The Relationship between Connexin Expression and Gap junction Resistivity in Human Atrial Myocardium

PS Dhillon[1] MRCP PhD, R Chowdhury[1] PhD, P Patel[1] BSc, R Jabr[2] PhD, AU Momin[3] FRCS, J Vecht[1] FRCS, R Gray[2] PhD, A Shipolini[3] FRCS, CH Fry[2] DSc, NS Peters[1] FRCP.

[1] National Heart & Lung Institute, Imperial College, St Mary's Hospital, London.

[2] Institute of Biosciences and Medicine, University of Surrey, Guildford, UK.

[3] Department of Cardiothoracic Surgery, The London Chest Hospital, London

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Correspondence: Professor Nicholas S. Peters

Waller Cardiac Department

St. Mary's Hospital

Praed Street

London

W2 1NY, UK

Fax number: +0044 20 7886 1763

Telephone number: +0044 20 7886 2468

Email: n.peters@imperial.ac.uk

Abstract

Background: The relative roles of the gap-junctional proteins connexin40 (Cx40) and connexin43 (Cx43) in determining human atrial myocardial resistivity is unknown. In addressing the hypothesis that changing relative expression of Cx40 and Cx43 underlies an increase in human atrial myocardial resistivity with age, this relationship was investigated by direct ex-vivo measurement of gap-junctional resistivity and quantitative connexin immunoblotting and immunohistochemistry.

Methods and Results: Oil-gap impedance measurements were performed to determine intracellular resistivity (R_i) and gap-junctional resitivity (R_j). Total Cx40 quantification by Western blotting correlated with R_i, (r=0.64, p<0.01, n=20), and R_j, (r=0.63, p=0.01, n=20). Although Cx43 expression showed no correlation with resistivity values, the proportional expression of the two connexins, (Cx40/[Cx40+Cx43]) correlated with R_i and R_j (r=0.58, p<0.01 for R_i and r=0.51, p=0.02 for R_j). Cx40 localised to the intercalated disks was quantified by confocal immunohistochemical imaging of *en-face* disks, and also correlated with R_j (r=0.66, p=0.02, n=12). Advancing age was associated with a rise in R_i (r=0.77, p<0.0001), R_j (r=0.65, p<0.001, n=23), Cx40 quantity (r = 0.54, p=0.01, n=20), and Cx40 gap junction protein per unit area of *en-face* disk (r=0.61, p=0.02, n=12).

Conclusion: Cx40 is the main determinant of human right atrial gap-junctional resistivity such that increased total, gap-junctional and proportional Cx40 expression increases gap-junctional resistivity. Accordingly, advancing age is associated with an increase in Cx40 expression and a corresponding increase in gap-junctional resistivity. These findings are the first to demonstrate this relationship and a mechanistic explanation for changing atrial conduction and age-related arrhythmic tendency.

Keywords: Gap junction, Connexin, Conduction, Arrhythmogenesis

Introduction

Gap-junction (GJ) channels are transmembrane proteins that in myocardium mediate cell-to-cell coupling required for ordered action potential (AP) propagation.[1-3] Each GJ channel is formed from two hemi-channels (connexons), in-turn composed of six connexin (Cx) subunit proteins. In the human atrium Cx40 and Cx43 are the major connexins, with small amounts of a third, Cx45, also expressed.[1-4]

The relative contributions of Cx40 and Cx43 in determining atrial conduction properties has been investigated in murine Cx-gene knock-out models, which suggested that reduction of Cx40 slowed conduction, and prolonged P-waves, whereas reduction of Cx43 had little effect.[5-7] In a human open-chested mapping study, conduction velocity (CV) during sinus rhythm and atrial pacing was correlated with Cx40 and Cx43 in matched tissue samples.[8] In contrast to the murine studies, CV was reduced as the proportion of Cx40 signal increased (and therefore that of Cx43 decreased). Consistent with these findings, a study using synthetic strands of neonatal and foetal murine atrial cardiomyocytes showed that Cx40 deletion ($Cx40^{-/-}$) was associated with increased CV, and genetic deletion of Cx43 ($Cx43^{-/-}$) was associated with a decrease of CV.[9] In light of these apparently conflicting results, and given the importance of a mechanistic understanding of this relationship specifically in humans, we investigated the relationship between human atrial connexin expression and gap-junctional resistance, the key intermediary and gap-junctional determinant of myocardial conduction velocity. Biophysical theory proposes that an increase of gap-junctional resistance (R_j) reduces CV.[10]

We used the validated method of myocardial oil-gap impedance measurements,[11] to measure R_j, and in the same human myocardial samples used quantitative Cx40 and Cx43 immunoblotting and immunohistochemistry, to address the hypotheses that there is a relationship between the relative expression of Cx40 and Cx43 and R_j, and that changes in the

relative expression of Cx40 and Cx43 underlie an increasing atrial myocardial resistivity with age.

