to characterize the early stages of atrial remodelling and its effects on the properties of the heart, at the genetic and electrophysiological levels, in order to clarify some of the pathophysiological effects of AF. A novel approach was used to analyse the atrial electrical signals, by means of an iterative fitting algorithm based on the Levenberg-Marquardt compromise that models the signal using a set of generalized Gauss functions.*

*An extensive experimental approach was devised, using rapid atrial electrical stimulation in laboratory animals to simulate the chaotic activation pattern of AF. During these experiments the electrical activity of the heart was monitored and samples of atrial tissue were collected for posterior RNA quantification of specific genes, encoding cardiac ion channels. The atrial recordings were correlated with the changes in expression of ion channels in the atrial tissue, to identify causality between them.*

## 1. Atrial Fibrillation: From Genetics to Signals

Atrial fibrillation (AF) is the most common sustained arrhythmia in humans, it is characterized by rapid ineffective atrial activity with irregular ventricular contractions.

AF occurs in approximately 0.4% to 1.0% of the general population and it affects more than 2 million people in the United States annually. Its prevalence increases with age and up to 10% of the population older than 80 years has been diagnosed with AF at some point.

The ever growing numbers justify the importance of AF and the growing interest in the underlying mechanisms and the investigation of prophylactic treatment of AF, especially with the projected growth of the elderly population.

Atrial fibrillation occurs when the electrical impulses in the atria degenerate from their usual organized pattern into a rapid chaotic pattern. This disruption results in an irregular and often rapid heartbeat that is classically described as “irregularly irregular” and is due to the unpredictable conduction of these disordered impulses across the atrioventricular node and to the ventricles.

Changes in the properties of functional expression of myocardial ion channels, resulting from inherited mutations in the genes encoding these channels or from myocardial disease, can lead to changes in action potential waveforms, synchronization, and/or propagation, thereby predisposing the heart to potentially life-threatening arrhythmias. These alterations appear, in many instances, as part of the homeostatic adaptive response to a primary abnormality, but often result in secondary cardiac dysfunction, including excessively rapid cardiac rhythms called tachyarrhythmias.

In AF the normal ionic currents that characterize the atrial action potential are disturbed by the arrhythmia and this fact might be determinant for the perpetuation of the disease.

During AF a formed atrial contraction does not exist and so one of its hallmark signs is the absence of normal P waves in the electrocardiogram.

Re-entry conduction occurs throughout the atria and ectopic pacemakers may be present. The conduction intermittently passes through the AV node into the ventricles, causing the QRS complexes to occur at irregular intervals. This leads to an irregular heart rhythm with ventricular contraction occurring at rates higher than 100 beats per minute. The resultant dilation facilitates disorganized atrial conduction that leaves the

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normal conduction pathway intermittently and circles back into the conduction pathway. This conduction is called a re-entry circuit. Re-entry circuits and ectopic foci represent the underlying conduction abnormalities of atrial fibrillation.

For many years the electrophysiological mechanism of atrial fibrillation was thought to involve several coexisting re-entrant wavefronts or wavelets continuously sweeping around the atria, repeatedly encountering excitable myocardium. The phenomenon of re-entry is a conduction anomaly promoted by decreased atrial refractory periods, slowed conduction and an increased mass of cardiac tissue.

Recent insights about the factors involved in the initiation and maintenance of AF suggest a more complex mechanism, including a set of possible triggers that induce AF and the substrate that has the ability to sustain it. Triggers include sympathetic or parasympathetic stimulation, bradycardia, atrial premature beats or tachycardia, accessory atrioventricular pathways and acute atrial stretch. Recently identified as triggers are ectopic foci occurring in "sleeves" of atrial tissue within the pulmonary veins or vena caval junctions. Initiation and maintenance of AF may depend on uninterrupted periodic activity of a few discrete reentrant sources localized to the left atrium, emanating from such sources to propagate through both atria and interact with anatomical and/or functional obstacles, leading to fragmentation and wavelet formation.

Once initiated, AF may be brief. A variety of factors may act as perpetuators, ensuring the persistence of AF for longer periods. One is persistence of the triggers and initiators that induce AF, but at some point, AF persists even in their absence. Persistence here may result from electrical and structural changes, characterized by atrial dilatation and shortening of the atrial efective refractory period. This combination, along with other changes, likely facilitates the appearance of multiple reentrant wavelets (a final common pathway for AF).

The longer AF persists, the more difficult it is to restore sinus rhythm and prevent recurrence. This is because AF induces a series of changes within the tissue. The process by which these alterations in electrophysiological, mechanical and structural properties of the tissue take place, caused by the arrhythmia itself is termed "atrial remodelling in AF". The net effect of these modifications is to decrease the atrial refractory period and possibly interfere with atrial conduction in a spatially heterogeneous way, i.e., the magnitude of the changes varies in different locations, increasing electrical heterogeneity and thus promoting fibrillation.

