

## **EUTRAF: The European Network for Translational Research in Atrial Fibrillation**

Atrial Fibrillation (AF) is the most common sustained cardiac rhythm disturbance, occurring in between 1 and 2% of the general population. It is estimated that over 10 million Europeans suffer from this arrhythmia and its prevalence is calculated to increase by at least 2 fold in the next 50 years as the population ages. Furthermore one in five of all strokes are attributable to AF. The ischemic strokes seen in association with the arrhythmia are often fatal, and those that survive are left more crippled by their stroke and more likely to suffer recurrent strokes. In summary, AF is associated with a 3 fold risk of heart failure, 2 to 3 times the likelihood of hospitalisation and double the mortality risk. At least one percent of the healthcare budget of Western European and North American countries is spent on the management of AF. In 2005 the annual direct costs of AF were estimated to be \$US 6.65 billion and previous estimates from France and the United Kingdom were similar per capita. Thus this disease presents a rapidly growing social, medical and public health problem in need of urgent solution.

The multiplicity of possible causes of AF presents a challenge in identifying those factors that are critical to the development of AF in specific contexts, such as hypertension or diabetes. In many instances not even simple associations have been determined and the possibility that these associations may have a mechanistic role is far from being understood. Thus, at present it is impossible to contemplate specific therapies that may target specific mechanisms that could then be eradicated or modified. Antiarrhythmic drug therapy is an excellent example of the resulting clinical approach to management.

Amongst those drugs that have been evaluated for use in AF some have been intended to inhibit the function of a particular ion channel, but this approach has not been successful because the drug target has not been specific for the atrium or for the arrhythmia. Instead, blunderbuss therapy such as amiodarone, which has a broad range of effects on ion channels and receptors etc., has proved the most useful therapy, but for reasons that we do not understand.

Similarly, although pulmonary vein isolation therapy was introduced with the specific intention of ablating or isolating the triggers for AF, it has now extended to include substantial modification of the substrate for AF. This therapeutic "advance" emphasises how little is known about the triggers for AF, substrate development and trigger-substrate interactions. Although it is well known that many cardiovascular diseases, pulmonary and systemic conditions, specific toxicities and perhaps subtle abnormalities of autonomic nervous function may predispose to AF the knowledge does not extend to any method of recognizing those at particular risk. No electrocardiographic or chemical biomarker has yet been identified that will accurately identify those who will develop AF. Thus there is little or no opportunity to initiate therapy sufficiently early to impede the onset of the arrhythmia or to properly prepare the patient for such a development. Often the first intimation of AF is the presentation of a patient with a devastating stroke.

The EUTRAF project is a unique European Network of highly successful investigators who have common interest in the pathophysiology and treatment of AF. By integrating the mutually complementary strengths and expertise from molecular and cellular electrophysiology to the study of large cohort of patients, this network will develop the most modern approaches toward increasing knowledge of the arrhythmogenic substrate, with the ultimate goal of developing novel treatments and diagnostic tools.

## **WP1: Identification of cellular and molecular mechanisms between stromal and parenchymal cells in AF**

### Insights into interactions between cardiomyocytes and myofibroblasts (Bern)

Mechanical interactions: After having shown in the past that cardiac myofibroblasts exert substantial arrhythmogenic effects on cardiomyocytes following establishment of heterocellular gap junctional coupling in vitro, we tested the hypothesis that mechanical strain acting on myofibroblasts may potentiate these effects. While application of up to 10% strain failed to affect impulse conduction in cardiomyocyte strand preparations despite causing a significant reduction of the resting membrane potential its constituent cells, it induced a substantial slowing of impulse propagation and maximal upstroke velocities in models of the fibrotic myocardium consisting of strands of cardiomyocytes coated with myofibroblasts. The apparently paradoxical finding that strain caused a substantial depolarization of both cardiomyocytes and myofibroblasts but had an appreciable effect on conduction only in the fibrosis model can be explained by taking into account the phenomenon of supernormal conduction. For cardiomyocyte strands displaying well polarized resting potentials under control conditions, strain related membrane depolarization did barely affect conduction velocity because it embraced the region of supernormal conduction. By contrast, resting potentials in cardiomyocytes coupled to myofibroblasts were depolarized beyond the region supporting supernormal conduction already under control conditions. Accordingly, any strain-related additional membrane depolarization induced considerable slowing of conduction. Overall, these findings suggest that presence of electrotonic crosstalk between stromal and parenchymal cells sensitizes cardiac tissue towards strain-induced conduction slowing which may contribute to an increased strain sensitivity of fibrotically remodeled myocardia. The results of this study have been published (Grand et al, Cardiovasc Res 2014).

Humoral interactions: Transforming growth factor-- $\beta_1$  (TGF-- $\beta_1$ ) is well established to play a pivotal role in fibrotic tissue remodeling by inducing fibroblasts to undergo a phenotype switch to myofibroblasts and by increasing secretion of extracellular matrix proteins. In the context of WP1, we found that TGF-- $\beta_1$  exerts substantial autocrine effects on cardiac myofibroblast electrophysiology (increase of transmembrane currents, membrane depolarization and increase of heterocellular gap junctional coupling to cardiomyocytes). In models of the fibrotic myocardium consisting of cardiomyocyte monolayers coated with myofibroblasts, TGF-- $\beta_1$  caused substantial slowing of impulse conduction and elicited high frequency ectopic activity. These arrhythmogenic effects of TGF-- $\beta_1$  were exclusively due to alterations in myofibroblast electrophysiology and an increase of gap junctional coupling to cardiomyocytes because TGF-- $\beta_1$  exerted little if any effects on single cardiomyocyte electrophysiology or on conduction in cardiomyocyte only monolayer preparation. These findings suggest that TGF-- $\beta_1$  may contribute to arrhythmogenesis during cardiac fibrotic remodeling by adversely affecting the electrophysiology of cardiac myofibroblasts and by increasing heterocellular gap junctional coupling to cardiomyocytes. (Manuscript ready for submission).

