Arrhythmogenic gene remodelling in elderly patients with type 2 diabetes with aortic stenosis and normal left ventricular ejection fraction

Running Title: Arrhythmogenesis and Genetic Change in Diabetes

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Word Count: 4876

References: 59

Subject area: Heart/cardiac muscle

Keywords: Diabetes, Potassium channels, Action potential, Repolarisation, Genetics

New Findings

- **What is the central question of this study?**

  Type 2 diabetes is associated with a higher rate of ventricular arrhythmias compared to the non-diabetic population, but the associated myocardial gene expression changes are unknown; furthermore, it is also unknown if any changes are due to chronic hyperglycaemia or a consequence of structural changes.

- **What is the main finding and its importance?**

  We found downregulation of left ventricular ERG gene expression and increased NCX1gene expression in humans with type 2 diabetes compared with control patients with comparable left ventricular hypertrophy and possible myocardial fibrosis. This was associated with QT interval prolongation. Diabetes and associated chronic hyperglycaemia may therefore promote ventricular arrhythmogenesis independently of structural changes.

Abstract

Type 2 diabetes is associated with a higher rate of ventricular arrhythmias, and this is hypothesized to be independent of coronary artery disease or hypertension. To investigate further, we compared changes in left ventricular myocardial gene expression in type 2 diabetes patients with patients in a control group with left ventricular hypertrophy. Nine

control patients and seven patients with type 2 diabetes with aortic stenosis undergoing aortic valve replacement had standard ECGs, signal-averaged ECGs and echocardiograms before surgery. During surgery, a left ventricular biopsy was taken, and mRNA expressions for genes relevant to the cardiac action potential were estimated by RT-PCR. Mathematical modelling of the action potential and calcium transient was undertaken using the O'Hara–Rudy model using scaled changes in gene expression. Echocardiography revealed similar values for left ventricular size, filling pressures and ejection fraction between groups. No difference was seen in positive signal-averaged ECGs between groups, but the standard ECG demonstrated a prolonged QT interval in the diabetes group. Gene expression of KCNH2 and KCNJ3 were lower in the diabetes group, whereas KCNJ2, KCNJ5 and SLC8A1 expression were higher. Modelling suggested that these changes would lead to prolongation of the action potential duration with generation of early after-depolarizations secondary to a reduction in density of the rapid delayed rectifier K+current and increased Na+–Ca2+ exchange current. These data suggest that diabetes leads to pro-arrythmogenic changes in myocardial gene expression independently of left ventricular hypertrophy or fibrosis in an elderly population

Abbreviations. 2D, 2-dimensional; **AP,** action potential; **BMI,** body mass index; **CACNA1c-d,** L-type voltage gated Ca^2 channel α subunit 1c-d**; CX40-43,** connexin 40-43; **EAD,** early after-depolarisation**; EF,** ejection fraction; **ERG,** ether-à-go-go-related gene; **DAD**, delayed after depolarization: **IFCC,** international federation of clinical chemistry and laboratory medicine**; HCN 1-4**, Na^+/K^+ hyperpolarisation-activated cyclic nucleotide gated channel 1-4; I_{CaL}**,** L type Ca^{2+} current; I_F, funny current; $I_{K,1}$**,** inward rectifying K^+ current**;** $I_{k,ach}$ **,** acetylcholine activated inward rectifying current**;** $I_{K,r}$**, r**apid delayed rectifier K^+ current; $I_{K,ur}$, ultra-rapid delayed rectifier K^+ current ; $I_{K,s}$, slow delayed rectifier K^+ current; I_{Na}, fast Na^+ current*;* $I_{Na/Ca}$, Na^+-Ca^{2+} calcium exchange current**;** I_{Ti}, late transient inward current; I_{to-f}, fast-transient outward K^+ current; I_{to-s}**,** slow transient outward K^+ current; **KChIP2**, K^+ voltage gated channel interacting protein 2; **KCNA4-5,** K^+ voltage gated channel subfamily A

member 4 and 5; **KCND2/3,** K^+ voltagegated channel subfamily D member 2-5; **KCNJ2/-5,** K^+ voltage gated channel subfamily J member 2- 5; **KCNQ1,** K^+ voltage gated channel subfamily Q member 1; **LV,** left ventricle; **MAPSE,** mitral annular longitudinal excursion**;** **MRI,** magnetic resonance imaging**; NCX1**, Na^+-Ca^{2+} calcium exchanger**; OCT,** optimal cutting temperature compound; **qPCR,** quantitative polymerase chain reaction measurement; **RYR2**, ryanodine receptor 2**; SAECG**, signal averaged ECG; **SCN5a**, Na^+ voltage gated channel subunit α5; **SERCA2a,** sarcoplasmic reticulum Ca^{2+}-ATPase**;** $\mathbf{T_1}$, spin–lattice relaxation time