### 1.1. Ionic Remodelling in Atrial Fibrillation

Whatever the initial cause of AF or the determinants for its recurrence, electrical remodelling is likely to be a final common pathway that ultimately supervenes. Recent advances in understanding ion channel function, regulation and remodelling, at the molecular level, have allowed a much more detailed appreciation of the basic determinants of AF.

The AF-promoting effect of AF is associated with a progressive decrease in atrial effective refractory period (ERP) and in the AF cycle length. Similar changes are observed after either electrically maintained AF or rapid atrial pacing in experimental models [1]. AF induces electrical remodelling primarily by virtue of a very rapid atrial rate.

The evolving information regarding atrial tachycardia-induced remodelling (ATR) has fundamental implications regarding the mechanisms of AF. Since all cases of AF involve very rapid atrial activation, tachycardia-induced remodelling will inevitably follow. Thus, even if AF begins as a result of other mechanisms, such as single reentrant circuits (mother waves) with fibrillatory conduction or rapid ectopic activity, tachycardia remodelling will act as a final common pathway to reduce the wavelength in a heterogeneous fashion and promote multiple-circuit reentry.

All these changes occuring in the heart tissue as a response to AF and in an analogous way in ATR are thought to be caused, initially by functional adaptation of the cardiac ion channels and latter by the regulation of their genetic expression levels. Of course there can be numerous possible mechanisms (transcriptional, translational, and posttranslational) that could be involved in regulating the functional expression and the properties of these channels.

The prominent changes in atrial refractoriness caused by AF (and atrial tachycardias in general) point to important alterations in the atrial action potential and particularly action potential duration (APD), the principle cellular determinant of the refractory period. These alterations include a loss of the plateau and decreased APD, as well as increased APD heterogeneity. These action potential alterations were subsequently observed to develop progressively in dogs as a result of pacing-induced atrial tachycardia (400 bpm). Furthermore, pacing-induced APD alterations in isolated cells correspond closely to refractory period changes in vivo indicating that cellular action potential modifications likely account for the refractory period changes that promote AF [2].

In addition to decreasing atrial ERP, long-term atrial tachycardia appears to slow intra atrial conduction, thus

tending to decrease the wavelength for reentry. Furthermore, changes induced by remodelling are spatially heterogeneous, increasing the heterogeneity in atrial refractory properties. The combination of decreased wavelength and increased heterogeneity would be expected to promote multiple circuit reentry [3].

Although results are not always confirmed by different available studies, using different animal models, most agree upon that long term ATR, as a simulation of AF remodelling, abbreviates atrial refractoriness by decreasing APD [1, 2, 4, 5]. This happens primarily by $I_{CaL}$ downregulation but also via increased inward rectifier $K^+$ currents such as the background current $I_{K1}$ and a constitutively active form of acetylcholine-dependent $K^+$ current ($I_{KACh,c}$), which is closely related with autonomic vagal activation. In addition, ATR impairs atrial contractility, principally by causing $Ca^{2+}$ handling abnormalities, which causes atrial dilation that further promotes reentry.

The cardiomyocyte resting membrane potential is set by background $K^+$ conductances, primarily inward rectifiers, and becomes more negative in AF. The inward rectifier $K^+$ current $I_{KACh}$ mediates cardiac vagal effects: Acetylcholine released from vagal nerve endings activates $I_{KACh}$, which causes APD abbreviation and cell membrane hyperpolarization. Increased vagal activity strongly promotes AF by stabilizing atrial reentrant rotors and clinical AF often begins under vagotonic conditions.

The transient outward $K^+$ current ($I_{to}$) is consistently decreased in ATR. The functional consequences of $I_{to}$ downregulation are unclear. However $I_{to}$ activates quickly and produces an outward-current component that can oppose inward $Na^+$ current during the action potential upstroke, so $I_{to}$ downregulation may facilitate wave propagation by indirectly increasing action potential amplitude. Decreased $I_{to}$ parallels reductions in both mRNA and protein expression of its pore forming Kv4.3 subunit. Atrial tachycardia also appears to reduce $I_{Na}$, possibly accounting for conduction slowing after prolonged periods of atrial tachycardia.

Decreased expression of connexins has also been reported as a consequence of rapid atrial pacing, possibly contributing to a slower conduction velocity of cardiac action potential throughout the heart. In addition to the expression changes, connexins can also have a deficient distribution across the cardiomyocite plasma membrane. In this case the lateralization of connexins further contributes to the decreased conduction velocity of the action potential [6].

Studies of ionic currents in patients with AF are complicated by the potential effects of concurrent cardiac disease and drug therapy. However the limited data available are generally in agreement with results in animal studies. Patients with persistent AF have significant decreases (average decrease ranging from 49 to 60%) in mRNA encoding L-type $Ca^{2+}$ channel a1c subunits. L-type $Ca^{2+}$ channel protein levels were also found to be reduced. The ionic changes seen in cells from patients with chronic AF are not observed in patients with sinus rhythm and a history of paroxysmal AF, suggesting that they are a result, and not the primary cause, of the arrhythmia.

