Electrophysiological and Genetic Insights into Atrial Fibrillation

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Electrophysiological and Genetic Insights into Atrial Fibrillation

Electrofysiologische en Genetische Inzichten in Boezemfibrilleren (met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

Atrial Fibrillation (AF), the most frequently encountered arrhythmia in clinical practice[1], is a multifactorial disease. A variable substrate, triggers and modulating factors among which the autonomic nervous system and genetic background, are creating a pathogenic triangle embracing AF (Fig 1)[2]. Although familial forms of AF have been recognized and AF can be secondary to underlying organic diseases, in almost 30% of patients with AF no detectable etiology or genetic predisposition is present and the onset of AF is rather unexpected[1]. This multifaceted nature of AF is reflected by the diversity of the clinical presentation and symptoms of AF and its unpredictable occurrence. Patients can have paroxysmal, persistent or permanent AF. Paroxysmal AF is defined as recurrent AF that terminates spontaneously within 7 days. Sustained AF lasting for at least 7 days or less but necessitating cardioversion is defined as persistent; and permanent AF is sustained AF in which cardioversion has either failed or not been attempted for more than 1 year.

The prevalence of all kinds of AF is up to 1% in the general population, increasing with age to 8% in those older than 80 years. AF is related to all-cause mortality and morbidity and frequent hospitalization due to its recurrent character and cardiovascular complications[1]. Therefore its treatment has always been a focus of attention. The Cox-Maze procedures (I, II and III) were highly effective in treating AF and restoring sinus rhythm (SR), as well as reducing the rate of cerebrovascular events[3]. However these procedures were not widely adopted due to their complexities and technical difficulties. Nowadays, besides the conventional treatment with antiarrhythmic drugs, new catheter ablation and surgical methods have been developed to treat AF[4-6]. Such methods have been described to be effective in up to 70% of the patients, with higher success in paroxysmal AF patients[7]. Accordingly, none of the treatment options mentioned above guarantee success in all patients with AF, many of them remain symptomatic. A better understanding of the pathophysiological and electrophysiological mechanisms of AF is necessary to improve our treatment strategies.

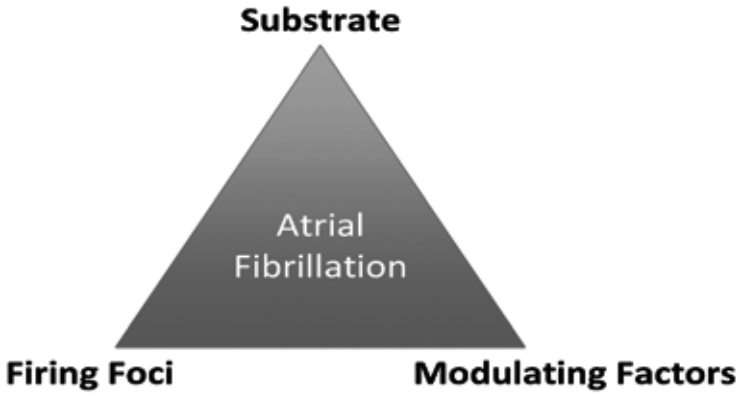

Figure 1: Triangle of Atrial Fibrillation: The combination of substrate, triggers and various modulating factors leads to the core problem of AF.

Mechanisms of Atrial Fibrillation

A well known theory for the mechanism behind the initiation and maintenance of AF is the multiple reentrant circuit theory, hypothesized by Moe et al[8] and experimentally confirmed by Allessie et al[9]. This theory suggests that multiple random wavelets of activation coexist to create a disorganized cardiac rhythm. Other hypotheses like "mother rotors" and "spiral waves" with fibrillatory conduction, have also been made to explain the mechanisms behind AF[10;11]. Finally, focal electrical discharges due to triggered activity or abnormal automaticity may trigger and maintain AF[4;7].

Substrate of Atrial Fibrillation

Structural Changes

Fibrosis

The prevalence of AF increases with age[1] and this can partly be explained by the age-dependent atrial fibrogenesis[12]. Fibrosis results from an excessive deposition of extracellular matrix and is a hallmark for arrhythmogenesis in AF[13;14]. Atrial fibrosis can result from a mechanical overload secondary to heart disease and conditions that lead to increased collagen production. This occurs when circulating and locally synthesized pro-fibrotic factors act on resident cardiac cells to increase collagen production without offsetting increases in collagen degradation[15]. However, whether atrial fibrosis precedes the onset of AF[16;17] or is induced by AF, as one of the most frequent histopathological changes[14;18], is not always clear. Atrial fibrosis impairs coupling of myocytes and leads to increased tissue anisotropy that may cause localized regions of conduction delay and increased conduction heterogeneity[13;19-21]. These conditions predispose to reentry and focal triggers[22], which are both related to AF. There is thus a clear relation between atrial fibrosis and AF occurrence and/or persistence.

Cell-to-cell coupling (gap junctions)

Effective cell-to-cell coupling of the myocardial cells is essential for rapid and uniform conduction of the action potential. Cell-to-cell coupling is directly related to the properties of connexins, which form the gap junctions, throughout the myocardium (Fig 2) [23].

In the human heart 4 main connexin isoforms are expressed: Cx40, Cx43, Cx45 and Cx37. Conductance and voltage sensitivity of the different connexin isoforms differ, conductance of Cx40 being largest (130 pS). In contrast, conductance of Cx45 is only 30 pS, whereas Cx43 has a value in between (60 pS). A gap junction can be homomeric- homotypic, which means that the adjacent connexons contain the same connexin isoform; or homomeric-heterotypic in which the adjacent connexons contain another isoform of connexin. Finally, heteromeric- heterotypic gap junctions are those in which both connexons are composed of different connexin isoforms. The different combinations of connexons result in differences in conductance of the assemblies. Cx40 and Cx43 are the major connexins of the atria and several studies have shown that their role is important for normal conduction properties of the atria[24-28]. The ratio of Cx43/[Cx40+Cx43] seemed to be directly

and Cx40/[Cx40+Cx43] inversely related to propagation velocity of the atria. That suggests that there is an interaction between Cx43 and Cx40 function and expression in the atria[25;29].

Although both Cx40 and Cx43 are important for impulse propagation in the atria, it has been shown that heteromeric connexons (with Cx40 and Cx43) are functionally insignificant for the atrial impulse propagation[30]. However, it is difficult to predict the behavior of each channel type. In addition, it has been shown that increased fibrosis[31] or high atrial rate[32], results in changes of expression levels and distribution patterns of connexins, leading to changes in gap junction functionality, that can disturb cell-to-cell communication. Altered connexin expression leads to modified electrophysiological properties of the atria, resulting in activation delay and creating conditions for reentry. In diseases like heart failure and non-ischemic dilated cardiomyopathy such alteration of the expression of Cx43 in the ventricles has been observed and associated with lethal ventricular arrhythmias[33;34]. Apart from ventricular disease, various features of gap junction organization and connexin expression have been implicated in the initiation and persistence of AF.

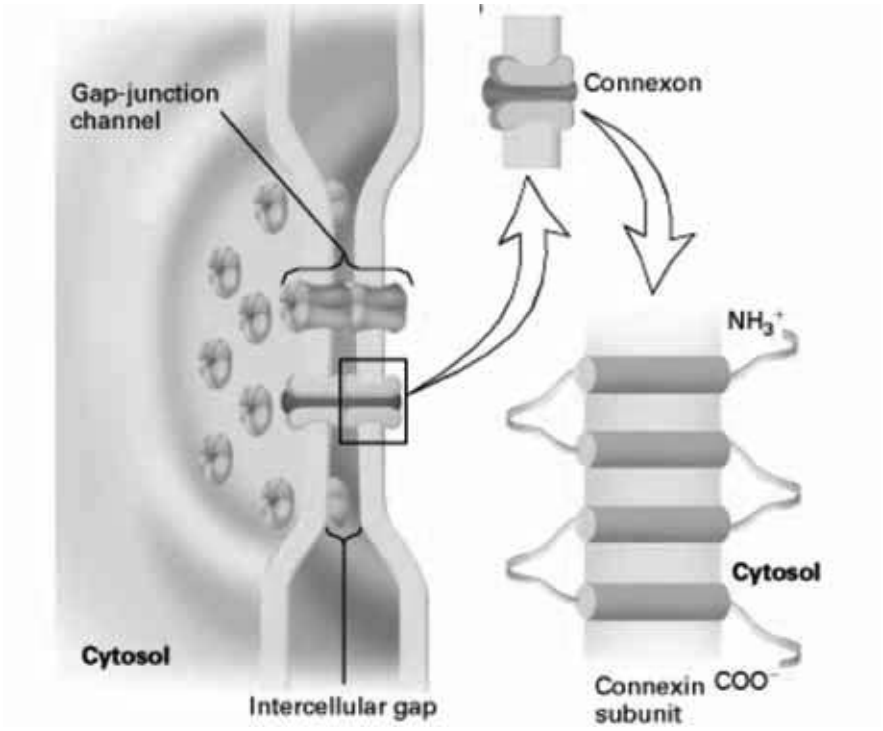

Fig 2: Structure of gap junctions. It is a cluster of channels between two plasma membranes that are separated by a gap (2-3 nm). Both membranes contain connexon hemichannels, cylinders of six dumbbell-shaped connexin subunits. Each connexin subunit has four transmembrane helices. Two connexons join in the gap between the cells to form a gap junction channel, 1.5-2 nm in diameter that connects the cytoplasm of the two cells (www.tainano.com).

Functional Changes

Enhanced dispersion of refractoriness

One of the key requirements for initiation of reentry is the presence of unidirectional conduction block. Heterogeneity of the refractory period at adjacent areas of the atrial myocardium can initiate unidirectional block in response to premature depolarization, which can start reentry[9]. Previous studies from our group have shown that enhanced dispersion of refractoriness, at least in the right atrium, may represent an arrhythmogenic substrate in patients with sporadic AF episodes related to the Wolf-Parkinson-White syndrome, without a history of other heart or systemic disease[35;36]. This indicates that enhanced dispersion of refractoriness may be an important substrate for AF initiation by providing a substrate for reentry. Dispersion of refractoriness, however, may not only lead to unidirectional conduction block, but may also generate obstacles around which the impulse can travel and thus perpetuate the arrhythmia[9]. Therefore, spatial heterogeneity of the atrial refractory period is a determinant of AF induction, but may also be important to sustain AF[37-39].

Ion channel remodeling

Ion channel remodeling can be caused by heart diseases, concomitant drug therapy or a rhythm with high rate, like AF. It is often difficult to separate cause and consequence of altered ion channel properties. It has been shown that sustained atrial tachycardia reduces I_{to} and I_{Ca}. The reduced I_{Ca} decreases action potential duration (APD). These cellular changes may account for the alterations in atrial refractoriness associated with enhanced ability to maintain AF[40]. Congestive heart failure, a disease that predisposes to AF, selectively decreases atrial $I_{to,}$ I_{Ca} and I_{Ks} but increases the Na^+/ Ca^+ exchanger (NCX) current[19]. The Na^+/ Ca^+ exchanger (NCX) current contributes to the action potential and plays a major role in the development of delayed afterdepolarization in the human atrial myocardium[19;41]. Delayed afterdepolarizations are an important mechanism for triggered activity.

Atrial remodeling due to AF

AF itself induces changes in atrial function and structure and provides a substrate that can perpetuate itself and results in a progressive disease: "AF begets AF"[42]. Prolonged episodes of AF result in electrical and structural remodeling that favors the reoccurrence or perpetuation of AF. Electrical remodeling is a reaction of the atria to the high rate of the atria, which is established within the first 24h of AF[42;43]. The micro- and macroscopic effects of structural remodeling manifest at a later phase than that of electrical remodeling[42].

Electrical remodeling

Electrical remodeling involves alterations in ion channel expression and/or distribution. In atria prone to AF it is associated with a decrease of the effective refractory period (ERP)[38;42;44;45] due to shortening of the atrial action potential and maladaptation of the APD. The shortening of the action potential is mainly due to a reduction in the L-Type Ca^{2+} current[14;40]. Spatial dispersion of refractoriness seems to be unaffected during high rate induced electrical remodeling of the atria[42]. Finally, shortly after the initiation of AF gap junction remodeling also occurs[29;46] which may impair impulse propagation and facilitate the occurrence of reentrant circuits.

Structural remodeling

AF-induced structural changes in atrial myocytes include: increase in cell size, perinuclear accumulation of glycogen, central loss of sarcomeres, changes in mitochondrial shape, fragmentation of sarcoplasmic reticulum, homogeneous distribution of nuclear chromatin and changes in quantity and localization of structural cellular proteins[14]. Such changes might be considered as the consequence of metabolic stress.

Firing foci of Atrial Fibrillation

Haissaguerre et al reported in 1998 that in humans AF is frequently initiated by firing foci in the pulmonary veins (PVs). Activity arose from the atrial myocardial extensions into the PVs (myocardial sleeves) and ablation of these foci could abolish AF[4]. Such foci can either initiate AF, or be so rapid that conduction to the atria is irregular (fibrillatory conduction)[47]. Also other areas of the atria, like the myocardial sleeves of the superior vena cava, the vein of Marschall, coronary sinus, crista terminalis, interatrial septum and atrial free wall, may harbor firing foci[48-51] that considered to be important in both the initiation and maintenance of AF. Firing foci is a term used to describe rapid impulse formation in cardiac fibers. Such firing foci can be either due to triggered activity, microreentry or abnormal automaticity.

Triggered activity

Triggered activity is the impulse formation in cardiomyocytes that is dependent on afterdepolarizations. These may occur either during phase 2 or 3 of the action potential (early afterdepolarization EADs) or phase 4 when repolarization is complete (delayed afterdepolarization DADs). If the amplitude of an afterdepolarization is large enough to reach the threshold potential for activation, a new action potential is generated, which is referred to as "trigger". Such triggered activity has been observed in various areas in the atria[51].

Microreentry and abnormal automaticity due to nonuniform anisotropy

The architecture of the myocardial sleeves within the PVs is rather complex. It consists of myocardial fibers with complicated arrangement and sudden changes of their directions[52]. Such areas are consistent with zones of activation delay and block that may result in microreentry[53] as the mechanism of the firing foci. The increased collagen deposition between the myocardial fibers of the sleeves may result in nonuniform anisotropy[20]. Nonuniform anisotropy involves the loss of intercellular coupling, mainly in the transverse fiber direction. At high values of intercellular coupling, a possible focus due to abnormal automaticity is suppressed by the load of the surrounding cells. If coupling is reduced by increased fibrosis, load is reduced and ectopic activity may be able to escape and activate surrounding myocardium[22].

Modulating Factors and Genetics of AF

Autonomic Nervous System

Coumel defined paroxysmal AF as vagotonic or adrenergic due to the autonomic tone before its onset[54]. However, other studies in which heart rate variability was studied as an index of the autonomic tone

before the onset of paroxysmal AF, revealed conflicting results regarding the role of the sympathetic and parasympathetic part of the autonomic nervous system (ANS) in AF initiation[55-58]. Only a restricted number of studies exist that assessed the relation between ANS and the substrate of AF. Studies in animals, that investigated the relation between ANS and dispersion of refractoriness showed that mainly the parasympathetic limb of ANS influences the heterogeneity of atrial refractoriness and facilitates sustained AF[37;59;60]. In a study in patients with supraventricular tachycardia, it was shown that autonomic blockade can blunt the effect of remodeling due to high intensity drive train stimulation[61].

Recently, it has been shown that complex fractionated atrial electrograms (CFAEs), which are seen during AF, correspond to areas that seem to represent a critical substrate for the maintenance of AF. Studies in a dog model showed that CFAEs were mainly located in areas of the heart where the intrinsic cardiac ANS is located (ganglionic plexi, GP)[62;63]. The ablation of GPs eliminated CFAEs. Also, in patients with AF, it has been shown that CFAE sites were mostly located around the four main GPs of the human atria, suggesting that there is a close relationship between CFAEs and ANS[64]. It has also been shown that pharmacological autonomic blockade can reduce CFAEs in the left atrium of patients with paroxysmal AF[65]. However, CFAEs can also represent areas with structural and functional changes that predispose to AF such as impaired conduction, anisotropy or anatomical obstacles due to fibrosis or connexin depletion[66]. Finally, rapid firing from the PVs, that trigger AF, can be induced and eliminated by stimulation and inhibition of the ANS respectively[63;67]. Up to now, the role of ANS as a possible substrate of AF in patients with idiopathic AF has never been studied.

Genetics

Assessment of genetic predisposition in all kinds of AF might be of increasing importance in predicting AF and perhaps even some of its most devastating complications such as stroke[68]. In a subset of patients, AF is a heritable arrhythmia associated with various gene mutations and/or polymorphisms. Familial AF has been shown to be related to potassium (K^+) channel gene mutations[69;70]. Studies have also shown a strong connection between AF and two sequence variants on chromosome 4q25[71]. The parental character of AF has also been described in the general population[72]. In addition, there are many candidate gene polymorphisms for AF (polymorphisms of angiotensin converting enzyme, potassium channel genes polymorphisms and sodium channel genes polymorphisms) [73-75]. Genes encoding connexins play a significant role in AF genetics. Studies correlate a minor polymorphism within the regulatory region of Cx40 gene, at nucleotides -44 (G→A) and +71 (A→G), to the substrate of AF[76;77] . In these cases changes in gap junction function are expected to occur because of decreased expression of the Cx40 protein. However, immunohistological data have never been shown in patients bearing this polymorphism.

Aim and outline of the thesis

The aim of the thesis is to further understand the role of the ANS as a possible substrate of AF and the role of Cx40 as a candidate gene in patients with AF.

Therefore, the thesis is divided into two parts. In the first part, we study the influence of ANS on the

electrophysiological properties in the atria during AF. The second part deals with the association of the Cx40 minor polymorphism and the occurrence of idiopathic AF and the expression of the Cx40 protein in the gap junctions of human atria free of AF.

Part I: Electrophysiological Insights

In **Chapter 2** the effect of autonomic blockade on spatial dispersion of refractoriness and refractory period, expressed as mean fibrillation intervals, is evaluated in two groups of patients: patients with long-lasting AF, and thus atrial (structural) remodeling, and patients with induced AF and thus in the early phase of atrial remodeling (mainly electrical remodeling).

In **Chapter 3** the influence of the ANS on the occurrence of complex fractionated electrograms (CFAEs), as a substrate of AF, is assessed. As we already mentioned, CFAEs seem to be critical for the maintenance of AF since they may represent areas with impaired conduction, anisotropy or anatomical obstacles imposed by fibrosis or connexin depletion[66]. All these factors are capable of sustaining AF.

In **Chapter 4** methods used for the detection of fractionated signals are evaluated. CFAEs are mainly studied with the use of filtered bipolar recordings during AF catheter ablation. We study the influence of technical restrictions on detected CFAEs and we compare the amount of CFAEs on bipolar and unipolar recordings.

Part II: Genetics Insights

In **Chapter 5** a review is presented of the relevant literature on the role of Cx40 on the substrate, the triggers and the modulating factors of AF.

In **Chapter 6** the role of Cx40 in patients with unexplained cerebrovascular ischemia is studied. The possible role of Cx40 minor polymorphism, as a marker to detect patients with silent idiopathic AF, is investigated.

In **Chapter 7** the influence of Cx40 minor polymorphism on *in vivo* expression of the protein is investigated. For this purpose patients undergoing cardiac surgery with no history of AF are included. Finally, we investigate if the presence of this polymorphism is correlated with the occurrence of post-operative AF.

Chapter 8 contains a discussion of our findings, possible explanations and future perspectives.

Chapter 9 provides a summary of this thesis.

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Chapter 2

The Influence of the Autonomic Blockade on Atrial Electrophysiological Properties in Patients with Idiopathic Atrial Fibrillation

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Submitted

Abstract

Background

The role of the autonomic nervous system (ANS) in initiation and perpetuation of atrial fibrillation (AF) is controversial. We studied the effect of pharmacological ANS blockade on mean fibrillation intervals (mFI) and dispersion of refractoriness in patients with idiopathic AF.

Methods and Results

Thirty-five patients (mean age 55±8, 27 men) underwent AF catheter ablation. In the 24hours preceding the procedure, 20 patients had AF (group 1) and 15 had sinus-rhythm (group 2). Twenty unipolar electrograms were recorded in the right (RA) and left atrium (LA). In group 2, AF was induced. In both groups a 100s segment of AF was recorded at baseline, 10min later with atropine infusion, and 30min later with metoprolol infusion. In group 2, five patients served as controls and did not receive pharmacological blockade (group 2B). The mFI and coefficient of dispersion (CD) were calculated between 15 and 30s. At baseline mFI were shorter in group 1 vs group 2 (RA: 143.2 ± 16.3ms vs171.2 ± 27.8ms, LA: 139.1 ± 12.4ms vs155.5 ± 32.1ms, $p<0.05$). CD did not differ. Parasympathetic blockade reduced mFI in both atria in group 2A ($p=0.024$) and the CD of the RA in group 1 ($p=0.02$).

Conclusions

Dispersion of refractoriness was not influenced by AF duration, while mFI decreased with increasing remodeling. CD was greater in LA compared to RA independent of AF duration. Parasympathetic blockade reduced mFI in early phase of AF and CD in RA in longstanding AF. Additional β-adrenergic blockade did not further affect mFI or CD.

Introduction

Atrial fibrillation (AF) is the most common arrhythmia in man with a variable clinical presentation.[1,2] The multiple wavelet reentry theory,[1] mother rotors, spiral waves and structural changes due to fibrosis, changes in cell-to-cell coupling mediated by gap junctions as well as alteration in ion channel expression/ characteristics, have been suggested as mechanisms to explain the maintenance of AF.[3-5] Focal activity (triggers) originating from the pulmonary veins was shown to initiate AF.[6;7] Studies in the right atrium (RA) of patients with idiopathic AF, demonstrated that increased dispersion of atrial refractoriness contributes to the initiation of reentry and thus of AF.[8;9]

The role of the autonomic nervous system (ANS), as a supplementary or principle mechanism for the initiation and maintenance of AF is controversial. Results of studies that examined the autonomic tone, before AF onset, are conflicting on the role of the sympathetic and parasympathetic part of the ANS in AF initiation.[10-14] Studies that investigated the influence of ANS on spatial dispersion of refractoriness also showed controversial results. Animal studies showed that either vagal stimulation[15;16] or sympathetic denervation increased the heterogeneity of atrial refractoriness and facilitated sustained AF.[17] On the other hand, a study in patients with supraventricular tachycardia showed that blockade of both parasympathetic and sympathetic limbs of ANS was necessary to blunt the effect of high drive stimulation on atrial refractoriness and its dispersion.[18]

With this study we investigated the influence of pharmacological ANS blockade on atrial refractoriness and dispersion of refractoriness in the right and the left atrium (LA) in patients with idiopathic AF.

Methods

Study population

Thirty-five patients with idiopathic AF (26 paroxysmal, 3 persistent and 6 permanent AF) were included in this study after having given informed consent. They were referred to our institution for radiofrequency catheter ablation because of ineffectiveness to or intolerance of antiarrhythmic drug therapy. Patients were included if structural heart disease ,such as coronary artery disease, heart failure, left ventricular hypertrophy (due to hypertension), all kinds of cardiomyopathy, and conditions that could affect cardiac electrophysiological properties were excluded based on medical history, physical examination, ECG, echocardiography, biochemical and hematological testing. Patients were excluded if the left atrial size was >50 mm (echocardiography parasternal long axis) or if they used amiodarone. Twenty-one patients with paroxysmal AF also underwent 48 hours (h) Holter recording, while on antiarrhythmic drugs, in order to evaluate AF episodes.

Antiarrhythmic medication was discontinued for >5 half lives before admission to the electrophysiology ward and telemetry (ECG monitoring) was performed in all patients for at least 24h before electrophysiological study (EPS). Patients were divided into 2 groups. Group 1 included 20 patients (14 male, mean age 56±8 years, range 33 to 66) with a history of long-lasting AF episodes

and persistent AF >24h before EPS. Six of them had permanent AF. Group 2 included 15 patients (13 male, mean age 53.6 ± 9.2 years, range 37 to 65) who were continuously in sinus rhythm (SR) in the 24h preceding EPS.

Study protocol

The study protocol was approved by the medical ethics committee of the University Medical Center Utrecht. All patients were studied in a fasting non-sedated state. After right and left femoral venous access, two 10-polar electrode catheters (Livewire[a], St. Jude Medical, Minnetonka, MN, USA) with an inter-electrode spacing of 5 mm were inserted. One was positioned along the anterior wall of the RA and the other in the LA at the superior/posterior wall through an open foramen ovale or after transseptal puncture. Catheter stability was monitored by fluoroscopy. Surface leads II and V1 together with 20 intracardiac unipolar electrograms were continuously recorded with an electrophysiological recording system (Cardiolab; Prucka Engineering Inc, Dallas, TX, USA). Intracardiac unipolar electrograms were bandpass-filtered from 0.05 to 500 Hz and amplified (2mV/cm). Pacing was performed with a programmable stimulator at twice diastolic threshold current and with 2ms pulse width. In all patients blood pressure and heart rate were continuously monitored.

Group 1: Muscarinic and β-adrenergic receptor blockade

Patients in this group had ongoing AF >24h before and during the EPS. When a stable position of the catheters was obtained, the study protocol started [time (t) 0] by recording a first 100s segment of AF (baseline). Then, atropine was administered intravenously (0.04mg/kg, maximal dose 3mg in 3 minutes). After a latency of 5 minutes to allow parasympathetic blockade, a second 100s AF segment was recorded at t = 10 minutes. Thereafter, metoprolol was infused intravenously (0.2mg/kg, maximal dose 20mg in 3 minutes) and a waiting time of 15 minutes followed to achieve additional β-adrenergic receptor blockade.[19] Finally at t = 30 minutes the third 100s segment was recorded [systemic muscarinic- β-adrenergic blockade ($M_{\beta}AB$)].

Group 2: AF induction and $M_{\beta}AB$

In group 2 patients, who were in SR in the 24h preceding the procedure, AF was induced by programmed atrial extrastimulation. Then in 10 randomly selected patients (group 2A), the same sequence of recordings and the same protocol for $M_{\beta}AB$ as in group 1 was used. The remaining 5 patients served as control (group, 2B) and did not receive $M_{\beta}AB$. Recordings were made at the same time intervals (t= 0, 10 and 30 minutes) as in patients with $M_{\beta}AB$.

Electrograms Analysis: Fibrillation intervals and dispersion of refractoriness

Custom made software based on Matlab 7.0 (The MathWorks Inc., Natick, MA, USA) was used for analysis and automatic detection of unipolar electrograms. In all recordings at least 8 electrograms per catheter had sufficient quality for analysis. Of each recorded 100s of AF, a 15s segment was analyzed. For this, the period between 15 and 30s was chosen.[9] Local activation time was defined as the point of steepest negative dV/dt in the unipolar tracings (dV/dt < -0.5V/s)[8;9] and this method was manually

checked by two independent observers in a subset of recordings. If a preceding or following activation time occurred within 50ms, the sharpest deflection was selected. This interval of 50ms was chosen based on studies in humans. Successive deflections with a timing <50ms are likely to represent areas of anatomical or functional delay or block, rather than true local activation due to tissue re-excitability.[20] As an index for the local atrial refractory period, we determined the mean fibrillation interval (mFI) for all 20 electrode terminals.[21-23] This allows to determine the time course of changes of the refractory period simultaneously at different sites of the atria. The mean and standard deviation (SD) of the mFIs at all sites were calculated for RA and LA. As a measure of dispersion of refractoriness in each atrium, the coefficient of dispersion (CD) was calculated as the SD of all mFIs, expressed as the percentage of the average mFI [(SDx100) / average mFI].[9] Values of CD above 3.0 are considered to represent enhanced dispersion of refractoriness.[8;9]

Statistical Analysis

All data are presented as mean values ± SD. For comparison between the groups we used one-way ANOVA test or chi-square test. The effect of $M_{\beta}AB$ on the mFI and dispersion of refractoriness was examined using the repeated measures analysis. If there was an effect for both analyses then all paired comparisons were done. A two-tailed p-value <0.05 was considered statistically significant. SPSS version 15.0.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for data analysis.

Results

Clinical characteristics:

Table 1 shows the clinical characteristics for the 2 groups. Estimated by the clinical history of the patients and 48h Holter recordings, group 1 patients had more episodes of AF compared to group 2. Other characteristics were not different between the groups.