Introduction

Type 2 diabetes is becoming increasingly common worldwide and is associated with ischaemic heart disease and other macrovascular diseases. A cardiomyopathy related to chronic damage from hyperglycaemia has been described and termed ‘diabetic cardiomyopathy’(Miki *et al.*, 2013). Higher rates of cardiac arrhythmias are observed in diabetes (Suarez *et al.*, 2005) and specifically in type 2 diabetes (Panova & Korneva, 2006) compared to control patients without diabetes, both in patients with established coronary artery disease and in the general population (Movahed *et al.*, 2007).

The mechanisms predisposing to increased arrhythmogenesis in patients with type 2 diabetes are complex but it does seem to be independent of coronary artery disease and cardiac ejection fraction (Junttila *et al.*, 2010; Eranti *et al.*, 2016). Suggested potential disease specific mechanisms from animal studies include an abnormal responses to catecholamines (Frier *et al.*, 2011), inhibition of the ERG channel (Zhang *et al.*, 2006) or alterations in cardiac ryanodine receptor number/function, as a result of chronic hyperglycaemia (Yaras *et al.*, 2005). In addition to effects of type 2 diabetes, the treatment of

this condition can potentially be arrhythmogenic, with hypoglycaemia as a result of treatment with insulin or sulfonylureas linked to arrhythmias (Lindstrom *et al.*, 1992) and specifically as sulfonylureas also have an effect on cardiac K^+ channels (Brady & Terzic, 1998). In addition to pro-arrhythmic cellular and channel level changes, arrhythmogenic structural changes such as left ventricular hypertrophy (LVH) and myocardial fibrosis are common in type 2 diabetes (Karagueuzian, 2011). Cardiac hypertrophy and fibrosis in the absence of hypertension or ischaemia, can be seen in other cardiac diseases such as valvular aortic stenosis (Weidemann *et al.*, 2009).

In this study, we measured the gene expression of key ion channels and associated molecules to investigate what changes may underpin the higher arrhythmic event rate in patients with type 2 diabetes and how, using mathematical modelling based on gene expression data the action potential may be affected. We undertook this study in patients with aortic stenosis, a condition which also causes LVH and myocardial fibrosis, to try and identify changes specific to diabetes.

Methods

Ethics approval

The study was approved by the NHS Liverpool East Research Ethics Committee (11/NW/0290) and conducted in accordance with the principles established in the Declaration of Helsinki sixth version. The study was not registered in a research database and this is an exception to clause 35 of the Helsinki declaration. All participants gave written informed consent.

Study population

Criteria for inclusion were patients over 18 years of age, male or post-menopausal female, with or without type 2 diabetes, undergoing aortic valve replacement for aortic stenosis (calculated aortic valve area of $1cm^2$ or less) with preserved left ventricular function defined

by European Society of Cardiology and an ejection fraction of over 50% (Nagueh *et al.*, 2009) at Liverpool Heart and Chest Hospital.

Exclusion criteria were patients in atrial fibrillation, patients with type 1 diabetes, chronic renal impairment with an estimated glomerular filtration rate of less than 30ml/minute (Levey *et al.*, 1999), need for surgery in addition to the aortic valve replacement other than coronary artery grafting, other conditions which may cause LV impairment such as regurgitant valvular lesions graded moderate or more, thyroid dysfunction and excess alcohol consumption.

Our control group had their available medical records reviewed for previous random blood sugar measurements and fasting samples. All patients in the control group had not had any abnormal fasting glucose measurements (>7.0mmol/L) or random samples over 11.0mmol/L with typical symptoms(American Diabetes Association, 2015).

Patients had their height, weight and waist circumference measured, body mass index (BMI) calculated and patients with diabetes had their most recent glycated haemoglobin(HbA1c) recorded using the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) method(John *et al.*, 2007). In addition, medications for each patient's diabetes treatment and any cardiac specific medication was recorded.

ECG assessment

All patients had a standard 12 lead ECG and their QT interval measured using the Bazett formula (Luo *et al.*). All patients underwent signal averaged ECG monitoring (SAECG) following a 10-minute rest in the supine position using a MAC 500 machine (GE healthcare,Madison, WI,USA) prior to cardiac surgery. Digital filtering was performed with a 40-250HZ band pass bidirectional filter and averages were taken over 250 beats with maximum acceptable noise of 0.5µV.