Patients and Methods

Specimen source, collection and handling. The use of human tissue conformed to the principles outlined in the Declaration of Helsinki and the study had local Ethical Committee approval with written informed consent. Atrial appendage biopsies were collected from patients undergoing cardiac surgery at The London Chest Hospital, London, UK. Twenty-three patients (15M) in sinus rhythm (age 68±8 years, range 53-80 years) undergoing coronary bypass surgery (CABG, n=15), aortic valve replacement (AVR, n=3) or both (n=5) were included. Patients were in sinus rhythm at the time of surgery as assessed by ECG and did not have any history of atrial arrhythmia. All patients underwent surgery using conventional cardiopulmonary bypass support, and biopsies were collected from the tips of the right atrial appendage immediately after the pericardium was opened and prior to exposure to cardioplegic agents. Samples were divided immediately upon collection; one part was snap-frozen in liquid nitrogen at -70ºC for subsequent Cx quantification and immunolabelling, and the remainder was transported to the laboratory in pre-oxygenated Tyrode's solution (4ºC) containing (mM) NaCl 118, KCl 4.0, $NaHCO_3$ 24, NaH_2PO_4 0.4, $MgCl_2$ 1.8, glucose 6.1, Na pyruvate 5.0 (pre-gassed with $95\%O_2/5\%CO_2$, pH 7.35 ± 0.03). Transportation time to the laboratory was approximately 15 minutes. On arrival at the laboratory the unfrozen specimens were gradually warmed to 37^{o}C and atrial trabeculae were dissected for physiological experiments. This approach permitted extraction of connexin protein for Western blotting, and parallel determination of gap junction resistance in intact myocardium. All chemicals were from Sigma, UK.

Measurements of longitudinal impedance and calculation of gap junction resistance. The method and its validation have previously been described in detail[11] and are available in our online supplement. Myocardial preparations (≤1 mm diam. 5-6 mm length) were placed in a three-chambered bath; the outer chambers were superfused with Tyrode's solution at 37ºC, whilst the muscle in the central chamber was coated with mineral-oil gel. Myocardial strips have been shown to remain metabolically and structurally intact during impedance measurements.[11,11a] Alternating current (0.25-150 kHz) was passed between platinum (Pt)-black electrodes in the outer chambers to constrain current to the intracellular pathway of the muscle within the oil-gap, with a fraction through a parallel extracellular shunt. The frequency-dependent intracellular resistance (r) of the preparation was recorded with a balanced Wien bridge (Wayne-Kerr, Wetherby, UK). Total preparation impedance, z, was modelled as $z = (z_i.r_{ec})/(z_i+r_{ec})$, where r_{ec} is the resistance of the extracellular shunt and z_i is the impedance of the intracellular pathway. r_{ec}, was measured separately as the resistance between two Pt-black needle electrodes, a known distance apart, placed in the muscle within the oil-gap to obtain true intracellular resistance, r_i, from $r_i=(r.r_{ec})/(r+r_{ec})$. Intracellular resistance itself has two components; the sarcoplasm, r_c, and gap junction resistances, r_j, (i.e. $r_i=r_j+r_c$), obtained separately by measuring the frequency-dependent component of r_i and the limiting value at high frequencies (Figure 1). Preparation length and radius in the oil-gap were measured after measurements. Lower case values of variables (r, x; $\Omega.cm^{-1}$) were converted to specific (R, X; Ω.cm) values by scaling to preparation cross-section area (CSA) and the proportion of CSA occupied by muscle. The non-muscle fraction of CSA was calculated from the value of r_{ec}, assuming it was filled with Tyrode's (49Ω.cm).[11]

FIGURE 1 NEAR HERE

Connexin protein quantification by Western blotting. Tissue homogenates were prepared from frozen tissue samples to give a solution of final concentration 0.5 mg/ml in sample buffer. Total protein (5.0 mg) from each sample was resolv

ed by polyacrylamide gel electrophoresis (BioRad, Hercules, USA) on a 12.5% gel (4.5% stacker). The gel was run at 60 V until the dye front was through the stacker and subsequently run at 150 V. The gel was electrophoretically transferred onto a polyvinylidene fluoride membrane at constant voltage, 30 V. The membrane was blocked in the dilution buffer (TBS / 0.1% Tween20 [Merck, UK] / 1% blot qualified BSA / 4% Marvel) for one hour, followed by incubation with the primary antibody for Cx43 and Cx40 for 1 hour.