Table 1: Clinical characteristics

	Group 1 (long-lasting AF)	Group 2 (induced AF)	p - value
N	20	15	…
Age (years), mean ± SD	56.2±8.4	53.7±9.2	0.41
Gender (male), n (%)	14 (70%)	13 (87%)	0.23
LA size (mm), mean ± SD	44.8±4.5	41.8±5.2	0.07
Patients with AF episodes*, n (%)			
per day (+ permanent)	16 (84%)	0 (0%)	<0.001
per week	2 (11%)	4 (27%)	
per month	2 (5%)	7 (46%)	
per year	0 (0%)	4 (27%)	

* The occurrence of AF episodes was estimated by clinical history of patients and 48h Holter registrations.
N: number, SD: standard deviation, LA: left atrium, AF: atrial fibrillation

Electrophysiologic parameters at baseline:

In group 2 patients, the mFI was longer in both atria compared to group 1 (RA: 171.2 ± 27.8ms vs 143.2 ± 16.3ms, p=0.001, LA: 155.5 ± 32.1ms vs 139.1 ± 12.4ms, p=0.049, figure 1A), and the mFI in RA was longer than in LA (171.2 vs 155.5 p= 0.005, figure 1A). In group 1 the mFI was not different between RA and LA.

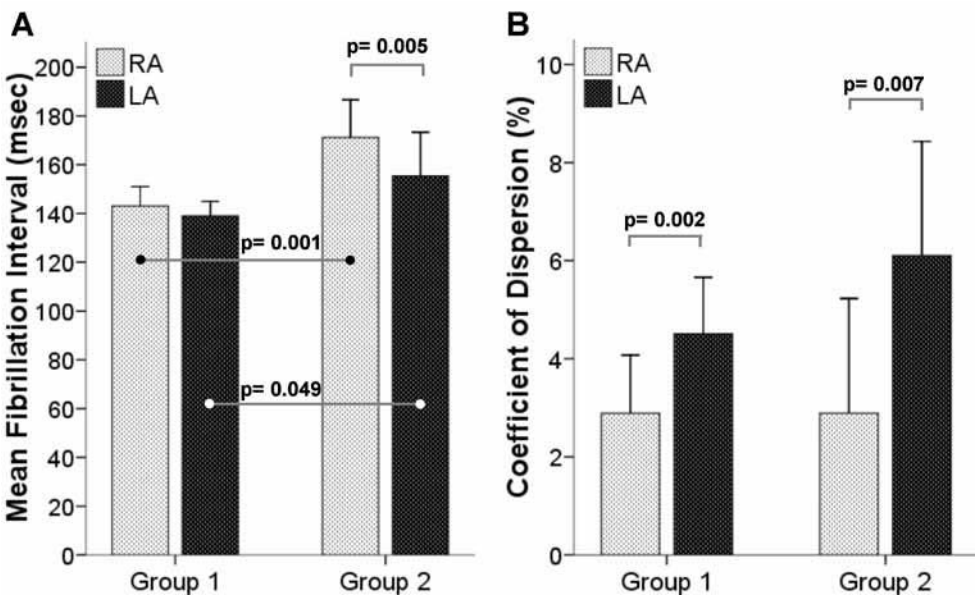
In both groups, the CD was significantly enhanced in LA compared to RA (group 1: p=0.002, group 2: p=0.007, figure 1B). Between the 2 groups, CD of RA and LA were not different (RA: 2.9 ± 2.5 vs 2.9 ± 4.2, LA: 4.5 ± 2.4 vs 6.1 ± 4.2, p=ns).

There were also no differences in clinical characteristics and electrophysiologic parameters at baseline between group 2A (patients who received $M_{\beta}AB$) and the control group 2B, table 2.

Effect of ANS on mean fibrillation interval (mFI):

The effect of atropine alone and of $M_{\beta}AB$ on mFI in groups 1 and 2A together with the changes in mFI over time in the control group 2B, are shown in figure 2. There was an interaction between

Figure 1: Baseline mFI and CD in RA and LA in both groups.



A: Mean fibrillation intervals, an index of atrial refractoriness, are shorter in both atria of group 1 compared to group 2. B: Dispersion of refractoriness, expressed as coefficient of dispersion, is significantly enhanced in LA compared to RA and it is not different between the 2 groups. RA: right atrium, LA: left atrium, mFI: mean fibrillation intervals, CD: coefficient of dispersion.

$M_{\beta}AB$ and study groups (p= 0.023). Consequently we tested the groups separately. In group 1 there was no effect of parasympathetic blockade or $M_{\beta}AB$ on mFI of both atria (p= 0.326, figure 2A). In group 2A patients, however, there was a significant effect of $M_{\beta}AB$ on mFI (p=0.024) (figure

2B). Parasympathetic blockade significantly reduced mFI in both atria (p=0.016), whereas $M_{\beta}AB$ restored mFI toward initial values. In control group 2B, the mFIs of the RA and LA did not change significantly from time 0 to 10min and 30min (p=0.468, figure 2C).

Table 2: Comparison of the subgroups of group 2

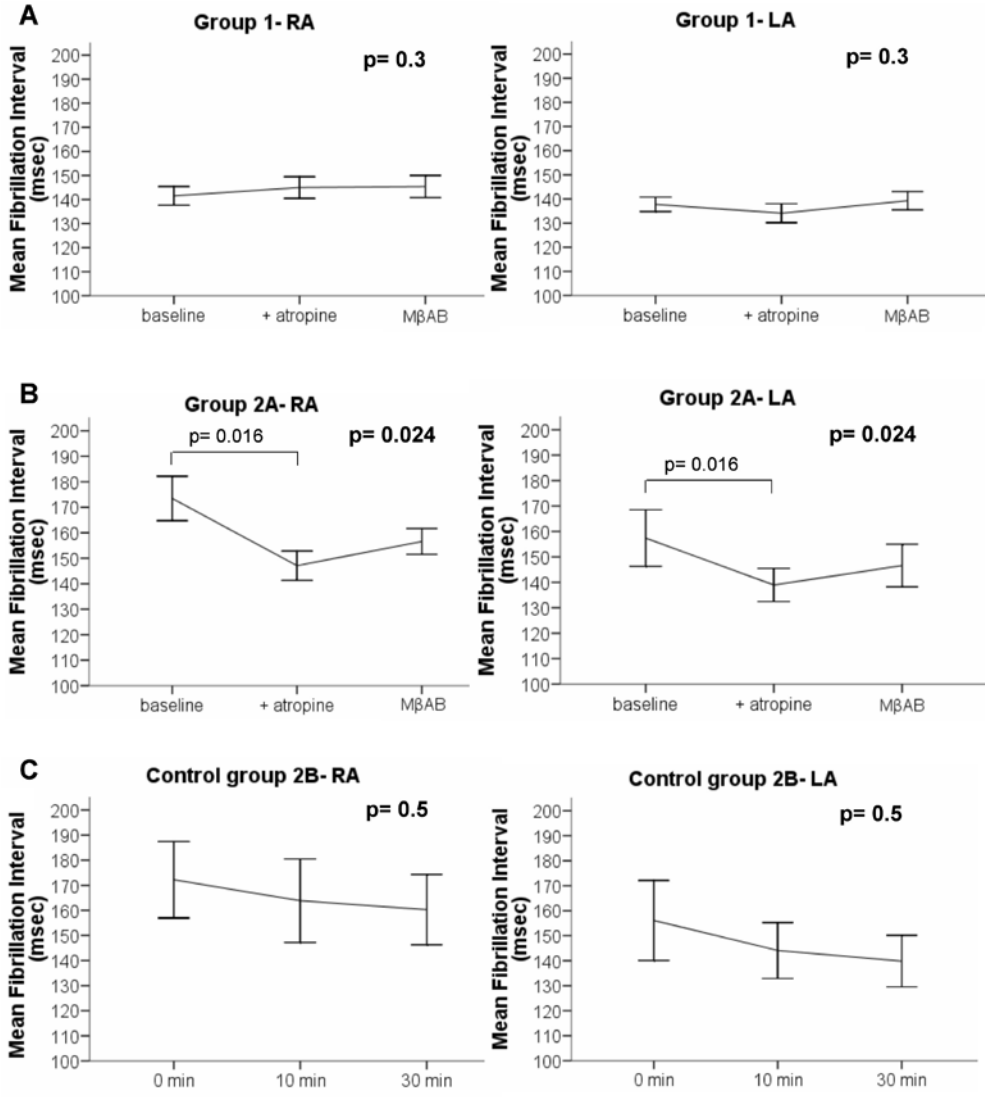
	Group 2A	**Control Group 2B**	**p - value**
n	10	5	…
Age (years), mean ± SD	50.8±9.1	59.4±6.9	0.09
LA size (mm), mean ± SD	42.4±4.4	40.6±6.9	0.55
Type AF, n: **Paroxysmal** **Persistent** **Permanent**	 8 2 0	 5 0 0	0.43
mFI, RA	170.7±26.1	172.2±34.0	0.93
mFI, LA	155.2±32.2	156.1±35.8	0.96
CD, RA	2.4±3.1	3.9±6.2	0.55
CD, LA	4.8±3.4	8.8±4.8	0.08

n: number, SD: standard deviation, LA : left atrium, AF: atrial fibrillation, mFI: mean fibrillation interval, RA: right atrium, CD: coefficient of dispersion

Effect of ANS on coefficient of dispersion (CD):

CD's were calculated for all groups during the 3 different recording segments. An interaction was found among the groups, the 3 different recording segments and the recording sites (p= 0.014). Thus the groups and the sites were analyzed separately (figure 3). In group 2A and 2B, the baseline values of CD of the LA seem different (figure 3B and 3C). However, this did not reach statistical significance (p=0.08). Moreover this is not relevant for our results because patients were used as their own controls. In group 2A, CD in both atria was not significantly affected by parasympathetic or $M_{\beta}AB$ (RA: p=0.264, LA: p= 0.578). In group 2B, CD did also not change over time (RA: p=0.503, LA: p= 0.182). In group 1, however, there was a significant effect of $M_{\beta}AB$ on CD of RA (p=0.02). Parasympathetic blockade reduced CD in RA (p=0.013), while $M_{\beta}AB$ restored CD toward initial values. The influence of $M_{\beta}AB$ on CD of the LA in group 1 patients was not statistically significant (p=0.08, figure 3A).

Figure 2: Effects of parasympathetic and β-adrenergic blockade on mFIs in groups 1 and 2A and the effect of time in control group 2B.



A: Both parasympathetic and β-adrenergic blockade do not influence mean fibrillation intervals in both atria of patients with long-lasting AF> 24h. B: Parasympathetic blockade significantly decreases the mFI in both atria of patients in group 2 (early phase of remodeling), while additional β-adrenergic blockade trend to increase them again. C: In control patients of group 2 mFI do not change significantly during the same time course. $M_{\beta}AB$: muscarinic- β-adrenergic blockade, other abbreviations as in figure 1.

Discussion

Main findings

The present study provides the first evaluation of the influence of ANS on mFI and dispersion of refractoriness in patients with idiopathic AF. Our study shows that:

1. Parasympathetic blockade reduced mFIs in both atria of group 2A patients (early phase of AF) and reduced dispersion of refractoriness in the RA of group 1 patients (AF>24h).
2. Systemic M_{β}AB did not affect mFI and CD in neither of the groups.
3. Mean fibrillation intervals were dependent on electrical remodeling of the atria, but dispersion of refractoriness was not.
4. Dispersion of refractoriness was higher in the LA compared to the RA.

AF duration and electrical remodeling

Electrical remodeling develops within the first days of AF. During remodeling, the atrial refractory period can shorten up to 40%, thus contributing to the increased stability of the arrhythmia.[5;24] During the first 24 hours of AF the atrial refractory period can shorten by as much as 12 to 35%, while the discontinuation of AF can restore the electrical properties of the atria.[5;25] Therefore, we selected 2 groups of patients based mainly on their rhythm status at inclusion and not based on the AF burden. As an index for the refractory period at each recording site we measured mFI. Group 1 patients had long-lasting AF >24h prior to the study. In these patients, mFI were shorter, indicating *advanced* electrical remodeling, while in group 2, patients in the early phase of an AF episode, mFI were longer indicating less electrical remodeling.

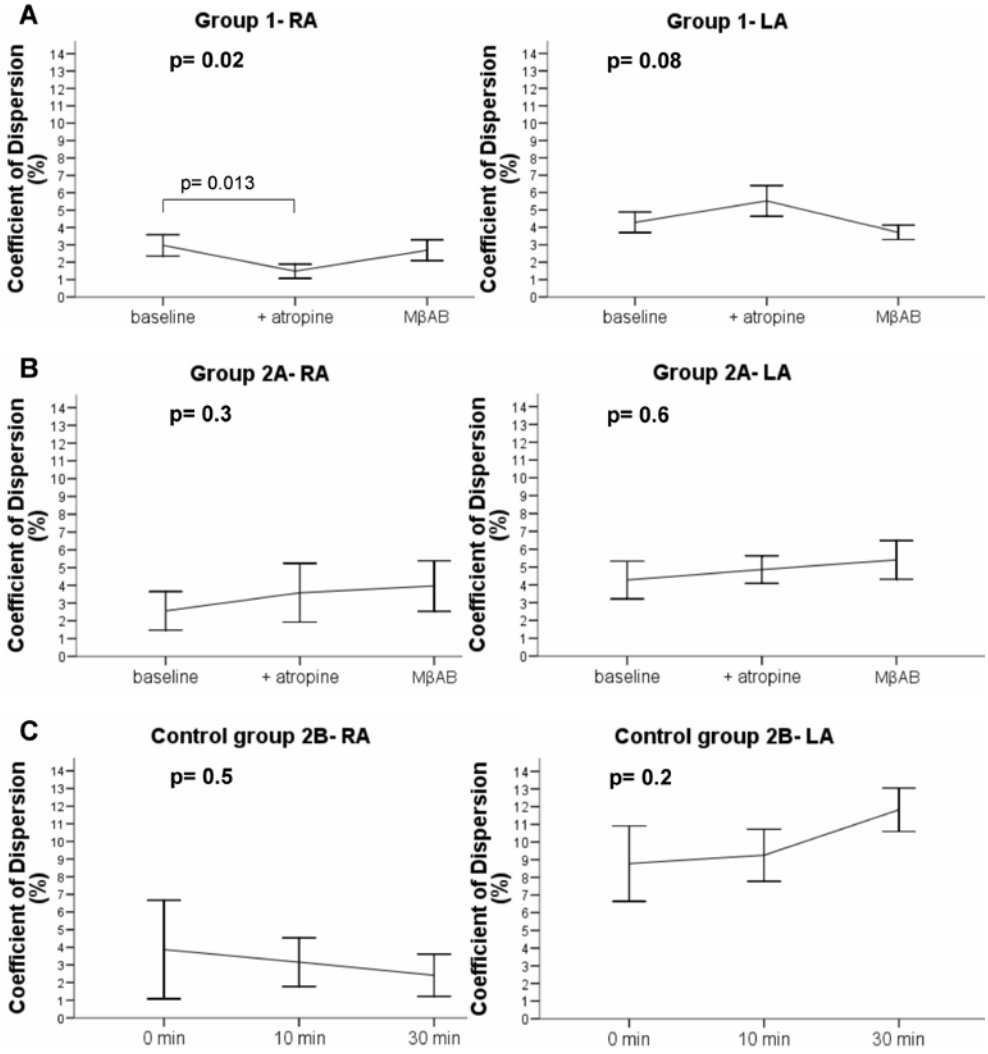
Influence of ANS on mean fibrillation interval and dispersion of refractoriness

The role of ANS on the atrial refractory period and its dispersion is controversial. While animal studies have suggested that the heterogeneity of atrial refractoriness can be influenced by vagal stimulation or heterogeneous sympathetic denervation alone,[15-17] a study in patients with supraventricular tachycardia has shown that blockade of both limbs is needed to blunt the effects of rapid stimulation on atrial refractoriness and its dispersion.[18]

The current study shows that the influence of M_{β}AB on mFI and CD depended on the degree of atrial electrical remodeling. In patients with advanced remodeling, the mFI, an index of the refractory period of the atria, was neither influenced by parasympathetic nor M_{β}AB. In patients with early remodeling, however, parasympathetic blockade significantly decreased mFI in both atria. This effect was different from that in the control patients where no significant changes occurred in the same time period. Sympathetic predominance due to inhibition of the parasympathetic limb probably accelerated the reduction of mFI, which subsequently increased again after M_{β}AB. This suggests that the initial decline of mFI was not associated with the changes due to a new AF episode, but due to parasympathetic blockade.

Dispersion of refractoriness, expressed as CD, was also differently influenced by M_{β}AB between the groups. In patients with early remodeling, parasympathetic blockade alone or M_{β}AB did not change

Figure 3: Effect of parasympathetic and β-adrenergic blockade on CD in groups 1 and 2A and the effect of time in control group 2B.



A: Parasympathetic blockade significantly decreases CD of RA, while CD of LA was not influenced. $M_{\beta}AB$ does not further influence dispersion of refractoriness, expressed as CD, of both atria. B: Parasympathetic and β-adrenergic blockade do not influence CD of group 2. C: CD does not change significantly in both atria of the control group. $M_{\beta}AB$: muscarinic- β-adrenergic blockade, other abbreviations as in figure 1.

CD. In patients with advanced remodeling, however, parasympathetic blockade decreased the CD in the RA, but it had no significant influence on CD in the LA. Although LA is more densely innervated than the RA[26-28], the fact that the LA showed no response to parasympathetic or $M_{\beta}AB$ indicates that the high dispersion of refractoriness in the LA, in patients with idiopathic AF, might rather be anatomically mediated.[29] Accordingly, it is not influenced by the dynamic changes of the ANS or the status of the electrical remodeling, as previously mentioned.

Electrical remodeling and dispersion of refractoriness

The influence of electrical remodeling on spatial dispersion of refractoriness is still unclear. Animal studies have shown that spatial dispersion of mFI due to atrial remodeling can increase[30] or remained unchanged.[5] In our patients the dispersion of refractoriness expressed as CD was similar in both RA and LA of both groups, despite different states of electrical remodeling as indicated by different mFI. This implies that dispersion of refractoriness was independent of the status of atrial electrical remodeling.

Dispersion of refractoriness as a substrate of AF initiation

Today, triggers from the myocardial sleeves of the pulmonary veins are thought to be essential for the initiation of AF.[6;7] However, not every AF patient reveals such triggers. Dispersion of refractoriness in the RA was found to be enhanced in patients with increased vulnerability to AF.[8;9] The importance of non-uniformity of recovery of excitability was studied by Allessie et al.[31] In isolated rabbit atrial muscle, they demonstrated that spatial dispersion of refractoriness could yield local conduction block after a premature atrial beat, thereby initiating circus movement tachycardia and AF. In our patients with idiopathic AF, CD was enhanced in LA compared to RA, which suggests that the LA plays an important role in the initiation of AF in these patients. The results from our study suggest that dispersion of refractoriness is independent of atrial electrical remodeling. It is well known that structural complexities in LA can lead to conduction disturbances.[32-36] This may also influence local refractory periods and may lead to enhanced dispersion of refractoriness in LA.

Limitations of the study

Recordings were made from one location in the RA and the posterior wall of the LA. Multiple recording sites could have provided more information. However, we chose one stable recording site for 2 main reasons. At first the consistency of the recording site during the 30 minutes time period and therewith better comparability of the recordings. Second, the study time was limited, because the protocol was conducted in patients prior to pulmonary vein ablation and different recording sites would have been time consuming.

As well, patients who underwent AB first received atropine for parasympathetic blockade and then b-blockade. The other way round, b-blocker infusion first and then atropine, was not performed. The cardiac ganglia in the posterior wall of the left atrium (LA), where we collected our recordings, contain abundant and predominantly parasympathetic neuronal cell which mainly influence the autonomic nervous status of the LA.[26] For this reason we found it interesting to see the effect of parasympathetic blockade on that side. In addition, the final result for parasympathetic and M_{β} AB would not be influenced if drugs administration was randomized because the recordings for autonomic blockade were made after both drugs had reached the Tmax (the maximum plasma concentration) after intravenous administration (4min for atropine en 15 min for metoprolol).[19]

We chose the mean fibrillation interval as an index for the atrial refractory period. We are well aware of the fact that the mFI may overestimate the actual refractory period.[21] However, this method allows to record changes simultaneously at different sites of the atria during AF and at the different stages of the M_{β}AB protocol. Finally, the analysis of the 15-30s period was chosen based on a previous

publication of our group.[9] Also, in patients in whom AF was induced we have chosen the 2nd 15s period for two reasons: Not earlier in order to ensure that the AF episode was stable and not later to avoid advanced atrial electrical remodelling at baseline.

Conclusion

This study investigated atrial refractoriness and its dispersion in the right and the left atrium in patients with idiopathic AF in the early and late phase of electrical remodeling, and the influence of systemic $M_{\beta}AB$ on these parameters. As expected, mFI decreased with increasing remodeling. However, dispersion of refractoriness was not influenced by AF duration. CD was greater in LA compared to RA independent of AF duration.

Total systemic $M_{\beta}AB$ did not affect mFI and CD in both early and late remodeling. However, parasympathetic blockade alone reduced mFI in both atria in the early phase of AF and reduced dispersion of refractoriness in RA in patients with longstanding AF.

Acknowledge

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Conflict of interest:

Dr. F. Wittkampf is a consultant for St. Jude Medical and Dr. A. Linnenbank receives a grant from the Netherlands Heart Foundation. All other authors have nothing to disclose.

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Chapter 3

Complex Fractionated Electrograms in the Right Atrial Free Wall and the Superior/Posterior Wall of the Left Atrium are Affected by Activity of the Autonomic Nervous System

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Abstract

Background: Complex fractionated atrial electrograms (CFAEs) are supposed to be related to structural and electrical remodeling. Animal studies suggest a role of the autonomic nervous system (ANS). However this has never been studied in humans.

Objective: Goal of this study was to investigate the influence of ANS on CFAEs in patients with idiopathic atrial fibrillation (AF).

Methods: Thirty-six patients (28 men, 55±9 years) were included before undergoing catheter ablation. In the 24h preceding the procedure, 20 patients were in AF (group 1) and 16 were in sinus rhythm (SR, group 2). With 2 decapolar catheters, one in the right atrium (RA) and one in the left atrium (LA), 20 unipolar electrograms were simultaneously recorded during a 100s AF-period (in group 2 after induction of AF). After atropine and metoprolol administration, a 2nd 100s AF-period was recorded 30 minutes later. Five patients of group 2 served as controls and did not receive atropine and metoprolol prior to the 2nd recording. CFAEs were assessed and the prevalence of CFAEs was expressed as percentage of the recording time.

Results: The prevalence of CFAEs was greater in group 1 than in group 2 in both RA and LA ($p=0.026$, $p<0.001$ respectively). Atropine and metoprolol significantly reduced CFAEs in group 1 ($p<0.001$) and prevented the time-dependent increase of CFAEs in group 2.

Conclusion: The prevalence of CFAEs is greater in long-lasting AF episodes. Atropine and metoprolol administration reduces CFAEs in both atria. Thus, CFAEs are at least partly influenced by the ANS.

Introduction

Recently, abolishment of complex fractionated atrial electrograms (CFAEs) was established as an additional ablation target to treat Atrial Fibrillation (AF)[1;2]. Areas of CFAEs seem to be critical regions for maintaining AF. Such areas are probably related to anatomic and electrical changes of the atrial myocardium, which result in conduction delay or block, micro- reentry and/or pivoting points in the atrium[3]. High frequency focal activity of the pulmonary veins may also provoke CFAEs[4].

It is well known that the autonomic nervous system (ANS) can influence AF onset. AF can be characterized as vagotonic and adrenergic according to the autonomic state of the patient[5;6]. Presumably, the ANS also plays a role in originating CFAEs and may contribute to rapid firing, at least from the superior vena cava[7]. The ANS is composed of axons and ganglia grouped into so-called ganglionated plexi (GP) within the epicardial fat pads of the heart[8]. During treatment of patients with AF an additional ablation of these GP improved the outcome of such treatment[9-11] Animal studies showed that activation of the ANS could induce CFAEs[12;13] suggesting that the substrate for CFAEs can be modulated by the ANS. Finally, a recent study showed that pharmacological autonomic blockade can reduce CFAEs in the left atrium of patients with paroxysmal AF[14].

This study sought to evaluates the impact of the ANS on the prevalence of CFAEs in both atria during idiopathic AF. In addition, we studied the spatial and temporal stability of CFAEs in long-lasting AF and in the early phase of AF.

Methods

Study population

Patients were recruited from those who underwent radiofrequency catheter ablation for drug refractory AF. Patients with idiopathic AF were prospectively included. Patients with structural heart disease and/or conditions that could influence the cardiac electrophysiological properties were excluded based on history, physical examination, ECG, echocardiography and biochemical and hematological testing. Patients were excluded if the LA was > 50mm on echocardiography (parasternal long axis) or if amiodarone was used. Telemetry was performed in all patients for at least 24h prior to the procedure.

A pilot study was performed in 6 patients in order to evaluate our study protocol. In patients included in the pilot study, additional recordings were made as described below.

Pilot study patients

Six patients (all men, mean age 53±13, five with paroxysmal and 1 with persistent AF) were included. All patients were in sinus rhythm (SR) prior to the ablation procedure.

Study patients

Thirty-six consecutive patients with idiopathic AF were included. Patients were divided into 2 groups according to the rhythm before the procedure. Group 1 consisted of 20 patients (14 men, mean

age 56 ± 8, range 33-66, 13 paroxysmal, 1 persistent and 6 permanent AF) with a long-lasting episode of AF (>24 hours) before the procedure. Group 2 consisted of 16 patients (14 men, mean age 53 ± 9, range 37- 65, 14 paroxysmal and 2 persistent AF) with SR at least during the 24h period before and at the time of the procedure.

Electrophysiological study (EPS)

The study protocol was approved by the local medical ethics committee of the University Medical Center Utrecht and all patients signed informed consent. The protocol was executed before pulmonary vein antrum isolation was performed. All patients were studied in a fasting non-sedated state and antiarrhythmic drugs had been discontinued for >5 half lives.

Pilot study

In patients selected for the pilot study, after femoral venous access, a monophasic action potential (MAP) catheter (Franz catheter, EP technologies) was positioned at the posterior or superior wall of the LA through an open foramen ovale or after transseptal puncture, in order to evaluate the refractory period during AF. This measurement was crucial for the detection and assessment of CFAEs. This contact electrode recording technique has been described and evaluated previously[15].

Stimulation protocol: After induction of sustained AF by programmed atrial extrastimulation, fixed rate bipolar pacing (cycle length, 600 ms) was performed via the MAP catheter with 10 times the diastolic threshold for approximately 2 minutes[16;17]. This protocol was repeated at 2 other LA sites.

Study protocol

After femoral venous access, two decapolar electrode catheters (Livewire, St. Jude Medical, Minnetonka, MN, U.S.A.) with an inter-electrode spacing of 5mm were inserted. One was positioned in the right atrium (RA), along the anterior wall, and the other in the LA at the superior/posterior wall, between the ostia of the pulmonary veins, through an open foramen ovale or after transseptal puncture (Figure 1). Steady catheter position was ensured by fluoroscopy and signal stability. Patients were anticoagulated with intravenous heparin to maintain an activated clotting time of 250-350 seconds. Surface ECG leads II and V1, and 20 intracardiac unipolar electrograms were continuously recorded with an electrophysiological recording system (Cardiolab; Prucka Engineering Inc, Dallas, TX, USA). Intracardiac unipolar electrograms were bandpass-filtered from 0.05 to 500 Hz and amplified (2mV/cm). Pacing was performed with a Pace-1A cardiac stimulator (Radionics, Burlington, MA, USA).

Group 1: Patients (n=20) had ongoing AF 24h prior to and during EPS. When a stable catheter position was obtained, the study protocol started (time 0) by recording the first 100s AF-period. Thereafter, intravenous atropine (0.04mg/kg, maximal dose 3mg) and metoprolol (0.2mg/kg, maximal dose 20mg)[18] were administered consecutively. After 30 minutes, which were necessary for the drug administration and the establishment of full blockade of the muscarinic and β-adrenergic receptors on the heart (M_{β}AB: muscarinic -β-adrenergic blockade), a second 100s recording of AF was obtained.

Group 2: Patients (n=16) were in SR 24h prior to and at the beginning of the procedure and

AF was induced by programmed atrial extrastimulation. Directly after the onset of AF (time 0), the first 100s episode was recorded. In 11 randomly selected patients atropine and metoprolol were given as described in group 1 and a 2nd 100s AF period was recorded. The remaining 5 patients served as control group and the 2nd 100s recording was obtained after 30 minutes without $M_{\beta}AB$. None of the patients reverted back to SR after AF induction.

For both groups full blockade was assumed when both drugs had reached the Tmax, the maximum plasma concentration, after intravenous administration (4min for atropine and 15 min for metoprolol) [18].