All ECGs were analysed for late potentials (Okin *et al.*, 1995).

Echocardiography

All patients underwent 2-dimensional (2D) echocardiography study prior to cardiac surgery using commercially available vivid Q, vivid 7 (GE healthcare, Hatfield, Hertfordshire, UK) and IE 9 (Phillips, Guilford, Surrey) systems and a 3.5 MHz transducer.

Assessment of strain was made using offline software (EchoPAC, GE healthcare) and measurements were made in apical 2, 3 and 4 chamber for global longitudinal strain with values from each segment being averaged for a mean score. Circumferential and radial strain was measured using a mean score from 6 segments in the parasternal short axis at the mid wall level as previously described (Hung *et al.*, 2010).

Several studies have reported that myocardial fibrosis can associated with higher rates of arrhythmias(de Jong *et al.*, 2011) and is widely reported in patients with diabetes (Picatoste *et al.*, 2013) and when trying to allow for this potential confounder we have tried to match our experimental group to a control group reported to also exhibit signs of myocardial fibrosis (patients with aortic stenosis). Myocardial fibrosis can be assessed by cardiac MRI (magnetic resonance imaging) or histological examination, but there have been studies in aortic stenosis (Weidemann *et al.*, 2009) and diabetes (Ng *et al.*, 2012) looking at the correlation of 2D speckle tracking strain rate using echocardiography with fibrosis in patients with normal ejection fraction values. A global contrast-enhanced myocardial T_1 mapping time (spin–lattice relaxation time) on MRI of less than 500ms has been shown to correlate with fibrosis(Iles *et al.*, 2008) and a T1 mapping time of less than 500ms has been correlated with a global longitudinal strain of less than -18% using 2d speckle tracking echocardiography (Ng *et al.*, 2012).

Left ventricular biopsy

Patients had a biopsy sample taken by a consultant cardiothoracic surgeon through the LV apex in the position normally used for transapical aortic valve implantation at the time of

aortic valve replacement surgery. The biopsy was performed using a single TRUCUT™ biopsy needle (Cardinal Health, Dublin, Ohio, USA) just prior to the insertion of cardioplegia solution. The sample was then taken and immediately placed in physiological Hartmann's solution (Baxter Health, Berkshire, UK) before immediate transfer to the lab for processing. All samples were mounted in OCT (optimal cutting temperature compound, Tissue Tek, Fisher Scientific, Loughborough, UK) before flash freezing in liquid nitrogen.

RNA isolation and quantitative polymerase chain reaction measurement (qPCR)

Briefly, from frozen tissue, RNA was isolated using *mir*Vana RNA isolation kit (Life Technologies, Paisley, UK) and then amplified to cDNA using high-capacity RNA to cDNA for quantitative PCR (Applied Biosystems, Warrington, UK).

Quantitative PCR was performed using custom preloaded low-density Taqman array micro-fluidic cards which contain multiple specific individual custom gene primer sequences and universal Mastermix II and a 7900HT fast real-time PCR system (all Applied Biosystems). Samples were run with one control sample with distilled water for every gene target run per card. Expression of the gene targets was referenced to an abundant abundant housekeeper gene, 18-S in this study. We chose 18-s as the housekeeper gene for this study based on previous experimental work(Sharma *et al.*, 2004; Pérez *et al.*, 2007) and preliminary animal data from a previous study performed within the department (Ashrafi *et al.*, 2016). Initial mRNA expression data was logarithmically transformed using the $2^{-\Delta\Delta CT}$ method as previously described (Livak & Schmittgen, 2001).

The list of target genes analysed with their context sequences is listed below in Table 1:

Gene	Gene name	Context sequences
18-S	Eukaryotic 18-S	CCATTGGAGGGCAAGTCTGGTGCCA
ATP2A2	Cardiac Sarcoplasmic Reticulum Ca^{2+}Activated- ATPase 2a	AGATGTCTGTCTGCAAGATGTTTAT
CACNA1C	L-type Voltage-Gated Ca^{2+} Channel Alpha Subunit 1c	ACCAATTCCAACCTGGAACGAGTGG