For Cx43, an IgG_1 raised in mice against a synthetic peptide corresponding to positions 252-270 of rat native Cx43 was used (Chemicon, USA; 1:500 in dilution buffer). For Cx40, a goat antibody raised against a human peptide mapping near the C-terminus of Cx40 was used (Santa Cruz Biotechnology, USA; 1:1000 in dilution buffer). Membranes were washed and incubated for one hour with secondary alkaline phosphatase conjugated anti-mouse for Cx43 and anti-goat for Cx40 (Pierce, Rockford, IL, USA; 1:2,500 dilution. The membrane was then incubated with alkaline phosphate buffer (0.1 M TRIS pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$), followed by incubation with freshly prepared Nitro blue tetrazolium chloride (NBT)/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) substrate solution (Promega Corp., Madison, WI, USA). Band intensities were quantified by a densiometric scan and all values corrected for protein loading using the actin band on a coomassie-stained gel run in parallel. For connexin quantification, one patient was randomly selected and arbitrarily scored as 100 units of Cx40 and Cx43 and all other values were scaled accordingly.

Immunofluorescence confocal microscopy. Frozen sections (10 µm) were mounted onto poly-L-lysine coated slides, fixed in 100% methanol at -20^{O}C and washed with phosphate buffered saline before blocking in 1% bovine serum albumin (BSA) for 30 minutes (for Cx43) or 1hr (for Cx40). The same primary Cx43 and Cx40 antibodies were used as for protein quantification. Incubation was at a 1:1000 dilution in BSA for 2 hours for both antibodies. After washing, a secondary antibody tagged with Cy3 fluorescent marker (Chemicon, USA) for Cx43 and FITC (Chemicon, USA) for Cx40 were used at a dilution of 1:500 and 1:50 respectively in BSA for 45 minutes. Sections were then washed and mounted with Vectasheild mountant (Vector Labs, CA). Immunolabelled sections were examined using a Zeiss LSM-780 confocal microscope.

To assess the degree of connexin lateralization, a previously published, blinded, semiquantitative visual method was used.[12] A simple, arbitrary score was derived as follows, based on the degree of clustering of immunolabeled gap junction. Longitudinal orientation by standard light microscopy of an adjacent section was first confirmed. Immunolabeled sections were then examined using phase-contrast transmission microscopy, coupled with fluorescence microscope settings that maintained cell outline visualization to further confirm longitudinal cell orientation, to assess the distribution pattern of immunolabeled gap junctions. Three randomly selected optical fields (~ 250,000 µm^2) of at least three separate tissue sections from each specimen were examined by a single experienced immunofluorescence microscopist blinded to the origin of the specimens. The scoring system assessed Cx40 and Cx43 label distribution, ranging from being confined exclusively to the normal, transversely orientated clusters at cell abutments (score 1), to a distribution of labelling within longitudinal arrays along the length of the myocyte, with markedly diminished labelling at the end-on abutments (score 5). Although only semi-quantitative, this

technique has proven useful,[12] and identifies changes that are relatively gross and of likely biological significance.

Measurement of connexin expression per unit area per en-face intercalated disc area

Transversely sectioned samples labelled with Cx40 were examined using a Zeiss LSM-780 confocal microscope. 15-to-20 *en-face* discs per patient were selected from at least five randomly selected fields (230 µm x 230 µm). Confocal images were converted into TIFF format using Fiji software and then analysed with Photoshop CS3 (abode) software. Immunolabelled pixels were counted electronically to allow measurement of both intercalated disc area and Cx40 area per *en-face* disk.

Measurement of connexin40 heterogeneity

For each sample, six randomly selected areas (230 µm x 230 µm) were imaged on a Zeiss LSM-780 confocal microscope. Using Photoshop CS3 (Adobe) a grid of 7x7squares was super-imposed on the image. The number of squares without label was counted and divided by the total number of squares to give percentage labelled fields as a marker of Cx40 heterogeneity.

Quantification of myocardial fibrosis. Elastin and collagen autofluorescence were measured with an argon ion laser at 488 nm.[13] 10µm frozen tissue sections were fixed and mounted at -20^{O}C as above and imaged using a confocal microscope (Zeiss Pascal). Quantification used the ImageJ programme (National Institute of Health) to calculate percentage fluorescence in six randomly chosen fields per sample.

Statistics. Data are presented as mean±SD. Comparisons between groups was performed with Student's t-test. Association between variables was analysed by Pearson's correlation

tests. A one-sided Spearman's rank correlation co-efficient, r_s, expressed the association between variables, with no assumed relationship. The null hypothesis was rejected at $p<0.05$. Impedance data were fitted to the equation of a circle using the program KaleidaGraph (Synergy Software). All other analysis was performed using Graphpad Prism 2.01 (Graphpad software).

Results

Patient population. The relevant clinical, electrocardiographic and echocardiographic characteristics of the patients are shown in Table 1. There was no association between ECG P-wave duration, left atrial diameter, and any physiological or immunohistochemical parameter.

TABLE 1 NEAR HERE

Specimens. Biopsy samples were of sufficient size to perform impedance measurements for all patients (n=23). However, connexin quantification by Western blot analysis was only possible in 20/23 samples. Fibrosis quantification and morphometry was performed in 16/23 samples and intercalated disc area and Cx40 gap junction protein per area *en-face* disc were measured in 12/23 patients.