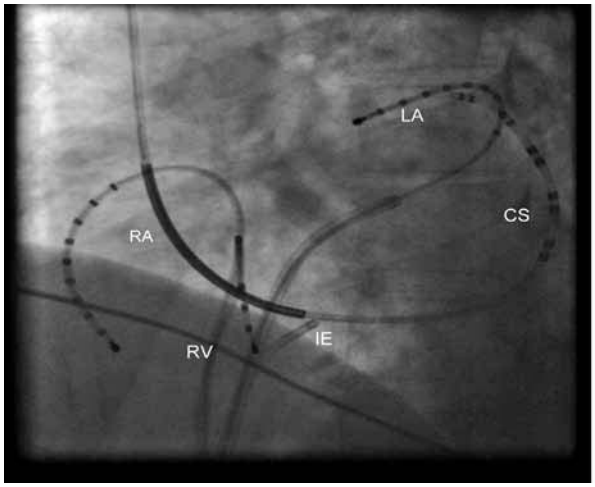
Electrogram analysis

Activation times were automatically detected with custom made software[19] based on Matlab 7.0 (The MathWorks Inc., Natick, MA, USA).

Pilot study-MAP recordings:

An action potential during AF was considered to be captured by the stimulus when the following 3 criteria were fulfilled: 1) the stimulated action potential had lower amplitude than the spontaneously occurring action potential, 2) the morphology of captured action potentials was similar and 3) a 30±10ms delay between stimulus and upstroke of the action potential occurred (Figure 2A)[17;20]. When a stimulus captured the atrium, we measured the capture interval between the upstroke of the preceding spontaneous action potential and the stimulus artifact (Figure 2B). On average 12±7 stimuli per 2 minutes were captured. Of all 2 minutes MAP recordings the shortest capture interval was determined. The mean shortest capture interval of all MAP recordings was 104±13 ms (range 85-110ms) in agreement with an earlier study[17]. This value was defined as a capture window of the atrial myocardium during AF in which atrial myocardium was excitable again and used as a cut-off to define CFAEs.

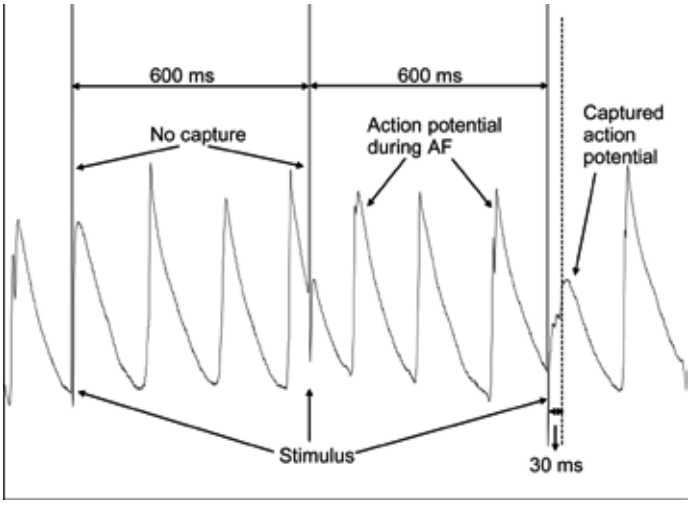
Figure 1: Position of the catheters in the right and left atrium



Position of the catheters in the right and left atrium: RA: right atrium catheter at the anterior wall, LA: left atrium catheter at the posterior wall, RV: right ventricle catheter, CS: coronary sinus, IE: indifferent electrode, positioned high in the interventricular septum in the right chamber.

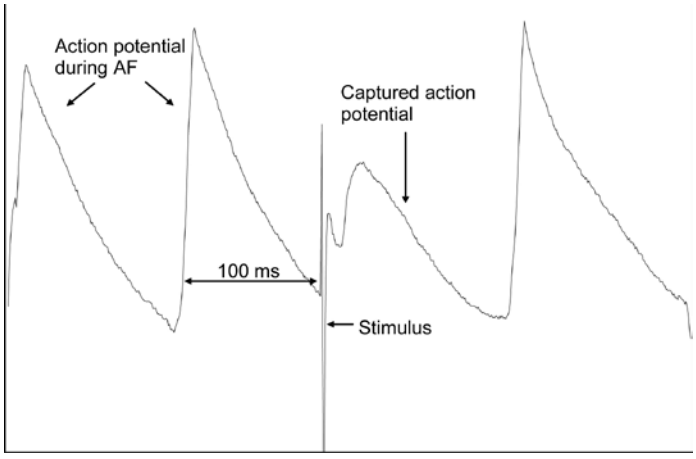
Figure 2: MAP recordings

A) Example of local capture during atrial fibrillation



A) During MAP bipolar pacing at the posterior wall of the LA (cycle length, 600ms) the atrium is considered to be captured if the action potential had lower amplitude and similar morphology with other captured action potentials and if a 30±10ms delay between the stimulus and the upstroke of the action potential was present.

B) Example of a Capture Interval



B) The shortest interval between the stimulus artifact that captured the atrium and the upstroke of the preceding spontaneously occurring action potential was determined.

Study Protocol- Atrial Electrograms:

Recordings were made in unipolar mode because maximal negative dV/dt has been shown to correspond with the upstroke of the cellular action potential under the electrode in unipolar but not in bipolar signals[21;22]. Also, data from our group have shown that bipolar electrograms often erroneously mark deflections as fractionated, although unipolar recordings reveal only a single downslope[23].

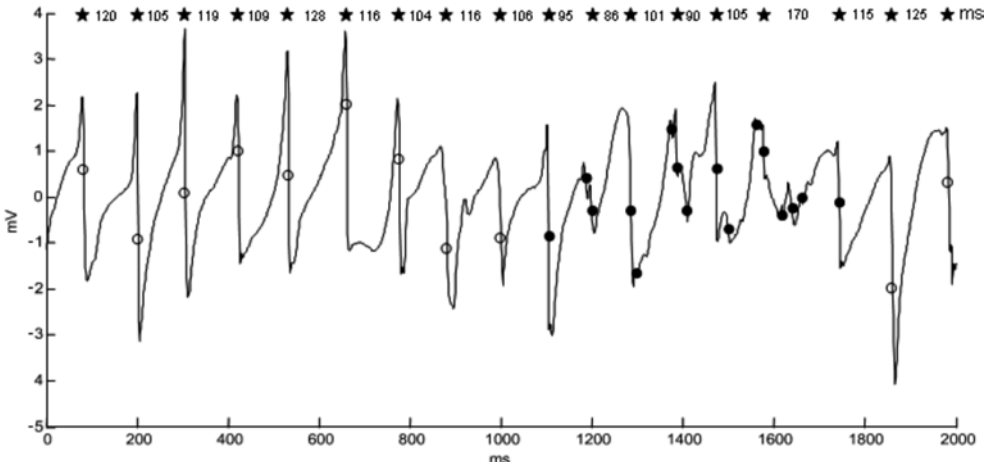
Determination of the mean AF cycle length (AFCL): For each patient, the mean AFCL was determined at all 20 electrode terminals (RA and LA) for the period between 15 and 30s of the 100s AF-recording before and after $M_{\beta}AB$. For determination of AFCL, local activation was defined as negative deflections with a dV/dt >-0.5V/s[24].

Detection of CFAEs: The detection of CFAEs has been a limiting factor in many studies and CFAEs are mainly determined based on visual judgment[1]. In our study CFAEs were automatically detected, by a custom made software[19], for all 20 electrode terminals for the 100s period per each patient before and after $M_{\beta}AB$. CFAEs were defined as periods of successive atrial deflections with the following properties: a) dV/dt < -0.04V/s, b) amplitude >2% of the highest unipolar electrogram recorded in every tracing and c) continuous atrial activation or deflections separated by an interval ≤ 104ms (Figure 3). The prevalence of CFAEs per 100s recording period was calculated per electrogram and expressed as percentage (CFAEs%). The values of dV/dt and amplitude were chosen based on data from two independent observers who analyzed a subset of this data manually. The chosen values gave the best match when used in the custom made software for automatic detection. As already mentioned, the period of 104 ms was chosen based on the MAP pilot study results. The detection of AFCL and CFAEs was done independently and automatically to ensure objectivity. (Figure 3)

Spatial heterogeneity of CFAEs: To assess the spatial heterogeneity of CFAEs in the area covered by the recording catheters for both atria, we calculated the standard deviation (SD) of the 10 values of CFAEs%. The SD can be used as an index of spatial heterogeneity and in this case it is used to evaluate whether $M_{\beta}AB$ homogenously affected the recording sites in both atria.

Short-term temporal stability of CFAEs: To evaluate the short-term temporal stability of CFAEs we compared the CFAEs proportion in the intervals 0-50s and 50-100s for every 100s recording.

Figure 3: Example of CFAEs during AF



Left part of the recording shows organized atrial activation (open circles). After approximately 1.1 seconds, activation became disorganized, illustrated by fractionated electrograms (closed circles). Circles indicate atrial deflections with dV/dt < -0.04V/s and amplitude >2% of the highest unipolar electrogram recorded in this tracing. Open circles: interval between deflections > 104ms. Closed circles: continuous atrial activation or deflections separated by an interval ≤104ms (CFAEs).

The asterisks indicate the deflections determined by the AFCL algorithm. In this example mean AFCL remains almost stable throughout the whole time of recording, although activation changes from organized to fractionated. 1-1000 ms (organized): mean AFCL = 114ms; 1000-2000 ms (fractionated): mean AFCL = 110ms.

Statistical analysis

All data are expressed as mean ± SD. Continuous variables between the groups were compared using ANOVA. Categorical values were compared by chi-square test. The mean values of the parameters acquired before and after $M_{\beta}AB$ were compared using repeated measures analysis. Probability values <0.05 were considered statistically significant. SPSS version 15.0.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for data entry and analyses.

Table 1: Clinical Characteristics:

	All patients	Group 1 AF> 24h	Group 2 early phase AF	p-value
N	36	20	16	
Age (years)	55 ± 9	56 ± 8	53 ± 9	ns
Male, n (%)	28 (78)	14 (70)	14 (87)	ns
LA diameter, (mm)	43.5 ± 5	45 ± 5	42 ± 5	ns
Duration of AF (year)	4.4 ± 2.4	4.7 ± 2.5	4.2 ± 2.4	ns
Type of AF*, n (%)				0.05
Paroxysmal	27 (75)	13 (65)	14 (87)	
Persistent	3 (8)	1 (5)	2 (13)	
Permanent	6 (17)	6 (30)	0 (0)	

* The occurrence of AF episodes was estimated by patient history and/or Holter recordings; LA: Left atrium, AF: Atrial Fibrillation

Results

Baseline characteristics:

The baseline characteristics for the 36 patients who were included in the study were not significantly different between the groups (table 1). Patients in group 1 (long lasting AF) had shorter AFCL in both atria compared to group 2 (SR 24h prior to the procedure) (RA: 143.2 ± 16.3 ms vs 171.2 ± 27.8 ms, p=0.001; LA: 139.1 ± 12.4 ms vs 155.5 ± 32.1 ms, p=0.049).

Influence of ANS on AFCL:

In group 1, $M_{\beta}AB$ did not affect AFCL (Figure 4). Both in the study and control patients of group 2, the AFCL in both atria tended to become shorter after 30 minutes AF compared to the initial values (time 0). This AFCL shortening was not different between control patients and patients receiving $M_{\beta}AB$ (RA: -11ms vs -14 ms, p= 0.5; and LA: -15ms vs -8 ms, p= 0.3). Thus, $M_{\beta}AB$ did not affect the mean cycle length of AF in any of the 3 groups.

CFAEs at baseline:

Before M_βAB the prevalence of CFAEs in group 1 was significantly greater in both atria compared to group 2 (Figure 5). In addition, in both groups, CFAEs prevalence was significantly greater in LA compared to RA. The spatial heterogeneity of the prevalence of CFAEs expressed as

Figure 4: The influence of Autinomic Nervous System on mean AFCL

SD, in the LA of patients with long-lasting AF was higher compared to that in the RA (16±7 vs 9±5, p=0.003). It was also higher compared to the LA of patients in the early phase of an AF episode (16±7 vs11±6, p=0.04).

Short- term CFAEs% stability*:* The prevalence of CFAEs of the 1st and the 2nd 50s interval of each 100s recording was not significantly different in neither of the groups.

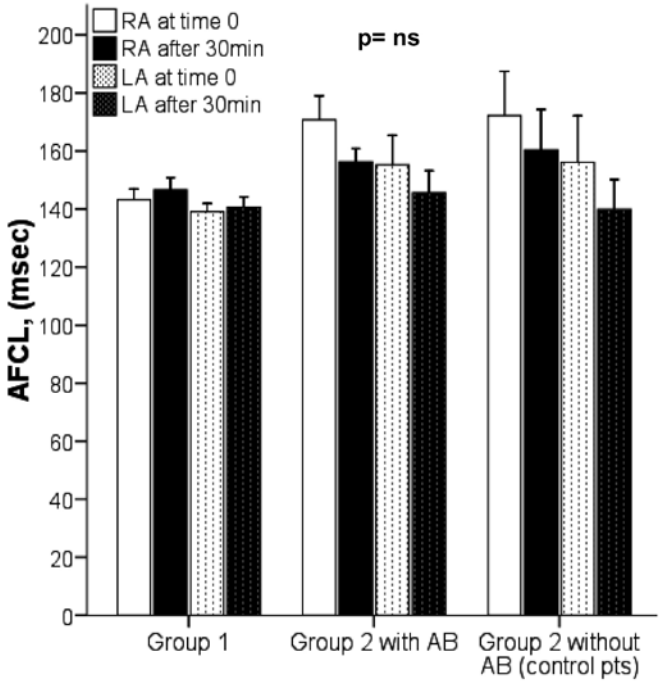
Influence of ANS *on* CFAEs:

There was an interaction between the groups and the influence of ANS (p< 0.001), that's why we studied the effect of ANS on the amount of CFAEs and SD separately for every group.

Effect of ANS on group 1 (patients with AF > 24h):

The effect of full M_βAB on the prevalence of CFAEs and their spatial heterogeneity in both atria is shown in Figure 6A. After M_βAB, CFAEs prevalence was significantly reduced in both atria (RA: 24±19% vs 21±18% and LA: 48±22% vs 43±22%, p<0.001) while spatial heterogeneity remained unchanged, indicating that the decrease of CFAEs was rather homogeneous.

Figure 4: The influence of Autinomic Nervous System on mean AFCL

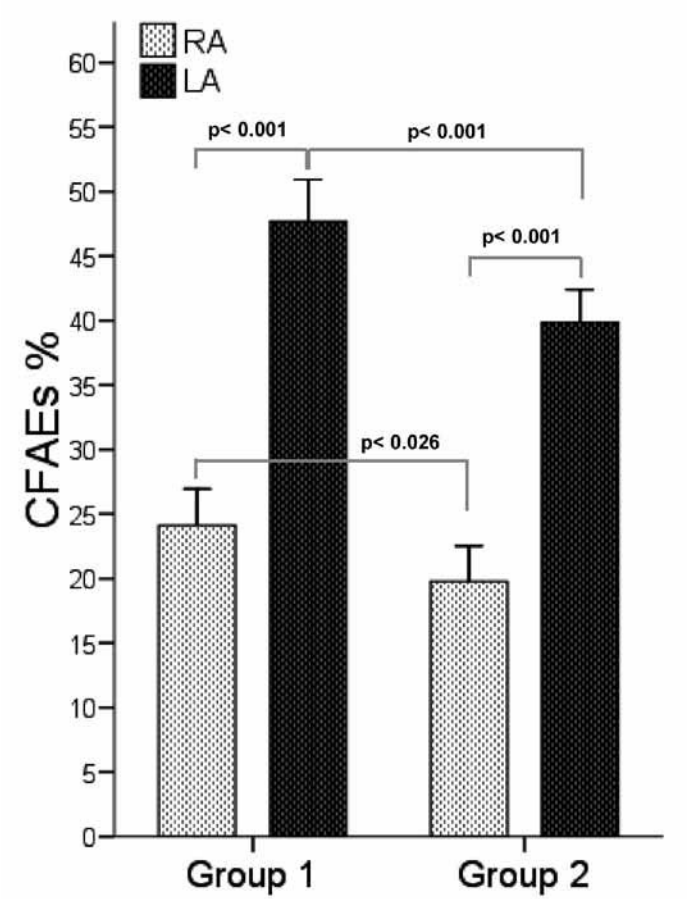


Group 1: patients with AF >24h + M_βAB. Group 2: patient in the early phase of AF + M_βAB. Control patients from group 2 without M_βAB. ANS did not influence the AFCL in all 3 groups. AFCL: mean Atrial Fibrillation Cycle Length, RA: Right Atrium, LA: Left Atrium, M_βAB: Muscarinic -β-Adrenergic Blockade. Error bars indicate 95% confidence intervals.

Effect of ANS on group 2 (early phase of AF):

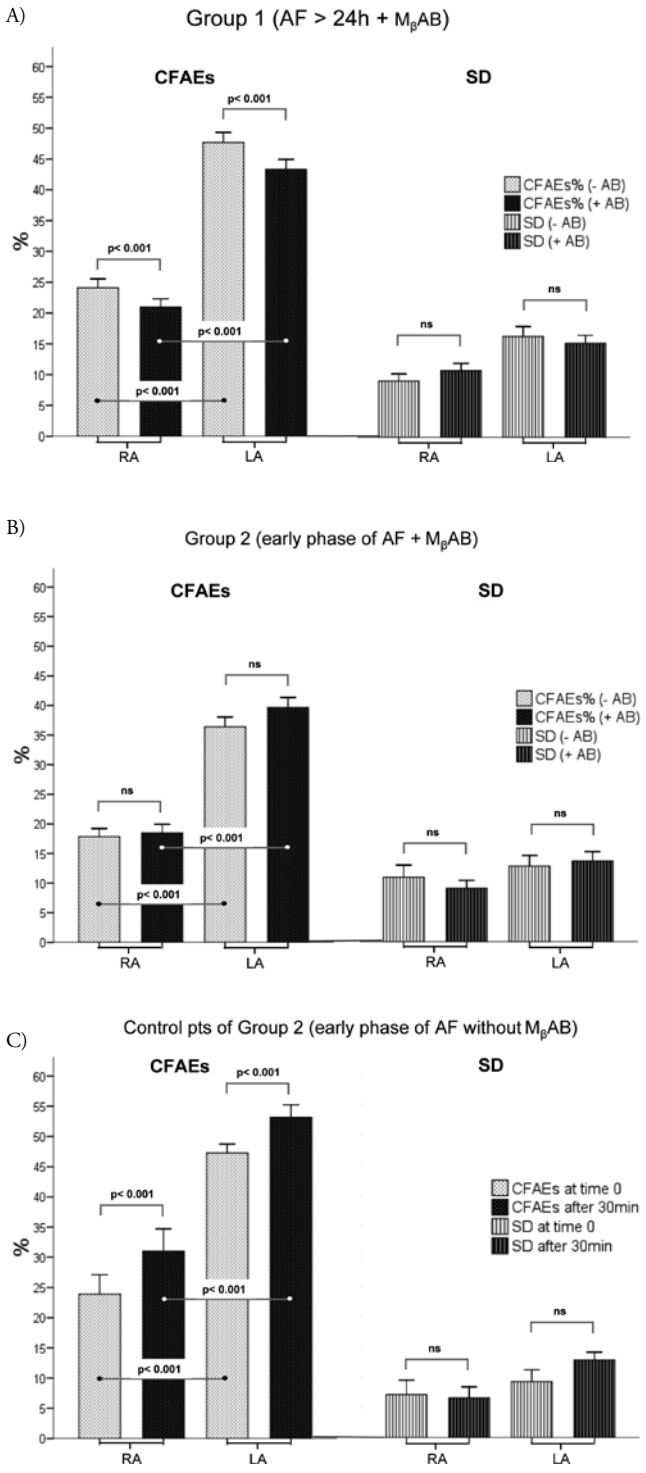
M_{β}AB did not significantly change the prevalence of CFAEs of both atria in group 2 patients (RA: 18±14% vs 19±15% and LA: 36±15% vs 39±17%, p=0.131 - Figure 6B). In contrast, in control patients who did not receive M_{β}AB, CFAEs prevalence increased significantly in both atria during the 30 minutes time interval (RA: 23±19 vs 31±26 and LA: 47±10% vs 53±15%, p<0.0001 - Figure 6C). The spatial heterogeneity was not changed with or without M_{β}AB, indicating that M_{β}AB homogeneously prevented the increase of CFAEs with time.

Figure 5: CFAEs during baseline before M_{β}AB



Group 1: patients with AF >24h + M_{β}AB at baseline. Group 2: patient in the early phase of AF at baseline. Prevalence of CFAEs in group 1 was significantly greater in both atria compared to group 2. CFAEs: Complex Fractionated Atrial Electrogram expressed as percentage (%), RA: Right Atrium, LA: Left Atrium. Error bars indicate 95% confidence intervals.

Figure 6: The influence of $M_{\beta}AB$ on CFAEs%



The influence of Autonomic Nervous System on CFAEs% A) In group 1: $M_{\beta}AB$ significantly reduced CFAEs prevalence in both atria. B) In group 2 with $M_{\beta}AB$: during $M_{\beta}AB$ the prevalence of CFAEs did not change. C) In control patients of group 2: CFAEs prevalence increased within 30 minutes. Spatial heterogeneity of CAFEs expressed as standard deviation did not change in all groups. SD: Standard Deviation of CFAEs used as an index of spatial heterogeneity, $M_{\beta}AB$: Muscarinic -β-Adrenergic Blockade, RA: Right Atrium, LA: Left Atrium.

Discussion

Main findings

The present study provides the first evaluation of the influence of the ANS on CFAEs in both atria in patients with idiopathic AF. $M_{\beta}AB$ significantly reduced the prevalence of CFAEs in patients with long-lasting AF and prevented the increase of CFAEs in the early phase of AF. The study also shows that in patients with long-lasting AF the prevalence of CFAEs was greater in both atria compared to patients in the early phase of AF, and the prevalence of CFAEs was greater in the LA compared to the RA.

Detection of CFAEs

Definition and detection of CFAEs has been controversial between studies. Some groups consider a single electrogram with multiple components as being "fractionated"[3;25], whereas others focus on regions with very short cycle lengths or continuous activation[1;2]. The present study used the definition of CFAEs introduced by Konings et al[3]. In contrast to many clinical studies[26-29], unipolar electrograms were used for the reasons stated above. Currently available mapping systems mainly use bipolar electrograms for detection of CFAEs. For unipolar recordings, the activation time is well defined. Simultaneous recordings from microelectrodes and extracellular electrodes from the same site of myocardial tissue during propagation of the action potential has shown that the upstroke of the action potential, which marks depolarization of the tissue at the recording site, coincides with the point of steepest negative dV/dt in the unipolar electrogram[30]. For bipolar recordings the activation time is less well defined because both amplitude and configuration of the signal are dependent on the direction of the activation front. Therefore, unipolar recording would be preferable for determining activation times and thus CFAEs. However, a disadvantage of the unipolar recording mode is that remote activation is detected as well. Unipolar recordings of the atrium will contain remote ventricular deflections, that will give rise to false activation times in an automatic detection system. Thus unipolar atrial recordings with ventricular activation subtraction (or manual correction) would be the preferable option to determine CFAEs. Whether bipolar recordings for CFAE estimation is a valuable replacement needs to be elucidated. [23 30]. Therefore, we used custom software and defined new criteria for automatic detection of fractionated electrograms. These criteria provided the best match between automatic detection and human observers.

Autonomic Nervous System and CFAEs

The present study evaluated the role of ANS on CFAEs in patients with idiopathic AF by systematic temporary $M_{\beta}AB$. We found that $M_{\beta}AB$ in patients with long-lasting AF reduced the prevalence of CFAEs in both atria independent of AFCL. Autonomic blockade in patients in the early phase of AF prevented a time-dependent rise in CFAEs prevalence, like seen in the control patients. These findings suggest that CFAEs are at least partly influenced by the ANS.

These results are in line with other experimental and human observations. Canine and human studies showed that CFAEs become more prominent closer to the GP[12;13;31]. In animal models CFAEs

could be eliminated by GP ablation[12;13]. Areas of CFAEs during AF have been attributed to conduction disturbances due to anatomic or functional block, anisotropy or meandering of rotors affected by excessive concentration of neurotransmitters[3;21;32;33]. High frequency firing of the pulmonary veins or adjacent atrial tissue, induced by hyperactivity of the ANS, has also been shown to generate CFAEs[4]. A systematic M_{β}AB may lead to reduced secretion of neurotransmitters at the GP and consequently to reduction of the proportion of CFAEs.

On the other hand, a recent study showed that pharmacological autonomic blockade reduced the proportion of CFAEs in patients with paroxysmal AF but not in those with persistent AF, and that this effect was probably mediated by prolongation of the AF cycle length[14]. This discrepancy can be explained by the fact that the patients included in that study had a different status of atrial electrical remodeling at the time of recording and also altered autonomic remodeling[34]. Thus, It is possible that the autonomic nervous system was augmented, which generated more CFAE and also made CFAE more susceptible to atropine and beta-blockers.

Electrical Remodeling and Temporal Stability of CFAEs

Studies in patients with persistent AF or sustained long-lasting AF, demonstrated that regions of CFAEs are relatively stable over time without changes of the AFCL[28;35;36]. On the other hand, a study in patients with paroxysmal AF in whom AF was induced showed that the prevalence of CFAEs increased together with a decrease of the AFCL in the first 30 minutes of AF[25]. In addition, we found that in patients with long-lasting AF (>24h) the prevalence of CFAEs was significantly higher and the mean AFCL was significantly shorter in both atria compared to patients in the early phase of an AF episode. This suggests that the increase of CFAEs prevalence may be mediated by AFCL shortening due to electrical remodeling in the early phase of AF, like seen in control patients. Experimental studies in goats and dogs showed that a substantial amount of electrical remodeling (shortening of AFCL) occurs during the first 24h of a new AF episode[37;38]. The present study also showed that M_{β}AB prevented the time-dependent increase of CFAEs prevalence in patients in the early phase of AF; and reduced CFAEs prevalence in patients with long-lasting AF, without influencing the AFCL. In group 2, however, the CFAEs prevalence did not decrease significantly after M_{β}AB, probably because there is a competition between the effect of the M_{β}AB and that of electrical remodeling due to a new AF episode.

Interatrial difference in prevalence of CFAEs

The study of Rostock et al[25] showed that in patients with paroxysmal AF the increase in the prevalence of the CFAEs after AF onset was different between RA and LA despite similar AFCL decrease. The investigators suggested that this was caused by different musculature architecture between the LA and the RA. In the present study, the RA also showed significantly less CFAEs prevalence than the LA. Despite the fact that the LA is more densely innervated[8;39;40], M_{β}AB did not differently influence the two atria. Our results, thus, also indicate that the difference in the prevalence of CFAEs between the two atria is more likely due to anatomical and structural differences.

Clinical Implications and Study Limitations

CFAEs during AF are associated with electrophysiological or structural changes of the atrium[3;21;33;41]. The results of the present study show that CFAEs are also significantly influenced by the state of the ANS. These findings help to explain the clinical success of radiofrequency ablation of CFAEs and GP in addition to pulmonary vein antrum isolation.

The major limitation of this study is that LA recordings of CFAEs were only obtained from one location at the posterior wall between the pulmonary veins. No recordings were made from other LA regions where CFAEs are also observed. That's why this study does not address whether M_{β}AB has also any effect on the global distribution of prevalence of CFAEs throughout the both atria. Since the recordings were made in patients prior to catheter ablation of AF, we were limited by the duration of the study protocol and the number of catheters. We chose the catheter position at the posterior wall because of a better catheter stability over time and because earlier studies showed that the posterior wall of the LA is a prominent area for CFAEs [28;36]. Finally, in this study we included 6 patients with permanent AF who were assigned in the long-lasting AF group. This may introduce some bias, such as magnifying the differences between the two groups.

Conclusions

The present study describes the influence of the ANS on CFAEs in the right and left atrium of patients with idiopathic AF. In patients with long-lasting AF the prevalence of CFAEs is greater compared to patients in the early phase of AF. M_{β}AB reduces the prevalence of CFAEs in both atria in patients with long-lasting AF. In the early phase of AF, M_{β}AB prevents the increase of CFAEs prevalence compared to control patients without M_{β}AB. From these findings we conclude that CFAEs are not only due to anatomical or structural mechanisms but are at least partly influenced by the activity of the ANS.

Disclosures

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Chapter 4

Detecting Complex Fractionated Atrial Electrograms in Atrial Fibrilllation: Unipolar versus Bipolar Recordings.