CACNA1D	L-type Voltage-Gated Ca^{2+} Channel Alpha Subunit 1d	GAATGGAAACCATTTGACATATTTA
HCN1	Hyperpolarisation Activated Gated K^+ Channel 1	ATCAGTGGGAGGAGATCTTCCACAT
HCN2	Hyperpolarisation Activated Gated K^+ Channel 2	CCCTACAGTGACTTCAGATTTTACT
HCN4	Hyperpolarisation Activated Gated K^+ Channel 4	ATGATGGCTTATTACAGTGGCAATG
KCNA4	K^+ voltage-gated channel, shaker-related subfamily, 4	ATGGGAGGCTTGCTGAACATGGATA
KCNA5	K^+ voltage-gated channel, shaker-related subfamily, 5	TCTAACAGCCGATCCAGTTTAAATG
KCND2	K^+ voltage-gated channel, Shal-related subfamily, 2	CACAACCAGTCGCTCCAGCCTTAAT
KCND3	K^+ voltage-gated channel, Shal-related subfamily, 3	CTCTGGCTCTGAGGAGCTGATCGGG
KCNH2	Human Ether Related a Go-Go	CAGTTCTTTCCTCAAGGAGACTCCA
KCNIP2	K_v channel-interacting protein 2	CCTGCGCCAGCAACAGGACATGTTC
KCNJ2	K^+ inwardly-rectifying channel, subfamily J, 2	AAGCTGCTCAAATCTCGGCAGACAC
KCNJ3	K^+ inwardly-rectifying channel, subfamily J, 3	GTGGAAGCCACAGGCATGACCTGCC
KCNJ5	K^+ inwardly-rectifying channel, subfamily J, 5	CACCCACATCTCACAGCTGCGGGAA
KCNQ1	K^+ voltage-gated channel, KQT-like subfamily, 1	CATTACTGCAGGCCACCTACTCATG
RYR2	Ryanodine Receptor 2	CCCGCCAGACACGACCACGCCATCG
SLC8A1	Na^+-Ca^{2+} Exchanger	TCACTGTCAGTGCTGGGGAAGATGA
SCN5A	Na^+ Channel, Voltage-Gated Type Vα	TGAGAAAGTGTACCACATCTGTGTG

Table 1: Gene targets and primer sequences

Mathematical modelling of the action potential

In this study we used the O'Hara-Rudy dynamic model (O'Hara *et al.*, 2011), which is a mathematical model simulating the human ventricular myocyte action potential transmurally, to look at the effect of the changes in mRNA seen in the diabetes group. This model has been used in a wide variety of experiments and experimental conditions with good validation(Bartos *et al.*, 2013). Using this model, channel conductance was scaled per the measured average ratio of mRNA between the control and the diabetes groups. In the control and the diabetes groups, the models were run for a 5-s period to obtain a stable state condition before a sequence of external stimulus pulses (with an amplitude of 0.8 nA, duration of 5 ms and frequency of 1 Hz) were applied, to evoke an action potential. To evaluate the relative role of each of the remodelled ion channels, simulations were also performed by looking at the change to each individual ion channel alone.

Statistical analysis

Patient characteristics are shown as mean and 95% confidence intervals. Experimental data are reported as mean ± SD .All single variable experimental work was analysed for significance using a Student's unpaired *t*-test comparing the control group to the type 2 diabetes group. As previously described multiple t-tests with a Benjamini-Hochberg false discovery rate correction (≤ 0.05) was used to assess the significance of the mRNA expression results (Tsay *et al.*, 2015). . Results were taken as being significant with a *P* value of ≤0.05.

Results

Patient characteristics

A summary of patients recruited and their mean baseline information is shown in Table 2 and there were no were no significant differences in baseline patient characteristics (*P*=ns). Patient medication breakdowns are shown in Table 3 with no significant difference in medications usage between groups for β-blockers, angiotensin blocking drugs (ACE/ARB) or statin use.

Table 2: Patient characteristics

Table 3: Patient medications

ECG assessment

Standard ECG assessment of the corrected QT intervals revealed a higher mean QT interval in the diabetes group (n=9) (467ms±2.32) compared with the control group(n=7) (451ms±5.26; *P*=0.011). SAECG assessment of our 2 groups yielded 1 patient in each group with a positive SAECG for late potentials.

Echocardiographic assessment

Our results show that both study groups were comparable in terms of traditional measurements of left ventricular function (ejection fraction (EF)) and left ventricular wall thickness (septal width). Both groups showed significant hypertrophy of the left ventricular septum when compared to reference values (Lang *et al.*, 2005) and the left ventricular hypertrophy seen was felt to be due to pressure loading from the aortic stenosis and therefore by European society of cardiology guidance is appropriate and not suggestive of hypertrophic cardiomyopathy(Authors/Task Force *et al.*, 2014). However longitudinal function was lower in the diabetes (n=9) group measured using mitral annular longitudinal excursion (MAPSE) and there was greater left atrial size compared to the control group (n=7), consistent with other studies (Table 4) (Ha *et al.*, 2007).