Connexin expression and myocardial resistivity. Table 2 shows values of physiological and histological parameters for each patient. Figures 2A and B show plots of right atrial Cx40 and Cx43 expression against corresponding R_i and R_j values. There were significant positive correlations between Cx40 expression and R_i, ($r=0.64$, $p<0.01$, $n=20$) as well as R_j, ($r=0.63$, $p=0.01$, $n=20$). Cx43 expression, however, did not correlate with either R_i or R_j and there was no correlation between either connexin or R_c (not shown). There was also a significant positive correlation between the proportional level of Cx40 expression (Cx40/[Cx40+Cx43]) and both R_i ($r=0.58$, $p<0.01$, $n=20$) and R_j ($r=0.51$, $p=0.02$, $n=20$), (Figure 2C).

FIGURE 2 TABLE 2 NEAR HERE

Cellular connexin distribution. Figure 3A and 3B shows immunolabelling for Cx40 and Cx43 respectively. Cx43 labelling was well distributed throughout all specimens but Cx40 distribution was heterogeneous and patchy, with some regions intensely labelled and some

regions displaying less intense labelling, as described previously.[8;14] Both connexins were located predominantly at the intercalated discs at the poles of the myocytes but there was presence of labelling at the lateral cell borders of all specimens. A semi-quantative method was used to grade connexin localisation ranging from being confined exclusively to the normal, transversely orientated clusters at cell abutments (score 1), to a distribution of labelling within longitudinal arrays along the length of the myocyte, with markedly diminished labelling at the end-on abutments (score 5). The mean score was 2.5 ± 1 indicating a degree of lateralisation in all samples. Figure 3D shows that there was no association between Cx43 lateralisation score and R_j for each patient.

FIGURE 3 NEAR HERE

Immunolabelled Cx40 gap junction protein per unit area of en-face disk and Cx40 heterogeneity. Given the relationship established between Cx40 expression and R_j, it was of interest to examine whether R_j was related to Cx40 expression specifically at the intercalated disc. Figure 3C shows enface views of a typical *en-face* intercalated disc. As described in the methods section, the quantity of Cx40 immunolabelled pixels were measured and expressed as a percentage of the area of the *en-face* disk. The mean intercalated disc area was 96 ± 37.8 μm^2, mean Cx40 area was 39.4 ± 10.2 μm^2, and mean Cx40 gap junction area per *en-face* disk was 45 ± 20%. Cx40 expression, measured by Western Blot analysis, did not significantly correlate with Cx40 gap junction per unit area of enface disc (p=NS, n=12) (Figure 4A), but there was a significant positive correlation between Cx40 gap junction per unit area of *en-face* disc and R_j, (r=0.66, p=0.02, n=12) (Figure 4B). The heterogeneity of Cx40 labelling, was examined by assessing the percentage of labelled cells per field for 12/23 patients, and values are shown in table 2. There was no significant relationship between Cx40 heterogeneity and junctional resistivity (r= -0.44 p=NS) (Figure 4C).

FIGURE 4 NEAR HERE

The relationships between age, connexin expression and myocardial resistivity. There was a significantly positive correlation between advancing age and both R_i (r= 0.77, p<0.0001) and R_j (r= 0.65, p<0.001, n=23) (Figure 5A), and a corresponding correlation between age and Cx40 expression (r=0.54, p=0.01, n=20) (Figure 5B), but not Cx43 expression. There was no relationship between age and R_c (not shown).

Myocardial fibrosis was quantified for 16/23 patient samples to provide average values from six randomly selected fields. There was no significant relationship between age and the percentage of myocardial fibrosis (Figure 5C). Advancing age was associated with an increase in Cx40 gap junction protein per unit area *en-face* disk (r=0.61, p=0.02, n=12) (Figure 5D) but not with Cx43 area or lateralisation score (Figure 5E).

FIGURE 5NEAR HERE

Discussion

The main findings of this study are that increased Cx40 expression, and an increase in the relative expression of Cx40 (Cx40/[Cx40+Cx43]), are associated with an increase in myocardial resistivity in the human right atrium, that it is the Cx40 specifically located in the intercalated disk which correlates with gap junctional resistivity, and that increased atrial myocardial resistivity with advancing age is associated with an increase in Cx40 expression.

On the background of our previous demonstration of a relationship between atrial connexin expression and conduction velocity measured in vivo in the human right atrium,[8] we now confirm a relationship between atrial connexin expression and directly measured gap junction resistivity in multicellular human right atrial preparations.