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P. Loh, J.M. de Bakker

Abstract

Introduction

Atrial sites revealing complex fractionated atrial electrograms (CFAEs) have been proposed as target sites for ablation of atrial fibrillation (AF). We determined whether the gold standard for CFAEs estimation (using activation times in unipolar recordings with QRS subtraction) could be replaced by CFAEs estimation in unipolar recordings without QRS subtraction or bipolar recordings.

Methods

Seven patients with AF (4 paroxysmal, 3 persistent) were studied. In 4 patients, 20 unipolar electrograms were recorded using 2 decapolar catheters positioned parallel to the atrial wall of the right (RA) and left atrium (LA). Bipolar electrograms were calculated. In 3 patients, 2 unipolar and 1 bipolar electrograms were recorded with a 4mm irrigated ablation catheter perpendicular to the LA wall (4 positions). For both unipolar and bipolar electrograms activation times were defined as points of steepest -dV/dt in deflections >0,2% of the largest amplitude, >20 ms apart and dV/dt < -0.04 V/s. Episodes with successive activation times <104 ms were considered as CFAEs. Intra class correlation (ICC) was used to quantify the degree to which activation times resemble those of the gold standard.

Results

For catheter positions parallel to the atrial wall, ICC for activation times was greater for unipolar electrograms without QRS contraction than for unfiltered bipolar electrograms (0.89 and 0.65 respectively). For catheter positions perpendicular to the atrial wall these ICC values increased to 0.96 and 0.78 respectively. Mismatches in activation times of unipolar electrograms without QRS subtraction were mainly due to extra activation times caused by remote ventricular deflections. Extra (parallel 20.2 %) and missed (parallel 14.4%) activation times caused the mismatches in activation times for bipolar electrograms. ICC for CFAEs intervals determined in unfiltered bipolar electrograms was greater than for unipolar electrograms without QRS subtraction (0.91 respectively 0.84), which was due to compensation of missed and extra activation times in the bipolar electrograms. ICC values for CFAEs were similar for catheter positions perpendicular to the atrial wall (unipolar, 0.93 versus bipolar 0.94).

Conclusions

ICC analysis of activation times showed that a unipolar recording without QRS subtraction is the best replacement for the gold standard and that ICC is higher if the catheter is perpendicular to the atrial wall.

Introduction

Recently a new catheter ablation strategy has been suggested for the treatment of atrial fibrillation (AF) that aims to eliminate areas in the atria where signals characterized as complex fractionated atrial electrograms (CFAEs) are recorded[1]. Such areas are supposed to be critical for the maintenance of AF, since they may represent regions acting as pivot points, zones of anisotropic or slow conduction and anatomical obstacles, all capable of sustaining AF[2-4].

Although CFAEs were initially visually identified[1], several methods have been proposed for their automatic detection[5-8]. These methods often use bipolar electrograms recorded between the 3.5 or 4mm tip electrode and the first ring electrode of a standard roving mapping and ablation catheter. CFAEs are defined as atrial electrograms having multiple deflections and local activation times. Activation times are usually determined by selecting points with the fastest negative down slope (max -dV/dt) in the multiple deflections of the signal[9]. Bipolar recordings are preferably used because they eliminate, in contrast to unipolar recordings, much of the far-field signals. However, max -dV/dt as a marker for local activation has been validated for unipolar signals only[10;11]. That is why unipolar atrial electrograms with subtracted QRS deflections can be considered as a gold standard for the detection of activation times to assess CFAEs.

Morphology and amplitude of bipolar recordings are highly dependent on the direction of the wave front and the position of the recording catheter. A bipolar catheter that is positioned parallel to the atrial wall will record local and remote activity at both poles. Deflections generated by remote activity will cancel, whereas deflections generated by local activation at the poles will be subtracted and generate a complex deflection as compared to a unipolar electrogram. If the catheter is perpendicular to the wall, the distal pole (tip) will record local and remote activity, whereas the proximal pole (ring), which is in the cavity, will record virtually remote activity only. Thus in this case, remote deflections will cancel to a large extent and the configuration of the bipolar recording would more resemble that of a unipolar one. A bipolar recording at which both poles touch myocardial tissue is, in contrast to a unipolar recording, direction dependent. If the wave front is parallel to the line between the poles of the electrode, the amplitude of the signal will be small and theoretically may become zero. These characteristics make the bipolar recording more complex than the unipolar one. We have included these aspects in our study. Another point of concern with regard to bipolar electrograms recorded in the clinical setting is that these electrograms are generally filtered by a 30Hz high pass filter, which will also influence signal morphology.

CFAEs determined from activation times in unipolar atrial electrograms with QRS subtraction were considered as the gold standard. The purpose of this study was to compare and correlate CFAEs estimated from activation times in fractionated unipolar electrograms without QRS subtraction and in bipolar electrograms, with CFAEs derived using the gold standard. In all recording modes CFAEs were based on activation times derived from max –dV/dt. The intra class correlation was used to quantify the degree to which activation times and CFAEs resemble those of the gold standard.

Methods

Study population

Seven AF patients (all male, 58±7 years old, range 50-70 years), left atrial (LA) size 45±5 mm (range 37-50 mm, echocardiography, parasternal long axis) were included. The study was approved by the local medical ethics committee and hospital board of administrators and written informed consent was obtained from all patients. Four patients had paroxysmal and 3 persistent AF. Two patients with persistent AF had a history of myocardial infarction. All patients were referred to our institute for radiofrequency (RF) catheter ablation because of ineffectiveness or intolerance of antiarrhythmic drug therapy. Patients were excluded if they were aged <18 or >70 years, if their left atrial size was > 50mm or if they used amiodarone. All antiarrhythmic drugs were discontinued at least 5 half-lives before the procedure. Six patients had AF and one patient was in SR at the time of the procedure. In the patient with SR, AF was induced with rapid atrial pacing prior to the measurements.

Study protocol

Patients were studied in a fasting non-sedated state. Intracardiac electrograms were recorded in all 7 patients before RF ablation and after transeptal puncture, while patients had ongoing AF. In four patients, two 10-polar electrode catheters (Livewire, St. Jude Medical, Minnetonka, MN, USA) with an inter-electrode spacing of 5 mm were inserted after right and left femoral venous access. One was positioned along the anterior wall of the RA and the other in the LA at the superior/posterior wall through an open foramen ovale or after transseptal puncture. All electrodes were parallel to the atrial wall (as verified by fluoroscopy) and a total of 20 unipolar simultaneous recordings of 100s were made per patient.

In the other three patients, three simultaneous recordings (2 unipolar and 1 bipolar) of 100s were made at four different positions of the LA with the use of a 4mm irrigated ablation catheter ("Cool path", St. Jude Medical, Minnetonka, MN, USA). This catheter was positioned perpendicular to the atrial wall. From the distal pair of electrodes a bipolar and 2 unipolar electrograms were recorded. One

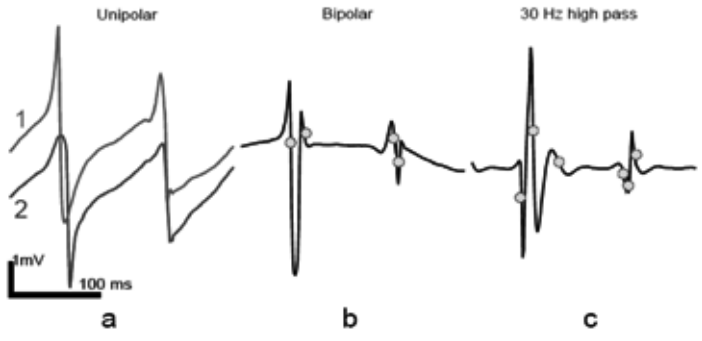

Figure 1: a) Unipolar electrograms from two neighboring electrode terminals of a catheter. The two biphasic deflections indicate that a single wave front is passing the electrode. b) Bipolar electrograms constructed by subtracting trace 2 from trace 1. Dots indicate the points of maximal negative deflections that were used as markers for the time of activation. Note that the second dot is extra and corresponds to a positive deflection in the signal from the indifferent (negative) lead (trace 2) and does not indicate a separate activation front close to the electrode. c) Bipolar electrogram from panel b but this time 30Hz high pass filtered. Note that the second (unipolar) complexes in panel a are almost simultaneously and that subtraction resulted in a much smaller and apparently fractionated complex although the unipolar leads show that there is only one wave front passing under each electrode.

catheter position was at the roof of the LA, the second at the posterior wall of the LA, the third at the ostium of the left superior pulmonary vein at the base of the LA appendage and the last against the interatrial septum from the side of the LA. Those areas are well described for having a high prevalence of CFAEs[12;13]. All electrograms were recorded with the Prucka electrophysiological recording system (Cardiolab; Prucka Engineering). Intracardiac unipolar and bipolar electrograms were bandpass-filtered from 0.05 to 500 Hz ("unfiltered") or 30 to 500Hz ("filtered"). Sample frequency was 1 kHz.

From the unipolar electrograms bipolar electrograms were constructed (in addition to recorded bipolar electrograms for catheter positions perpendicular to the atrial wall) by subtracting the signals from the distal tip- and the first ring electrode of the most distal electrode terminals. Digital filtering was applied to mimic the conventional 30Hz high pass filter setting of clinical bipolar leads (figure 1).

Activation detection

The activation detection algorithm comprised three thresholds, one for the interval between activations (ms), one for the steepness (max -dV/dt) and one for the amplitude of the signal (mV) (figure 2). All points where the signal exceeded the steepness and amplitude thresholds were marked as activation times. If two points were closer than the interval threshold or on the same downslope, only the steepest one was retained. Cut-off values used in this study were: steepness of the deflection < -0.04 mV/ms; amplitude of the deflection > 2% of the largest amplitude in the signal; minimum interval between deflections > 20 ms.[9]

Thresholds were the same for all unfiltered and filtered recording modes to simulate an automatic procedure. Channels where the standard threshold could not be applied were rejected, in order not to skew the results.

We recorded electrograms from electrode pairs that were both touching the atrial wall and from pairs where only the tip made contact with the wall and the proximal ring electrode was within the atrial cavity. The former was the case when multipolar (>=10 electrodes) catheters were used. When using a roving catheter to construct a 3D model of the atria (NavX, Carto) and/or a 3D activation map, the catheter is often positioned perpendicular to the wall. The recorded bipolar signals in the dataset with the catheter perpendicular to the atrial wall were also used to validate our digitally computed filtered bipolar leads.

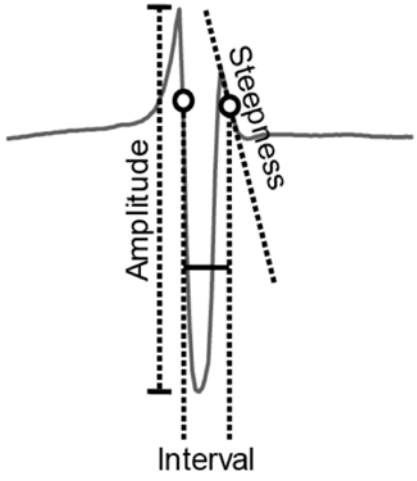

Figure 2: Definition of the amplitude, interval and steepness parameters for determining activation times. Thresholds used in the study were: steepness of deflection < -0.04 mV/ms; amplitude larger than 2% of the largest down slope in each tracing; minimum interval between deflections more than 20 ms. Two possible activation times are marked by open circles.

Activation times of unipolar electrograms with QRS subtraction (gold standard) were compared with those in bipolar recordings and unipolar recordings without QRS subtraction. Matching

activation times were identified by selecting activation in the standard set and finding the closest activation in the target set. Mean difference in identification of matching activations were computed as well as the percentage of activations that were found by one method but not the other.

QRS subtraction

Template matching was used to automatically identify the QRS complexes in a surface lead. For each atrial recording the mean value of all time points from onset QRS on the surface lead to well within the T-wave was determined of all episodes that matched the QRS template. Mean values were subtracted at each time point to cancel the far field ventricular signals.

Fractionation detection

As a measure of fractionation we used the percentage of the recording time that fractionated electrograms were recorded. Fractionation was defined as successive automatically detected deflections occurring within 104 ms. The threshold of 104 ms was chosen because this was the mean of the shortest refractory periods during AF (determined by stimulation during AF) we measured in 5 patients with paroxysmal AF. Fractionated intervals were grouped together. A new fractionated interval started whenever a deflection was more than the 104ms later than the last one of the preceding interval. The percentage of CFAEs was computed by adding all fractionated intervals and dividing the total fractionated interval by the total length of the recording and multiplying by 100.

Statistics

The aim of the present study was to assess whether CFAEs determination from bipolar and unipolar atrial electrograms without QRS subtraction "agree" well enough with the gold standard (CFAEs determined from unipolar electrograms with subtracted QRS complexes) to be interchangeable. For measuring the consistency between 2 different methods the intra class correlation (ICC) was used. ICC was determined between the different recording methods and the gold standard for: 1) the number of matching activation times and 2) the percentage of the tracings that revealed CFAEs. ICC for matching activation times is most sound, because it only compares values that match between the gold standard and the alternative method. ICC for the percentage of tracings that reveal CFAEs may misrepresent, because extra and missed activation times may compensate.

A random effects analysis of variance was performed, which is appropriate for a nested design. A 3-level nested model is used to estimate the components of variance at each level of clustering. The 3 levels are patients, locations, and electrodes. Each of the effects is considered to be random. The MLwiN software, version 2.02, was used for the analyses. Parameters were estimated using the residual, or restricted, iterated generalized least-square.

Results

To validate our digital filtering and bipolar electrogram reconstruction technique, simultaneously recorded unipolar and bipolar electrograms were obtained from recordings with the catheter positioned perpendicular to the atrial wall. Applying digital filtering to bipolar electrograms computed from the two unipolar electrograms showed that reconstructed bipolar electrograms were identical to the recorded ones and that our filtering technique could also be applied to the signals from the decapolar catheters (figure 3).

All activation times were related to activation times determined in unipolar atrial electrograms with removed QRS complexes i.e. the gold standard for determining atrial activation times. Activation times were considered to be extra if they were present in the target electrogram but not in the gold standard, missing if the activation time was present in the gold standard but not in the target electrogram and shifted if the activation time of the target was from the same deflection as the activation time of the gold standard, but shifted in time (> 5 ms).

Catheters positioned parallel to the atrial wall

These measurements were made with 2 decapolar catheters (inter-electrode distance 5 mm). From these catheters 18 bipolar signals were computed (figure 4). Thus in 4 patients, a total of 80 unipolar and 72 bipolar signals were analyzed. Of 5 signals (7%) the automatic detection of activation times using the standard parameters was considered unsatisfactory because of a too high interference level. These 5 signals were excluded from further analysis.

Unipolar electrograms without QRS subtraction

For catheter positions parallel to the atrial wall 88.6% of the activation times determined in unipolar electrograms without QRS subtraction matched with activation times in the gold standard (unipolar electrograms deprived of QRS complexes; see table 1). 18.9% of the activation times were extra and due to remote ventricular deflections; only 4.5% of activation times were missing and 6.3% were shifted compared to corresponding activation times in the gold standard.

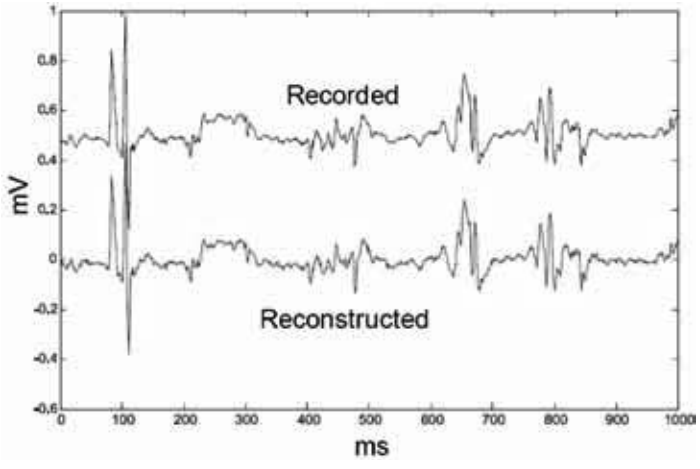

Figure 3: Comparison of measured and reconstructed bipolar signals. The upper trace shows a bipolar electrogram recorded with a 30 Hz high pass filter. The lower trace is reconstructed by subtracting the simultaneously recorded unipolar signals at adjacent electrode terminals and applying a first order 30Hz digital Butterworth high pass filter.

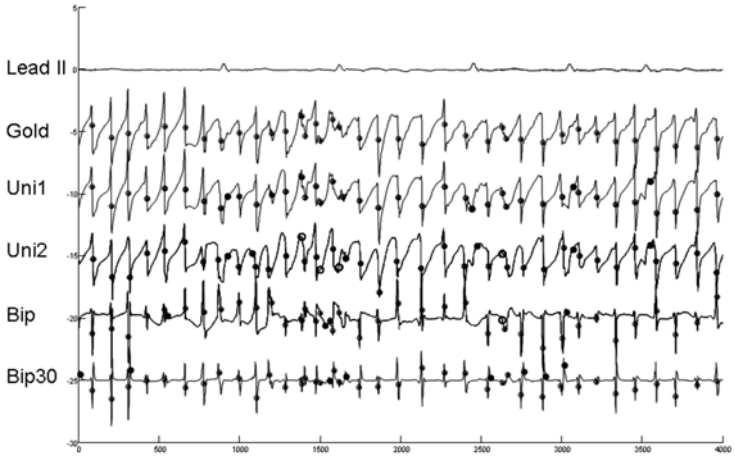

Figure 4: Electrograms recorded with a catheter positioned parallel to the atrial wall. Activation times were detected by applying the following criteria: dV/dt <-0.04 mV/ms, amplitude larger than 2% of the largest deflection in the entire signal and a distance of at least 20 ms between activation times. Lead II: surface lead two showing ventricular complexes. Gold: Golden standard recording comprising a unipolar recording with QRS subtraction. Activation times are indicated by circles with a central black dot. Uni1 and Uni2 are unipolar electrograms without QRS subtraction of the distal tip and the second ring electrode respectively. Circles with the central black dots correspond with activation times in the Gold tracing; thick black dot are extra activation times (not present in the gold standard) and open circles are missing (present in the Gold standard but not in the Uni-recordings) activation times. Bip and Bip30 are bipolar recordings without and with 30 Hz high pass filtering. Dots are activation times as described before. Numbers along the x-axis indicate times in ms.

Bipolar electrograms

74.4% of the activation times of the unfiltered (0.05 to 500 Hz) and 61.9% of the filtered (30 to 500 Hz) bipolar signals matched to the activation times of the unipolar signals with removed QRS complexes (table 1). Mismatch was due to extra activation times [20.2% (unfiltered) and 27.8% (filtered)] and missing activation times (14.4% and 19.4% respectively). Of the matched activation times, 11.1% in the bipolar electrograms without filtering and 18.7% in bipolar signals with filtering were shifted >5 ms.

Table 1: Percentage of matched, shifted (>5ms), extra and missed activation times as compared to activation times determined in the same unipolar atrial recordings after QRS subtraction (gold standard).

Parallel

	unipolar	**bipolar**	**bip. 30Hz**	**uni. 30 Hz**
match	88.6±9.5	74.4±10.7	61.9±13.5	66.8±15.1
shifted	6.3±4.6	11.1±6.2	18.7±8.8	16.4±6.5
extra	18.9±9.9	20.2±15.3	27.8±18.6	32.0±19.9
missed	4.5±4.0	14,.4±7.9	19.4±11.2	16.8±10.7

Perpendicular

	unipolar	**bipolar**	**bip. 30Hz**	**uni. 30Hz**
match	89.5±6.4	77.2±9.8	57.7±14.3	61.6±15.3
shifted	4.9±3.7	5.4±2.5	9.7±5.7	12.5±6.3
extra	15.8±8.9	7.6±5.6	6.5±4.8	15.3±17.3
missed	5.6±5.3	17.4±9.4	32.6±17.4	25.9±13.5

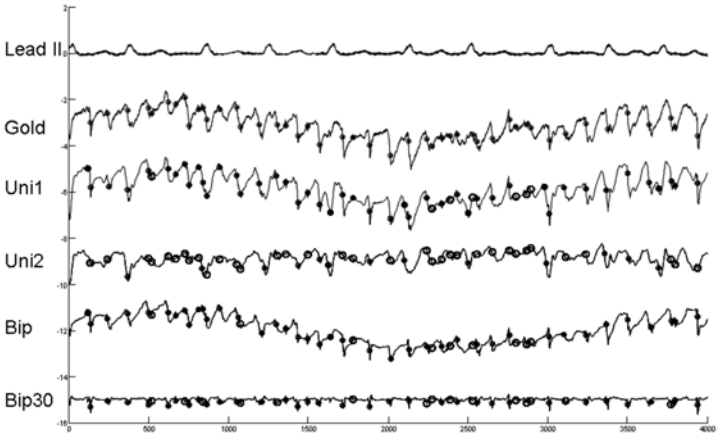

Figure 5: Electrograms recorded with a catheter positioned perpendicular to the atrial wall. Further details are similar to those described in the legends of figure 4

Catheter positioned perpendicular to the atrial wall.

Measurements with the catheter perpendicular to the atrial wall were done with the distal pair of an ablation catheter (distance between the poles 2 mm). Unipolar and bipolar recordings from the distal pair of electrodes were obtained.

Unipolar electrograms without QRS subtraction

Results were similar to those obtained when placing the decapolar catheters parallel to the atrial wall (table 1).

Bipolar electrograms

With the catheter positioned perpendicular to the atrial wall, the percentage of matched activation times in bipolar electrograms increased to 77.2% (74.4% with the catheter parallel to the atrial wall). The percentage of extra activation times decreased from 20.2% to 7.6 % (without filtering) and the percentage of missed activation times slightly increased from 14.5% to 17.4% (Figure 5). Only 5.4% of activation times without filtering and 9.7% with filtering were displaced more than 5 ms.

ICC for alternative measurements

ICC was determined for: 1) activation times and 2) the percentage of the tracings that exhibit CFAEs.

Table 2A shows the ICC values for matched activation times. For catheter positions parallel to the atrial wall, ICC is greater for unipolar electrograms without QRS subtraction (0.89) than for bipolar electrograms (0.65). ICC values are lower if electrograms are filtered. ICC values increase if the catheter is positioned perpendicular to the atrial wall.

Table 2B illustrates the ICC for CFAEs intervals. Interestingly, ICC for bipolar electrograms is higher than for unipolar electrograms without QRS subtraction if the catheter position is parallel to the atrial wall (0.91 vs 0.84). ICC for unipolar and bipolar electrograms is virtually the same for catheter positions perpendicular to the atrial wall. Filtering of the electrograms reduces all ICC values.

Table 2A: Intra class correlation for matched activation times:

	Parallel	Perpendicular
methods	ICC	ICC
Unipolar (no QRS subtraction) vs gold standard	0.89	0.96
Bipolar vs gold standard	0.65	0.78
unipolar (no QRS subtraction) + 30Hz filter vs gold standard	0.60	0.59
bipolar + 30Hz filter vs gold standard	0.35	0.48

Table 2B: Intra class correlation for CFAEs intervals:

	Parallel	Perpendicular
methods	ICC	ICC
Unipolar (no QRS subtraction) vs gold standard	0.84	0.93
Bipolar vs gold standard	0.91	0.94
unipolar (no QRS subtraction) + 30Hz filter vs gold standard	0.64	0.78
bipolar + 30Hz filter vs gold standard	0.74	0.86

ICC: intra class correlation, Parallel: catheter positioned parallel to atrial wall, Perpendicular: catheter positioned perpendicular to atrial wall

Discussion

Main findings

ICC data indicate that activation times derived from unipolar electrograms without QRS subtraction are the best replacement for the gold standard. Thus, for CFAEs estimation too, unipolar electrograms without QRS subtraction are the best substitute. ICC values are bigger if the catheter was positioned perpendicular to the atrial wall as compared to catheter positions parallel to the wall. Extra, missed and moved activation times contributed to the mismatch with the gold standard. For catheters positions parallel to the atrial wall, extra activation times were the major cause of mismatch for both unipolar and bipolar recordings. For bipolar recordings, also missed activation times highly contributed to the mismatch. For electrodes perpendicular to the atrial wall the percentage of extra and missing activation times reduced.

Interestingly, ICC value of CFAEs intervals was greater for bipolar than unipolar recordings in cases the catheter was parallel to the atrial wall, although this is most likely a misrepresentation. ICC values always reduced in case electrograms were filtered with a first order 30 Hz high pass filter.

Activation times

Activation times are only well defined and verified for unipolar recordings. It has been shown that even

during ischemia the steepest point of negative dV/dt in the unipolar electrogram corresponds with the upstroke of the action potential and marks the time the myocardium underneath the electrode is activated. For bipolar electrograms the detection of activation times is more complex because the configuration of the bipolar electrogram depends on the direction of the wave front and so does the activation time. This becomes even more complicated if the signals are fractionated. Detection of activation times in unipolar electrograms too is not always straightforward. Because of the large field of view of the unipolar recording mode, remote activity may generate additional deflections and activation times. For that reason subtraction of the ventricular deflection in atrial tracings is required.

For unipolar recordings the percentage of missing activation times marginally changes with catheter position. For bipolar recordings, the percentage of extra activation times is greater for parallel than for perpendicular positions. This might be due to the fact that if the electrode is parallel to the wall, the recording is true bipolar, whereas it is semi bipolar (approaching unipolar) in case the position is perpendicular to the atrial wall.

Intra Class Correlation

ICC values for activation times reveal that unfiltered unipolar electrograms without QRS subtraction are the best replacement for the gold standard.

The observation that ICC for CFAEs intervals scores better for bipolar than unipolar electrograms without QRS subtractions (at least for catheter positions parallel to the atrial wall), is most likely caused by the fact that extra and missed activation times in bipolar recordings compensate (20% and 14% respectively). Additional activation times are caused by the differentiating nature of the bipolar recording mode, which may generate additional deflections as illustrated in figure 1. In the unipolar atrial leads, additional activation times (caused by the remote ventricular deflections) are as frequently present (19%), but only few activation times are missing (4.5 %). Thus, less compensation will occur for unipolar activation times. Filtering with 30 Hz virtually always decreases ICC, because this type of signal processing increases the number of deflections as illustrated in fig. 1. All ICC values for catheter positions perpendicular to the atrial wall are higher than for catheter positions parallel to the wall.

Although ICC for CFAEs intervals in unfiltered bipolar recordings is better than in unipolar recordings without QRS subtraction, it does not mean that activation times of bipolar recordings match better with the gold standard than activation times of the unipolar recordings, which can be appreciated from table 1.

Clinical implications

Areas presenting complex fractionated electrical activation may represent a target for RF catheter ablation of AF. The most accurate technique to identify these areas is unipolar recordings with subtraction of remote ventricular activity. However, in most electrophysiology laboratories CFAEs are determined using filtered bipolar recordings. The results of this study indicate that with the bipolar technique, some areas with CFAEs may be missed while other areas may be erroneously identified as presenting CFAEs. Furthermore, orientation of the catheter relative to the endocardial wall may also

influence CFAEs detection. CFAEs derived from activation times in unipolar recordings without QRS subtraction and a catheter placed perpendicular to the endocardial atrial wall come closest to the gold standard (ICC 0.96).

Limitations

We used the same threshold values for all the techniques to determine activation times and CFAEs. Adapting threshold values, however, may further optimize individual techniques. In addition, we only recorded electrograms at a number of fixed locations in the left atrium. It cannot be excluded that contact of the electrode with the atrial wall varies with different locations and that, as a consequence, the estimation of activation times might be location dependent. A problem that may arise due to QRS subtraction is that QRS morphology may change during atrial fibrillation because of abnormal activation of the ventricles, due to bundle branch block. This may result in a suboptimal subtraction of the ventricular signal.

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Chapter 5

The Role of Connexin 40 in Atrial Fibrillation

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Abstract

Connexin40 is the major gap junction protein in the atrial myocardium. In the heart, gap junctions are responsible for cell-to-cell conduction of the action potential. During several cardiac diseases, the expression of connexins is changed and is associated with increased propensity for arrhythmias. Atrial fibrillation (AF) is the most common arrhythmia in man with a diverse clinical presentation, different underlying mechanisms and difficult treatment. The vulnerability to arrhythmias of the heart is determined by the combined presence of an arrhythmogenic substrate and initiating triggers. The arrhythmogenic substrate is formed by reduced effective refractory period, enhanced spatial dispersion of refractoriness or abnormal atrial impulse conduction. Initiating triggers for AF most frequently originate from firing foci in the pulmonary veins and/or superior caval vein. Prolonged episodes of AF result in electrical and structural remodeling that favors the reoccurrence or perpetuation of AF. This electrical remodeling embodies changes in Cx40 expression and distribution, both in the atrial myocardium itself and in the thoracic veins. In addition, Cx40 gene mutations or polymorphisms give an inherited predisposition to AF.