Table 4: Standard echocardiographic parameters with mean and SD

Speckle tracking strain analysis is a more detailed measurement of myocardial function and pathology(Gorcsan & Tanaka, 2011) as it measures myocardial deformation using tissue tracking and this is more accurate than looking at simple movement (MAPSE) or volume change (ejection fraction) to truly understand the myocardium's tissue function. Strain analysis was undertaken between the groups to look for subtle left ventricular dysfunction/pathology and was assessed in the circumferential, radial and longitudinal directions. Global circumferential strain in the diabetes group was nearly 50% lower (11.6±6.2) compared to the control group (-20.5±1.9; P=<0.01) and similarly longitudinal strain was lower in the diabetes group (-12.7±1.09) compared to the control group (-16.5± 0.75; P=0.013). As myocardial fibrosis progresses, replacing tissue between myocytes, this leads to reduced myocardial deformation which can be measured using strain analysis. In our control and diabetes groups we observed longitudinal strain measurements (below -18%) in the range associated with myocardial fibrosis in diabetes (Ng *et al.*, 2012) and in aortic stenosis alone when a normal ejection fraction has been recorded in patients without

diabetes (Weidemann *et al.*, 2009). These figures suggest our diabetes group and control group are similarly matched with regards to potential fibrosis.

Ventricular mRNA expression

Results from the gene expression part of the study are summarized in Table 5, with significant increases in the diabetes group for *SLC8A1*, *KCNJ2* and *KCNJ5*. There was a reduction in the diabetes group in the expression of *KCNH2* and *KCNJ3*.

Table 5: mRNA expression with mean and SD. Raw P values and adjusted P values for false discovery

Action potential modelling

Using the mRNA results discussed above, we used the relative percentage difference in gene expression to alter current density in the diabetes group and utilising the O'Hara-Rudy dynamic model we were able to produce action potentials (AP) for the control and diabetes group at both the endocardial and epicardial levels (Table 6).

Figure 1 shows a modelled Aps from the endocardium, A, and epicardium, B. In both modelled wall layers, changes in the diabetes groups produced increased amplitudes of the action potential, elevation in the plateau phase and prolongation in the AP. We were also able to show at the endocardial layer modelled creation of an early after-depolarisation (EAD) at the plateau phase, the trigger for many types of ventricular arrhythmias.

In Figure 2 panels A (for the endocardium) and B (for the epicardium) the effects of all the individual currents are modelled to identify which of the currents are responsible primarily for the AP prolongation and EAD generation we have seen at the endocardial layer. Most of the currents modelled individually do not have much input into the AP prolongation suggested above, apart from I_{NaCa} encoded by *SLC8A1* and IKr coded by *KCNH2*. There was also a very slight acceleration in the final phase of repolarisation, creating a steeper rate of descent in the diabetes group which appeared to be created by the modelled increase in $I_{K,1}$.

Discussion

In this study, we report for the first-time lower left ventricular expression of *KCNH2* and increased *SLC8A1* in humans with type 2 diabetes and aortic stenosis (compared to non-diabetic patients with the same condition) with the corresponding clinical sequelae of prolongation of the QT interval. Whilst QT interval elongation in humans with type 2 diabetes (Rutter *et al.*, 2002) and *KCNH2/SLC8a1* mRNA cardiac expression alteration in animal models of diabetes (Hattori *et al.*, 2000; Zhang *et al.*, 2007) have been reported previously, the combination has not been reported in humans before. The other novel aspect of our study was the use of a control group with a condition other than diabetes that produces LVH and myocardial fibrosis (aortic stenosis(Weidemann *et al.*, 2009) and this helps support the theory that gene expression changes in our diabetes group were as a result of diabetes/hyperglycaemia not just a final common downstream pathway linked to fibrosis and LVH.

The major gene expression change found was a marked reduction in ventricular expression of ERG mRNA which encodes the pore forming subunit of the $I_{K,r}$ channel, the major outward K^+ delayed rectifying current with a marked prolongation of the modelled AP. $I_{K,r}$ is a K^+ voltage gated current which begins to be activated as the membrane potential drops at the end

of phase 2, the plateau phase, reaching a current maximum at -40mV during phase 3 allowing for a large outward current. $I_{K,r}$ is at positive voltages in quickly inactivated maintaining the AP plateau, but is also quick to recover from inactivation, allowing the generation of the large outward current which is largely responsible for phase 3 in humans and limits the AP duration(Vandenberg *et al.*, 2012).