Connexin40 expression and atrial resistivity. Connexin-knockout mice have been shown to have changes in conduction velocity in atrial myocardium[7] and in the specialised conduction system[15] providing evidence that connexins are a key determinant of impulse propagation. However, by contrast with the present study, murine Cx-gene knock-out models have indicated that reduction of Cx40 slows atrial conduction with corresponding prolongation of the P-wave on the ECG.[5-7] Further, we previously demonstrated an inverse relationship between human right atrial Cx40 expression and CV, measured in open-chested mapping studies of patients undergoing cardiac surgery.[8] Given that CV is determined by several factors including gap-junctional coupling,[16] depolarising currents,[17] and tissue architecture,[18] it is an important finding of the current study that the inverse relationship between Cx40 expression and CV is mediated by an increase in atrial gap junction resistivity. Indeed, given that the unitary conductance of pure Cx40 channels is higher than that of pure Cx43 and Cx45 channels,[19;20] it might be expected that with the naturally occurring variations in Cx40

expression in the human heart, a higher level of Cx40 expression would result in a lower myocardial resistivity.

However, when a pair of myocytes expresses two or more connexins channels the different potential combinations of connexins (homomeric homotypic, homomeric heterotypic, heteromeric homotypic or heteromeric heterotypic),[21;22] result in a corresponding variety of functional properties of the gap-junctional channels. In-vitro studies have confirmed that Cx40 and Cx43 interact in the formation of heteromeric/heterotypic gap junction channels,[23] and we have previously confirmed co-localisation of Cx40 and Cx43 within human right atrial gap junction plaques,[8] although co-localisation does not prove that Cx40 and Cx43 form mixed channels. Cells which co-express Cx40 and Cx43[23-25] are known to be more susceptible to uncoupling than cells expressing a single connexin type.[25] Therefore an increase in Cx40 expression may result in the formation of either non-functional or low conductance channels, which result in an increase in overall gap junctional resistivity. It is also possible that an alteration of right atrial Cx40 expression may somehow alter Cx43 function. A study of cultured atrial myocytes with heterozygous ($Cx40^{+/-}$ or $Cx43^{+/-}$) or suppressed ($Cx40^{-/-}$ or $Cx43^{-/-}$) connexin expression showed that reduced Cx40 expression was associated with increased CV and also increased Cx43 labelling in gap junctions.[9] However, a study of cultured murine Cx43 ablated cells ($Cx43^{-/-}$) demonstrated that ablation of Cx43 reduced electrical conductance between myocytes as well as the expression of Cx40 and Cx45.[25a] Furthermore, a study of the effects of a novel frameshift Cx43 mutation discovered in man, on murine cultured cells, confirmed a reduction of Cx40 gap junction conductance thought due to abnormal connexin protein trafficking to the intercalated disc,[25b] These studies highlight the fact that interactions between co-expressed connexins are not straightforward and predictable, and therefore the functional consequences of altered

connexin expression associated with age or disease states such as atrial fibrillation, may not be predictable from simple measurement of connexin quantity alone.

We did not observe a direct relationship between the quantity of Cx43 expression and R_j. However it remains possible that Cx43 may influence on Rj in a manner not identified by our current techniques.

Advancing age, atrial connexins and resistivity. We,[26] and others,[27] have shown an age-associated slowing of right atrial CV in human endocardial mapping studies although the mechanism underlying this phenomenon remains unknown. Animal studies have confirmed that atrial fibrosis with advancing age is associated with conduction slowing and increased rate of arrhythmogenesis,[28;29] and human studies have demonstrated a correlation between levels of atrial fibrosis and advancing age [29a, 29b]. We previously have shown that human atrial CV did not directly correlate with endomysial connective tissue content,[8] we did not find any association between age and quantity of myocardial connective tissue in this present study. A finding similar to previous reports. [29c, 29d] It remains possible, however, that the pattern of connective tissue deposition in older humans may change and result in greater separation of atrial bundles.[30] Our present study instead demonstrated an age associated increase in Cx40 expression, Cx40 gap junction protein per unit area of enface cells, and R_j. There have been no previous functional studies of the effects of ageing on cardiac gap junctions in the human atrium. However, a study of the effects of ageing on canine atrial myocardium,[31] comparing young (6-12 months) and older (6-10 years) dogs, demonstrated that gap junction distribution became progressively more polarized with increasing age and localized to the cell termini. There was no change in the cellular distribution of gap junctions in the human right atrium with advancing age in our present study, although the area of Cx40 gap junction protein per unit area of *en-face* disk increased with advancing age.

Insights from correlations of connexin expression with junctional resistivity. Cx43 quantity, measured by immunofluorescence, has been previously correlated with direct measurements of intercellular electrical conductance (the inverse of resistivity) in engineered rat myocytes.[32] Our study now provides a unique insight into human connexin biology, in particular the importance of Cx40 in determining atrial resistivity, and the effects of aging on atrial connexin expression and resistivity. Our results may also explain the finding that increased right atrial Cx40 expression in sinus rhythm is a predictor of the risk of developing post-operative AF,[14] and that an up-regulation of right atrial Cx40 has been described in association with atrial fibrillation in man.[33] However, although little is currently known about the mechanisms that underlie age-related atrial remodelling, or the control mechanisms or modulators of Cx40 expression, in the era of pharmacological[34] and genetic modulation[35] of connexin expression, human atrial Cx40 expression may be a potential therapeutic target in the prevention of atrial arrhythmias.