These observations point to decreases in mRNA levels, likely owing to transcriptional downregulation, as the molecular mechanism of tachycardia induced changes in atrial ionic current expression.

In contrast to the well-defined alterations of cellular and molecular electrophysiology underlying chronic AF, investigated in chronic AF patients and long term animal models of ATR, the initial changes and their time course are currently unknown, although they might be interesting targets for therapeutic interventions. In humans, even AF of several minutes (10 minutes) shortens atrial refractoriness. This shortening is considered to result from physiological responses to the intracellular $Ca^{2+}$ overload by high rate atrial excitation, as this shortening recovered rapidly within several minutes after cessation of AF.

The only short term data available on the molecular level showed a transient increase in Kv1.5 ($I_{Kur}$) mRNA and protein and a reduced mRNA expression of Kv4.2 and Kv4.3 ($I_{to}$) without changes in protein levels after short periods of RAP in rats [7].

## 2. Materials and Methods

### 2.1. Animal Experimental Model

In the reported study experiments were performed in 65 Wistar Rats, aged more than 10 weeks, anesthetized with sodium pentobarbitone (Pentothal, 60 mg/kg, intra peritoneal) supplemented as necessary. The femoral artery and vein were cannulated for monitoring arterial blood pressure (Neurolog, Digitimer) and the injection of drugs, respectively. The trachea was cannulated below the larynx and the animal was ventilated with $O_2$-enriched air applied after induction of neuromuscular blockade with Vecuronium bromide (Norcuron, 4 mg/kg, Intra Venous) using a positive pressure ventilator (Harvard Apparatus Ltd).

An adequate level of anesthesia was maintained by ensuring the absence of a withdrawal reflex before the neuromuscular blockade. During this blockade, the level of anaesthesia was monitored by recording blood pressure and heart rate. Rectal temperature was main-

tained at 36.5−38 °C by a servo-controlled heating blanket (Harvard Apparatus Ltd). The electrocardiogram (ECG) was recorded (Neurolog) from subcutaneous electrodes placed in the limb's origin. Heart rate was derived from the ECG.
A mid-line thoracic incision was made to expose the heart in order to record and stimulate the atria. Two recording electrodes were placed in the right and left atrial surface respectively and a concentric bipolar stimulating electrode was placed in the right atrial surface in order to perform rapid atrial pacing. Stimulation at a frequency of 50 Hz or 3000 bpm was performed for periods of 30 minutes, 2 hours and 4 hours, using 1 ms rectangular pulses, with the use of a programmable stimulator and a constant current source (Master-8, IsoFlex).
At the end of the experiment, the animals were killed with an overdose of anesthetic and samples of the right and left atria were collected for RNA quantification.
The animals were classified into seven groups according to the recording and pacing duration. In thirty animals only atrial recordings were perform, in thirty animals atrial recordings and atrial pacing were perform and in five animals only the surgical procedures were perform.

Messenger RNA quantification was made for the set of ten genes shown in table 1, which code for membrane proteins involved in the generation and conduction of action potentials throughout the cardiac tissue, including connexins and ion channels. The measured quantities of mRNA are not a sole indication of genetic expression, for there are other post-transcription mechanisms that influence the amount of functional protein produced. Even so, for simplicity, the terms expression, underexpression and overexpression will be used to refer to measured mRNA levels.

| Current | Channel | Genes | Effects |
|---|---|---|---|
| all | connexin-40,43 | Gja5, Gja1 | conduction velocity |
| $I_{Na}$ | Nav1.5 | Scn5a | phase 1 slope |
| $I_{to}$ | Kv4.2, Kv4.3 | Kcnd2, Kcnd3 | AP amplitude |
| $I_{Kur}$ | Kv1.5 | Kcna5 | $APD_{20}$ |
| $I_{CaL}$ | L-type $Ca^{2+}$ | Cacna1 | plateau/APD |
| $I_{KAch}$ | Kir3.1, Kir3.2 | Kcnj3, Kcnj6 | vagal modulation |
| $I_{Cl}$ | Cftr | Cftr | |

**Table 1. Analysed Genes**

## 2.2. Data Analysis

In order to extract the subtle variations in atrial MAP a non-linear fit method was used. This method applies a template composed of a set of generalized Gauss functions parameterized by five coefficients, describing relative location, width, amplitude, skew and curtosis. The fitting is accomplished using an iterative convergence method based on the Levenberg-Marquardt compromise.
The model for each segment of the signal consists of a function composed by the sum of several generalized Gauss functions, expressed by:

$$M\left(\underset{\sim}{\theta},t\right) = \sum_{i=1}^{n} G_g\Big(a_i,c_i,d_i,e_i,f_i,t\Big) \qquad (1)$$

Where each Gauss function is expressed by:

$$G_g(a,c,d,e,f,t) = f \,.\, e^{\left(\frac{\pi^2(t-a)^2}{d^2(\pi+2atan(-e(t-a)))^2}\right)^c} \qquad (2)$$

Where $a$ is the time location or relative position of the Gauss function, $c$ is the kurtosis which is a measure of the "peakedness" of the function, $d$ is width or aperture, $e$ is the skew or inclination, $f$ is the amplitude of the Gauss function and $t$ is time.
The template is a first rough description of the measured signal, which is defined by an initial set of parameters for each Gauss function. It is this first approximation that will be adapted to fit the signal as perfectly as possible, by the use of the Levenberg-Marquardt iterative method.