### Mechanical control of ion channel expression in atrial myocytes

Atrial myocytes are continuously submitted to various mechanical stresses including shear stress generated by the movement of the interstitial fluid between adjacent myocytes in the myocardial wall. We developed a system to reproduce in vitro the shear stress experienced by atrial myocytes. Increasing shear stress from 0.5dyn.cm<sup>-2</sup> to 4dyn.cm<sup>-2</sup> activates a large outward potassium current from atrial myocytes which is due to the increase in the density of the functional potassium channel, Kv1.5 at the plasma membrane as evidenced by single channel recording and TIRF microscopy. Shear stress stimulates channel exocytosis from the recycling endosome and requires an intact microtubule

network and involves the integrin/FAK signaling pathway. During atrial dilation in a model of heart failure and of AF susceptibility, The up-regulation of integrin/FAK signaling pathway is associated with a reduced response to shear stress in hemodynamically overloaded atria (Boycott et al. PNAS 2013). We also identified a new partner of the dystrophin complex, CASK, which organizes a subpopulation of sodium channel at the lateral membrane of atrial myocytes (Eichel et al. submitted).

#### Adipose tissue: a new player in the formation of the AF substrate

The amount of adipose tissue that accumulates around the atria is associated with a high risk and a poor outcome after treatments of AF. This association is not coincidental but it is due to complex and direct effects of the adipose tissue on the neighbouring atrial myocardium. We found that the epicardial tissue secretes cytokines, such as Activin A, that can cause the fibrosis of the atrial myocardium (Venteclef et al. Eur Heart J 2015).

Fatty infiltrates can be observed in the atrial subepicardium of adult patients. They are replaced by fibrosis during permanent AF both in human and in a sheep model contributing to the substrate of the arrhythmia. An immune response mediated by cytotoxic T-lymphocytes is involved in fibrosis of the adipose tissue (Haemers et al. Eur Heart Journal 2015).

#### Computational model to study the impact of the various determinants of the substrate for AF on the conduction of the electrical impulse

In the past, numerous computer simulations of cardiac tissue have corroborated the pro-arrhythmic effects of myofibroblasts on conduction. However, these studies have not explored the heterocellular interactions between cardiomyocytes and myofibroblasts at a sufficiently high spatial resolution permitting to resolve these interactions at the level of the individual cell. In order to characterize and predict the specific effects of individual myofibroblasts and of the detailed cellular arrangement of myocytes and myofibroblasts on conduction, we developed a high spatial resolution computer model of cardiac tissue. The model was constructed and validated as rigorously as possible based on experimental measurements conducted in Bern (e.g., cell dimensions, capacitance and intercellular coupling conductance). The model can be used as benchmark to predict the arrhythmogenic effect of specific heterocellular configurations.

One major advantage of our tissue model is that it allows the detailed examination of phenomena at the cellular scale, which is not possible in computational frameworks in which cardiac tissue is considered to have a homogeneous structure. Our tissue model can be readily extended by incorporating homo- and heterocellular humoral interactions, offering the perspective to study such interactions in detail. Importantly, the discrete cellular nature of our model also offers the possibility to examine the effects of interventions that affect each cell individually (e.g., gene therapies which are typically successful in only a fraction of cells), opening the prospect for extended applications of the model in the future.

#### **WP2: Identification and validation of novel ion channels and transporters for treatment of persistent AF**

The primary objective of WP2 was the characterization of molecular indicators ('biomarkers') and regulators of abnormal function of ion channels and transporters in the context of AF (e.g.  $I_{Ca,L}$ ,  $I_{K1}$ ,  $I_{K,ACH}$ ,  $I_{Kur}$ , RyR2 and NCX1). Multiple studies from all partners involved in WP2 have successfully addressed this objective. For example, EUTRAF partners could show that shear stress increases membrane availability of the Kv1.5-subunit underlying  $I_{Kur}$  from subcellular pools through an integrin-dependent pathway, thereby shortening atrial action potential duration<sup>1</sup>. Activation of this pathway

that enhances Kv1.5 trafficking may explain the increase in  $I_{Kur}$  despite reduced total Kv1.5 expression during chronic hemodynamic overload. These findings highlight the complexity of AF-related ion-current remodeling and further suggest  $I_{Kur}$  as a potential drug target. The selectivity and properties of several  $I_{Kur}$  blockers were evaluated as part of EUTRAF WP2.

There is also accumulating evidence for altered intracellular  $Na^+$ -handling and downstream  $Na^+$ -dependent ion-channel regulation in AF. We could show that both IK1 and  $I_{K,ACh}$  are regulated by intracellular  $Na^+$  in human atrial myocytes of sinus rhythm patients, whereas  $Na^+$ -dependent regulation of  $I_{K,ACh}$  was lost in chronic AF patients. Patients with chronic AF also have slightly reduced peak but enhanced late  $Na^+$  current, the latter being potentially arrhythmogenic in AF. We also identified novel ion channels contributing to atrial repolarization potentially representing novel drug targets for mechanism-based AF therapy. For instance amplitude of atrial-selective  $K_{2p3.1}$  currents was higher in right-atrial cardiomyocytes of chronic AF patients, resulting in shortened  $APD_{90}$  compared to patients in sinus rhythm.

Recent EUTRAF studies have furthermore identified a prominent role for  $Ca^{2+}$ -handling abnormalities including increased sarcoplasmic reticulum (SR)  $Ca^{2+}$ -leak and spontaneous SR  $Ca^{2+}$  releases leading to delayed after depolarizations in AF patients.