This review focuses on the the role of Cx40 in atrial fibrillation, showing that abnormal Cx40 expression is correlated to both trigger formation from the thoracic veins as well as enhanced vulnerability of the atrial myocardium to AF.

1. Introduction

Atrial fibrillation (AF) is the most commonly encountered sustained arrhythmia in man with a variable clinical presentation[1]. Besides AF secondary to hypertension, coronary artery diseases (CAD), valvular abnormalities, cardiothoracic surgery, cardiomyopathy, inflammatory or infiltrative processes, endocrine disorders and drug abuse, idiopathic AF is observed in 30% or more of the patients[1]. In addition, Cx40 gene mutations or polymorphisms give an inherited predisposition to AF.

In general, the vulnerability to arrhythmias of the heart is determined by the combined presence of an arrhythmogenic substrate and initiating triggers, both of which can be modulated by the autonomic nervous system or drugs[2]. In patients prone to AF the triggered activity originates mainly from the thoracic veins[3]. These triggers combined with an atrial arrhythmogenic electrophysiologic substrate, caused by reduced effective refractory period[4-7], enhanced spatial dispersion of refractoriness[8] or abnormal atrial impulse conduction[9;10] lead to the initiation of AF.

AF is a self-perpetuating progressive disease in which "AF begets AF"[6]. Prolonged episodes of AF result in electrical and structural remodeling that favors the reoccurrence or perpetuation of AF. Fast atrial rhythms and AF give rise to electrical remodeling, i.e. changes in ion-, and gap junction channel expression[4-7]. Structural remodeling, found at later stage, involves changes in mitochondrial size and the disruption of sarcoplasmic reticulum at the subcellular level, myocardial cell hypertrophy at the cellular level and fiber disarray and increased collagen deposition at the tissue level[5].

Electrical remodeling associated with AF lead to changes in the effective refractory period (ERP)[4-7]. As part of this electrical remodeling, changes in gap junctions and connexins in AF have been reported[11-14], but this do not fall in to a consistent pattern. Gap junctions are clusters of transmembrane channels that link adjoining cells and mediate cell-to-cell electrical coupling and communication. They are formed by the joining of two *connexons* (=hexameric hemi-channels), which are composed of six integral membrane subunits, connexins (Cx), that surround the central aqueous pore[15]. In the human heart 4 main isoforms are expressed[16]. Cx43 is expressed in all chambers of the heart but predominantly in the ventricles, Cx45 is found in the conduction system of the heart and at low levels in the atrial and ventricular working myocardium[17] and Cx37 is located in the endothelial gap junctions in many vessels. Finally, Cx40 is expressed mainly in the atrial working myocardium, the conduction system and the vasculature. Cx40 was first described in a range of animal species [18;19], and subsequently mapped to human chromosome 1. It became apparent that Cx40 was expressed in the atrioventricular conduction system[20;21] and abundantly expressed in the atrial but not in the ventricular gap junctions[22;23]. Recently a new connexin was described in the mouse heart, i.e, Cx30.2 (the human equivalent is Cx31.9) which in mice seems responsible for slowing of impulse conduction in the atrioventricular node (AVN)[24]. However, the role of Cx31.9 in the human heart is unclear, for it is not detectable in the human cardiac conduction system[25].

Several reviews described the mechanisms of AF[26-29] or changes in Cx expression in cardiac disease[30;31].

This review focuses on the the role of Cx40 in atrial fibrillation, showing that abnormal Cx40 epxression is correlated to both trigger formation from the thoracic veins as well as enhanced vulnerability of the atrial myocardium to AF.

2. Contribution of Cx40 to the atrial electrical propagation

Cell-to-cell coupling by gap junctions is an essential determinant in uniform and successful propagation of the action potential and determined by the distribution and specific properties of connexins throughout the myocardium[32]. In the human atria, Cx40 and Cx43 are the major connexins[16] and several experimental studies attempted to elucidate their role in myocardial conduction. Studies in Cx43 haploinsufficient mice have shown that P-wave duration is not affected by Cx43 levels, suggesting unchanged conduction velocity in the atria[33]. Even levels of only 10% Cx43 did not significantly increase P-wave duration in the atria of mice[34]. These studies indicated that Cx43 is not a principal determinant for atrial impulse conduction in the presence of normal Cx40 levels.

Several studies in Cx40 knockout mice ($Cx40^{-/-}$) indicated that Cx40 is the dominant connexin for impulse conduction in the atria and the conduction system as summarized in table 1. The majority of these studies demonstrated that full deficiency for Cx40 prolonged P-wave, PQ/PR-interval, QRS and QTc duration in the surface electrocardiogram[35-42]. Epicardial mapping revealed that the prolonged P-wave and PQ/PR-interval was due to reduced conduction velocity (CV) in the atria, while the prolonged QRS complex was caused by right bundle branch block and reduced conduction velocity in the left bundle branch[42;43]. Typically, Cx40 knockout mice were susceptible to atrial tachyarrhythmias[35-38;42].

Although in mice, all studies are unambiguous, in respect to electrical propagation, Beauchamp and coworkers showed in synthetic strands of neonatal and fetal murine atrial cardiomyocytes that Cx40 deletion ($Cx40^{-/-}$) was associated with *increased* electrical propagation velocity and genetic deletion of Cx43 ($Cx43^{-/-}$) produced a decrease in propagation velocity[44]. In addition, a study that investigated the correlation between Cx40 and Cx43 expression in the atria and the atrial conduction properties in humans showed that the propagation velocity in the atria is related to the interactions between Cx40 and Cx43 expression[45]. A higher expression of immunodetectable Cx40 in the right atrium, in the presence of Cx43, reduced CV, while Cx43 alone was not directly correlated with propagation properties. The ratio of Cx43 to total Cx immunosignal (Cx43/[Cx40+Cx43]) was directly, and the ratio of Cx40 to total Cx (Cx40/[Cx40+Cx43]) inversely related to propagation velocity. These findings may be explained by the fact that heterotypic Cx40/Cx43 gap junction channels may be present, which have much lower conductance than either Cx40 or Cx43 homotypic gap junction channels[46;47]. These data, however are in contrast with the previously discussed mouse data, in which haploinsufficiency for Cx40 did not alter atrial impulse conduction and full deficiency for Cx40 is associated with lower impulse conduction. The apparent discrepancy between $Cx40^{-/-}$ mouse data and the latter in vitro and human data may be explained by specific expression patterns in adult mouse myocytes[44], and the fact that in transgenic animals altered expression of genes other than those targeted may occur.

Table 1: Studies on Cx40 Knockout mice

Author, *year*	**SCL**	PQ/PR	**QRS**	**QTc**	**Pdur**	**AF/AT induction**	**Remarks**
Simon et al [39], 1998	↔	↑	↑	↑	↑	ND	
Kirchhoff et al [38], 1998	↔	↑	↑	↑	↑	↑	
Hagendorff et al [37], 1999	↑	↑	↑	↑	↑	↑	
Verheule et al [42], 1999	↔	↔	↑	↔	↑	↑	CV ↓ (30%)
Bevilacqua et al [36], 2000	↑	↑	↔	↔	↔	↑	
Tamaddon et al [40], 2000	↔	↑	↑	NS	↔	NS	
Vanderbrink et al [41], 2000	↔	↑	↑	↔	↔	NS	AH ↑, HV ↑
Van Rijen et al [43], 2001	↔	NS	NS	NS	NS	NS	RBBB, LBB CV ↓
Bagwe et al [35], 2005	↔	↑	↑		↑	↑	CV ↓
Mean:	↔	↑	↑	?	↑	↑	CV ↓

↔: unchanged, ↑: increased, ↓: decreased, SCL: Sinus node Cycle Length, PR: PR-interval, QRS: QRS duration, QTc: corrected QT-interval, Pdur: P wave duration, AF: Atrial Fibrillation, AT: Atrial Tachycardia, NS: not studied, CV: Conduction Velocity, AH: Atrial-His time, HV: His-Ventricle time, RBBB: Right Bundle Branch Block, LBB: Left Bundle Brunch

3. Role of Cx40 distribution in maintenance of AF

The first studies to investigate the role of gap junctions in AF were carried out in animal models of AF (table 2A). Induction of persistent AF (lasting > 2 months) in a goat model was reported to lead to heterogeneous spatial distribution of Cx40 while the expression of Cx43 remained unchanged[12]. Heterogeneous expression was defined as the non uniform labeling pattern of Cx40: patches of cells virtually devoid of Cx40, next to areas with almost normal expression of Cx40[12]. This heterogeneous distribution occurred after 2 weeks of high rate atrial pacing[48]. Interestingly, discontinuation of AF resulted in reverse remodeling and gradual normalization of the altered distribution pattern of gap junctions and Cx40 expression[49]. Total Cx40 protein levels were unchanged or reduced in long lasting AF with unchanged levels of Cx40 mRNA[12;48;50].

In humans, several studies investigated connexin expression and distribution during AF (table

Table 2: Cx40 expression and distribution in atrial myocardium during AF

AF type	Cx40 protein level	mRNA	Cx40 distribution	Remarks	Author, *year*
A. Animal models					
Goats, AF>2 months	↔	↔	Heterogeneous	Cx43 ↔, CV ↔	Van der Velden et al[12], *1998*
Goats, AF 1, 2, 4, 8, 16 wk	↓	↔	Heterogeneous	Cx43 ↔, Cx40/Cx43 ratio ↓	Van der Velden et al[48], *2000*
Goats,AF 13.9±5.2 wk	NS	↔	NS		Thijssen et al[50], *2002*
B. Human studies					
postAF (CAD)	↑	↑	Heterogeneous	Cx43 ↔, LA< 40 mm	Dupont et al[52], *2001*
CAF >1yr (CAD, MVD, AVD)	↑	NS	Lateralization	Cx43 ↔ & lateralization	Polontchouk et al[54], *2001*
CAF > 1 yr (mini-maze)	↓	NS	Heterogeneous, Lateralization	Cx43 ↓, LA 63±11.9 mm	Kostin et al[53], *2002*
CAF > 5 mths (MVD)	↓	↓	Heterogeneous	Cx43 ↔, PhCx40 ↑, LA 55±7 mm	Nao et al[55], *2003*
CAF > 6 mths (MVD)	↔ or ↓ in AF with complex activation	NS	Heterogeneous	Cx43 ↔	Kanagaratnam et al[11], *2004*
LAF and AF (MVD)	↑	NS	NS	LA (LAF) 43 mm, LA (MVD) 55 mm	Wetzel et al[58], *2005*
Persistent AF > 3m	↓	↔	*Homogenous*	LA 45±4 mm	Wilhelm et al[59], *2006*
CAF > 3 mths (MVD)	↔	NS	Heterogeneous	LA 54±9.8 mm	Takeuchi et al[57], *2006*
Post- AF (CAD)	↔	NS	Heterogeneous	Cx43 ↔, fibrosis ↑, NF-κB ↑, LA 38.3	Li et al[56], *2008*
CAF > 1 year (CAD, MVD, AVD, mini-maze)	↔ Ca^{++} < 2.2 mM ↑ Ca^{++} > 2.2 mM	↔	Lateralization	Cx43 ↑ (unaffected of Ca^{++}), LA 49±1	Dhein et al[51], *2008*

↔: unchanged, ↑: increased, ↓: decreased, NS: Not Studied, CAD: Coronary Artery Disease, LA: Left Atrium, CAF: Chronic Atrial Fibrillation, LAF: Lone Atrial Fibrillation, MVD: Mitral Valve Defect, AVD: Aorta Valve Defect, NF-kB: Nuclear Foctor ϰB

2B) under different conditions. During SR, gap junctions are expressed mainly at the true end of the cells[51-54] although side-to-side dispositions can also be common in the atria[54]. During AF, however, Cx40 expression was predominantly side-to-side[51;53;54] and heterogeneously distributed[11;53;55-57] while the expression of N-cadherin and Desmoplakin remained normal[53]. In patients, with ischemic heart disease and no history of arrhythmias, post-operative AF was more likely when pre-existing higher levels of Cx40 protein were present[52]. Other studies, however, that investigated changes in Cx40 expression and distribution in patients with long-lasting AF (at least 3 months and thus atrial remodeling) showed inconsistent results with respect to the amount of Cx40 protein level during AF. Some of those studies showed that the Cx40 protein levels were increased with lateralized expression in the atria, independent of AF aetiology[54;58]. Other studies found that the expression of Cx40 during AF was significantly reduced[11;53;55;59], while various studies showed no difference in Cx40 protein expression levels between patients with sinus rhythm (SR) and long lasting AF[11;56;57] or that Cx40 expression levels were dependent on extracellular Ca^{++} level[51].

These seemingly different findings from the studies to date may in part be due to the different methodological approaches and experimental design. First, both polyclonal rabbit anti-rat Cx40 (S15) and anti-human Cx40 specific antibodies (Y2IY) were used in different studies for the quantification of Cx40. The affinity of both antibodies to Cx40, however, was shown to be comparable[60].Secondly, the different methods of Western Blot or immunofluorescent confocal analysis, which were used for Cx40 analysis, both have restrictions and limitations for the interpretation of the amount of Cx40 (reviewed in [29]). Finally, the use of small, non-representative atrial tissue for connexin analysis, and the inclusion of patients with different pathophysiological causes of AF may play an additional role in the conflicting results.

However, almost all studies pointed out that Cx40 gap junctions are heterogeneously distributed in the atria of patients with AF (table 2). Such an expression of connexins may result in heterogeneous intercellular coupling leading to conduction defects and nonuniform anisotropic characteristics that can facilitate reentrant circuits and therefore predispose to atrial tachycardias[61]. A second effect of inhomogeneous uncoupling might be the increased dispersion in refractoriness. Uncoupling was shown to lead to a dramatic increase in activation recovery intervals[62].

Interestingly, in heart failure, heterogeneous expression of Cx43 is associated with both dispersion of impulse conduction[63;64] and dispersion of refractoriness[63].

4. The role of Cx40 in the arrhythmogenic properties of the thoracic veins in AF

Triggers emerging from the thoracic veins,i.e., the PVs[3] and the superior vena cava (SVC)[65] are important factors in the initiation and perpetuation of AF[3;65]. So called myocardial sleeves, the extensions of atrial myocardium into the PVs and SVC, are well described[66] and identified as the underlying substrate for these triggers. The mechanisms behind the ectopic activity from the thoracic veins are thought to be based on either automaticity or microreentry and the possible role of Cx40 in

this arrhythmogenic behaviour has been subject of several studies (table 3).

Ectopic activity from the PVs are the most prominent triggers for AF[3]. Saito et al[67] studied the anatomy of the PVs in human hearts and showed that the myocardial cells in the PVs are separated from the muscular media of the veins suggesting that the trigger from the PVs must originate from the myocardial sleeves. Arora et al[68] showed that conduction at the proximal part of canine PVs was considerably slower than in the remaining LA. Also in canine PVs, Hocini et al[69] found zones of activation delay that were related to sudden changes in fiber direction that could result in microreentry and suggested that Cx distribution and expression might play a supplementary role. Evidence for this was provided by Verheule et al[13] who demonstrated that, although the myocytes in the canine PVs were similar to those in LA, the gap junctions in the myocardial sleeves expressed mainly Cx43 and that the levels of Cx40 were significantly lower than in the LA. On the other hand, spontaneous electrical activity was observed in PVs isolated from guinea pig hearts[70]. The response of this spontaneous activity to perivascular nerve stimulation was similar to that seen at SAN[70]. Node-like cells were identified in myocardial sleeves of PVs of adult rats and the intercalated disk of those cells were composed of small gap junctional specializations comparable to those seen in the SAN[71;72]. Furthermore these myocardial sleeves correspond to areas of the conduction system in embryonic myocardium and originate from the sinus venosus segment of the heart from which also the SAN originates[73;74]. Cells of the PV sleeves originate from mesenchymal stem cells and are not recruited from atrial cells[75].

Studies in dogs with atrial remodeling due to mitral valve regurgitation or rapid atrial pacing demonstrated that Cx40 protein expression in the PVs was downregulated which may be important for maintenance of AF[76;77]. In summary, in PVs, both automaticity and activation delay resulting in microreentry may form the source of triggers for AF. Gap junction remodeling seems to play an important role in two ways. Firstly the fact that the PVs contain autorhythmic cells, which share a sinus nodal like gap junction expression, may be able to drive the atria. Secondly, abnormal and discontinuous gap junction expression with rapid changes in fiber direction may facilitate microreentry, resulting in preexcitatatory triggering of the atrial myocardium.

In the SVC, myocardial sleeves also extend from the RA-SVC junction up to 2 to 5 cm into the SVC[78;79]. Yeh et al[80] studied the electrical properties of the SVC in canine hearts that are structurally comparable to human SVC. In SVC myocardial sleeves, gap junctions composed of Cx40, Cx43 and Cx45 were exclusively found at the intercalated disk. The distribution of Cx40 was homogeneous throughout the myocardial sleeves. In the proximal part of the sleeves, atypical areas were present that extended to 1 cm distally from the RA-SVC junction where Cx43 expressed in the center, surrounded by Cx40 spots. Interestingly, the Cx expression pattern of these atypical areas is analogous to that reported for SAN of dogs[81]. Automaticity of SVC cells was evidenced by the findings of Chen et al[82], who studied the electrical properties of the myocardial sleeves in the SVC. The SAN-like Cx expression pattern may favor the exit of activity from spontaneously active cells to surrounding myocardium. The investigators not only demonstrated pacemaker activity of some of the cardiomyocytes in the SVC, but also showed the presence of delayed after depolarization in a large percentage of cells suggesting that triggered activity plays an additional role in ectopic activity in SVC.

In a dog model of rapid atrial pacing, pacing for 2 and 6-8 weeks resulted in electrical

Table 3: Cx40 expression and distribution in the thoracic veins

Author, *year*	**Type of the study**	**Cx40 protein level**	**Distribution**	**Cx43**	**Remarks**
Superior caval vein					
Yeh et al [80], 2001	Mongrel dogs (n=8)	↓	Center area Cx43 Periphery Cx40	↑	Cx expression similar to SAN
Yeh et al [14], 2006	Post pacing mongrel dogs (n=16)	↓ (2 wk), ↑ (6-8 wk)	Lateralization	↑	Collagen ↑
Lee et al [83], 2005	RAP canine dogs	NS	NS	NS	ERP ↓, CV ↓, AF inducibility ↑ } ER
Pulmonary veins					
Verheule et al [13], 2002	Mongrel dogs (n= 8)	↓	Homogenous	↔ (↑)	
Sun et al [76], 2008	MR dogs with AF (n= 5)	↓	NS	↓	Interstitial fibrosis ↑
Zhang et al [77], 2008	RAP beagle dogs with MR (n= 11)	↓	NS	↔	mRNA ↔, Fibrous collagen ↓

↔: unchanged, ↑: increased, ↓: decreased, NS: Not Studied, SAN: Sinoatrial Node, RAP: Rapid Atrial Pacing, ERP: Effective Refractory Period, CV: Conduction Velocity, AF: Atrial Fibrillation, ER: Electrical Remodeling, MR: Mitral Regurgitation

and structural remodeling of the myocardial sleeves in SVC[14]. Beside changes in size, arrangement and proliferation of myocytes, there was perceptible remodeling of the gap junction distribution and Cx expression. During the first 2 weeks of continuous pacing an up regulation of Cx43 and a down regulation of Cx40 occurred and Cx were redistributed to the lateral borders of individual cardiomyocytes. Rapid pacing also resulted in shortening of the refractory period, decreased conduction velocity of the myocardial sleeves and increased vulnerability to AF[83]. Alterations in cell-to-cell coupling may contribute to this observed change in velocity[84].

The specific pattern of Cx expression, combined with the intrinsic automaticity of SVC myocytes may determine the mechanism for triggers emerging from the SVC[69] under normal conditions. During AF, however, the alteration in the expression and distribution of Cx40 may change the electrical characteristics of the SVC and cause inhomogeneous and discontinuous propagation of the impulse as well as activation delay through the myocardial sleeves, a substrate supporting reentry.

5. The Cx40 gene mutations and polymorphisms and AF predisposition

Atrial fibrillation has a variable clinical presentation and character, which may result from a genetic substrate with different gene mutations and/or polymorphisms. Familial forms of AF, due to gene mutations or polymorphisms have been described. An autosomal dominant trait of AF was first described in a small family in Spain by Brugada et al[85]. Loss or gain of function mutations in several potassium (K^+) channel genes, (KCNQ1, KCNE2, KCNJ2, and KCNA5) have been described in familial forms of AF[86-89]. Furthermore, relatives of probands with lone AF are at substantially increased risk of developing this arrhythmia as well, suggesting a hereditary origin[90-94]. Besides gene mutations, many gene polymorphisms are described in idiopathic AF, such as polymorphisms of angiotensin converting enzyme[95], potassium channels gene polymorphisms[96] and sodium channels gene polymorphisms[97].

Besides ion channels, abnormalities in the Cx40 gene (GJA5) have been reported to be associated with atrial arrhythmias. Groenewegen et al[98] were the first to connect the Cx40 gene with a rare atrial arrhythmia in humans. They showed that atrial standstill, a disease characterized by lack of electrical and mechanical activity of the atria, was due to a combination of a rare polymorphism of the promoter of the Cx40 gene at nucleotides -44 (G→A) and +71 (A→G), that occurs in 7% in the population, with a novel mutation in the sodium channel gene SCN5A. They demonstrated that patients with either the SCN5A mutation or the presence of that Cx40 polymorphism did not show evidence of atrial standstill. Only the combined effect of those genetic variants led to an additive and progressive pathological response and the presence of atrial standstill.

Firouzi et al[99] were the first to correlate the vulnerability for AF to this Cx40 promotor polymorphism in patients without structural heart disease in the absence of atrial remodeling. They compared the electrophysiologic characteristics of 30 patients with supraventricular tachycardia (SVT) and very rare episodes of AF with those without evidence of AF or history of episodes with irregular heartbeat. They illustrated that homozygous carriers of the minor haplotype (-44AA/+71GG) were more prone to both inducibility of AF by programmed electrical stimulation and spontaneous occurrence of AF episodes.

This predisposition to initiation of AF appeared to be related to enhanced dispersion of atrial refractoriness. Juang et al[100] showed the same relation in Taiwanese patients with paroxysmal or permanent AF. They showed that patients with AF (n= 173) had a significant higher Cx40 (-44AA / +71GG) genotype frequency compared to the control group (232 patients). Finally, Gollob et al [101] studied 15 patients with idiopathic AF with early onset (±45 yrs) and refractory to pharmacological therapy. They identified four novel, 3 somatic and 1 gernline, heterozygous mutations in the Cx40 gene in four of those patients.

Functional studies in cell lines have shown that promoter activity of Cx40 with the minor allele (-44A/+71G) was significantly reduced compared to the major allele (-44G/+71A)[98;100]. To what extend the levels or distribution of Cx40 protein is decreased due to this polymorphism is unclear yet. Further studies are needed to correlate the presence of the minor allele (-44A/+71G) to changes in the Cx40 protein level. Atrial sample of patients with mutations in the Cx40 gene showed abnormal gap-junction formation with intracellular accumulation of Cx40[101]. This abnormal expression and distribution of Cx40 protein presumably leads to heterogeneous impulse propagation that may increase AF vulnerability.

6. Factors modulating Cx40

Since abnormal expression of Cx40 is closely related to the vulnerability to AF, normalisation of Cx40 expression may be a successful therapeutic avenue. However, little is known about the mechanisms that underly atrial remodeling in general (e.g. reviewed by Brundel BJ et al[102]) and factors that specifically modulate Cx40 function during AF. Recently, Sarrazin et al[103] showed that oral administration of n-3 polyunsaturated fatty acids can result in reduced vulnerability to induction of AF in dogs[64]. This protection against AF was mostly related to reduced Cx40 expression levels. Agents that modify the phosphorylation state of connexins would be a potential pathway for regulating cell-to-cell coupling during AF. For Cx43 it is known that dephosphorylation is associated with intercellular redistribution and electrical uncoupling[104-107]. Only one study has directly shown the improvement of electrical coupling through Cx40 based gap junctions by cAMP-mediated phosporylation[108]. Wether this also occurs *in vivo* is unclear at current.

A potential new antiarrhythmic agent, the so called antiarrhythmic peptides (AAPs) have been described to improve gap junctional conductance with antiarrhythmic potential[109]. They were mainly studied to explore their antiarrhythmic character in the ventricular myocardium which resulted in reduced dispersion of action potential duration[110-112] and enhancement of gap junctional conductance[113-117]. The effect of Rotigapitde (also known as ZP123, a potent AAP analog with improved plasma stability) on atrial fibrillation was subject of several studies. In a rabbit model of volume overload induced AF, rotigaptide increased atrial conduction velocity, however, without reduction of AF vulnerability[118]. The expression levels of Cx43 and Cx40 were down regulated in the model, but remained unaltered after Rotigaptide treatment. Similarly, in a dog model of AF resulting from either atrial or ventricular tachypacing, Rotigaptide improved conduction velocity, without altering AF duration or vulnerability[119]. However, in a dog model of AF due to myocardial ischemia, the addition of Rotigaptide prevented ischemia induced conduction slowing and reduced AF duration. Similar results were obtained in a

canine sterile pericarditis model, in which GAP-134 (like rotigaptide a antiarrhythmic peptide analog) was able to reduce AF[120]. Additional studies in rats showed that ZP123 prevents the reduction of atrial conduction velocity during metabolic stress[118;121], however, the drug had no effect under normal physiological conditions[121].

Many studies have shown that Cx function can be modified[122-124]. However, further research, is needed to establish which Cx modifier is most succesful for the treatment of AF.

7. Conclusion

In this review, we have focused on the role of Cx40 in the initiation and maintenance of AF. Direct manipulation of the Cx40 amount in mice is related to conduction slowing and vulnerability to AF (in the absence of other structural or electrical remodeling). In patients with AF, Cx40 expression is heterogeneous, which may lead to abnormal impulse formation and conduction, which may form the substrate for AF. Altered Cx40 expression and distribution in the myocardial sleeves of the thoracic veins may be the substrate for abnormal impulse formation and/or micro-reentry, underlying the trigger for AF initiation. At the same time, a genetic predisposition, due to Cx40 gene polymorphism or other short mutation, seems also to be related to the initiation of AF. One last point of discussion may be the redundancy of connexins in the heart. For Cx43 it has been shown that a 50% reduction in Cx43 does not alter ventricular impulse conduction[125;126] and mice haploinsufficient for Cx40 do not have an electrical phenotype. Therefore, changes in Cx40 expression alone may not be sufficient for conduction slowing and arrhythmogenesis. The etiology of AF is not equal between patients, as a result of which the contribution of Cx40 abnormalities may vary. Other factor, such as enhanced fibrosis, as often found during AF[53;127], may be prerequisite for conduction slowing and enhanced arrhythmogenesis. Finally, therapies involving enhancement of Cx function using anti-arrhyhtmogenic peptides have been proven succesful, underlining the role of Cx40 as potential target for AF therapy.

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All authors have nothing to declare.

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Chapter 6

Absence of Connexin 40 Gene Polymorphism as a Marker of Undetected Atrial Fibrillation in Patients with Unexplained Cerebral Ischemic Events

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Abstract

Background

Atrial fibrillation (AF) is a major cause of cerebral infarction. Idiopathic AF is strongly associated with the human minor Connexin 40 (Cx40) promotor polymorphism. We examined the prevalence of the minor Cx40 allele in patients with cerebral ischemia and no other cardiovascular disease (CVD) as an indication of underlying idiopathic AF.

Methods

In patients with cerebral ischemia without prior CVD (n=225), DNA analysis of the Cx40 minor allele (-44 G→A) was performed. Patients were divided into those with a normal ECG (group A, n=164), with ECG abnormalities (group B, n=51) and those with normal ECG and documented episodes of AF (group C, n=10). Based on echocardiography data (ECHO) availability, further subgroups were defined: normal ECG and ECHO (group D, n=45); ECG or ECHO abnormalities (group E, n=22); and normal ECG and ECHO and documented AF episodes (group F, n=8). The prevalence of Cx40 promotor polymorphism was compared among all subgroups.

Results

The average age was 58.7 years (± 11.5) and 64.4% were men. Patients with episodes of AF and those with abnormal ECG or ECHO results (B+C or E+F) did not demonstrate a higher prevalence of the minor allele genotype (AA vs. GG) compared to the normal ECG/ECHO groups (A or D) [OR= 1.04, 95%CI: 0.26;4.11, group A and OR= 0.38, 95%CI: 0.04;3.63 group D].

Conclusions

In patients with cerebral ischemic events, without prior cardiovascular disease, a higher prevalence of the Cx40 gene polymorphism as a marker of underlying idiopathic AF appeared to be absent.