Combining the properties of the Gradient method, that is robust in terms of the initial conditions but slow in the final approach to the solution, with the properties of the Gaus-Newton method that is sensitive to the initial conditions but fast once it gets close to the solution, it is possible to create a new method that combines the advantages of both without the limitations of either one. The method is currently known as the Levenberg-Marquardt compromise and it interpolates between the Gauss-Newton algorithm and the method of gradient descent. The director function for this method derives from geometrical interpretation of the director functions of the Gradient and Gauss-Newton methods and it combines both functions.

$$\underset{\sim}{\nu}_M \equiv \underset{\sim}{\delta}_\theta = \left(\underset{\sim}{J}_M^{T^{(i-1)}} . \underset{\sim}{J}_M^{(i-1)} + \zeta \underset{\sim}{I}\right)^{-1} . \underset{\sim}{J}_M^{T^{(i-1)}} \left(\underset{\sim}{z} - \underset{\sim}{M}^{(i-1)}\right) \qquad (3)$$

The parameter $\zeta$ is a positive constant called damping coefficient. When $\zeta$ is small or zero the director function in 3 becomes identical to the director function for the Gauss-Newton method, and when the damping coefficient is bigger, the same function becomes identical to the director function for the Gradient method.
The damping coefficient $\zeta$ normally starts with a bigger

value, close to one, in order to take advantage of the robust behavior of the Gradient method when far from the solution. It is progressively reduced, once the paremeters estimation gets closer to the solution and the linear approximation given by the Gauss-Newton method improves. This way the final convergence takes advantage of the increased convergence velocity of the Gauss-Newton method when it gets close to the solution.
In order to quantify the quality of the linear model derived by the Gauss-Newton method the linearity coefficient $\rho_\iota$ is defined as:

$$\rho_\iota = \frac{\chi(\hat{\underset{\sim}{\theta}}^{(i-1)}) - \chi(\hat{\underset{\sim}{\theta}}^{(i-1)} + \underset{\theta}{\delta})}{\underset{\theta}{\delta}^T \left[ \underset{M}{J}^{T(i-1)} . \left( \underset{\sim}{z} - M^{(i-1)} \right) + \zeta \underset{\theta}{\delta} \right]}, \qquad (4)$$

Where $\chi$ is the cost function and $\chi_\iota$ is the cost function for the linearized model.
Which mean that for $\rho\iota > 0$ the iterative process evolves in the direction of the minimization of the cost function. This desired behavior can be sated as a condition for the next iteration:

$$\hat{\underset{\sim}{\theta}}^{(i)} = \hat{\underset{\sim}{\theta}}^{(i-1)} + \underset{\theta}{\delta} \Leftarrow \rho_\iota > 0. \qquad (5)$$

Based on the arguments stated above the evaluation procedure of the damping coefficient as an alternating factor between the Gradient and Gauss-Newton methods can be formulated as:

| | | |
|---|---|---|
| calculate | : | $\underset{\sim}{M}^{(i)}, \underset{M}{J}^{(i)}, \rho_\iota$ |
| $\rho_\iota > 0$ | : | $\hat{\underset{\sim}{\theta}}^{(i+1)} = \hat{\underset{\sim}{\theta}}^{(i)} + \underset{\theta}{\delta}$ |
| | : | $\zeta = \zeta \,.\, \max\left(1/\kappa 2,\ 1 - (\kappa 1 - 1)(2\,\rho_\iota - 1)^{\kappa 3}\right)$ |
| | : | $\kappa = \kappa 1$ |
| $\rho_\iota \leq 0$ | : | $\zeta = \zeta \,.\, \kappa 4$ |
| | : | $\kappa 4 = 2 \,.\, \kappa 4$ |

**Table 2. Damping coefficient adaptation algorithm as proposed by Nielsen**

In this algorithm the constants $\kappa_1 \ldots \kappa_4$ are initialized as: $\kappa_1 = 2$; $\kappa_2 = 3$; $\kappa_3 = 3$ and $\kappa_4 = 2$.