Work in transgenic mouse lines with specific defects in  $Ca^{2+}$ -handling recapitulated key components of the proarrhythmic  $Ca^{2+}$  handling in AF patients, validating the potential pathophysiological role of altered  $Ca^{2+}$  signaling in AF. Most important, the molecular underpinnings of  $Ca^{2+}$ -handling abnormalities were distinct between patients with paroxysmal AF (increased SR  $Ca^{2+}$ -uptake and  $Ca^{2+}$ -load, RyR2 expression and RyR2 open probability; unaltered NCX) and chronic AF (unaltered SR  $Ca^{2+}$ -load and RyR2 expression; increased RyR2 phosphorylation, RyR2 open probability and NCX). On the other hand, sustained tachycardia and AF as such have also been demonstrated to induce changes which reduce the likelihood of  $Ca^{2+}$ -related proarrhythmic cellular events ( $Ca^{2+}$  signaling silencing). It is likely that  $Ca^{2+}$  handling instability prevails in some cohorts, whereas  $Ca^{2+}$ -handling silencing prevails in other subpopulations of AF patients, pointing to a need for tailored therapeutic targeting of intracellular  $Ca^{2+}$  signaling in individual AF patients. Although these studies have identified important novel factors potentially contributing to AF pathophysiology, further studies are needed to establish that tailored therapy based on specific molecular alterations in  $Ca^{2+}$  handling improves outcomes in different AF groups.

### **WP3: Etiology-specific Mechanisms in Atrial Fibrillation**

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia. In about 90% of cases, AF occurs in the presence of other cardiovascular diseases like hypertensive heart disease, diabetes mellitus, and congestive heart failure. Due to the high prevalence in the general population, hypertension and diabetes are major predisposing factors. The aim of this work package is to determine the specific impact of hypertension, type-2 diabetes mellitus, ageing, and gender on atrial molecular biology, electrophysiology, and function. We hypothesize that these etiological factors cause distinct atrial remodeling and therefore require different therapeutic approaches to prevent the formation of the AF substrate.

The detailed characterization of atrial remodeling in the two rat models of hypertension (SHR) and type-2 diabetes mellitus (T2DM, ZDF) has revealed – in line with our main hypothesis – that there is disease-specific remodeling of atrial tissue. Atrial remodeling in T2DM is characterized largely by increased oxidative stress, inflammation and apoptosis. By contrast, atrial remodeling in hypertension

involves early and prominent augmentation of ET-1 and ANGII signaling associated with increased fibrosis and altered Ca<sup>2+</sup> signaling.

Based on our data, we identified the ET system as a potential therapeutic target in hypertensive heart disease to reduce/reverse atrial pro-arrhythmic remodelling: (1) the ET system was activated in SHR with increased expression of ET-1 and the ETAR; (2) ET-1 increased Ca<sup>2+</sup> transients in SHR atrial myocytes (to a larger extent than in WKY controls) and elicited arrhythmogenic SR Ca<sup>2+</sup> release (thus providing a potential trigger for atrial arrhythmias); (3) ET-1 and hypertensive heart disease are associated with atrial remodelling including hypertrophy, fibrosis and inflammation (thus providing a substrate for atrial arrhythmias). Finally, the ET system represents a "drugable" target as highly specific, small molecule inhibitors for ET receptors are available and used in the clinics already. Based on these considerations, we pursued the hypothesis that augmented ET-1 signalling in SHR atria contributes to atrial pro-arrhythmic remodelling and triggering of atrial tachyarrhythmias and that inhibition of ET signalling would ameliorate the atrial modelling. We tested this hypothesis in an in vivo study using the recently approved dual ET receptor antagonist, Macitentan.

In the DOCA-treated pig, an animal model of secondary hypertension, we could demonstrate that chronic hypertension was associated with higher AF inducibility. Reduced atrial contractility in vivo was related to Ca<sup>2+</sup> handling abnormalities at the myocyte level. Interestingly, we found different regulation of NCX function in LA and RA as a consequence of hypertension-induced remodelling. Atrial site-specific remodeling modulates the outcome of NCX inhibitors on restoring atrial function in hypertension.

Furthermore, we established a pig model of chronic hypertension to study hypertension-related atrial remodelling in a large animal model. We found that, in a porcine model of AF at an early stage, hypertension leads to structural remodeling, atrial myocyte hypertrophy and interstitial fibrosis, and increases AF stability but does not increase AF complexity. We therefore conclude that in this model of secondary hypertension, higher AF stability after 2 weeks of rapid atrial pacing is associated with both structural and electrical remodeling. Furthermore arterial hypertension triggers hypertrophic and profibrotic pathways which contribute to AF progression early in the disease and which may serve as early therapeutic targets in AF.

Transcriptome analysis revealed that there is a pacing-dependent induction of a specific adipocyte/adipositas-related gene panel in the atria. Applying a unique variety of different experimental models of AF (pig, ZDF rat, HL-1 cells) and tissue samples from patients with AF/SR we were able to demonstrate that RAP/AF has a clear, although minor effect on the induction of adipocyte/adipositas-related gene expression. Concomitant disease and AF risk factors substantially activate this gene panel and, thereby, mediate structural atrial remodeling, specifically the atrial adipocyte infiltration and lipid accumulation. We offer an explanation to the established beneficial effects of sartans or ACEi by demonstrating a substantial reversal of the AF/RAP/AF risk factor – induced adipocyte/adipositas-related gene expression. This supports the view that a combination of sartans/ACEi with other substances (e.g. PPAR $\gamma$  agonists) will add potentially to their therapeutic/preventive activity.

#### **WP4: Identification and preclinical testing of new 'upstream' therapeutic targets for sustained AF**

The overarching theme of WP4 was to identify and test the importance of mechanisms upstream of atrial remodelling in atrial fibrillation (AF). To date pharmacological strategies to restore sinus rhythm (SR) in patients with AF have been marred by poor efficacy and safety concerns related to their propensity to induce life-threatening ventricular arrhythmias. Similarly, whether AF ablation

techniques have long-lasting effects on the restoration of SR and a beneficial impact on patient outcomes remains to be demonstrated. Underlying AF resistance to treatment, is the ability of the arrhythmia to sustain itself by inducing electrical and structural remodelling of the atria, which, in turn, promotes AF maintenance and increases vulnerability to relapse. The mechanisms leading to atrial remodelling in AF are poorly understood and identification of atrial-specific molecular targets upstream of this process has been focus of our investigations.