Introduction

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice accounting for approximately one third of hospitalizations for cardiac rhythm disturbances.[1] The estimated prevalence of AF is up to 1% in the general population, increasing with age to 5% in those older than 65 years and to 8% in those older than 80.[1] Several factors predispose to AF. Alcohol and caffeine use, endocrine disorders, cardiac surgery, inflammatory or infiltrative myocardial diseases, and structural heart disease, such as valvular heart abnormalities, coronary artery disease and hypertension, are the most important. However, there is a substantial proportion of patients, approximately 30 to 40%, without any detectable underlying disease. These patients have, so-called, idiopathic or "lone" AF, a term which applies to individuals younger than 60 years old without evidence of structural cardiopulmonary disease or other conditions that predispose to AF.[2] No matter the cause of this arrhythmia, morbidity and mortality is increased due to hemodynamic impairment and thromboembolic events. An embolic complication (mostly ischemic stroke) or exacerbation of congestive heart failure (CHF) can be the initial presentation of AF. The Framingham Heart Study, together with other studies[3;4], reported a fourfold increased risk of stroke in patients with AF, independent of other factors.[5]

Genetic predisposition to AF is more frequent than previously recognized, not only in familial forms of AF, where a variety of gene loci has been identified, but also in the general population. Recently, a strong association of the minor allele of a human Connexin 40 (Cx40) promotor polymorphism and idiopathic AF has been demonstrated.[6;7] It has been shown that in healthy subjects the homozygous minor allele [-44 (G→A) linked to +71 (A→G) polymorphism] was present in only 7%, whereas in patients with episodes of supraventricular tachycardia (SVC) and sporadic short episodes of AF, its prevalence was nearly 30%.[6] Also, in the Taiwanese population it has been shown that patients with history of AF had a significantly higher Cx40 (–44A) haplotype frequency than the control group (37.6% vs 28.4%) and OR 1.51 (95% CI 1.13–2.04).[7] Those studies demonstrated for the first time an association between AF and Cx40 minor allele.

In many patients with cerebral ischemia (transient ischemic attack or ischemic stroke) without prior cardiovascular disease (CVD) the underlying cause is never identified. We hypothesized that undetected AF may be an important factor. AF detection is often difficult in patients with rare episodes of AF which are often asymptomatic.

The aim of this study was to assess if patients with unexplained cerebral infarction and normal electrocardiographic (ECG) and/or echocardiographic (ECHO) findings have a higher prevalence of the minor Cx40 gene polymorphism, as an indication of underlying idiopathic AF, compared to those with explicable cerebral infarction (abnormal ECG/ECHO findings).

Methods

Study population

A substudy within the main Second Manifestations of ARTerial disease (SMART) study was designed. The SMART-study is a single-center ongoing prospective cohort study among patients referred to the University Medical Center Utrecht with clinically manifest atherosclerotic disease or cardiovascular risk factors. The design of the study has been described in detail previously[8]. Briefly, at enrollment an extensive questionnaire was filled out and a baseline examination was performed including a twelve-lead resting electrocardiogram (ECG), ultrasound of the abdomen, body mass index (BMI), blood pressure measurements and various blood and urine measurements. Cardiovascular risk was also estimated. For all patients, blood and urine samples were stored at -80°C temperature for future research.

The study started in 1996 and at the time we carried out this substudy (January 2007), 6200 patients were included. For the current substudy all patients with cerebral ischemia, free of other clinically manifest vascular disease, and blood samples available for DNA analysis were selected from the SMART study cohort. Finally, 225 patients were included (3.6% of the whole SMART population at that moment). Those 225 patients were subcategorized into three groups according to their ECG recordings. Group A (n=164) included patients with a normal ECG. Group B (n=51) consisted of patients with an abnormal ECG, indicating evidence of myocardial infarction or ischemia, left ventricular hypertrophy or left atrial enlargement, all situations which can predispose to an embolic event[9]. Two patients had left bundle branch block, which can mask underlying coronary artery disease and left ventricular hypertrophy. Group C (n=10) consisted of patients who had recorded episodes of AF without evidence of structural heart disease. Evaluation of the ECGs was performed independently by two experienced investigators and in case of disagreement the decision was made by a third investigator.

Further subanalyses were carried out by restricting the number of patients according to whether they had additionally undergone ECHO at the time of their cerebral ischemic event. We collected the official reports of all transthoracic (TTE) or transesophageal (TEE) echocardiograms made in our hospital (71 reports) or in the referring hospitals (4 reports), and used them to redefine the groups. In total, the number of patients with ECHO recordings were 75 (47 patients had only TTE, 19 only TEE and 9 had both). In this subgroup analysis, group D (n=45) included patients with a normal ECG and ECHO. In group E (n=22) patients had evidence of heart disease on their ECG or structural abnormalities on their ECHO examination that could be potential sources of cardioembolism. The main abnormalities on ECHO in group E were: patent foramen ovale, atrial septal aneurysm, akinetic/dyskinetic ventricular wall segment, left ventricular hypertrophy, mitral valve incompetence, dilated left ventricle and aortic-arch atheroma. Group F (n=8) included patients with recording at least one episode of AF and no evidence of structural heart disease based on their ECG and ECHO.

Cx40 polymorphism analyses

The 225 DNA samples were genotyped for the Cx40 polymorphism at nucleotide position -44 (G→A) [linked to +71 (A→G)], which is located within the regulatory region of the Cx40 gene. Sequencing was performed on purified polymerase chain reaction (PCR) products and the samples were run and analyzed on an ABI 3730 automated sequencer (FosterCity, USA)[10]. Genotyping was carried out blinded to subgroup classification.

Data analysis

Descriptive analyses were carried out with baseline risk factors in the different subgroups. The main analyses consisted of two comparisons. The prevalence of Cx40 gene genotype was compared among groups A, B, and C and among groups D, E, and F, using Chi-squared tests. Additionally B+C were grouped together as well as E+F and compared with the reference groups A and D respectively. Odds ratios (ORs) and 95% confidence intervals (CIs) were computed to estimate the associations. A two-tailed *p*-value <0.05 was considered significant. Assuming that the group of patients with atrial fibrillation would be small (N=10), a prevalence of 40% of the homozygote genotype of the minor allele (-44AA) of Cx40 in this group, and a prevalence of 7% of this genetic variant among the approximately 150 patients with a normal ECG, the anticipated odds ratio would be 10 with a 95% confidence interval of 2.5 to 18.

Results

The mean age of patients in this study (n=225) was 58.7 years (± 11.5) and 64.4% were men. Table 1 shows the baseline characteristics for groups A, B and C. Patients in group A were the youngest in contrast to groups B and C (mean age 57.5 ± 11.7 vs 61.2 ± 10.3 vs 66.2 ± 10.8). There was a lower proportion of hypertension, hyperlipidemia, a history of cerebrovascular ischemia, and a higher proportion of smokers in group A compared to C, but not significant. All the other baseline characteristics were not different between the groups. Similar baseline characteristics were also shown for groups D, E and F.

Table 2 shows the results for the homozygous minor genotype (-44AA) in the initial groups A, B and C (n=225) and the subgroups D, E and F (n=75). There was no significant difference in prevalence of the homozygous Cx40 minor allele (-44AA) in any of the subgroup comparisons. In addition, it is shown that with the presence of the homozygous and the heterozygous genotype for the minor allele, there was no significantly increased risk (expressed with odds ratios) in those patients with cerebral ischemia without prior CVD with abnormal ECG or ECHO results and neither in those with episodes of AF (groups B+C and E+F).

Table 1: Baseline characteristics of the cerebral ischemic event cohort of the SMART study

	Group A (N=164)	*Group B (N=51)*	*Group C (N=10)*	*p-value*
Baseline caracteristics				
Age (years), mean±SD	57.5 ± 11.7	61.2 ± 10.3	66.2 ± 10.8	0.015
Male gender, % (n)	65% (106)	65% (33)	60% (6)	0.9
Risk factors				
Body mass index (kg/m2), mean±SD	26.5 ± 4.0	26.3 ± 3.8	27,4 ± 2.7	0.7
Obesity (BMI ≥30.0), % (n)	14% (23)	10% (5)	20% (2)	0.6
Waist-Hip ratio, mean±SD	0.89 ± 0.08	0.89 ± 0.08	0.89 ± 0.09	0.9
Hypertension, % (n)	54% (88)	63% (32)	70% (7)	0.4
Diabetes mellitus, % (n)	15% (25)	6% (3)	10% (1)	0.2
Total cholesterol (mmol/L), mean±SD	5.1 ± 1.0	5.2 ± 1.1	5.3 ± 1.0	0.7
LDL-C (mmol/L), mean±SD	3.0 ± 1.0	3.2 ± 1.0	3.1 ± 1.0	0.4
HDL-C (mmol/L), mean±SD	1.4 ± 0.5	1.3 ± 0.5	1.5 ± 0.5	0.2
Triglycerides (mmol/L), median (IQR)	1.3 (1.0,2.0)	1.4 (0.9,2.3)	1.5 (1.3,1.9)	0.9
Hyperlipidemia, % (n)	56% (91)	57% (29)	70% (7)	0.6
Current smoking, % (n)	31% (50)	22% (11)	10% (1)	0.4
Ever smoking, % (n)	43% (70)	57% (29)	50% (5)	0.4
Current alcohol consumption, % (n)	77% (126)	67% (34)	80% (8)	0.7
Previous conditions, [%, (n)]				
Previous cerebrovascular ischemia	38% (63)	41% (21)	70% (7)	0.1
Renal insufficiency	4% (6)	4% (2)	0% (0)	0.8
Peripheral arterial disease	2% (3)	4% (2)	0% (0)	0.6
Abdominal aortic aneurysm	1% (1)	2% (1)	0% (0)	0.6
Diagnosis at baseline, [%, (n)]				
TIA	59% (97)	53% (27)	40% (4)	0.8
Ischemic stroke	34% (55)	41% (21)	50% (5)	
Amaurosis fugax	5% (8)	4% (2)	10% (1)	
Retina infarction	2% (4)	2% (1)	0% (0)	

Group A: Patients with normal ECG, **Group B**: Patients with ECG abnormalities,
Group C: Patients with recorded episodes of AF without evidence of structural heart disease on ECG.
SD, Standard Deviation; BMI, Body Mass Index; LDL-C, Low-Density Lipoprotein Cholesterol; HDL-C, High-Density Lipoprotein Cholesterol; IQR, Interquartile Range; TIA, Transient Ischemic Attack.

Table 2: Prevalence of the Cx40 gene polymorphism and the association between the prevalence of the homozygous and the heterozygous genotype for the minor allele and abnormal ECG/ECHO results as well as those with episodes of AF

Genotype	**Group A**	**Group B**	**Group C**	**B vs A**	**C vs A**	**B+C vs A**
N	164	51	10			
GG	66%	65%	60%	ref.	ref.	ref.
AG	29%	29%	40%	1.0 (0.5-2.1)	1.5 (0.4-5.6)	1.1 (0.6-2.1)
AA	5%	6%	0%	1.2 (0.3-4.9)	*	1.0 (0.3-4.1)
	Group D	***Group E***	***Group F***	***E vs D***	***F vs D***	***E+F vs D***
N	45	22	8			
GG	60%	59%	63%	ref.	ref.	ref.
AG	31%	26%	37%	1.2 (0.4-3.5)	1.2 (0.2-5.6)	1.2 (0.4-3.2)
AA	9%	5%	0%	0.5 (0.1-5.1)	*	0.4 (0.1-3.6)

Group A: Patients with normal ECG, **Group B**: Patients with ECG abnormalities, **Group C**: Patients with recorded episodes of AF without evidence of structural heart disease on ECG, **Group D**: Patients with normal ECG and ECHO, **Group E**: Patients with ECG and/or ECHO abnormalities, **Group F:** Patients with recorded episodes of AF without evidence of structural heart disease on ECG and/or ECHO. All values are Odds ratios (OR) and 95%CI; *OR could not be calculated because of zero values.

Discussion

In this study we did not demonstrate a statistically significant difference in the Cx40 genotype prevalence between patients with cerebral ischemic events and with or without any underlying disease which can predispose to those events. The percentage of the homozygous carriers of the minor Cx40 allele (-44 G→A) was not significantly different (5.4%) among the groups and similar to that in the general population (7%) and the relative risk (expressed as odds ratios) was not increased. The significantly younger age in patients in groups A and D could be consistent with our hypothesis that the idiopathic AF could be the reason for their ischemic episode, since the age of its onset is known to be lower in patients with idiopathic AF.[1;2] However, we were unable to find a higher prevalence of Cx40 minor allele in groups A and D.

Two independent studies demonstrated an increased prevalence of Cx40 minor polymorphism in a population with AF. In patients with supraventricular tachycardia (SVT), mainly with WPW syndrome, the homozygous carriers of the minor haplotype (-44AA linked to +71GG) were those who were more prone to develop AF and they had already experienced sporadic episodes of idiopathic AF.[6] In addition, in a Taiwanese population,173 patients with AF and 232 control patients without, it is shown that the AF group had a significantly higher Cx40 (–44A) genotype frequency than the control group (37.6% vs 28.4%) and the risk for this patients to develop AF was 2.7-fold increased.[7]

Functional studies in cell lines have shown that promoter activity of Cx40 with the minor allele (-44A/+71G) was significantly reduced compared to the major allele (-44G/+71A).[7;11] It is speculated that such a change would have a concomitant effect on the total Cx40 protein levels and distribution in the gap junctions in vivo. Such abnormal expression could lead to heterogeneous impulse propagation that may increase AF vulnerability. However, to what extend the levels and/or distribution of Cx40 protein is influenced by this polymorphism is unclear yet. Further studies are needed to correlate the presence of the minor allele (-44A/+71G) to changes in the Cx40 protein expression.

AF, nevertheless, is an independent and one of the main risk factors for embolic and non-embolic strokes.[5;12] Even idiopathic AF is considered a medium-risk source of cardioembolic ischemic event.[13] Moreover, clustering of strokes has been observed at the time of the onset of idiopathic AF and during the first years after diagnosis, while it is still paroxysmal, and not only during permanent AF.[14-17] There is no significant difference in the probability of stroke between patients with permanent idiopathic AF and those with recurrent episodes or even a single episode.[2;18;19] AF can lead to temporary or permanent cerebral defects due to different mechanisms. A thrombus formation is the most common reason due to hemostasis because of absence of wall contraction during AF or increased hemoconcentration and multiple disturbances of the coagulation system.[20-24] Finally, a reduction of cerebral blood flow during AF can contribute to the development of brain ischemic events and neurological manifestations.[25-27]

In our study, however, we could not prove our hypothesis that AF could have been the cause of the unexplained cerebral ischemic events, based on analysis of a genetic marker. However, some limitations have to be taken into account. The power of our study was low because we could identify only a small group of patients with episodes of AF. In addition, in only 36.5% of the patients echocardiography was performed, restricting our subgroup analysis. Finally, sensitivity of the Cx40 minor allele as a marker for undiagnosed AF may be too low to demonstrate a significant difference when study groups are relatively small. Clinically subjects with only sporadic episodes of idiopathic AF without symptoms are difficult to identify before they develop a more advanced state of the arrhythmia.

The present study did not demonstrate a higher prevalence of the minor allele of Cx40 in patients with unexplained cerebral ischemia. Although Cx40 gene polymorphism was found to be associated with AF, its value for excluding AF in patients with cerebral ischemic events still needs to be established.

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Chapter 7

Reduced Connexin (Cx) 40 Protein Expression in Atria of Patients Bearing the Minor Cx40 Allele (-44 G→A)

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Submitted

Abstract

Background: The occurrence of a Connexin40 (Cx40) minor polymorphism (-44 G→A) was increased in patients with idiopathic AF though its effect on atrial Cx40 protein expression is unknown. Therefore, we studied the effect of this polymorphism on Cx40 expression and distribution in patients without any history of AF and in patients that developed post-operative AF.

Methods: Hundred-eight patients (mean age 67±9 years), without a history of AF or conditions that predispose to AF, were included. During heart surgery, 10cc blood were collected for DNA genotyping and the right atrial appendage was partly excised. Ten patients (9%) were homozygous for the minor allele (AA, group 1), 30 (28%) were heterozygous (AG, group 2) and 68 (63%) were non-carriers (GG, group 3). Ten age and sex matched tissue samples per group were analyzed for Cx40 expression by: 1) Q-PCR, 2) Western blotting, and 3) immunohistochemistry on cryosections.

Results: Q-PCR showed no significant differences of Cx40 mRNA between the groups. Western Blot analysis however, revealed a reduction in Cx40 protein in groups 1 (-36.4%) and group 2 (-39.5%) as compared to group 3. Immunohistochemistry confirmed this reduction but indicated no differences with respect to subcellular distribution of the remaining Cx40. Expression of other structural proteins was unchanged. Incidence of post-operative AF (28%) was age-dependent but unrelated to presence of the polymorphism or fibrosis.

Conclusion: Presence of the Cx40 minor allele (-44 G→A) resulted in a uniform down-regulation of Cx40 expression in the atria which, however was not related to development of post-operative AF.

Introduction

Efficient cell-to-cell coupling of myocardial cells is essential for rapid and uniform action potential propagation. Cell-to-cell coupling is directly related to expression, distribution, localization and the specific properties of gap junction channels throughout the myocardium.[1] These channels consist of connexin proteins and connexin 40 (Cx40) and Cx43 are the major isoforms found in the atrial myocardium. Several studies have shown that their presence is crucial to maintain normal atrial conduction properties.[2-6] Atrial Fibrillation (AF), manifests in 1-2% of the general population.[7] This arrhythmia may induce changes in distribution, intercellular orientation and expression of gap junctions, resulting in so-called "gap junction remodeling".[5;8-15] Gap junction remodeling is associated with impaired cell-to-cell communication and modified electrophysiological properties of the atria that finally may lead to activation delay creating a substrate for reentry which allows to sustain AF.

On the other hand, primary dysfunction of gap junctional communication in the atria has also been associated with AF. This includes both mutations in the coding region of Cx40[16] and polymorphisms in the Cx40 promoter *A* region of the gene. For the latter, studies in patients presenting idiopathic AF have shown that prevalence of a rare Cx40 polymorphism (-44 G→A) was increased, in comparison to the general population.[17;18] Studies in vitro indicated that this minor Cx40 haplotype (-44A) significantly reduced (up to 65%) the promoter activity when transfected in a rat smooth muscle cell-line (A7r5).[19] Another in vitro study showed, however, no difference in promotor activity under basal conditions when constructs were transfected in human HEK293 cells. In this study, promotor activity of the -44AA polymorphism was only reduced under influence of co-transfected Sp1 and GATA4.[20] To date, the relation between this Cx40 polymorphism and Cx40 protein expression in atria of patients free of AF-induced gap junction remodeling is still unknown.

In this study, we investigated the possible influence of this Cx40 polymorphism on expression of Cx40 protein in humans in vivo and related this to a potential contribution for development of post-operative AF. Patients without any existing history of AF were used to avoid secondary gap junction remodeling. This study shows that the presence of the Cx40 minor allele (-44 G→A) resulted in a uniform down-regulation of Cx40 expression in the atria. However, post-operative AF appeared not related to the observed reduction in Cx40 expression.

Materials and Methods

Study population

For this mono-center observational cross-sectional study, 108 patients were included. Patients scheduled for coronary artery bypass grafting (CABG) and/or aortic valve surgery were selected and written informed consent was obtained. Patients were excluded if preoperative AF was diagnosed based on medical history and/or electrocardiographic findings. Also patients with conditions that could predispose to AF, such as mitral valve abnormalities and/or left atrial (LA) size more than 50mm (echocardiogram parasternal long axis view), and patients younger than 18 years were excluded. Prophylactic use of b-blocker therapy to avoid post-operative AF was absent.

Study protocol

The study protocol was approved by the Medical Ethical Committee of the University Medical Center Utrecht. Through the central line during surgery, 10cc blood were collected and used for DNA analysis and genotyping. In addition a small part (~1x1 cm) of the right atrial appendage (RAA) was excised while patients went on cardiopulmonary bypass. Tissue was directly snap-frozen in liquid nitrogen and stored at -80^{o}C until use. Collected tissue permitted isolation of total RNA (for analysis by real-time quantitative Polymerase Chain Reaction (Q-PCR)), extraction of proteins (for Western blot analysis) and cryo-sectioning for immunohistochemistry.

Genetic analysis

Genomic DNA was extracted from peripheral blood samples. DNA sequencing was performed on purified PCR products using the Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequence products were purified using Agencourt CleanSEQ reagent and subsequently separated on a 3100 ABI automated sequencer (Applied Biosystems). Data were analyzed with Mutation Surveyor v3.1 software (Softgenetics). DNA samples were genotyped for the Cx40 polymorphism within the regulatory region of the Cx40 gene at nucleotide position -44 (G→A) which is tightly linked to +71 (A→G). Primers: 5'-TGAGGACAAGGACAACAGGCAG-3' and 5'-CTTCCTCTGGCTACTTCATATC-3'

Tissue analyses

Patients were divided into 3 groups based on their genotype at position -44: AA genotype (group 1), AG (group 2) and GG (group 3). Atrial tissue samples of ten age and sex matched patients from each of those 3 groups were judged large enough for the following 3 types of analysis:

Real-time Q-PCR analysis of mRNA levels

Total cellular RNA was isolated from human atrial appendage tissue using the TRIzol Reagent (Invitrogen). Residual genomic DNA was digested through incubation of the RNA preparation in 1x reaction buffer for 60 minutes at room temperature with 0.5 units of RNase-free DNase-I per μg of total RNA. One microgram of DNase-I-treated RNA was reverse transcribed using Superscript

II (Invitrogen) and oligo-dT primers. The resultant cDNA was diluted 25-fold prior to PCR amplification. Quality of the cDNA preparation was checked first with a standard PCR reaction for the internal reference gene GAPDH. A reverse transcriptase minus reaction served as a negative control. Cx40 and GAPDH (control) mRNA levels were quantified by SYBR Green Real-Time Q-PCR (MyIQ, Biorad) using the following protocol: an initial step at 95°C for 3 min., followed by 40 cycles of 95°C for 15 sec. and 55°C for 30 sec., then 95°C for 1 min., and finally in 61 steps of 0.5°C from 65°C to 35°C (melting curve). Relative expression levels of Cx40 mRNA in all patient samples were assessed with the $2^{-\Delta\Delta Ct}$ method.[21]
The nucleotide sequences of PCR primers used are listed in Supplemental Table 1.

Western Blotting and Immunohistochemistry
Total protein from the tissue of all patients in the 3 groups was isolated and processed as described previously.[22] Protein concentration was quantified using the BCA-assay. Equal amounts of protein (9 pooled per group since in one group one sample was lost, 30µg/lane) of each group were separated on 10% SDS-polyacrylamide gels and transferred by electrophoresis to nitrocellulose membrane (Biorad). Ponceau S staining assessed equivalence of protein transfer. After first and second antibody incubation, immuno-reactivity was detected using the ECL chemiluminescence kit (Amersham).
To quantify Cx40 protein expression, differences in signal intensity were determined as follows. Processed films and Ponceau S staining (gray scale scanned) were imported into ImageQuant software to measure separate protein band intensity. Cx40 protein band signal was controlled to exclude saturation. Unequal protein loading was corrected against total applied protein; the ratio Cx40/ Poncea S signal intensity represented the actual Cx40 protein concentration of the different lanes.
Primary antibodies used were raised against Cx40 (rabbit, 1:250, Invitrogen), Cx43 (mouse, 1:250, Transduction Laboratories), N-cadherin (rabbit, 1:1000, Sigma), α-actinin (mouse, 1:1000, Sigma), and β-catenin (mouse, 1:7000, Transduction Laboratories).

Unfixed cryo-sections (10µm) of the right atrial appendix of the same patients analyzed by Western blotting were serially collected on slides and labeling was performed as described previously.[22] The following primary antibodies were used: rabbit anti-Cx40 (1:250, Invitrogen), mouse anti-Cx43 (1:250,Transduction Laboratories), rabbit anti-N-Cadherin (1:1000, Sigma), and mouse anti-a-actinin (1:1000, Sigma). Secondary antibodies used were Donkey anti-Mouse-Texas-Red (1:150, Jackson) and Goat anti-Rabbit-FITC (1:250, Jackson).
Sections were mounted with Vectashield and examined with a Nikon Eclipse 801 microscope equipped for epifluorescence and representative areas were digitized into 256-leveled grayscale pictures. For quantification, a threshold was identified for each set of pictures to clearly distinguish the high-intensity signal from background in the optical field.[23] Signal intensity of immunolabeling was calculated as percentage of total tissue area (%) using ImageJ 1.40g software (2008, NIH, Bethesda, MD, USA).
For analysis of the heterogeneity of Cx40 and Cx43 labeling, a cut-off level of 35 and 90 for Cx40 and Cx43, respectively, was installed and a custom written script in Matlab (The MathWorks Inc, USA)

was used to assess for each black pixel (Cx40 or Cx43) the shortest distance to the next black pixel in a virtual circle around that pixel. Standard deviation of all shortest distances of all pixels was used as a measure of heterogeneity in each labeling.

Statistical analysis

Categorical data are given as absolute as well as corresponding relative frequencies and were compared with χ^2- test. Data are presented as mean values ± SD. Clinical characteristics between groups were tested with one-way ANOVA. Statistical significance of differences between immunohistological data was determined using nested ANOVA. The analysis was carried out using the Univariate ANOVA in SPSS. Nested ANOVA was used to account for the fact that multiple samples from the same heart of the patients are not fully independent of one other. In the nested ANOVA, the dependent variable was partitioned as the sum of variances due to differences between the group genotypes, between patient's heart of the same group, and between the four samples selected from each heart. P-value <0.05 was considered statistically significant. SPSS version 15.0.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for data analysis.

Results

Clinical characteristics

Table 1 shows the basic characteristics of the patient population. Ten patients (9%) were homozygous for the Cx40 polymorphism (AA, group 1), 30 patients (28%) were heterozygous for the minor allele (AG, group 2) and 68 patients (63%) were non-carriers (GG, group 3). Ten patients in each of the 3 groups were matched for age, gender, type of surgery, LA dimension, and the occurrence of post-operative AF (Table 2). Tissue samples of those patients were used for histological and molecular analysis

Analysis of Cx40 mRNA expression levels

Q-PCR data were calculated according to the instructions of the manufacturer using the $2^{-\Delta\Delta Ct}$ method.[21] Expression levels of Cx40 mRNA, normalized for the internal reference gene GAPDH, revealed no significant differences between the 3 groups (Figure 1).

Table 1: Basic characteristics of all included patients

Patient Characteristics	
n	108
Male, n (%)	83 (77%)
Age, (year) mean±SD	67±9
LA, (mm) mean±SD	43±4
Reason for admission, n (%) **CABG** **Aortic valve surgery**	 87 (81%) 21 (19%)
Genotype, n (%) **GG** **AG** **AA**	 68 (63%) 30 (28%) 10 (9%)
Post operative AF, n (%)	30 (28%)
AF Onset, Days After Surgery, mean±SD	4±2

LA: Left atrium, CABG: coronary artery bypass grafting, AF: atrial fibrillation, SD: standard deviation.

N-Cadherin, Cx43 and Cx40 protein expression

Western Blot analysis for Cx40, Cx43, N-Cadherin and a-actinin, as depicted in Figure 2, demonstrated that Cx40 protein levels for the AA and AG genotype were reduced (- 36.4% and -39,5%, respectively) as compared to the GG genotype. Levels of the other proteins were not different between the three groups.

Immunohistochemical analysis confirmed the results obtained with Western Blot. Labeling intensities of Cx43 (Figure 3 left panels, green signal) and N-cadherin (right panels, green signal) showed equal ratios of Cx43/N-cadherin (black bars) for all three groups (Figure 4A, $p=0.45$). In contrast, presence of the A minor allele significantly reduced the amount of Cx40 protein (Figure 3 red signals) as expressed in ratio to N-Cadherin (Figure 4A, grey bars). Cx40 signal in group 1 (AA) was 63.4% reduced ($p<0.001$) and in group 2 (AG) 43.8% ($p<0.01$). The amount of Cx40 protein was not significantly different between the AA and AG group ($p=0.67$). Despite of this lower protein level of Cx40, the distribution and heterogeneity of both Cx40 and Cx43, was not different between the 3 groups (Figure 4B). ECG analysis revealed no significant differences between the groups with respect to PR-interval and P-wave duration (Table 2).

Table 2: Patients characteristics of the 3 groups

patient characteristics	Group 1 AA genotype	Group 2 AG genotype	Group 3 GG genotype
n	10	10	10
Male, n	9	9	9
Age, (year) mean±SD	64±8	65±8	64±7
LA, (mm) mean±SD	44±4	43±4	43±3
P-wave (ms) mean±SD	98.3±12	97.9±15	102.6±13
PR (ms) mean±SD	164.6±23	164.8±22	164.0±15
CABG, n	9	9	9
Post-operative AF, n	4	3	3
AF Onset, Days After Operation, mean±SD	4±2	3±1	4±1

LA: left atrium, P: p wave, CABG: coronary artery bypass grafting, AF: atrial fibrillation, SD: standard deviation.