## 3. Results

### 3.1. Gene Expression Analysis

Gene expression analysis was performed in groups, designated pools, comprising the samples referent to five animals subjected to the same experimental conditions. For each experimental condition - three different periods of pacing and three different periods of control - two five animal pools were analysed. The gene expression analysis by mRNA quantification was performed in both the right and left atria. Although only the right atria were subjected to rapid pacing, its effects were also investigated in the left atria.
This approach allows for normalization of the results from pacing experiments, not only against the control genes but also against the experimental control groups that underwent similar surgery periods. The experimental approach is therefore cancelled, by this normalization procedure, as a possible contributing factor for the gene expression variations.
The graphic in figure 1 shows the averaged results of the two pools, normalized for both the expression of a control gene and the expression levels of experimental control groups, in a logarithmic scale, where zero stands for unchanged expression, negative values stand for underexpression and positive values for overexpression of the analysed genes. In the right atria, there seems to be some genes that are almost unaffected by the rapid pacing, like the ones that code for connexions, Gja1 and Gja5. On the contrary, some genes have quite significant changes in expression levels like Cacna1, Kcnj6 or cftr. Analyzing the results of the right atria in a time evolution perspective, it is possible to identify genes like Kcnj3 and Kcnj6 which had a clear evolution of underexpression throughout the different periods of pacing, showing a quite significant drop in expression levels, to less then half, at the end of four hours of pacing. Other genes expression levels have a less linear evolution, like Kcnd2, Kcnd3 and Cacna1, which start by being underexpressed at thirty minutes of pacing, close to unchanged expression after two hours and again underexpression at the end of four hours.
None of the gene expression levels reported surpasses a $\pm$ 75% change regarding the control condition.

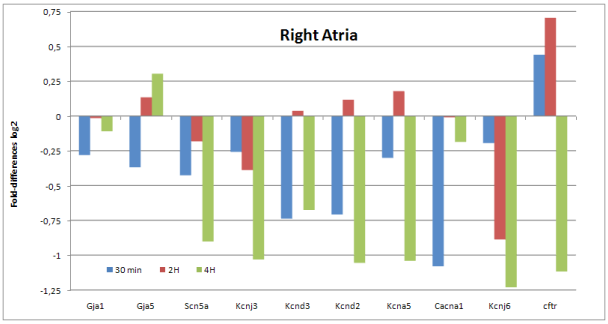


**Figure 1. Right Atria Gene Expression Results**

### 3.2. Signal Measurements

Atrial activation signals were measured using the unipolar monophasic action potential method. The measurement setup was developed during the course of the experiments, evolving from initial electrogram-like biphasic signals to the action potential-like monophasic signals in the final experiments. The quality of the measured signal depends greatly on the position and pressure exerted by the recording electrode placed on the atrial surface. For this reason, equivalent signals measured in different experiments can have different morphology. Some of the measures did not meet the requirements for the parametric signal analysis and thus were not used.

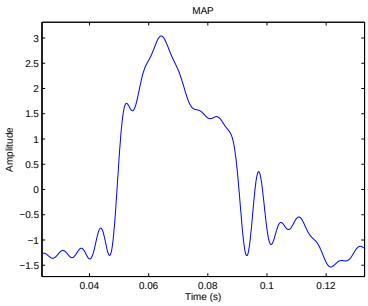


**Figure 2. Examples of measured MAP signals**

The feature that is maintained throughout most of the recordings, which is the most important for the evaluation of MAP, is the main accident's shape. The wide positive deflection contains most of the useful information about duration, phase 1 upslope and phase 4 down slope of the underlying action potential.
Recordings in which this deflection was not reasonably conserved were not considered for the parametric signal analysis.

### 3.3. Data Analysis

The algorithm was applied to 50 segments of atrial MAP signal corresponding to small time periods before and after rapid atrial pacing. This procedure allows for a comparison between the model parameters found before and after atrial pacing and consequently, a characterization of the atrial ionic remodelling that could have happen in between.
The iterative fitting process converged to a solution in all the tested signals and the quality of this solution can be evaluated by the quadratic error of the final approximation. The error averaged 1.9167, SD = 2.4934.

The results represented in figure 3 show the evolution of the analysed parameters for the Gauss function that models the main positive deflection of the atrial MAP. This analysis was performed for three different pacing periods, 30 minutes, 2 hours and 4 hours. The results were normalized against the same parameters obtained for the control experiments in order to discard the possible influence of experimental condition and isolate the influence of rapid atrial pacing.

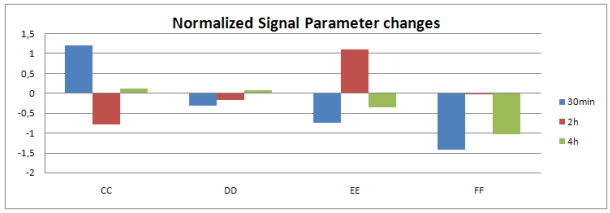


**Figure 3. Right Atria Parameter Changes in log scale**

It is possible to notice in the graphic on figure 3 that most of the parameters analysed do not have a clear progression throughout the three pacing periods.