We first examined the role of atrial reactive oxygen species (ROS) in predicting the new onset of post-operative AF and other complications after cardiac surgery, and evaluated the contribution of individual sources of ROS in the development of these complications in 303 patients. We found that patients who developed post-operative AF had significantly higher atrial ROS and that both basal and NOX2-derived ROS were strongly associated with the development of post-operative AF, even after adjusting for age, use of beta blockers and Euroscore.

To assess whether preoperative treatment with statins may have a beneficial effect on myocardial redox state in 42 patients and investigated the molecular mechanisms underlying this effect in 28 samples of the right atrial appendage obtained at the time of cardiac surgery. Statin therapy (simvastatin 20 mg od) for 3 days before surgery was sufficient to reduce atrial ROS production (vs. placebo). To further explore the mechanisms by which statins affect the myocardial redox state, we incubated right atrial samples from 26 patients with atorvastatin 20 $\mu$ mol/L for 1 hour. Atorvastatin caused a mevalonate-reversible reduction of myocardial Rac1 activity and ROS (derived from NOX2) generation. We also investigated time-dependent changes in the expression and activity of atrial ROS-generating systems in the right (RA) and left atrial (LA) myocardium of goats after 14 days (i.e., at a stage when electrophysiological remodelling is completed but structural changes are minimal) and 6 months (when atrial structural remodelling is fully established) of pacing-induced AF, and in their controls in sinus rhythm (SR) with or without atrial structural remodelling secondary to 4 weeks of complete atrioventricular block (AVB) induced by AV node ablation. These experiments showed that increased NOX2 activity account for the increase in atrial ROS production at 14 days after AF induction but no longer account for the increased atrial oxidative stress after 6 months. Together these findings suggest that NOX2 inhibition by statin therapy may reduce the new onset of AF and other post-operative complications in patients undergoing cardiac surgery, as suggested by several small studies. However, in the Statin in Cardiac Surgery Trial (STICS) perioperative statin treatment did not have a significant effect on the incidence of postoperative AF in 1992 cardiac surgery patients randomized to rosuvastatin (20 mg od) or placebo. Furthermore, acute kidney injury was significantly more common in the rosuvastatin group (absolute excess 5.4%; SE 1.9%;  $p=0.0047$ ); most of this excess was stage 1 acute kidney injury, but there was also a significant excess of stage 2 or 3 acute kidney injury (absolute excess 1.8%; SE 0.7%;  $p=0.0196$ ).

Mitochondria are also an important source of ROS in the atrial myocardium and we have observed an increase in mitochondrial ROS in atrial tissue from a goat model of long-term AF and in patients with permanent AF. A mismatch between perfusion and metabolic demand may account for these changes and our data have uncovered a reduced perfusion reserve in fibrillating atria of a pig model of AF. However, in pigs with one week of AF (early metabolic remodelling), we have observed reduced mitochondrial ROS production and an enhanced propensity for opening of the mitochondrial permeability transition pore, suggesting increased mitochondrial fragility.

Nitric oxide (NO) is known to regulate atrial electrical properties and exert anti-fibrotic and anti-thrombotic actions. Short-term AF has been reported to induce a profound reduction in atrial nitric oxide (NO) release in animal models and inconsistent changes in the "endothelial" isoform of NO synthase (eNOS). A "neuronal" NOS isoform (nNOS) is also constitutively expressed in the sarcoplasmic

reticulum and sarcolemmal membrane of cardiomyocytes (as part of the dystrophin-associated glycoprotein complex) where it regulates intracellular calcium fluxes and sarcolemmal ion conductance and prevents arrhythmic death in mice after myocardial infarction. Basal blood flow in the human coronary vascular bed and perfusion of the exercising muscle are also regulated by nNOS-derived NO and loss of sarcolemmal nNOS in the skeletal muscle of patients with Duchenne muscular dystrophy (DMD) leads the dystrophin-deficient muscle to ischemia during contraction, suggesting that subcellular localization of nNOS signalling may be an important function of the dystrophin-associated glycoprotein complex. We identified myocardial nNOS depletion as a novel molecular mechanism upstream of the atrial electrical substrate that sustains AF in humans. Upregulation of microRNA (miR)-31 in the human atrial myocardium is an early event in the natural history of AF that results in a profound reduction in atrial NO synthesis by accelerating nNOS mRNA decay, and repressing dystrophin translation, leading to altered nNOS subcellular localization and increased protein instability.

In conclusion, despite promising observations in experimental studies and small clinical trials, NOX2 inhibition by perioperative statin therapy did not prevent postoperative AF or perioperative myocardial damage in patients undergoing elective cardiac surgery and had an adverse effect on acute kidney injury. Conversely, by impairing muscle perfusion, altering Ca<sup>2+</sup> homeostasis and increasing membrane fragility, and cell death, atrial miR-31 upregulation and related dystrophin and nNOS depletion may impact on all aspects of AF-induced atrial remodelling. As upregulation of miR-31 is atrial-specific and its effects on atrial electrical properties are rapidly reversible, interventions targeted to this pathway may provide an effective and safer therapeutic option for patients with AF. More advanced delivery strategies (e.g., tissue-specific vectors and promoters) as well as the identification of mechanisms leading to atrial miR-31 upregulation by AF may circumvent the lack of cell-specific targeting that is currently a major drawback of miR manipulation for therapeutic purposes.

#### **WP5: Translating genetic contributors to AF into novel therapeutic targets**

Work package 5 made use of translational approaches to decipher the genetic components contributing to atrial fibrillation. Based on prior knowledge that patients with sodium channel mutations can develop atrial fibrillation at very young age, we studied the mechanisms leading to atrial fibrillation in mice carrying a sodium channel mutant in the late sodium channel (D-KPQ-SCN5A). We demonstrated that a prolongation of the atrial action potential, especially in bradycardic conditions, provokes afterdepolarizations and triggered activity in mice expressing that mutation. Furthermore, sodium channel inhibitors suppress these arrhythmias very effectively in mice 4, and there is some change in the late component of the late sodium current in atrial cardiomyocytes from patients with atrial fibrillation. Thus, inhibition of sodium currents with available antiarrhythmic drugs is an excellent way to prevent atrial fibrillation in patients who carry mutations in the cardiac sodium channel, and may have the potential to be used in personalized approaches to rhythm control therapy. We are currently exploring whether quantifiable markers on the ECG, e.g. the PR interval, can be used to identify patients who will benefit from sodium channel inhibitor therapy.