Our AP modelling shows that down-regulation of ERG creates EADs at the endocardial level at, which has been reported previously in long QT syndromes with normal cellular coupling(Viswanathan & Rudy, 2000), this being thought to be due to reactivation of $I_{Ca,L}$, created by extension of the plateau phase due to reduced ERG. The endocardium seems to be more vulnerable to EADs than the epicardium predominantly as the sharper electrical gradient between the epicardium and mid-myocardium allows formation of a 'sink' for excess current in the epicardium reducing EADs in the mid- and epicardial layers(Viswanathan & Rudy, 2000). Reduced ERG expression and or ERG blockade clinically in arrhythmogenesis(Gong *et al.*, 2007) has been previously reported, particularly in patients with diabetes. (Zhang *et al.*, 2006) In previous studies looking at ERG down-regulation, it has been shown that both myocardial hypertrophy(Hu *et al.*, 2011) and myocardial fibrosis (Chu *et al.*, 2012) are associated with reduced ERG and it was not clear that diabetes had a specific effect on ERG expression within the myocardium or whether down-regulation of ERG was a function of hypertrophy/fibrosis. As discussed above by comparison with a similar control group there seems to be a specific effect of diabetes on ERG expression within the myocardium and that this may go some way to explaining higher arrhythmia rates in patients with diabetes, particularly in the light of the modelled EAD formation and longer QT intervals seen.

As well as reduced ERG expression, we also observed NCX1 over expression which would prolong the AP as was shown in our modelling and this with reduced $I_{K,r}$, leads to EAD generation at the endocardial level.

As well as EAD formation, prolongation of the action potential leads to stimulation of NCX1 forward mode, and generation of a late transient inward current, $I_{TI,}$ in phase 3 of the action potential, which is more likely with a sharper membrane potential drop as we have observed in our modelled AP (largely the result of increased I_{K1}) and in patients with hyperglycaemia secondary to increased Na^+ levels. $I_{TI,}$ can cause delayed after depolarizations (DADs) if the current generated is large enough.

NCX1 expression has been seen to be increased in LV hypertrophy(Menick *et al.*, 2013) and by comparing our diabetes group who would be expected to have increased LV mass, with a control group with LV hypertrophy (as a result of aortic stenosis) we are able to show support for the theory that diabetes specifically does seem to be associated with NCX1 overexpression.

The role of NCX1 on the AP has been the subject of much discussion and findings have been variable in differing experiments due to the forward and reverse modes of NCX1. It has been shown previously in failing canine hearts with high Na^+ that NCX 1 acts in the outward mode to shorten the AP but in low Na^+ states it acts in the inward mode to prolong the AP(Armoundas *et al.*, 2003). In similar pathological states with hypertrophy and fibrosis, increased NCX1 has been shown prolong repolarisation at the end of the AP(Wang *et al.*) and over expression of NCX1 in diabetes has been shown in animal models to prolong the AP and increase systolic function(LaRocca *et al.*, 2012). There has been conflicting data on NCX1 expression in the streptozocin rate model with several papers showing a reduction in NCX1 expression(Zhao *et al.*, 2014; Akhtar *et al.*, 2016). In these studies, the studied rats were comparatively young with significantly lower body weights and left ventricular function and our results may reflect a different diabetes process related to insulin resistance and obesity.

Contrary to the above changes in *KCNH2/SLC8A1*, the diabetes group had an up-regulation of *KCNJ2*, the gene responsible for the main component of $I_{K,1}$ channel, which sets the rest-

ing membrane potential. Our AP modelling has shown that this probably has only a small effect on the AP, most obviously in acceleration in phase 3 of repolarisation to give a sharper terminal part of the AP and acts to limit the AP duration. The role of $I_{K,1}$ this setting is difficult to be certain of but it is possible that the increase in mRNA may be compensatory in the face of the AP prolongation caused by $I_{K,r}$ and NCX1

The final significant mRNA changes observed were in *KCNJ3/5*, with reduced expression of *KCNJ3* in the diabetes group but increased *KCNJ5*. *KCNJ3* codes for a tetrameric, acetylcholine activated inward K^+ rectifying channel ($I_{K,Ach}$) that is expressed in the atria and ventricles. Most prior experimental work is mainly on the atrial effects of $I_{K,Ach}$ and we did not incorporate this into our biophysical model as the channel is only active with acetylcholine. In atrial studies, inhibition of the $I_{K,Ach}$ channel resulted in prolongation of the action potential in the atria(Bingen *et al.*, 2013). Whether this effect would be reproduced in the ventricle would require physiological testing but seems in keeping with the prolonged AP we have modelled. *KCNJ5* codes for the remaining two of the four subunits of the $I_{K,Ach}$ channel and has stretch related properties and in high stretch situations inhibits channel function (Tamargo *et al.*, 2004) This upregulation of the stretch sensitive subunits would suggest, that the higher LV filling pressures commonly seen in diabetes, may present a mechanism for further $I_{K,Ach}$ channel inhibition which would merit further physiological testing.