Kurtosis represented by the parameter CC is significantly increased after 30 minutes of rapid pacing. At 2 hours of pacing it is considerably decreased and after 4 hours it returns to near control levels. A decreased kurtosis corresponds to a more pointy triangular shaped potential with slower slopes.
The width of the modelling Gauss function, represented by the DD parameter is decreased in the two first periods of pacing and slightly increased at 4 hours of pacing. Additionally an increasing tendency can be noticed. The width of the modelling function is closely related to the duration of the cardiac action potential being modelled.

The skew of the function represented by EE is reduced after 30 minutes of rapid pacing and increased after 2 hours. At 4 hours this parameter is slightly decreased again. The skew of the curve measures the tilt or inclination of the Gauss function. This parameter was negative for all segments analysed which indicates a left tilt of the function. It can be related with the slope of the repolarization phase of the action potential. Decreased skew means less left tilt, which in term means a faster repolarization phase.
The amplitude of the Gauss function is considerably reduced in relation to control experiments at 30 minutes and 4 hours of pacing. After 2 hours it remains unchanged. The amplitude of the signal is correlated with the amplitude of the action potential.

## 4. Discussion

### 4.1. Gene Expression Analysis

The results of the gene expression analysis add significant information concerning the early stages of atrial ionic remodelling and show that even in such short periods of time the genetic regulation machinery can, in some cases, start to have measurable effects in the expression of cardiac ion channels. These results are in most cases in agreement with similar data published in recent literature. Since most studies of this type are performed in animal models of chronic rapid atrial pacing ( 1 day) or patients with long term persistent AF, the comparison can only be made by interpolation of the results found for the three short pacing periods evaluated.

Connexins Cx40 and Cx43 are the most abundant connexins in the heart tissue and its influence in atrial remodelling was already investigated in the context of AF in a goat model of chronic atrial pacing [6]. This study reported a reduced amount of protein (up to 50%) for both species accompanied by heterogeneous distribution of Cx40. The protein underexpression was not validated by decreased mRNA levels, these remained unchanged. This author also measured the protein levels and its distribution along the atrial miocytes, which were found to be heterogeneous after atrial pacing.
The expression changes presented in the previous section for Cx43 are quite small and due to the considerable statistical dispersion cannot be interpreted as significant. The maintenance of mRNA levels for Cx43 points to the hypothesis of sole post-transcriptional downregulation of this protein, from short to long periods of pacing.
Connexin Cx40 showed more pronounced changes in expression levels, leaning in the direction of overexpression. This tendency is contrary to the ones reported in the referred study, suggesting that the early response of this protein's genetic regulation to atrial pacing can be opposite to the one observed after extended pacing periods (underexpression of Cx40).

The expression regulation of the sodium channel coded by the Scn5a gene is in agreement with similar available studies, like to one by Yue [2] in a dog model of AF which reports significant downregulation of the gene coding for the $\alpha$ subunit of the sodium channel after 7 and 42 days of rapid pacing.
Similar results were reached after much shorter periods of just 4 hours of pacing (see figure 1), which confirms the relevance of short term modulation of $I_{Na}$ in atrial ionic remodelling in the rat model. This significant decrease in mRNA if accompanied by similar reduction in the concentration of functional protein can originate low amplitude and low velocity cardiac action potentials.

The genetic expression regulation of Kv4 channel ($I_{to}$), coded by the Kcnd2 and Kcnd3 genes, is investigated in a variety of studies, using several animal models and also humans, and ranging from short to long periods of rapid atrial pacing. Yue [2] reports 71.6% decline in protein concentration of the Kv4.3 specie and 74% in mRNA concentration for the corresponding gene after 42 days of rapid atrial pacing in dog models of AF.
Yamashita et al. [7] investigated the mRNA and protein concentrations of Kv4.2 and Kv4.3 after periods of pacing raging from 30 minutes to 8 hours in a rat model of AF. Their results show a progressive underespression of the two proteins starting at 1 hour of pacing but only reaching significant values at 2 and 4 hours for the Kv4.2 and Kv4.3 subunits respectively.

The results found for this genes are shown in figure 1 and in general confirm the underexpressin tendency reported in the mentioned studies. The progression is however peculiar and difficult to justify, since the initial underexpression is absent after 2 hours and returns after 4 hours of pacing. Reduction in the expression of these channels and its consequent decrease in $I_{to}$ could in theory prolong the action potential and add to its amplitude, by decreasing the notch repolarization in phase 2 of the cardiac action potential.

The expression changes of Kv1.5 channel ($I_{Kur}$) in the context of AF are not consensual over the available literature. Studies in human AF patients [8] show slight underexpression, although the study by Yamashita, referred before, reports significant overexpression right since 30 minutes of pacing, reducing to unchanged expression after 8 hours of pacing, in rats.

In this particular case it cannot be said there is agreement between the measured results and the ones available in the literature since the latter do not agree between each other. Either way, comparing with the results by Yamashita, in which the experimental conditions are closer related, it can be stated that they disagree almost totally.