Limitations

Specimens were of insufficient size to perform immunohistochemical experiments for all patients. Tissue was only collected from the atrial appendages and it is possible that the gap junction remodelling described in this paper is limited to the appendage only and not reflective to changes in other atrial regions. It is possible that the pre-operative medication taken by patients in this study could have influenced gap junction conductance. Although our results demonstrate an association between Cx40 expression, gap junction resistivity and advancing age they not prove a direct causality between these variables.

Conclusion

In the human right atrium, Cx40 expression and Cx40 gap junction protein per unit area of *en-face* disks, are directly related to gap junctional resistivity. Advancing age is associated with an increase in Cx40 expression and gap junction resistivity, and these findings may explain the association of advancing age with atrial arrhythmias.

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Disclosures

None

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Table 1 Patient characteristics.

Patient	Age (years)	Sex	Operation	History	Medication	LV EF (%)	LA (mm)	P-wave duration (s)
1	64	M	AVR	AS	BB	30	34	0.12
2	80	F	CABG AVR	IHD AR HT	CCB	30-40	45	0.12
3	54	F	AVR	AR	ARB	>50	44	0.12
4	69	M	CABG	IHD DM HT	BB CCB ACE	>50	40	0.08
5	53	M	CABG	IHD DM HT	CCB	>50	42	0.1
6	72	F	CABG	HT	BB ACE	>50	40	0.12
7	68	M	CABG	MI HT	BB ACE	40-50	38	0.12
8	78	F	CABG AVR	MI AS HT	BB CCB ACE	40-50	36	0.08
9	77	F	CABG	IHD	BB ACE	>50	30	0.12
10	75	M	CABG	MI HT	BB ACE	>50	45	0.08
11	65	F	CABG AVR	IHD AS DM	CCB ARB	>50	47	0.1
12	55	M	CABG	MI HT	BB ACE	40-50	42	0.08
13	67	M	CABG	MI HT	BB	>50	40	0.08
14	76	F	CABG AVR	AS AR		35-40	37	0.08
15	76	M	CABG	IHD	CCB	>50	41	0.12
16	68	M	CABG	MI DM HT	ACE CCB	>50	49	0.12
17	74	M	CABG	MI DM	BB ACE	35	39	0.08
18	72	M	CABG AVR	IHD AR	BB ACE	>50	31	0.12
19	73	M	CABG	MI	BB CCB ACE	>50	43	0.08
20	72	M	CABG	MI	BB	>50	40	0.16
21	60	M	CABG	IHD	BB ACE	>50	38	0.18
22	55	F	AVR	AS		>50	41	0.16
23	62	M	CABG	IHD	ACE	>50	33	0.12

LV EF = Left Ventricular Ejection Fraction; LA = Left Atrium; PWD = P wave duration; CABG = Coronary Artery Bypass Grafting; AVR = Aortic Valve Replacement; MI =

Myocardial Infarction; IHD = Ischaemic Heart Disease; AS = Aortic Stenosis; AR = Aortic Regurgitation; HT = Hypertension; DM = Diabetes Mellitis; BB = Beta Blocker; CCB = Calcium Channel Blocker; ACE = Ace Inhibitor; ARB = Angiotensin Receptor Blocker.

Table 2 Physiological and histological parameters

Patient	R_i(Ω.cm)	R_c(Ω.cm)	R_j(Ω.cm)	Cx43 units	Cx40 units	Fibrosis (%)	Cx40 area/disc (%)	Cx40 labelling per field (%)
1	320	194	126	110	78	N/A	N/A	N/A
2	375	230	145	103	114	N/A	N/A	N/A
3	303	227	76	22	N/A	N/A	N/A	N/A
4	397	208	189	21	170	31.6±12.4	36.0±10.9	74.0±10.0
5	329	173	156	24	80	N/A	N/A	N/A
6	404	216	188	94	151	39.4±12.6	35.7±10.7	86.4±11.0
7	344	225	119	117	48	32.1±15.5	34.5±14.4	80.6±8.6
8	474	182	292	104	150	33.0±13.9	N/A	N/A
9	420	181	239	29	202	19.4±9.9	49.6±16.32	83.1±12
10	443	154	289	30	146	52.0±12.8	N/A	N/A
11	402	225	177	11	121	51.0±10.9	23.9±7.8	85.4±8.1
12	291	178	113	100	100	26.2±12.1	36.5±10.5	92.1±7.2
13	315	208	107	104	34	73.7±20.9	N/A	N/A
14	456	218	238	10	210	56.7±7.6	55.9±5.8	81.1±7.4
15	396	246	150	18	130	34.2±22.4	N/A	N/A
16	426	165	261	86	102	18.8±7.1	51.2±9.7	80.6±6.4
17	488	227	261	25	96	42.1±29.6	57.0±9.7	87.6±5.0
18	467	213	254	42	152	21.8±3.5	51.3±10.0	80.8±8.3
19	386	183	180	138	106	35.3±16.1	56.1±12.1	80.9±7.0
20	497	182	315	99	109	27.7±9.3	56.4±15.3	74.1±10.1
21	290	199	91	N/A	N/A	N/A	N/A	N/A
22	265	158	107	N/A	N/A	N/A	N/A	N/A
23	356	237	119	53	66	N/A	N/A	N/A