As with many studies similar to ours, a major limitation is the small sample size and before extrapolation to the wide human population with diabetes, larger studies would be needed. In our study population, we have had a relatively older group of patients with mean ages over 70 years in both groups and fewer women than men which is an important limitation as age (Ocorr *et al.*, 2007; Rabkin, 2014) and gender (Cheng, 2006) are asscoaited with differences in cardiac repolarisation. A larger study would be needed to assess the importance of these factors in the changes we have observed and whether the results could be extrapolated in younger people and by gender specifically. One area of potential limitation is in the

medication background of our diabetes group as sulphonylureas have been shown to have some effect on the cardiac ATP dependent K^+ channel(Aronson *et al.*, 2003) and whilst this channel was not assessed in this study, some overlap with the other K^+ channel genes studied here cannot be excluded. Similarly, as metformin has wide ranging effects on growth factors and anti-fibrotic effect (Bai *et al.*, 2013), some reduction in the gene expression changes we may have seen in the study could have occurred. In this study, we used a combination of QT prolongation on the ECG with AP modelling and mRNA change to look at the effect of diabetes and confirmatory protein measurement of the mRNA results with direct electrophysiology measurement of myocytes from the diabetic human hearts would be required for confirmation of the effects reported here. Review of the existing literature currently suggests that there is reasonable correlation of mRNA with protein measurements(Ellinghaus *et al.*, 2005; Brioschi *et al.*, 2009; Ferdous *et al.*, 2016) and with electrophysiological measurements(Howarth *et al.*, 2009) but that correlation is not universal and post-transcriptional mechanisms do play an important role in multiple settings(Panguluri *et al.*, 2013), something which would need further analysis.

Conclusion

Patients with type 2 diabetes have a prolonged QT interval compared to a control population with similar levels of LV hypertrophy and that this is associated with gene expression changes likely to cause AP prolongation. These findings may partly explain the higher rates of ventricular arrhythmias seen in patients with diabetes.

Funding and Competing Interests

This work was funded by Aintree University Hospital NHS Foundation Trust. There are no competing interests.

Author contributions

All laboratory experiments were performed at the University of Manchester. RA, GH, MRB, GKD and JPHW conceived the study and designed the project methods. RA PM, DMP, AYO, KJ, HZ, JYG, GH, MRB, GKD and JPHW contributed to acquisition, analysis, or interpretation of data and to the drafting of the manuscript and its revision.

Acknowledgements

We would like to acknowledge the patients who kindly took part in this study for their generosity without which the study would not have been possible.

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Group	Number	Caucasian	Age (Years)	Male	Waist (Cm)	BMI (kg/m^2)	HbA1c (mmol/mol^{-1})
Control	9	9	78.8 (8.29)	6	35.8 (2.73)	27.8 (2.28)	Not recorded
Diabetes	7	7	74.9 (12.80)	4	39.7 (8.81)	31.2 (6.86)	57.9 (14.57)

Table 2: Patient characteristics. Values are means (SD). Abbreviations: BMI, body mass index and HbA_{1c}, glycated haemoglobin

Group	Number	Beta blocker	ACE/ARB	Insulin	Metformin	Sulphonylureas	Statin
Control	9	3	5	0	0	0	4
Diabetes	7	3	5	0	4	2	6

Table 3: Patient medications. Abbreviations: ACE, angiotensin-converting enzyme and ARB, angiotensin receptor blocker

Echocardiographic parameter	Control group	Diabetes group	*P* value
Ejection fraction (%)	63.9 (6.53)	58.2 (2.59)	0.09
LV septal width (cm)	1.6 (0.23)	1.7 (0.44)	0.53
End systolic volume (mls)	31.6 (14.5)	37.8 (6.22)	0.39
End diastolic volume (mls)	76.2 (26.5)	89.6 (15.59)	0.33
MAPSE (cm)	1.6 (0.23)	1.1 (0.20)	**<0.01***
Left atrial area (cm^2)	20.4 (2.20)	26.9 (5.52)	**0.01***
E/A ratio	0.9 (0.21)	0.9 (0.23)	0.81
E/e' lateral	11.2 (3.27)	12.8 (3.42)	0.44

Table 4: Standard echocardiographic parameters. Values are mean (SD). Abbreviations: LV, left ventricle and MAPSE, mitral annular longitudinal excursion. *= statistically significant.