Bosch el al. [4] studied the expression changes of five subunits of the L-type calcium channel and found most of them to be underexpressed after 6 to 12 hours of atrial pacing. This underexpression was confirmed by matching, although less intense, decreases in protein concentration.

The gene coding this channel was found to be underexpressed at 30 minutes and 4 hours of pacing (figure 1), which is in agreement with literature results. The hypothesised reduction in calcium channels would de-

crease $I_{CaL}$, which in turn can contribute to a shorter action potential phase 3 plateau and consequent reduced action potential duration and ERP.

The role of the Acetylcholine (Ach) dependent potassium channels in ionic atrial remodelling is still unclear and only a few studies have investigated the expression of these channels in the context of AF. Dobrev et al. [9] have reported downregulation of the Kir3.4 subunit in human patients of chronic AF.
However studies with animal models of AF using rapid atrial pacing show unchanged expression levels of protein subunits of the Ach dependent potassium channels. Although these ion channels expression has found to be unchanged or decreased, the $I_{KAch}$ current was reported augmented. This fact suggests a complex regulation of this kind of current mediated not just by the number of functional proteins.

The results found for the Kcnj3 and Kcnj6 genes coding for Ach dependent potassium channels point in the same underexpression direction as those found in human patients of AF. Although clear underexpression results were found, it is not possible to draw any conclusion about the effect of this reduced expression in the cardiac action potential, since the number of functional channels cannot in this case be connected to the respective current intensities.

It was not possible to find results in the literature concerning the expression of any $Cl^-$ cardiac ion channel, in the context of AF.
The $Cl^-$ current is activated mainly by physical stress and exhibit outward rectification, or are predominantly activated at depolarized voltages and, thus, contribute significantly to shortening of the action potential duration. The action potential shortening by $Cl^-$ current activation may not only perpetuate reentry by shortening the refractory period in a reentry pathway, but may also prevent the development of early afterdepolarization and triggered activity caused by the prolongation of action potentials [10].
The initial increase in Cftr expression can be justified by the stress condition of rapid atrial pacing. The pronounced underexpression of this gene after 4 hours of pacing is quite more puzzling, but it can be explained by prolongation of rapid pacing.

The agreement between mRNA and protein concentrations reported in most of the referred studies suggests that, although there are other contributing factors, the reduced transcription is the major mechanism of ion channel downregulation, which characterizes the electrophysiological alterations by which AF leads to its own perpetuation. It also allows the analysis of results based solely on mRNA quantification since an agreement between this parameter and protein concentration can, in most cases, be assumed.

The time periods analysed over this study are insufficient to clarify the phenomena of ionic atrial remodelling, but they add information concerning the early stages of this process. An investigation of the same nature reaching longer periods of rapid atrial pacing would be very useful to clarify the direction of expression regulation. However, this kind of long term intervention cannot be accomplished with the same surgical approach, and for that reason an implantable stimulation device is currently being developed by a colleague, that should allow for long term rapid atrial stimulation.

The results presented are quite noisy and statistically disperse, especially the ones concerning the shorter 30 minutes periods, therefore constitute insufficient grounds to draw a clear conclusion from, only an indication of progression can be extracted. The experimental procedures and mRNA quantification analysis were performed in a precise and methodical manner and most probably do not contribute to the dispersion of the results. This problem of statistical dispersion of the two pools analysed can instead be due to inter-individual variability and in that case a possible solution would be to increase the number of subjects. Accounting for this fact an additional set of experiments, comprising one more 5 animal pool, is currently underway, as an attempt to decrease the soaring standard error associated with the quantifications for the first two pools. Another justification for the result dispersion can be the short time scale of the study when compared to the long time scale of the phenomena being analysed. There is no doubt that atrial remodelling is a near immediate phenomena in the sense that changes to the electrophysiological properties of the heart start to happen after only a few minutes of rapid pacing. However, the relevance of the genetic regulation increases in time and the initial changes are most probably not predominantly genetic in nature, but functional.

Although several studies have demonstrated that mRNA quantification closely correlates with functional protein quantification in the context of long term, chronic rapid pacing experiments, translational and post-translational mechanisms can play an important role in the properties of the final protein, especially in the early stages of exposure to the disturbance, when functional changes can be viewed as the first adaptive response.
In order to account for the translational and post-translational, folding associated mechanisms a proteomics approach would be needed. An immunoblotting study would help to identify the protein subunits

that make up the ion channels and probable functional changes that take place in short term adaptation to atrial pacing. In addition, mass spectroscopy methods can be used to quantify the protein species that interfere in the regulation of cardiac ion currents.
The regulatory networks and signaling pathways that control the expression of the ion channels should also be investigated, for in most cases they are the most likely target for pharmacological modulation and are very important for the understanding of the physiological processes of tissue response.