Post-operative AF

Thirty patients (28%) developed post-operative AF 4±2 days after surgery (Table 1). Neither the prevalence of the minor allele -44 G→A (Figure 5A), nor the level of interstitial fibrosis (data not shown) was significantly different between the patients who developed AF and those who did not. Only age appeared to be a predictive factor for development of AF since those patients that developed AF appeared significantly older than those without (71±8 vs 65±9, p=0.003). In the 30 atrial samples selected for tissue analysis, 10 patients (34%) developed post-operative AF. Protein levels and subcellular distribution (Figures 5B and 5C respectively) of Cx40 and Cx43 were not significantly different between AF and non-AF although there was a tendency to a reduced Cx40 level in post-operative AF patients.

Discussion

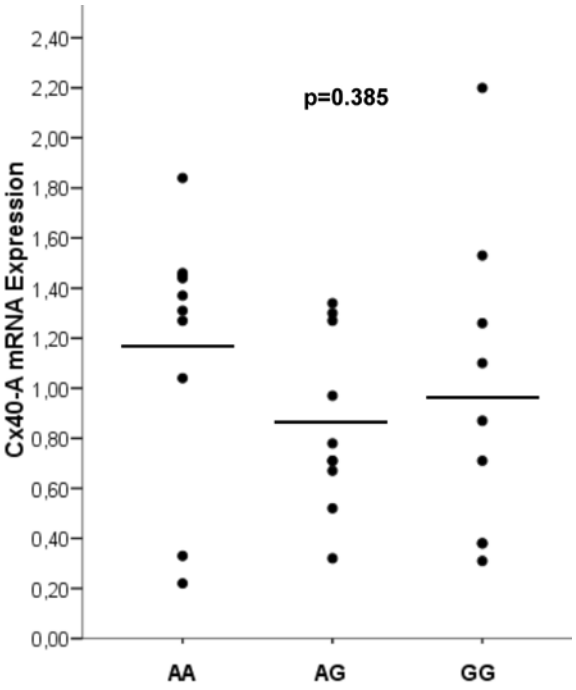
Main Findings

This is the first study to demonstrate that patients without any history of AF that carry the minor allele (-44 G→A) in the Cx40 promoter *A* region of the gene, have decreased expression of Cx40 protein in their atria. Other important structural proteins appeared unaltered and heterogeneity of Cx40 and Cx43 expression was similar between carriers and non-carriers of this polymorphism. Despite reduced Cx40 protein levels in patients carrying this Cx40 minor allele, the occurrence of post-operative AF is not related to its presence.

Connexin 40 protein expression

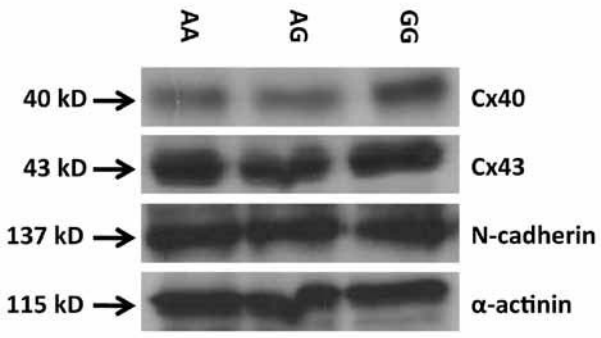
The studied Cx40 polymorphism is located within the basic promoter *A* region of the Cx40 gene. In a

Figure 1:Q- PCR analysis of Cx40 mRNA expression in the three groups.



Q-PCR analysis indicated that patients bearing the Cx40 minor allele (-44 G→A) polymorphism expressed similar Cx40 mRNA levels as the wildtype (-44G).

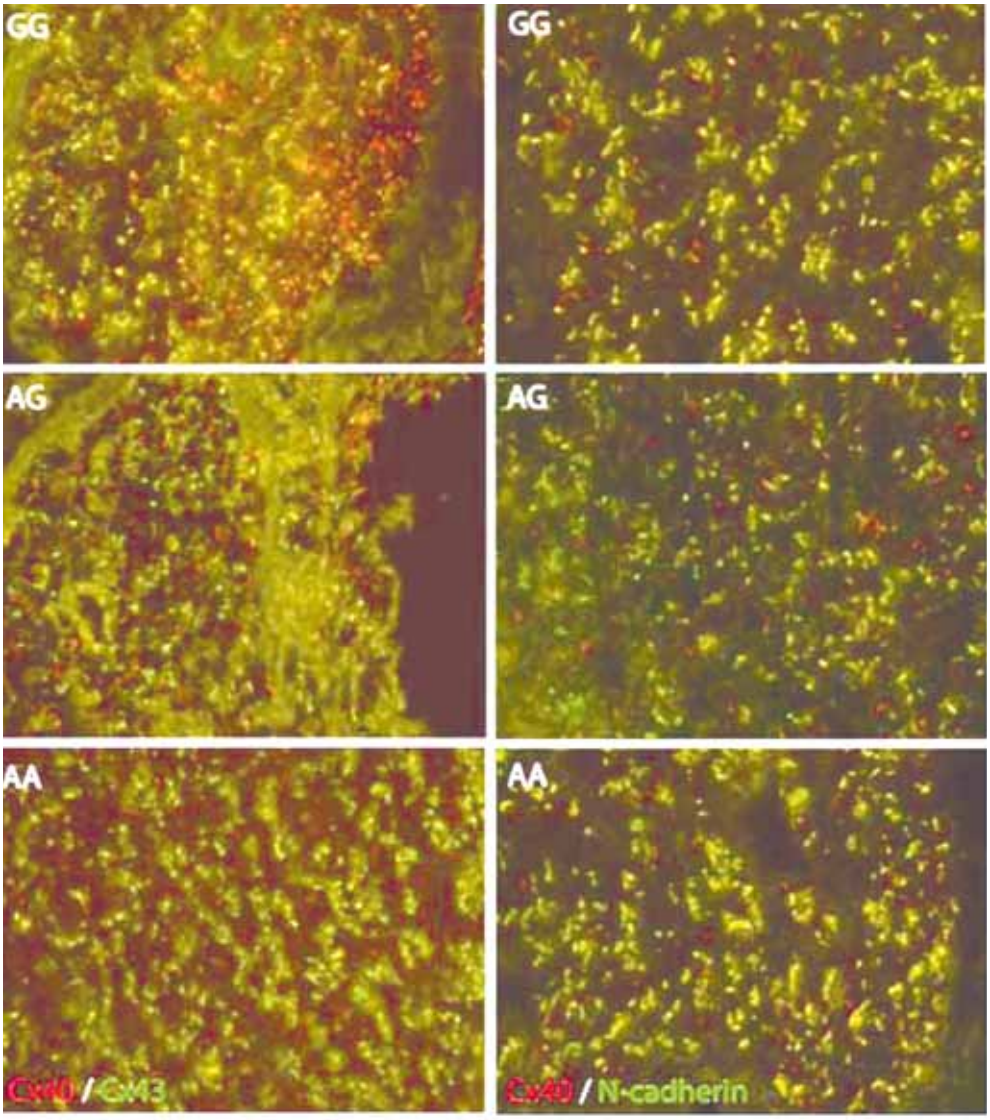
Figure 2: Immunoblot analysis of protein expression in the 3 groups.



	AA	*AG*	*GG*
Cx40	1.52	1.45	2.39
Cx43	5.31	4.79	5.06
N-cad	2.11	2.04	2.59
α-act	4.11	3.67	3.41

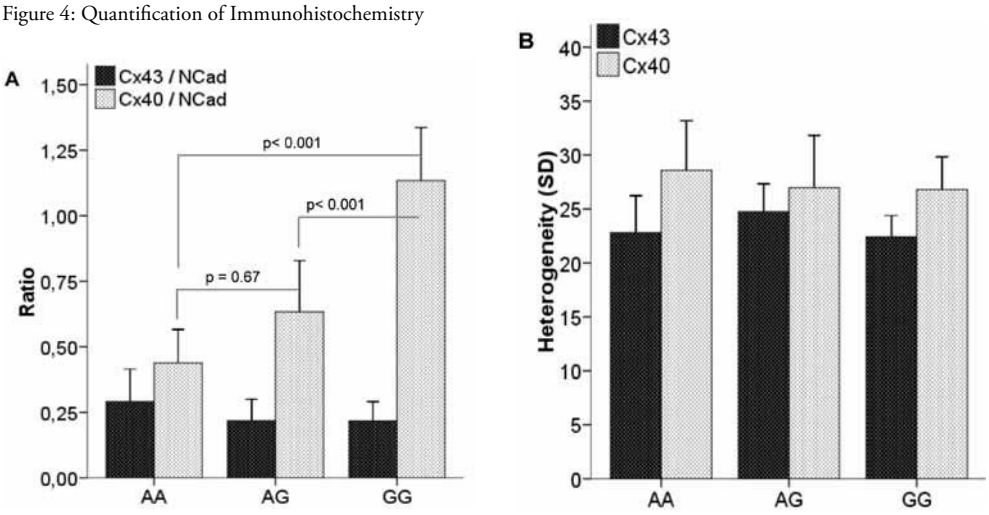
Western Blot analysis of the genotype groups GG, AG and AA with isolated protein pooled per group (n=9/group). The table shows the quantified densities. Cx40 appeared the only protein to be decreased in patients bearing the minor allele compared to non-carriers. The other analyzed proteins were not different between the 3 groups.

Figure 3: Immunohistochemistry



Low magnification (20x) of atrial samples double labeled for Cx40/ Cx43 and Cx40/ N-Cadherin. As indicated, Cx40 staining is seen in red fluorescence while Cx43 (left panels) and N-Cadherin (right panels) are depicted in green. As compared to atrial samples with GG genotype Cx40 is markedly reduced in AG and AA samples. Both Cx43 and N-Cadherin signals are not different between the groups. Distribution of all 3 proteins is not different between the groups.

Figure 4: Quantification of Immunohistochemistry



Quantified immunohistochemical signals indicate that Cx40 protein in ratio to N-Cadherin is significantly decreased in the AA and AG groups as compared to the GG group. B) Quantified heterogeneity of immuno signals shows that the distribution pattern of Cx40 and Cx43 is not different among the groups.

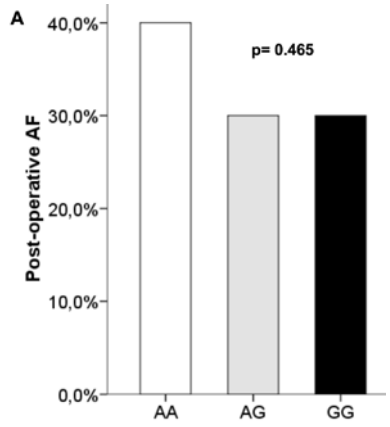
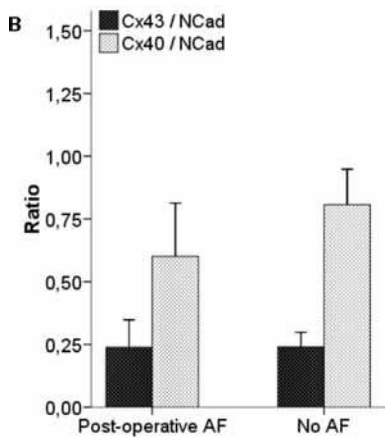
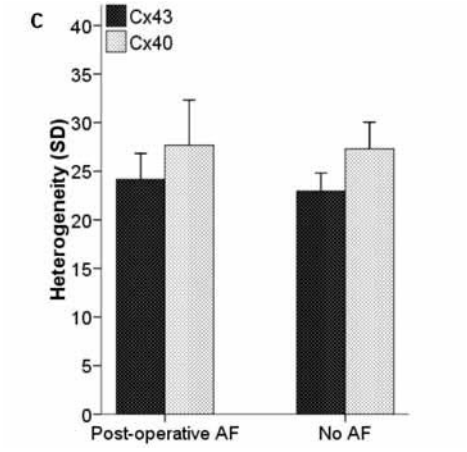


Figure 5 : Genotype and protein expression between patients with and without post-operative AF.

A: The presence of the minor genotype (AA and AG) is not significantly enhanced in the group that developed post-operative AF. B, C: No differences exist in the amount (B), and heterogeneity (C) of Cx40 and Cx43 between patients who developed post-operative AF and those who did not.





previous *in vitro* study, this Cx40 gene polymorphism induced an almost 65% reduction in luciferase gene expression compared to the common Cx40 haplotype (-44 G)[19], while in another *in vitro* study the reduction could only be uncovered through co-transfection of the transcription factors Sp1 and GATA4.[20] Whether *in vivo* the amount of Cx40 protein expression was influenced by the presence of this polymorphism was until now never investigated and only speculative. Very recently, Wirka *et al.* reported unaltered Cx40 mRNA levels in atrial tissue collected from AF patients bearing the -44AA genotype.[24] In contrast to the initial *in vitro* data, but in line with the data of Wirka *et al,* our study also shows that in patients without any history of AF, presence of this minor polymorphism does not lead to a significant reduction of Cx40 mRNA.[24] Strikingly, however, patients bearing this minor polymorphism do have reduced protein levels of Cx40 compared to non-carriers. This reduction in Cx40 protein could be demonstrated both with Western blot analysis of atrial tissue samples and through quantification of immunolabeled cryo-sections obtained from tissue samples adjacent to the ones used for Western blotting. The fact that any history of AF, or other conditions that predispose to AF were exclusion criteria in the present study is a very important prerequisite since AF itself may result in gap-junction remodeling and changes in Cx40 expression.[10-15;25-28] Reduced atrial Cx40 protein, thus, is strongly and exclusively related to the genotype of the patients. Currently, the mechanism underlying the reduction in Cx40 protein is still unknown.

The down-regulation of Cx40 protein or other unidentified effects related to the AA genotype, however, appeared to have no influence on protein levels of other important structural and gap-junction proteins since Cx43, N-Cadherin and a-actinin remained unchanged in all 3 groups. Since heterogeneity of Cx40 is not different between the groups, this indicates that down-regulation of Cx40 in those patients was rather uniform throughout the atrial myocardium and presence of the Cx40 polymorphism did not affect the distribution pattern of Cx40 protein. Finally, also the distribution pattern of Cx43 protein was not significantly different between carriers and non-carriers.

Expression of Cx40 and Cx43 and atrial conduction properties

Although studies in $Cx40^{-/-}$ knockout mice have shown that a complete deletion of Cx40 is responsible for atrial conduction disturbances[5;29], a 50% reduction of atrial Cx40 (comparable to our study) did not alter atrial conduction.[2] In humans, a partial decrease in expression of a particular connexin isoform is not necessarily related to impaired conduction velocity. A reduced Cx40 expression was even associated with increased electrical propagation velocity,[6;30] while presence of high levels of Cx40 protein played an essential role in establishing interatrial conduction velocity heterogeneity.[31]

In synthetic strands from atrial murine myocytes deletion of Cx40 was associated with a significant increase of Cx43 immunosignal in the gap junctions.[6] In the same study it was also shown that the total cellular Cx43 content remained unchanged, which is in agreement with the data presented in our study. In our study, patients bearing the minor allele had lower levels of Cx40 protein compared to non-carriers and as such higher relative levels of Cx43 (since absolute Cx43 levels were indifferent between the groups). According to the studies of Kanagaratnam *et al*[30], and Beauchamp et al[6], these relative levels of Cx40 and Cx43 correspond to high atrial conduction velocity. Previously we identified no difference in P-wave duration between controls and patients with idiopathic AF bearing

the -44AA genotype.[17] Differences in PR interval were also absent in a study where we compared controls to patients bearing the sole -44AA genotype or this polymorphism in combination with a sodium channel mutation (SCN5a-D1275N)[19]. Both observations suggest that conduction over the atria was not affected. The present study also shows that there is no difference in P-wave duration or in PR interval between carriers and non-carriers which strengthens the suggestion that the conduction velocity is at least unaffected.

Post-operative AF

The current study demonstrates that presence of the minor Cx40 genotype (AA or AG) is not related to an increased occurrence of post-operative AF in our patients. Cx40 protein expression and distribution was also not significantly different between the patients that developed post-operative AF and those who did not. These results suggest that alteration of connexin expression is not solely required for initiation of AF after cardiac surgery, at least. Wirka and co-workers also excluded a direct relation between presence of the -44 G→A polymorphism and occurrence of AF using a large cohort of patients (merely with a history of AF).[24] These observations, however, contrast with those reported by Dupont *et al.* which is the only study, to our knowledge, that also reports levels of Cx40 in post-operative AF. Although they also observed that the expression and distribution of Cx43 was not different between the non-AF and AF group, they found that in patients with post-operative AF, the amount and heterogeneity of Cx40 protein was significantly increased.[10] Other studies, however, have shown that post-operative AF may result from hyperactivity of the autonomic nervous system (explaining why administration of β – blockers appears to be an effective treatment[32]), or of an inflammatory response triggered by cardiac surgery.[33-35] The first suggestion may also be an explanation why the occurrence of AF is most prevalent 2 to 3 days after surgery and is commonly not persistent.[36;37] In the present study, and also in that by Dupont et al., post-operative AF occurred at least 2 to 10 days after surgery. If a pre-existing enhanced level of Cx40 protein was required for the occurrence of AF, it may be discussed why AF did not occur immediately after cardiac surgery but only after at least 48h.

Study limitations

The fact that no significant reduction in Cx40 mRNA was detected is, as mentioned, in line with the study of Wirka *et al.*[24] The absence of significant changes might, however, also be related to the fact that using the Q-PCR technique would imply an expected (based on the ≈40% reduction in Cx40 protein and a 65% reduction in promoter acivity *in vitro*[19]) difference in Cx40 mRNA signal of less than one PCR-cycle. Due to inter-individual variation between the patients within one group, such a potential difference may be too small to result in a significant change.

Conclusion

This is the first study to show that presence of the Cx40 minor allele (-44 G→A) in the promoter area of the Cx40 gene, leads to a uniform reduction of Cx40 protein expression in the atria of patients without any history of, or predisposition to AF. ECG analysis suggests that atrial conduction is not

affected by this reduction. Finally, the prevalence of the Cx40 polymorphism and the reduced amount of Cx40 protein appeared not directly related to the occurrence of post-operative AF.

Disclosures

None

Supplemental Table 1:

primer	forward	reverse
Cx40 exon2 148F-313R	5'-CCTCTTCATATTCCGTATGCTCGT-3'	5'-GATGATCTGCAGCACCCAGTAGCG-3'
GAPDH 856F-1059R	5'-CCTGCCAAATATGATGACATCAAG-3'	5'-GCCAAATTCGTTGTCATACCAGGA-3'

Nucleotide sequences of PCR primers used for Q-PCR

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Chapter 8

General Discussion

Electrophysiological insights into atrial fibrillation

The Impact of the Cardiac Nervous System on the Initiation and Maintenance of Atrial Fibrillation

In the human heart a richly developed neural network containing up to 700-1500 epicardiac ganglia on the atria and ventricles is divided into ganglionated plexi (GP) located in the epicardiac fat pads. Every plexus is composed of: 1) pre-ganglionated nerves that extended from the heart hilum into a ganglionated field, 2) a ganglionated field, in which ganglia occupy the restricted locality in atria and ventricles and 3) post-ganglionated nerves that proceed into discrete innervation regions by their specific pathway.[1] Atrial GP are identified on 1) the superior surface of the right atrium (RA), 2) the superior surface of the left atrium (LA), 3) the posterior surface of the RA, 4) the posterior surface of the LA and 5) the inferior and lateral aspect of the posterior LA[2] (Fig 1). Ventricular GP are located 1) surrounding the aortic root, 2) at the origins of the right and left coronary arteries, 3) at the origin of the posterior descending coronary artery, 4) adjacent to the origin of the right acute marginal coronary artery and 5) at the origin of the left obtuse marginal coronary artery.[2]

Regulation of cardiac neural activity is achieved at multiple levels. In higher brain centers, through baroreceptor mechanisms and finally in the intrinsic cardiac nerves themselves. Intrinsic cardiac nerves appear to provide local neural coordination independent of the higher brain centers.[3] The intrinsic cardiac nervous system, can control the heart rate, electrical properties of the myocardial tissue, such as conduction velocity and effective refractory period, and is involved in various arrhythmic disorders of the heart.[4-7] The concept that neural activity exerts a potent influence on arrhythmogenesis has been studied extensively in both human and animal models.[8;9] The role of the autonomic nervous system (ANS) in the pathogenesis of atrial fibrillation (AF) has also been suggested in multiple settings.

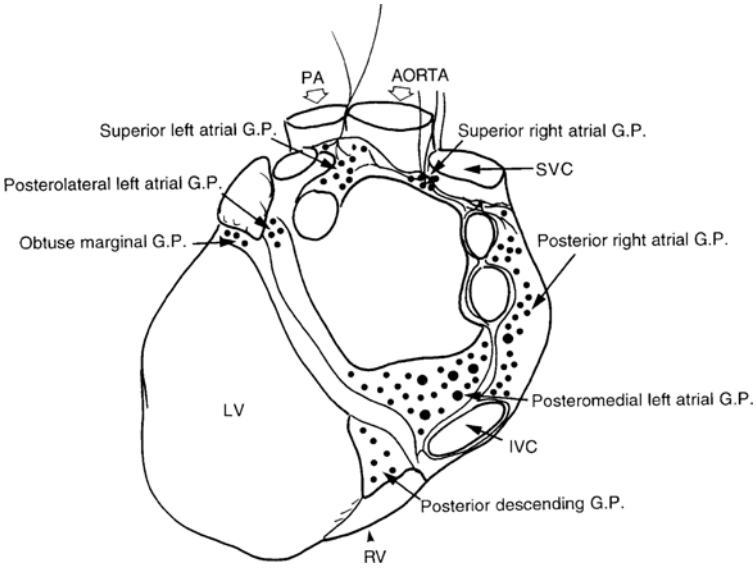

Figure 1: A posterior view of the human heart. Illustration of the locations of all the atrial and some of the ventricular ganglionated plexuses. PA: pulmonary artery, SVC: Superior vena cava, IVC: inferior vena cava RV: Right Ventricle, LV: Left Ventricle, GP: ganglionated plexus (Armour JA et al. Anat Rec1997, permission granted)[2]

Coumel et al[10] were the first to define paroxysmal AF as vagotonic or adrenergic according to the autonomic tone before its onset. Vagal AF was related to patients who developed AF when slow heart rates occurred during sleep; and adrenergic AF in those patients whose AF was associated with sympathetic states, during exercise or emotional stress. Hou et al.[11] also showed that the presence of an interconnecting neural network in the LA may provide a substrate for focal AF. Furthermore, it has been shown that the ANS innervating the pulmonary veins (PV), can also play an important role in the arising of the premature complexes, seen in the PV and associated with the initiation of AF.[12] Activation of the ANS markedly shortened pulmonary vein action potential duration, decreased refractoriness and initiated rapid firing due to early after-depolarization.[13] The influence of the ANS, however, on dispersion of refractoriness and the occurrence of complex fractionated electrograms is not very clear. The first part of this thesis attempted to identify this role of the ANS.

Dispersion of Refractoriness and Autonomic Nervous System

Studies have shown that the nerve distribution in the atria and in the region of the PV is rather heterogeneous.[14;15] Such spatial heterogeneity of nerve distribution could result in profound heterogeneity of myocardial excitability and refractoriness[15-17]. Dispersion of refractory periods is considered a major factor in both induction and persistence of reentrant arrhythmias in the ventricles and the atria.[18-21] However, the role of ANS on the dispersion of refractoriness is still poorly understood.

The first study that investigated the effect of ANS on dispersion of refractoriness was done in dogs and it was mainly focused on the effect of sympathetic stimulation on dispersion of refractoriness in the ventricles during ventricular fibrillation (VF). [22] They showed that dispersion of VF intervals increased after sympathetic stimulation, but not significantly in every heart. A later study examined the effect of both sympathetic and vagal limbs of ANS on dispersion of atrial refractoriness, during a new episode of AF.[23] They showed that the variability in atrial refractoriness was increased mainly due to vagal stimulation. The sympathetic stimulation was much less effective. These results were also confirmed by another study.[24] It showed that regional sympathetic denervation increased dispersion of refractoriness and facilitated sustained AF. The authors also concluded that this regional sympathetic denervation indirectly changed the vagal effects on refractoriness through changes in the sympathetic modulation on vagal effects. Finally a study in dogs with sustained AF showed that dispersion of refractoriness was unchanged with both sympathetic and/or parasympathetic stimulation.[25]

The only study in humans that investigated the effect of ANS on dispersion of atrial refractoriness was done in patients with supraventricular tachycardia, without a history of AF, and during high intensity stimulation. Nevertheless, they showed that the increase of the dispersion of refractoriness, caused by stimulation, was abolished after total autonomic blockade.[26] Thus, these results indicated, that both limbs of the ANS were needed to influence dispersion of refractoriness.

These studies actually pointed out that there is no consensus on the role of ANS on dispersion of refractoriness. Contradictory results of these studies, however, may be due to different methodology and study protocols. In the first study the ventricles and the sympathetic ANS were mainly investigated. The studies that followed were predominantly focused on dispersion of atrial refractoriness, but they used different study models: Dogs with new episode of AF were studied, or dogs with sustained AF,

or even dogs without AF at all. In the human study, as well, the patients who were included had no history of AF, and no AF was induced. Such methodological differences between the studies may be an explanation of the different results.

We were the first to evaluate the influence of ANS on dispersion of refractoriness in patients with idiopathic AF and different degree of atrial remodeling. The atrial electrical remodeling, however, did not influence the amount of dispersion of refractoriness. On the other hand, the influence of ANS on dispersion of refractoriness during AF depended on the degree of electrical remodeling and the anatomic site. In patients with advanced atrial remodeling the dispersion of refractoriness decreased, mainly due to parasympathetic blockade and only in the RA. Additional sympathetic blockade had no further influence. In patients in the early phase of an AF episode, the dispersion of refractoriness was not influenced by either parasympathetic or total autonomic blockade. The results of our study differ from those discussed above. However, our study population was also different. Patients with idiopathic AF and different degree of atrial remodeling were never studied before, that's why we can not compare our results with that of the previous studies.

Our results suggest that the role of ANS on dispersion of refractoriness in patients with idiopathic AF is rather limited. Changes of the ANS alone are not enough to explain the amount of dispersion of refractoriness in those patients.

Complex fractionated electrograms (CFAEs) and Autonomic Nervous System.

Although the definition of complex fractionated electrograms (CFAEs) seen during AF was induced by Koning et al[27], the first to point out the importance of CFAEs in the treatment of AF were Nademanee et al.[28] Since then, CFAEs have been proposed as a new target of AF management. The definition and detection of CFAEs, however, has been controversial among studies. CFAEs are defined as fractionated electrograms composed by different morphologies and more than 2 deflections, electrograms with a short cycle length (≤120ms)[28], or electrograms with continuous signals.[27-29] Such fractionation of atrial electrograms can be determined by different substrates:

1) The presence of tissue structural complexities[30] and specific anatomical characteristics, can lead to activation delay and thus to the genesis of CFAEs.[27] Such anatomical changes can be due to increased fibrosis, tissue anisotropy or heterogeneous coupling changes due to altered connexin expression in the gap junctions.[30-34] 2) Spatial dispersion of refractoriness. It has been shown that in areas with longer effective refractory period, action potential formation may be blocked or conduction velocity may decrease if early excitation occurs in the absolute or relative refractory period, respectively. For this reason, this difference in atrial refractoriness can lead to the genesis of disorganized electrograms in the areas with longer refractory period.[35] 3) Increase meandering of the rotor driving AF can reduce activation manifestation and increase fractionation.[36] 4) Finally, it has been proposed that CFAEs are areas close to GP, where the local vagal stimulation shortens the local effective refractory periods and causes the formation of CFAEs.[37]

However, the role of ANS on the genesis of CFAEs is not yet completely investigated. Two experimental studies that investigated the effect of ANS during sustained AF in dogs showed that CFAEs resulted from the activation of the intrinsic cardiac ANS; and that ablation of GP attenuated

CFAE.[38;39] One clinical study showed that pharmacological autonomic blockade can reduce CFAEs in the left atrium of patients with paroxysmal AF. [40]

In this thesis, we showed that ANS significantly reduced the prevalence of CFAEs in patients with long-lasting AF and prevented the increase of CFAEs in patient in the early phase of a new AF episode. Such findings indicate that the occurrence of CFAEs may also be due to hyperactivity of the intrinsic cardiac ANS.