Starting from the known role of the cAMP responsive element binding protein (CREB) and cAMP responsive element modulator (CREM) for cardiac gene regulation, we studied the mechanisms leading to spontaneous atrial fibrillation in mice with cardiomyocyte-directed expression of CREM-IbΔC-X. CREM-TG mice develop spontaneous atrial fibrillation, atrial dilation, and myocardial thinning. These changes were combined with loss of sarcomeres and a decreased number of mitochondria and a non-typical pattern of myocardial hypertrophy markers up-regulated on the mRNA level. The remodeling of atria can be regarded as a key event in the development of AF in this mode.

Interestingly, both H1b1 and H1b2 can also be translated from other Crem mRNAs including the recently described small ICER (smICER) isoforms which are expressed from the Crem gene in an inducible manner. Interestingly, CREB and CREM target genes are dysregulated in human atrial with AF. Hence, CREM-TG may in part reflect the cardiac transcriptional response to the stimulation of the cAMP-dependent signal transduction pathway by plasma catecholamines elevated under stress and pathological conditions and may thereby reflect consequences of AF-related alterations of genetic regulation due to inhibition of CREB targets. We further identified genes differentially expressed along with the development of AF in CREM-TG and identified 63 genes consistently up- or down-regulated on both mRNA- and protein levels, at week 7 of life. A detailed biomathematical analysis revealed important signaling pathways regulating cardiac growth, the extracellular matrix and cardiac ion channels. Selected genes, up- or down-regulated in CREM-TG atria were further studied and tested in novel genetic mouse models. Annexin A4, up-regulated in CREM-TG atria, was identified as a novel regulator of the adenylyl cyclase, laying the foundation for further studies of CREM and annexin dependent genes for the development of AF. Based on these observations, we tested the potential of histone deacetylase (HDAC) inhibitors to counteract phenotypic changes in CREM-TG atria as well as alterations in atrial cardiac gene regulation due to the CREM transgene. This supports the idea that HDAC inhibition can antagonize effects of CREM repressors.

The most impactful and common gene variants found in patients with atrial fibrillation are single nucleotide polymorphisms on chromosome 4q25, close to the PITX2 gene. PITX2 is a transcription factor regulating left-right differentiation in the thorax and in the heart. Its function during embryologic development of the heart and lungs has been studied in detail, but there was very little data on the functional relevance of PITX2 in the adult heart at the start of the project. We therefore studied mice with a reduced expression of PITX2 and found that they are prone to inducible atrial fibrillation. The main functional change in this model is a shortening of the left atrial action potential without marked structural changes. Importantly, we described that PITX2 is a gene that is almost exclusively expressed in the left atrium, but not in other parts of the heart. We thus performed a systematic analysis of left atrial genes in the mouse atrium, and verified the selective expression of these genes in human left atria. PITX2 dependent genes are involved in ion channel homeostasis, inflammation, metabolic regulation, and other areas. Based on these insights we have started a research programme evaluating the functional role of PITX2 in the left atrium, supported by the British Heart Foundation and by Leducq Foundation. We have preliminary data that suggest that the atrial expression of PITX2 markedly modifies the response to antiarrhythmic drug therapy, and that such information can help to develop personalized therapy for atrial fibrillation. We will also test the relevance of PITX2 dependent genes in the response of the atria and the heart to injury and chronic exposure to damaging conditions such as hypertension and diabetes.

This information has, among other impacts that are outlined in the "EUTRAF summary publication", shaped published proposals for a pathophysiological classification of atrial fibrillation patients. Clearly, there is a need to determine clinically useful markers for different types of atrial fibrillation – caused by different pathologies – in patients. Such a classification of AF patients requires more research to better inform the clinical management of AF patients. We look forward to perform some of that work within the CATCH ME consortium ([www.catch-me.info](http://www.catch-me.info)) that has started to work in May 2015.

#### **WP6: New Diagnostic Tools for AF**

The electrophysiological mechanisms underlying AF are very heterogeneous. AF may be caused by the activity of ectopic foci, may be perpetuated by rotating wavefronts or by multiple wavelets. Also the degree of electrical dissociation and incidence of conduction block may vary from patient to patient.



Such differences likely have an impact on the efficacy of rhythm control strategies but usually are not taken into account during therapeutic decision-making. The most important reason for this is that we do not have sufficient diagnostic tools for the invasive and non-invasive classification of AF. Therefore the objectives of this WP were:

- To develop methods for the non-invasive assessment of the complexity of the AF substrate,
- To determine the predictive value of the non-invasive assessment of the AF substrate,
- To identify novel invasive tools to identify relevant targets for AF ablation,
- Develop a classification of AF based on the quantitative analysis of the AF substrate.

#### Non-invasive assessment of the complexity of the AF substrate

A software framework to analyse atrial activity extracted from 12-lead ECGs was created, and body surface potential maps (BSPM) of patients with AF (Lankveld et al. Heart, 2014). The software can extract the complexity of AF as well as the frequency behaviour of atrial electrical activity in various regions of the atria. For this purpose a large variety of time and frequency domain parameters are determined.

A novel, patented technique has been developed to determine AF complexity based on oesophageal ECGs. This technique allows for quantification of AF complexity in the posterior left atrial wall which is not well accessible for surface ECG characterisation. Also, echocardiographic surrogate parameters determined by tissue velocity imaging may serve as a tool for assessment of fibrillation wave complexity.