R_i = Intracellular resistvity; R_j = Gap junction resistivity; R_c = Sarcoplasmic resistivity;

Figure Legends

Figure 1. A plot of intracellular resistance, R_i, as a function of measuring frequency. The dotted lines represent the frequency-independent values of R_i at high and low frequencies. The smaller value at high frequency corresponds to cytoplasmic resisitivity, R_c; the difference between the two values is a function of gap junction resistance, R_j.

Figure 2. The relationship between Cx40 and Cx43 expression, as well as relative Cx ratio (Cx40/Cx40+Cx43), with R_i (open symbols) and R_j (closed symbols). A, Increasing Cx40 expression was significantly associated with both R_i/R_j ($p<0.01$ and $p=0.01$ respectively). B, Cx43 expression did not correlate with R_i/R_j. C, The relative Cx ratio (Cx40/Cx40 +Cx43) was significantly associated with to R_i/R_j ($p<0.01$ and $p=0.02$ respectively).

Figure 3. A and B, Cx40 (A) and Cx43 (B) immunolabelling showing predominant labeling at the intercalated discs at the poles of the cells. White arrows indicate cell alignment along longitudinal axis and white bar = 25 μm. C, Cx40 immunolabelling of enface discs allowing measurement of Cx40 area in each disc (white bar = 10 μm). D, A plot of gap junctional resistivity, R_j, against Cx43 lateralization score (p=NS).

Figure 4. Plots of Cx40 gap junction per unit area of *en-face* disk and: A, Cx40 expression determined by Western Blot analysis (p=NS); and B, gap junctional resistivity (p=0.02). C, plot of R_j against Cx40 heterogeneity measured in percentage of labeled cells per field.

Figure 5. Plots of age against: A, R_i (open symbol) and R_j (closed symbol) ($p<0.001$ and $p<0.01$ respectively); B, Cx40 expression (p=0.01); C, percentage fibrosis per field (p=NS); D, Cx40 gap junction protein per unit area of *en-face* disk (p=0.03); and E, Cx43 lateralisation score (p=NS).