CAFEs detection

We also discussed the advantages and the disadvantages of detecting CFAEs with unipolar en bipolar signals. For unipolar recordings, the local activation time is well defined. Simultaneous recordings from microelectrodes and extracellular electrodes from the same site of myocardial tissue during propagation of the action potential have shown that the upstroke of the action potential, which marks depolarization of the tissue at the recording site, coincides with the point of steepest negative dV/dt in the unipolar electrogram.[41] For bipolar recordings the activation time is less well defined because both amplitude and configuration of the signal are dependent on the direction of the activation front. Therefore, a unipolar recording would be preferable for determining activation times. However, remote ventricular deflections can give rise to false activation times, particularly if an automatic detection system is used. Therefore, unipolar atrial recordings with QRS subtraction are considered the gold standard to determine CFAEs. The results of this thesis indicate that with the bipolar technique, some areas with CFAEs may be missed while other areas may be erroneously identified as presenting CFAEs. Furthermore, orientation of the catheter relative to the endocardial wall may also influence CFAEs detection. CFAEs derived from activation times in unipolar recordings without QRS subtraction and a catheter placed perpendicular to the endocardial atrial wall come closest to the gold standard.

Genetic insights into atrial fibrillation

The Role of Connexin 40 on the Substrate of Atrial Fibrillation

Studies in animals have shown that a decreased amount of Cx40 is related to conduction slowing and vulnerability to AF. Also, altered Cx40 expression and distribution in the myocardial extensions in the thoracic veins may be the substrate for abnormal impulse formation and/or micro-reentry, underlying the trigger for AF induction. In patients with AF, mainly the heterogeneous expression of Cx40 protein, and not its amount, seemed to lead to abnormal impulse formation and activation delay, which may form a substrate for AF.[42] Nevertheless, a genetic predisposition, due to Cx40 gene polymorphism or other kinds of mutations, seemed also to be related to the initiation and maintenance of AF.[43-45]

A previous study from our department has shown that a minor allele (-44 G→A) in the promoter region of the Cx40 gene was present in patients with Wolf-Parkinson- White (WPW) syndrome and rarely occurring episodes of AF. This polymorphism was related to increased dispersion of refractoriness in the absence of atrial remodeling.[43] An other study from Taiwan also showed that the prevalence of this minor Cx40 polymorphism was increased in patients with secondary AF (~60% related to hypertension) compared to the control group.[45] Finally, there is also a study to identify four heterozygous mutations, three somatic and one germline, in the Cx40 gene that were related to

idiopathic AF.[44] They also pointed out the important role of Cx40 on the substrate of AF.

In the second part of the present thesis we investigated the role of the Cx40 polymorphism in two different populations with AF: 1) Patients with suspected idiopathic AF, presented with an ischemic cerebrovascular event probably as the first presentation of AF, and 2) Patients with post-operative AF and without history of heart rhythm disorders prior to cardiac surgery.

Although we were able to show that the presence of this minor polymorphism led to uniform reduction of Cx40 in the atria, we were not able to find that the prevalence of this polymorphism was advanced in the different types of AF. We conclude, thus, that the presence of the minor allele (-44 G→A) in the promoter region of the Cx40 gene is not necessary for the occurrence of idiopathic or post-operative AF. The reason why our results are in contrast to that seen in previous studies may be due to the fact that different populations were investigated. The first study investigating this polymorphism[43] mainly included patients with AF related to WPW syndrome. AF occurs in up to 10 - 32% of patients with WPW syndrome and can be related to other mechanisms than intrinsic atrial vulnerability.[21] The initiation of AF in patients with WPW syndrome and also in those with concealed AP can be related to spontaneous degeneration of atrioventricular reciprocating tachycardia (AVRT) into AF and the electrical properties of the accessory pathway (AP).[46-48] The incidence of spontaneous degeneration of AVRT into AF has been reported to be 16-26%.[49] Furthermore, after surgical excision or catheter ablation of the AP, more than 90% of the patients are free of AF during long-term follow up, which indicates that the AP most probably plays a role in AF initiation.[50-53]

The second study which evaluated the role of the polymorphism, included patients with AF secondary to hypertension.[45] The presence of hypertension, however, might have influenced the results substantially, since it has also been demonstrated that Cx40 polymorphism can form a genetic susceptibility factor for hypertension alone.[54] The etiology of AF in those populations with WPW syndrome or hypertension, is therefore different from patients with idiopathic or post-operative AF.

General conclusion

With this thesis we showed that ANS did not affect both atrial refractoriness and its dispersion in both early and late atrial remodeling during AF. Furthermore, the prevalence of CFAEs in the atria, areas that seem to represent a critical substrate for AF maintenance, is greater in patient with long-lasting AF compared to patients in the early phase of an AF episode. Atropine and metoprolol administration reduced the prevalence of CFAEs in patients with long-lasting AF and prevented the increase of CFAEs in the early phase of AF. The most accurate technique to identify CFAEs areas are unipolar recordings with subtraction of remote ventricular activity. The use of filtered bipolar recordings could lead to missing areas of CFAE's or erroneously identifying areas as CFAE's. Unfiltered bipolar recordings from a catheter placed parallel to the endocardial wall turned out to be a good alternative.

In addition we showed that the presence of the Cx40 minor allele (-44 G→A) resulted in a uniform down-regulation of Cx40 expression in the atria. However, in patients with cerebral ischemic events, without prior cardiovascular disease, a higher prevalence of the Cx40 gene polymorphism as a marker of underlying idiopathic AF appeared to be absent. Also its presence was not related to the development of post-operative AF.

Future Perspectives

In search of the most effective treatment of AF, new techniques have been developed, targeting the triggers and the substrate of AF. Such techniques include ablation of the triggers in the PV that can initiate AF[12], anatomical circumferential ablation of the PV in the LA[55], or ablation of the CFAEs.[28] All these methods seem to help patients with AF to remain free of symptoms, but they are not always effective and long-term efficacy is not known. With the studies described in this thesis, we showed that the ANS can also influence CFAEs. Ablation of the ANS with elimination of the GP additional to the conventional ablation methods may improve results. Such attempts have already been made during surgical and catheter ablation treatment of AF in a small number of patients with AF, with favorable primary results.[56-58] However, such an approach has to be tested in larger randomized studies, before definitive conclusions can be made.

In addition, we showed that the presence of the Cx40 polymorphism lead to uniform reduced expression of Cx40 protein in the atria. How this reduction influences the conduction properties of the atria is not known. In the ventricles reduction of Cx43 was not followed by conduction disturbances. Maintenance of large gap junctions supported safe ventricular conduction.[59] If such a protected response can also happen in the atria, as a result of decreased Cx40 protein is not yet clear. Future studies that would evaluate the electrophysiological properties of the atria bearing this polymorphism are required to show the effect of the Cx40 minor allele (-44 G→A) on the atrial electrical propagation and eventually its association with the substrate of AF. Finally, further investigation of other mutations or polymorphism on the Cx40 gene may lead to the detection of new important gene variations related to AF.

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Chapter 9

Summary

In this PhD thesis we evaluated the role of the autonomic nervous system (ANS) and the genetic background on atrial substrates required for the initiation and perpetuation of atrial fibrillation (AF). **Chapter 1** is an introduction to the work of this thesis. We described the structural and functional substrate of AF together with the triggers and the modulating factors responsible for the initiation and maintenance of AF. In **chapter 2** was shown that dispersion of refractoriness, an electrophysiological substrate for the initiation and perpetuation of AF, is not influenced by the electrical remodeling during AF and it is mainly enhanced in the left atrium (LA) of patients with idiopathic AF. The influence of the ANS on dispersion of refractoriness is rather limited and dependent on the degree of electrical remodeling and the atrial site. In patients with early remodeling dispersion of refractoriness is not influence by autonomic blockade (AB); while in patients with advanced remodeling due to long-lasting AF, dispersion of refractoriness after AB is only decreased in the right atrium (RA). However, the influence of ANS on complex fractionated electrograms (CFAEs) is greater. Areas of CFAEs can represent critical regions for maintaining AF sites, since they can be related to anatomic and electrical changes of the atrial myocardium. As shown in **chapter 3,** CFAEs are also influenced by the systemic blockade of the ANS. Such blockade reduced the prevalence of CFAEs in the atria of patients with long-lasting AF, and prevented the time-dependant rise of CFAEs in patients with a new episode of AF and thus in the early- phase of AF. The presence of CFAEs is thus, at least partly, influenced by the status of the ANS. The detection of CFAEs, however, has been controversial among studies. The most accurate technique to identify these areas is unipolar recordings with subtraction of remote ventricular activity (gold standard). In **chapter 4** was shown that with the bipolar technique, some areas with CFAEs may be missed while other areas may be erroneously identified as presenting CFAEs. Furthermore, orientation of the catheter relative to the endocardial wall may also influence CFAEs detection. CFAEs derived from activation times in unipolar recordings without QRS subtraction and a catheter placed perpendicular to the endocardial atrial wall come closest to the gold standard.

In the second part of this thesis we attempted to enlighten the role of connexin (Cx) 40 protein in AF and the presence of a Cx40 minor polymorphism as a genetic factor of AF. In **chapter 5**, based on literature, it was shown, that heterogeneous distribution of Cx40 protein in the atria during AF can contribute to the stabilization of AF. In addition, altered expression of Cx40 protein in the myocardial sleeves of the thoracic veins may be the substrate for the firing foci that can trigger AF. Finally, it was shown that the Cx40 polymorphism is associated with the presence of AF in patients with Wolf-Parkinson-White (WPW) syndrome and patients with hypertension. In **chapter 6**, was shown that Cx40 polymorphism was not present in a big cohort of patients with unexplained ischemic cerebrovascular events and it could not be used as a genetic marker in order to predict idiopathic AF in this population. Additionally, in **chapter 7** the specific effect of Cx40 polymorphism on the expression of Cx40 protein in the atria of patients without AF was shown. Patients bearing this polymorphism had a reduced expression of Cx40 protein in their atria. However, the presence of this polymorphism was not associated with the prevalence of post-operative AF. The role thus of Cx40 polymorphism in the occurrence of AF is not the same in the different types of AF. Finally, **chapter 8** is a discussion about our findings in comparison with those from other studies, a possible clarification for the differences noticed and future perspective.

Chapter 9

Samenvatting

In dit proefschrift hebben wij de rol van het autonome zenuwstelsel (AZS) geëvalueerd en de genetische achtergrond van atriale substraten die nodig zijn voor de initiatie en de instandhouding van boezemfibrilleren (AF). **Hoofdstuk 1** betreft de introductie van dit proefschrift. Wij beschrijven zowel het structurele als het functionele substraat van AF alsook de uitlokkende en modulerende factoren die verantwoordelijk zijn voor het initiëren en onderhouden van AF. In **hoofdstuk 2** werd aangetoond dat de heterogeniteit van de refractaire periode van verschillende posities in de boezem, de zogenaamde *'dispersion of refractoriness'*, een elektrofysiologisch substraat voor het initiëren en in stand houden van AF, niet beïnvloed wordt door de elektrische remodellering die ontstaat tijdens AF. "Dispersion of refractoriness" was voornamelijk toegenomen in de linker boezem van patiënten met idiopathisch AF. De invloed van het AZS op de *'dispersion of refractoriness'* bleek slechts beperkt en afhankelijk van de mate van elektrische remodellering en de lokatie in de boezem. Bij patiënten met recent ontstaan AF, en dus vroege atriale remodellering, wordt de 'dispersion of refractoriness' niet beinvloed door autonome blokkade (AB), terwijl bij patiënten met gevorderde remodellering als gevolg van langdurig AF, *'dispersion of refractoriness'* na de AB alleen is verminderd in het rechter atrium (RA). Echter, de invloed van het AZS op de zogenaamde complexe gefractioneerde elektrogrammen (CFAEs) was belangrijker. Gebieden van CFAEs weerspiegelen gebieden in de boezems met anatomische en elektrische veranderingen die cruciaal zijn voor het behoud van AF.
Zoals weergegeven in **hoofdstuk 3** worden CFAEs ook beïnvloed door de systemische blokkade van het AZS. Een dergelijke blokkade verminderde de prevalentie van CFAEs in de atria van patiënten met langdurig AF, en voorkwam de tijdsafhankelijke toename van CFAEs bij patiënten met een nieuwe episode van AF en dus in de vroege fase van AF.
De aanwezigheid van CFAEs wordt derhalve, in ieder geval gedeeltelijk, beïnvloed door de status van het AZS. Uit verscheidene studies blijkt dat de detectie van CFAEs echter omstreden is. De beste methode om deze gebieden te identificeren is het gebruik van unipolaire recordings met subtractie van farfield ventriculaire activiteit (gouden standaard).
In **hoofdstuk 4** werd aangetoond dat met het gebruik van bipolaire recordings enkele gebieden van CFAEs mogelijk werden gemist, terwijl andere gebieden mogelijk ten onrechte werden aangeduid als CFAEs. Bovendien was de oriëntatie van de catheter ten opzichte van de endocardiale wand mogelijk van invloed op de detectie. CFAEs die werden afgeleid van activatietijden bij de unipolaire recordings zonder QRS subtractie, met de catheter loodrecht op de endocardiale atriale wand, evenaarden de gouden standaard nog het meest.

In het tweede deel van dit proefschrift hebben we de rol van het connexine (Cx) 40 eiwit in AF en de aanwezigheid van een Cx40 minor polymorfisme bestudeerd, als een genetische factor van AF. **Hoofdstuk 5** is een literatuurstudie over de aanwijzingen dat de heterogene verdeling van het Cx40 eiwit in de atria tijdens AF bijdraagt aan de stabilisering van AF. Ook is het zo dat veranderde expressie van het Cx40 eiwit in myocardiale uitlopers in de longaders (myocardial sleeves) het substraat kunnen zijn voor de vurende foci die kunnen leiden tot AF. Tot slot werd besproken dat het Cx40 polymorfisme geassocieerd is met de aanwezigheid van AF bij patiënten met Wolf-Parkinson-White (WPW) syndroom en patiënten met hypertensie. In **hoofdstuk 6** werd aangetoond dat Cx40

polymorfisme niet significant verhoogd was in een groot cohort van patiënten met onverklaarde ischemische cerebrovasculaire accidenten en dat het niet kon worden gebruikt als een genetische marker om idiopathisch AF te voorspellen in deze populatie. In **hoofdstuk 7** werd het specifieke effect van Cx40 polymorfisme op de expressie van Cx40 eiwit in de atria van patiënten zonder AF getoond. Patiënten met dit polymorfisme hadden een verminderde expressie van Cx40 eiwit in hun atria. Echter, de aanwezigheid van dit polymorfisme was niet geassocieerd met de prevalentie van post-operatief AF. De rol van Cx40 polymorfisme bij het optreden van AF is derhalve niet dezelfde in de verschillende types van AF. Ten slotte vormt **hoofdstuk 8** een discussie over onze bevindingen, een mogelijke uitleg voor de discrepanties tussen onze bevindingen en andere studies en een perspectief voor de toekomst.

Chapter 9

Περίληψη

Σε αυτή τη διδακτορική διατριβή αξιολογήσαμε το ρόλο του Αυτόνομου Νευρικού Συστήματος (ΑΝΣ) και το γενετικό υπόβαθρο στους κόλπους της καρδίας που είναι απαραίτητοι παράγοντες για την εμφάνιση και την διαιώνιση της κολπικής μαρμαρυγής (ΚΜ). Το **Κεφάλαιο 1** είναι η εισαγωγή της εργασίας αυτής. Περιγράφεται το δομικό και λειτουργικό υπόστρωμα της ΚΜ, οι τρόποι πυροδότησης και οι διαμορφωτικοί παράγοντες που είναι υπεύθυνοι για την εμφάνιση και συντήρηση της ΚΜ. Στο **Κεφάλαιο 2** αποδεικνύεται ότι η ετερογένεια της ηλεκτρικής ανερέθιστης περιόδου του μυοκαρδίου των κόλπων, ένα ηλεκτροφυσιολογικό υπόστρωμα για την εμφάνιση και διαιώνιση της ΚΜ, δεν επηρεάζεται από την ηλεκτρική αναδιαμόρφωση του μυοκαρδίου των κόλπων, που συμβαίνει κατά τη διάρκεια της ΚΜ, και εμφανίζεται κυρίως στον Αριστερό Καρδιακό Κόλπο (ΑΚΚ) των ασθενών με ιδιοπαθή ΚΜ. Η επιρροή του ΑΝΣ στην ετερογένεια της ηλεκτρικής ανερέθιστης περιόδου του μυοκαρδίου των κόλπων είναι μάλλον περιορισμένη και εξαρτάται από τον βαθμό της ηλεκτρικής αναδιαμόρφωσης και σε ποιόν καρδιακό κόλπο αναφέρεται. Σε ασθενείς με περιορισμένη ηλεκτρική αναδιαμόρφωση του μυοκαρδίου, η ετερογένεια της ηλεκτρικής ανερέθιστης περιόδου δεν επηρεάζεται από τον αποκλεισμό του ΑΝΣ. Σε ασθενείς με έντονη ηλεκτρική αναδιαμόρφωση του μυοκαρδίου, λόγω της μακράς διάρκειας της ΚΜ, η ετερογένεια της ηλεκτρικής ανερέθιστης περιόδου ύστερα από αποκλεισμό του ΑΝΣ μειώνεται μόνο στο Δεξιό Καρδιακό Κόλπο (ΔΚΚ). Ωστόσο η επιρροή του ΑΝΣ στα περίπλοκα κλασματοποιημένα ηλεκτρογράμματα [Complex Fractionated Atrial Electrograms (CFAEs)], που καταγράφονται στη διάρκεια της ΚΜ, είναι μεγαλύτερη. Τα τμήματα του μυοκαρδίου των κόλπων με CFAEs είναι δυνατόν να εκπροσωπούν σημαντικές περιοχές για τη διατήρηση της ΚΜ, καθ' ότι φαίνεται να συσχετίζονται με ανατομικές και ηλεκτρικές αλλαγές του μυοκαρδίου. Όπως φαίνεται στο **Κεφάλαιο 3**, τα CFAEs επηρεάζονται επίσης από το φαρμακευτικό συστηματικό αποκλεισμό του ΑΝΣ.

Ο αποκλεισμός του ΑΝΣ στους κόλπους της καρδίας των ασθενών με ΚΜ μακράς διάρκειας μείωσε την επικράτηση των CFAEs, και στους κόλπους της καρδίας των ασθενών με νέο επεισόδιο ΚΜ, δηλαδή στην αρχική φάση της ΚΜ, απέτρεψε την χρονικά εξαρτώμενη εμφάνιση των CFAEs. Οπότε η παρουσία των CFAEs επηρεάζεται τουλάχιστον μερικώς από το ΑΝΣ. Παρόλα αυτά η ανίχνευση των CFAEs είναι αμφιλεγόμενη μεταξύ των διαφόρων ερευνητών. Η πιο ακριβής μέθοδος ανίχνευσης των CFAEs είναι η χρήση μονοπολικής ηλεκτρογραφικής καταγραφής με αφαίρεση της απομακρυσμένης κοιλιακής δραστηριότητας (χρυσή μέθοδος – gold standard). Στο **Κεφάλαιο 4** φαίνεται ότι η χρήση διπολικών καταγραφών θα μπορούσε να οδηγήσει σε παράλειψη περιοχών CFAEs ή σε λανθασμένα προσδιορισμένες περιοχές ως CFAEs. Επιπλέον ο προσανατολισμός του καθετήρα στο ενδοκαρδιακό τοίχωμα μπορεί να επηρεάσει την ανίχνευσή τους. Ανίχνευση CFAEs που προκύπτουν από μονοπολικές καταγραφές χωρίς την αφαίρεση της απομακρυσμένης κοιλιακής δραστηριότητας και με τον καθετήρα κάθετο στο ενδοκαρδιακό τοίχωμα είναι η πιο αξιόπιστη μέθοδος μετά τη χρυσή (gold standard).

Στο δεύτερο μέρος αυτής της διατριβής προσπαθήσαμε να διευκρινίσουμε το ρόλο της connexin (Cx) 40 πρωτεΐνης στην ΚΜ και την παρουσία του υπολλειπόμενου πολυμορφισμού στο Cx40 γονίδιο ως ένα γενετικό παράγοντα της ΚΜ. Στο **Κεφάλαιο**

5, βασισμένοι στην βιβλιογραφία, αναδείχθηκε ότι η ετερογενής κατανομή της Cx40 πρωτεΐνης στους κόλπους της καρδίας κατά τη διάρκεια της ΚΜ, μπορεί να συμβάλλει στη σταθεροποίησή της. Επιπρόσθετα, μεταβαλλόμενη έκφραση της Cx40 πρωτεΐνης στις μυοκαρδιακές προσεκβολές εντός των θωρακικών φλεβών μπορεί να είναι το υπόστρωμα για τις εστίες που πύροδοτούν την ΚΜ. Τελικά, αναδεικνύεται ότι ο υπολλειπόμενος πολυμορφισμός του Cx40 γονιδίου σχετίζεται με την παρουσία της ΚΜ σε ασθενείς με σύνδρομο Wolf-Parkinson-White (WPW) και σε ασθενείς με υπέρταση. Στο **Κεφάλαιο 6**, αποδεικνύεται ότι ο υπολλειπόμενος πολυμορφισμός του Cx40 γονιδίου δεν είναι παρών σε ένα μεγάλο πληθυσμό ασθενών με ιδιοπαθή ισχαιμικά αγγειακά εγκεφαλικά επεισόδια, και δεν θα μπορούσε να χρησιμοποιηθεί ως γενετικός δείκτης για την πρόβλεψη της ιδιοπαθούς ΚΜ σε τέτοιο πληθυσμό. Επιπλέον, στο **Κεφάλαιο 7** μελετήθηκε η επίδραση του υπολλειπόμενου πολυμορφισμού στο Cx40 γονίδιο στην έκφραση της Cx40 πρωτεΐνης στους κόλπους της καρδίας των ασθενών χωρίς ΚΜ. Ασθενείς που εμφανίζουν αυτόν τον υπολλειπόμενο πολυμορφισμό είχαν περιορισμένη έκφραση της Cx40 πρωτεΐνης στους κόλπους της καρδίας. Παρόλα αυτά η παρουσία αυτού του υπολλειπόμενου πολυμορφισμού δεν συσχετίσθηκε με την παρουσία ΚΜ στους μετεγχειρητικούς καρδιοχειρουργικούς ασθενείς. Ο ρόλος επομένως του υπολλειπόμενου πολυμορφισμού στο Cx40 γονίδιο στην ύπαρξη της ΚΜ δεν είναι ο ίδιος στους διαφορετικούς τύπους της ΚΜ. Τέλος, το **Κεφάλαιο 8** είναι η συζήτηση αυτής της διδακτορικής διατριβής σχετικά με τα αποτελέσματα της έρευνάς μας σε σύγκριση με αυτά άλλων μελετών, πιθανές ερμηνείες για τις διαφορές που παρατηρήθηκαν και μελλοντικές προοπτικές.

Chapter 9

Dankwoord

Σα βγεις στον πηγαιμό για την Ιθάκη, να εύχεσαι νάναι μακρύς ο δρόμος,
γεμάτος περιπέτειες, γεμάτος γνώσεις. Τους Λαιστρυγόνας και τους Κύκλωπας,
τον θυμωμένο Ποσειδώνα μη φοβάσαι, τέτοια στον δρόμο σου ποτέ σου δεν θα βρεις,
αν μέν' η σκέψις σου υψηλή, αν εκλεκτή συγκίνησις το πνεύμα και το σώμα σου αγγίζει.
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αν δεν τους κουβανείς μες στην ψυχή σου, αν η ψυχή σου δεν τους στήνει εμπρός σου.

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που με τι ευχαρίστησι, με τι χαρά θα μπαίνεις σε λιμένας πρωτοειδωμένους·
να σταματήσεις σ' εμπορεία Φοινικικά, και τες καλές πραγμάτειες ν' αποκτήσεις,
σεντέφια και κοράλλια, κεχριμπάρια κ' έβενους, και ηδονικά μυρωδικά κάθε λογής,
όσο μπορείς πιο άφθονα ηδονικά μυρωδικά σε πόλεις Αιγυπτιακές πολλές να πας,
να μάθεις και να μάθεις απ' τους σπουδασμένους.

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Αλλά μη βιάζεις το ταξείδι διόλου. Καλλίτερα χρόνια πολλά να διαρκέσει·
και γέρος πια ν' αράξεις στο νησί, πλούσιος με όσα κέρδισες στον δρόμο,
μη προσδοκώντας πλούτη να σε δώσει η Ιθάκη.

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ήδη θα το κατάλαβες η Ιθάκες τι σημαίνουν. (Κ.Π. Καβαφης).

Vertaling:

Als je de tocht aanvaardt naar Ithaka, wens dat de weg dan lang mag zijn,
vol avonturen, vol ervaringen. De Kyklopen en de Laistrygonen,
de woedende Poseidon behoef je niet te vrezen, hen zul je niet ontmoeten op je weg
wanneer je denken hoog blijft, en verfijnd, de emotie die je hart en lijf beroert.
De Kyklopen en de Laistrygonen, de woedende Poseidon zul je niet treffen
wanneer je ze niet in eigen geest meedraagt, wanneer je geest hun niet gestalte voor je geeft.

Wens dat de weg dan lang mag zijn. Dat er veel zomermorgens zullen komen
waarop je, met grote vreugde en genot zult binnenvaren in onbekende havens,
pleisteren in Phoenicische handelssteden om daar aantrekkelijke dingen aan te schaffen
van parelmoer, koraal, barnsteen en ebbehout, ook opwindende geurstoffen van alle soorten,
opwindende geurstoffen zoveel je krijgen kunt; dat je talrijke steden in Egypte aan zult doen
om veel, heel veel te leren van de wijzen.

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K.P. Kavafis (1863-1933 Alexandrië)

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Chapter 9

Curriculum Vitae

The author was born on October 31st 1979 in Athens, Greece. Secondary school was completed in 1997 at Varvakeio High school in Athens. In this year she started her study in biology at the University of Athens. The next year she started her study in medicine and in 2004 she graduated from the Medical School of the University of Athens. In September of that same year she did a 2 months research program at the department of Cardiology of the University Medical Center Utrecht under the supervision of prof.dr. R.N.W. Hauer. After this she went back to Athens, where she did research on hypertension under supervision of prof.dr. D. Cokkinos in the Onassis Cardiac Center. From September 2005 until September 2009 she came back to the University Medical Center Utrecht as a PhD student/ electrophysiology fellow, where most of the time she was to be found in the electrophysiology laboratory. Her supervisors were prof.dr. R.N.W. Hauer, prof.dr. J.M.T. de Bakker, prof.dr. P.A.F.M. Doevendans, dr. P. Loh and dr. F. Wittkampf. In January 2010 she started her cardiology training at the St. Antonius Hospital Nieuwegein under the supervision of dr. W. Jaarsma and dr. J. ten Berg. She started with one year of internal medicine at the Evaggelismos Hospital in Athens under supervision of prof.dr. E. Diamadopoulos. One year later she continued her internal medicine at the St. Antonius Hospital under supervision of dr. A.B.M. Geers. Starting January 2012 she will continue her cardiology training at the Gelre Hospital in Apeldoorn and in January 2013 she will come back to Nieuwegein in order to complete her cardiology training.

Chapter 9

Publications

Detecting Complex Fractionated Atrial Electrograms in Atrial Fibrillation: Unipolar versus Bipolar Recordings. Short title: Detecting Fractionated Electrograms in AF.
A.C. Linnenbank, S.M. Chaldoupi, A. Elvan, M.W. van Bemmel, P. Loh, J.M. de Bakker.
Manuscript in preparation.

The Influence of the Autonomic Blockade on Atrial Electrophysiological Properties in Patients with Idiopathic Atrial Fibrillation.
S.M. Chaldoupi, F.H.M. Wittkampf, A.C. Linnenbank, P.A. Doevendans, H. van Wessel, V.J.H.M. van Driel, A.M. de Vos, P. Westers, R.N. Hauer, J.M. de Bakker, P. Loh
Submitted.

Reduced Connexin (Cx) 40 protein expression in atria of patients bearing the minor Cx40 allele (-44 G→A).
S.M. Chaldoupi, L.E.G. Hubens, D.A. Smit Duijzentkunst, L. van Stuijvenberg, M.F.A. Bierhuizen, E.E.H.L. van Aarnhem, M. Nelen, J.M. de Bakker, R.N. Hauer, H.V. van Rijen, P. Loh, T.A.van Veen
Submitted.

Complex Fractionated Electrograms in the Right Atrial Free Wall and the Superior/Posterior Wall of the Left Atrium Are Affected by Activity of the Autonomic Nervous System.
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