To detect electrophysiological alterations before the occurrence of persistent AF we also developed a software package for p-wave complexity analysis of BSPMs of patients in sinus rhythm (SR). A first analysis in patients with paroxysmal AF revealed increased substrate complexity in these patients compared to patients without a history of AF.

#### Predictive value of the non-invasive assessment of the AF substrate

The non-invasive assessment of AF complexity proved to be a promising new tool to predict AF treatment outcome in a variety of clinical settings (Lankveld et al, Heart, 2014). Success of pharmacological cardioversion was better predicted by a combination of ECG-derived AF complexity parameters than by conventional clinical predictors. The same was true for SR maintenance after electrical cardioversion of patients with early persistent AF. Finally, AF termination and long-term success of catheter ablation were equally well predicted by ECG-parameters and known clinical predictors. The combination of both classes of parameters further improved prediction of outcome. This technique may help in the future to select the appropriate rhythm control therapy for individual AF patients.

#### Novel invasive tools to identify relevant targets for AF ablation

Bipolar complex fractionated atrial electrograms (CFAE) have been suggested as suitable targets for catheter ablation of AF. However recent studies could not confirm the clinical value of CFAE. We systematically studied all commercially available algorithms for CFAE identification and found that they correlated poorly with the underlying electrophysiological substrate of AF. Therefore, we developed a novel unipolar fractionation index that accurately reflected fibrillation wave complexity. In collaboration with the partners from Osypka GmbH also new catheters for endocardial mapping were developed. These tools will help to better identify more reliable targets for AF ablation.

## Classification of AF

We designed a classification of AF based on the quantitative analysis of fibrillation wave front propagation. A novel, fully automated algorithm was developed and validated to detect atrial deflections and construct fibrillation waves (Zeemering et al. IEEE, 2012). In goat models of AF, increased complexity of the AF substrate was associated with increased endo-epicardial dissociation, increased incidence of transmural breakthrough and the development of a 3-dimensional AF substrate (Eckstein et al. Circ AE, 2013, Maesen et al. Circ AE 2013, Verheule et al. Circ AE 2013). In human AF, conduction pattern complexity was comparable between paroxysmal AF and acute AF, but higher in persistent AF. Also here, endomysial fibrosis was a strong determinant of atrial conduction disturbances. The data demonstrate that AF complexity is clearly enhanced in persistent AF but that the class 'paroxysmal AF' contains a very heterogeneous group of patients with very variable degrees of AF complexity.

The mechanisms and substrate responsible for the persistence of atrial fibrillation (AF) remains largely unknown. We aimed in WP-7 at providing better understanding, mapping and treatment strategies for this epidemic condition. Our achievements are summarized in respect to 4 major themes. Our first goal to characterize AF EP substrate using Monophasic action potentials (MAP) had to be abandoned as the MAP catheter was recall for safety reasons.

- 1) Validation of MRI imaging of atrial fibrosis
- 2) Computer model/in vivo experiment for substrate ablation
- 3) Optimization of curative AF treatment, new approaches
- 4) Clinical studies

## Validation of MRI imaging of atrial fibrosis

In an effort to better characterize AF substrate in patients, we studied 190 unselected consecutive patients referred for cardiac MRI with high resolution aDE imaging. The aim was describe the incidence and characteristics of atrial fibrosis. The population comprised 60 AF patients (26 persistent), and 130 patients without AF, including 75 with structural heart disease (SHD). After left atrial segmentation, aDE was quantified using adaptive thresholding. Regression analysis was performed to identify predictors of the extent of aDE. Areas of aDE were registered on an atrial template to study the aDE distribution in specific subpopulations. In the total population, age, AF and SHD were independently associated with aDE. Extent of aDE was increasingly observed from 11.1±4.7% in patients with no SHD nor AF, 18.8±7.8% in SHD and no AF history, 22.9±7.8% in paroxysmal AF, to 27.8±7.7% in persistent AF. Among non-AF patients, age and SHD were independently associated with aDE. Among AF patients, female gender and AF persistence were independently associated with aDE. aDE was variably distributed but was more frequently detected in the posterior left atrial wall.

We demonstrated that age, history of AF and SHD are the most powerful clinical predictors of atrial fibrosis, as detected by MRI, in a general population of cardiology patients. If atrial fibrosis is variably distributed, it predominates in the posterior LA wall.

## Computer model/in vivo experiment for substrate ablation

Isolating the pulmonary veins (PV) with radiofrequency ablation (RFA) is the cornerstone for treating atrial fibrillation (AF), but few proven alternative strategies exist for when it fails, particularly for persistent AF. To help develop and guide novel RFA strategies, phase mapping (ECGM) was recently introduced into the clinic for locating reentrant electrical waves (rotors) that perpetuate AF (see 3). We developed a novel computational model of the human atria suitable to rapidly and ethically test

various RFA strategies for terminating AF rotors identified with phase mapping. Specifically, we developed a novel bilayer model of the human atria that simulates both normal and AF electrophysiology, while also being computationally efficient. Model parameters were tuned and image-based fibrosis incorporated into the model in order to simulate monophasic action potential duration and activation patterns observed in healthy and AF patients. AF was initiated in the AF model by rapidly pacing the PVs during sinus rhythm, and rotor locations/dynamics quantified with phase analysis. Three RAF strategies were applied to determine their effect on the initiated AF: i) PV isolation, ii) guided RFA with non-conductive line, circle, broken circle, and cross lesions of various sizes placed far and near rotor locations/pathways identified with phase mapping, and iii) streamlining activation patterns obtainable with ECGM by performing linear RFAs paralleling activation.

This computer model showed the limits of the traditional PV isolation RFA strategy as it failed to terminate AF because PV isolation did not eliminate the AF rotor(s). Linear lesions at the roof and mitral lines were also unsuccessful and only the RF lesions placed on the trajectory of the rotor(s) were successful. Various RFA shapes with lengths or diameters from 0.5-1 cm were successful at terminating AF, though lines and broken circles performed differently than solid circles or crosses. In particular, the former was most successful at some LA locations, while the latter performed better at other LA locations. This model could be used when personalized to the patient by including the pre-ablation MRI, to guide the ablation strategy as tailored by the aDE localization and characteristics of the fibrosis as suggested by the work done in collaboration with Nathalia Trayanova.