Gene	Channel	Control mean and SD	Diabetes mean and SD	Raw P-value	Adjusted P-value
ATP2A2	SERCA2a	4.68×10^{-8} (1.76×10^{-7})	9.61×10^{-9} (1.06×10^{-7})	0.630	0.795
CACNA1C	$Ca_v1.2$	0.000302 (0.0000795)	0.000346 (0.000192)	0.551	0.795
CACNA1D	$Ca_v1.3$	2.47×10^{-7} (1.94×10^{-7})	2.61×10^{-7} (1.53×10^{-7})	0.876	0.876
HCN1	HCN1	7.1901e-007 (1.19×10^{-6})	5.48×10^{-5} (0.00000820)	0.105	0.249
HCN2	HCN2	0.000119 (0.0000411)	6.00×10^{-5} (0.0000454)	0.0173	0.0546
HCN4	HCN4	3.27×10^{-5} (0.0000237)	1.62×10^{-5} (0.0000164)	0.139	0.273
KCNA4	$K_v1.4$	3.04×10^{-5} (0.0000156)	2.40×10^{-5} (0.0000162)	0.438	0.756
KCNA5	$K_v1.5$	5.63×10^{-5} (0.0000432)	5.11×10^{-5} (0.0000243)	0.759	0.811
KCND2	$K_v4.2$	2.80×10^{-7} (2.33×10^{-7})	5.86×10^{-7} (3.83×10^{-7})	0.0679	0.184
KCND3	$K_v4.3$	3.23×10^{-5} (0.0000257)	5.55×10^{-5} (0.0000343)	0.143	0.273
KCNH2	ERG	0.000327 (0.0000747)	0.000114 (0.0000684)	0.00004	0.000750*
KCNIP2	KChIP2	0.000109 (0.0000822)	0.00014957 (0.000187)	0.567	0.795
KCNJ2	Kir2.1	0.000165 (0.0000348)	0.000306 (0.0000832)	0.000400	0.00254*
KCNJ3	Kir3.1	2.06×10^{-5} (0.00000629)	2.94×10^{-6} (0.0000124)	0.00223	0.0106*
KCNJ5	Kir3.4	6.02×10^{-6} (1.06×10^{-6})	1.45×10^{-5} (4.96×10^{-6})	0.000190	0.00184*

KCNQ1	K_vLQT1	0.000136 (0.0000413)	0.000128 (0.0000561)	0.770	0.811
RYR2	RYR2	0.00409 (0.00111)	0.003601 (0.00249)	0.669	0.795
SCN5A	Na_v1.5	0.000787 0.000225)	0.000952 (0.000927)	0.610	0.795
SLC8A1	NCX1	0.000312 (0.000179)	0.00107 (0.000637)	0.00394	0.0150*

Table 5: mRNA expression. Values are mean (SD). Raw P values and adjusted P values for false discovery. *=Statistically significant.

Channel	**Current**	**Expression change in the diabetes Group**
Na_v1.5	I_{Na}	+21.08%
Ca_v1.2 and 1.3	$I_{Ca,\ L}$	+14.18%
K_v1.4, 4.2 and 4.3	I_{To}	+26.04%
ERG	$I_{K,\ r}$	-65.24%
K_vLQT1	$I_{K,\ s}$	-5.38%
K_{ir}2.1	$I_{K,\ 1}$	+85.23%
NCX1	I_{NaCa}	+243.83%
RYR2	SR Ca^{2+} release	-10.07%

Table 6: Relative mRNA expression changes in the diabetes group used to mathematically model the AP

Fig 1: Modelled APs at the endocardial level, A and epicardial level, B for the control and diabetes groups using relative mRNA expression changes. Run with external stimulus pulses at an amplitude of 0.8 nA, duration of 5 ms and frequency of 1 Hz.

Fig 2 Individualised APs from the endocardium, A and epicardium, B analysing the effect of each current singularly on the AP with control in black and diabetes group in red with scaled current change labelled. Run with external stimulus pulses at an amplitude of 0.8 nA, duration of 5 ms and frequency of 1 Hz.