### 4.2. Signal Measurements

Although the signals measured are not ideal representations of the actual action potential, they present all the properties of a unipolar monophasic action potential, and thus, are approximate illustrations of the action potential, suitable for correlation analysis with any kind of parameter that influences the electrophysiological properties of the tissue.
Recordings from the left atria were attempted, but due to experimental difficulties owing to the inaccessible position of this cavity, these recording had rarely the quality necessary for analysis.
The morphology of this kind of measurement is quite sensitive to variations of the surgical conditions, like position of the heart, position of the electrode in the surface of the heart or conductance variations of the electrode's tip. All these variables are, despite the efforts undertaken, very difficult to control in such minute experimental conditions and are therefore considered as a source of error.

### 4.3. Signal Analysis

A relatively high number of Gauss function was needed to completely model and fit the morphology of the measured signals. This fact brings problems when it comes to interpret the results due to the high number of produced parameters. Looking solely at the parameters for the Gauss function modelling the main positive deflection of the MAP, it is possible to follow the evolution of the signal's shape throughout the different pacing periods.
Unfortunately it is not possible to establish a univocal correspondence between the progression of a given parameter and the expression of a gene. However, a possible relation between the overall shape of the action potential, translated by the Gauss function parameters, and the relative expression of the different genes will be explored.

The reported results for gene Kcna5 coding for channel Kv1.5 through which flows the ultra rapid outward rectifying $I_{Kur}$ current can be related with the results of some of the Gauss function parameters that model the atrial MAP. This outward current when augmented induces a triangular shaped cardiac action potential, with a less pronounced plateau and decreased duration. It is possible to correlate the significant underexpression of this gene, after 4 hours of pacing, with the increased kurtosis represented by the parameter CC, which in turn corresponds to a more square signal, or in the case of the atrial action potential, a more pronounced plateau.

Genes Kcnd2 and Kcnd3 which code for Kv4 channel, influence the outward $I_{to}$ current, which in turn has an indirect effect over the cardiac action potential's amplitude. The expected boost in MAP amplitude caused by the decreased expression of $I_{to}$ channels at 30 minutes and 4 hours of pacing, is not corroborated by amplitude changes translated by FF parameter's evolution. These in fact point in the opposite direction.
The amplitude is a parameter greatly influenced by experimental variables, and measures carried out with several hours between them cannot be guaranteed to have happened in the same conditions.

Calcium, specifically the L-type calcium current $I_{CaL}$, is pointed as the major factor responsible for the action potential duration (APD), decrease observed in chronic AF remodelling. In this study it was found to be decreased but mainly after 30 minutes of pacing.
In the parametric signal analysis, the DD parameter, which translates de width or duration of the main Gauss function modelling the atrial MAP, is found to be decreased at 30 minutes of pacing, but with a rising tendency, reaching after 4 hours a level superior to control. Although this tendency is contrary to data presented on chronic studies of AF, it can be related with the expression results for several genes. The Cacna1 gene expression, related with the aforementioned L-type calcium current, is found to be considerably decreased after 30 minutes of pacing returning to near control levels in the other two pacing periods.
Also influencing the duration of the cardiac action potential are the Kcnj3 and Kcnj6 genes that code for an acetylcholine dependent inward rectifier potassium channel. These are progressively reduced throughout the three pacing periods. The decreased vagal modulation caused by this fact allows for a raise in APD, counteracting the effect of the calcium and possibly accounting for the observed progressive increase in DD.
The chloride current mediated by the Cftr channel may also play a role in the duration of the cardiac action po-

tential. Amplified $I_{Cl}$ can shorten the action potential and that fact is confirmed in the results for the 30 minutes and 2 hours pacing periods. The role of connexins and $I_{Na}$ is mainly in the action potential's conduction velocity and thus cannot be measured in a single locus action potential recording.

Although the fitting algorithm as show to be robust and reliable in terms of convergence, the interdependency of the Gauss functions used to model the data greatly complicates the analysis of the results. This problem could be avoided by decreasing the number of interdependable Gauss functions used to model the data. That would imply an increased fitting error if used in signals with complex morphology like to ones recorded in this study, but could be a possible solution in cleaner, more action potential like, MAP signals. In that case only two Gauss functions would be necessary to correctly model and fit the signals.
In order to isolate the influence of each type of ion current in the morphology of the measured signal, a selective pharmacological blockage of the corresponding ion channels could be used. This way, the model parameters associated with the expression changes of each ion channel could be identified.

The investigation of atrial remodelling should take into account the multi-scale dimension of the phenomena, trying to clarify and correlate all the steps, from its genetic or molecular origin to their cellular, tissue or organ manifestations. A complete approach would include the study of signal pathways, genetic regulatory networks and their effects on control of ion channels gene expression, translational and post-translational mechanisms of protein functional modulation, single channel pharmacological blockage to quantify parametrically the influence of each current in the MAP and an experimental pacing setup ranging from short to long periods of atrial stimulation.

Ideally AF recurrence should be prevented at the early stages of the disease, before it becomes permanent, using therapeutic strategies aimed at the factors and mechanisms that create the atrial conditions for fibrillatory conduction.

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