Using optical mapping, we characterized acute and persistent AF in a sheep model. The first experiments showed that AF propagation is a 3D phenomenon with a range of transmural discordance. However, global parameters like dominant frequency and regularity index are well conserved across the transmural wall. In this acute model of AF, the arrhythmia is mostly driven by short-lived meandering rotors. We also demonstrated that dominant frequency was significantly higher in sheep model of chronic and that RF ablation significantly reduced DF.

We also developed in addition to the initial WP a collaboration with the group of Uli Schotten. P Pasdois from Bordeaux worked with S. Verheule from Maastricht, looking at the metabolic consequences of AF. They demonstrated for the first time that rapid atrial pacing induces a metabolic shift on the ventricle as well as on the atria. Some parameters showed that AF could lead to a less oxidative environment in the ventricle, a metabolic shift from fatty acid to glucose oxidation (at least at the mitochondrial level) and an increased capacity to buffer hydrogen peroxide. Although some of the effects were not statistically significant they suggest that sustained AF could lead to a metabolic remodeling in the atrial and ventricular mitochondria.

#### Optimization of curative AF treatment, new approaches

AF remains a challenging arrhythmia to treat. Drugs are not very effective and catheter ablation could be improved. Better mapping tools being desirable for both atrial tachycardia (AT) and AF.

The role of ECGM was addressed in both conditions:

Various AT (de novo and post-atrial fibrillation ablation) were mapped using ECGM followed by standard-of-care electrophysiological mapping and ablation in 52 patients. The ECGM consisted of recording body surface electrograms from a 252-electrode-vest placed on the torso combined with computed tomography-scan-based biatrial anatomy (CardioInsight Inc., Cleveland, Ohio).

Comparison between ECM and electrophysiological diagnosis could be accomplished in 48 patients (48 AT). ECGM correctly diagnosed AT mechanisms in 44 of 48 (92%) AT: macro-re-entry in 23 of 27;

and focal-onset with centrifugal activation in 21 of 21. The region of interest for focal AT perfectly matched in 21 of 21 (100%) AT. ECGM was demonstrated to accurately diagnose the mechanism of AT and the location of focal arrhythmia.

This technology was also assessed in persistent AF, coupled with phase mapping. We hypothesized that by allowing to visualize AF sources, it would simplify dramatically the ablation procedure. 103 consecutive patients with persistent AF were mapped. The maps showed incessantly changing beat-to-beat wave fronts and varying spatiotemporal behavior of driver activities. Reentries were not sustained (median, 2.6 rotations lasting  $449\pm 89$  ms), meandered substantially but recurred repetitively in the same region. In total, 4720 drivers were identified in 103 patients: 3802 (80.5%) reentries and 918 (19.5%) focal breakthroughs; most of them co-localized. Of these, 69% reentries and 71% foci were in the left atrium. Driver ablation alone terminated 75% and 15% of persistent and long-lasting AF, respectively. The mean radiofrequency delivery to AF termination was  $28\pm 17$  minutes versus  $65\pm 33$  minutes in the control group ( $P<0.0001$ ). At 12 months, 85% patients with AF termination were free from AF, similar to the control population (87%). Using ECGM, we could demonstrate that persistent AF is maintained predominantly by drivers clustered in a few regions, most of them being unstable reentries. (Circulation. 2014;130:530-538.)

We also developed in collaboration with Biosense Webster a new multipolar irrigated circular ablation catheter, the nMARQ. It was used for paroxysmal AF ablation in 39 pts in Bordeaux. 98% of the PVs could be acutely isolated using solely the nMARQ catheter by applying a mean total of  $10.0\pm 4.6$  min of RF energy. The mean total procedure duration was  $86\pm 29$  min, and the mean fluoroscopy time was  $22.2\pm 6.5$  min, respectively (Europace (2014) 16, 1296–1303). These numbers are approximately half of what is reported in the literature.

This catheter was also used in a multicenter study including 374 pts and confirmed the initial benefit (JCE 2015).

#### Clinical studies

We sought to assess the role of drugs on AF complexity, using ECGM and ECG complexity in collaboration with U Schotten. 43 of the 50 included pts were analyzed. Flecainide was shown to reduce dramatically the number of rotors and the number of regions hosting rotors. The ECG AF complexity was also dramatically reduced by Flecainide. Interestingly, the ECGM rotors resistant to Flecainide were at the site of AF termination during ablation in 80% of pts. In 2 patients, Flecainide had no impact on rotor and ablation failed to terminate AF. This work suggests that ECGM combined with Flecainide infusion prior to persistent AF ablation could be used to guide RF delivery to the rotors resistant to Flecainide and therefore further shorten the procedure. This test could also be used to screen good candidates for catheter ablation pending confirmation of the present observation.

We also investigated whether Eplerenone, a major anti-fibrosis drug, could stabilize or reverse the atrial remodeling in high cardio-vascular risk patients. 50 pts were randomized to Eplerenone (27) or control (23). MRI including aDE were performed at inclusion and after 6 month of treatment. The atrial dimensions were unchanged and the impact on fibrosis is under evaluation.

#### **WP8: Development of the IT infrastructure for knowledge discovery**

Work Package 8 achieved three major tasks:

- 1) Supporting consortium partners by developing a web-based collaboration environment to facilitate scientific communication between the partners via data collection, data warehousing and integrated data analysis for basic and clinical research;
- 2) Generation of a new knowledge and insight on Atrial fibrillation (AF) and its complications through the application of novel machine learning and data mining methods and algorithms on clinical and experimental datasets;
- 3) Developed a risk model based on data mining, and the pertaining clinical decision support system (CDSS) to assess the risk of AF and its complications.

MITIS has developed a perfect platform, which has served both the basic scientists as well the clinicians in the consortium for data sharing, data analysis, communication, and collaboration. MITIS has established very close collaborations with the partners by engaging in various collaborative data mining studies over different clinical data sets. One major achievement was with the FLEC-SL data set, as it has been possible to discover new knowledge that was not detected by standard biostatistical methods. The results have been reported in a manuscript, which has been submitted to the European Heart Journal. The collaborative interaction with Prof. Andreas Goette's group on the ANTIPAF data set has also generated new scientific knowledge and insight, and has led to the preparation of another manuscript to be submitted to a major journal. The data mining studies with the Dresden Group with the lead of Prof Ursula Ravens have produced two manuscripts, one of which has already been published.

AF is the most frequent arrhythmia and causes high morbidity and mortality. The major morbidity is stroke; the probability of which increases five times with AF, and stroke associated with AF has a much more severe course. Oral anticoagulation has been shown to reduce the risk of stroke substantially. However the risk of bleeding associated with oral anticoagulant use begets the need of a risk-benefit evaluation and several risk score systems have been used so far for this purpose. Unfortunately, none of the hitherto developed score systems has been ideal for the identification of both high risk and low risk patients effectively, and has been able to optimize the risk tradeoff of stroke prevention by anticoagulation, and bleeding due to anticoagulation. We have had the privilege of accessing the National Claims Database of the Turkish Social Security Institution, and as part of the EUTRAF project, we were able to analyze the data of 545,008 patients with AF. This is the largest cohort in the world to date, and it gave us the opportunity of involvement and developing expertise in "Big Data Analysis", which is relatively new in Europe. Employing novel Big Data technologies and methods, we were able to bring new knowledge and insight to several aspects of the epidemiology of AF.

Indeed the results of analysis have been game changing in many ways. First of all, the findings related to the co-morbidities and the contributions of co-morbidities to the stroke risk have been helpful to solve many controversial issues in AF. For example coronary and peripheral arterial diseases under the name of vascular diseases have been suggested to increase the risk of stroke and included in CHA2DS2-VASc scoring system. However we were not able to show this relation in our large data set (Table 2). More interestingly, over the time frame of the EUTRAF project, this same finding has been repeatedly shown by others in the post-hoc analysis of ROCKET-AF, ATRIA Study and the preliminary analysis of the GARFIELD Registry. We could not show any relations to the increased risk of stroke with COPD, CKD and hyperthyroidism. On the other hand we have found that the age of the patient was the main driver of the risk in stroke in AF and we have found a stepwise increase between the age 64-75, 75-79 and over 80. History of previous stroke was another very strong predictor of stroke, and gender difference did not matter under the age of 65. We have concluded that the risk for women was increased after the age of 65. Based on these findings, we developed a new risk score system to assess the risk for stroke in AF, which we formulated as CHADS-F (Table 3). As seen in Table 3, we have

given the age a special emphasis by three levels and the female gender gets 1 point only after the age of 65. We give prior stroke 3 points, as per the very high risk attributed to the prior stroke in the cohort. Using propensity score matching, and harm/utility analysis from decision theory, we have shown that the CHADS-F scoring system and its risk stratification scheme in fact better optimize the risk-benefit trade-off of stroke prevention and hemorrhage due to anticoagulation compared to the existing CHADS2 and CHA2DS2-VASc scoring systems.

In addition to the above, we have also performed a wide variety of data mining exercises and logistic regression analysis on the datasets and we determined the contribution of several clinical and demographic parameters in prediction of stroke.

Based on the results of data mining and Cox regression analysis in our large dataset, we have developed a clinical decision support system (CDSS). The aim of such a system is to help the practicing physician to reach correct clinical decision in the risk evaluation of their patients with AF and give oral anticoagulation to the appropriate patients with high risk who will get benefit and not anti-coagulate those with low risk for stroke and put harm on them. The "CDSS" is currently the most detailed assessment tool created to date for predicting the risk of stroke/TIA/thromboembolism in non-valvular AF patients. It is also the tool that is based on the largest patient cohort in the world, and that uses the most recent and widely-accepted data analysis methods.

The CDSS is also innovative in terms of its data paradigm as well as its architecture. It has been designed in an adaptive, service-based architecture to automatically learn from new datasets that are added to the database. This will be a crucial tool as we will be growing the TRAF registry very soon with a new batch of data, and will also be integrating other global databases in it as well. It has been important to build a self-learning and self-updating system that can scale with new batches of data.

The CDSS client, built as a mobile application for all iOS and Android devices, is now available for a free download on App Store iTunes and Google Play. We firmly believe that such an aid will be very helpful to the practicing clinician for decision making in a busy practice and provide greater awareness and insight to the risks of AF and its complications.

#### **WP9: Dissemination and Exploitation**

The EUTRAF partners were very active in publishing project results once approved by the Exploitation Manager and the whole through peer-reviewed publications, press-releases, posters at relevant conferences and exhibition events such as worldwide meetings for the American Academy of Cardiology (AAC), American Heart Association (AHA), or the European Society of Cardiology (ESC), in addition to national events such as the British Cardiovascular Society (BCS) and Heart Rhythm Congress (HRC) in the UK.

The consortium published 70 articles describing original data obtained during the EUTRAF project. The total impact factor of the articles is 496. The 16 articles with the highest impact factors have a sum impact factor of 240.

However in total the partners published 137 articles which mention EUTRAF funding.

The final PUD was written and this included the project exploitation agreement.

#### **WP11: Ethical Monitoring**

An external Ethics Committee was appointed. The committee was constituted of a chair and two additional people (1 expert in animal research and 1 expert in human research) and a procedure was agreed to properly handle Ethical matters.

Each partner presented their own ethical requirements/study to their own ethical committee for consideration. As soon as an ethical document was raised this was sent to David Cartlidge (Heri) who inputted the information on to the Ethics map and also stored on the EUTRAF project portal.

During the project there were Twenty Five (25) Ethics Approvals for Humans, Thirteen (13) Ethics Approvals for Animals, Seven (7) Patient Consent Forms.

### **Consortium**

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