Figure 1

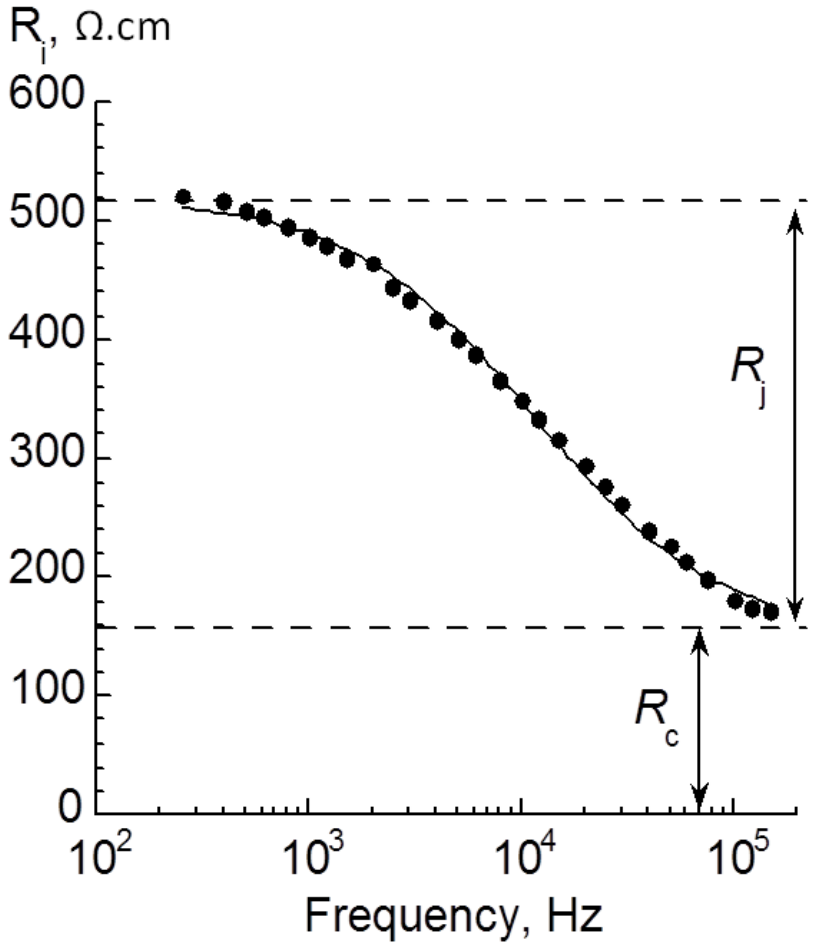
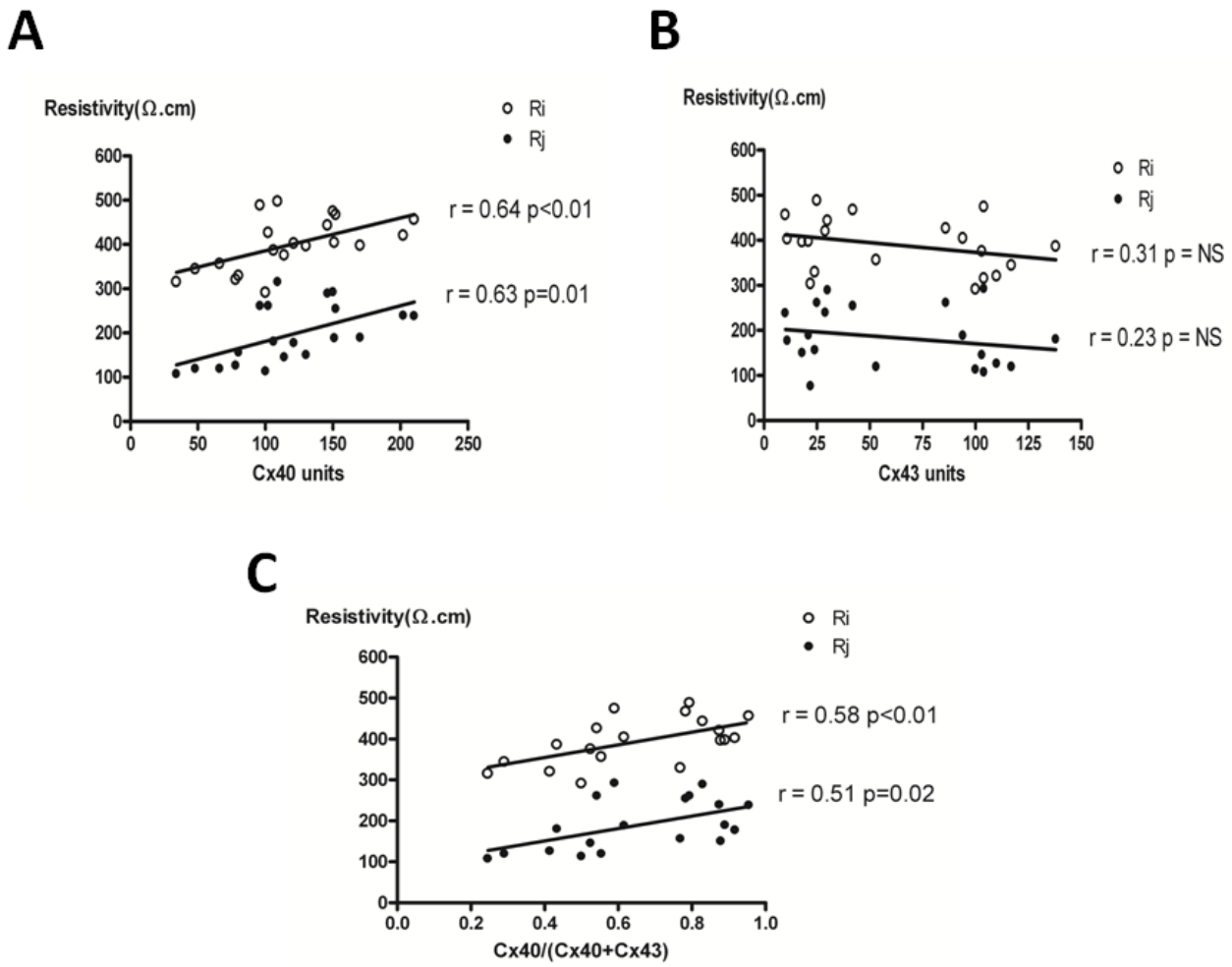

R_i, Ω.cm
600
500
400
300
200
100
0
R_j
R_c
10^2
10^3
10^4
10^5
Frequency, Hz

Figure 2


A
Resistivity(Ω.cm)
Ri
Rj
r = 0.64 p<0.01
r = 0.63 p=0.01
Cx40 units
B
Resistivity(Ω.cm)
Ri
Rj
r = 0.31 p = NS
r = 0.23 p = NS
Cx43 units
C
Resistivity(Ω.cm)
Ri
Rj
r = 0.58 p<0.01
r = 0.51 p=0.02
Cx40/(Cx40+Cx43)

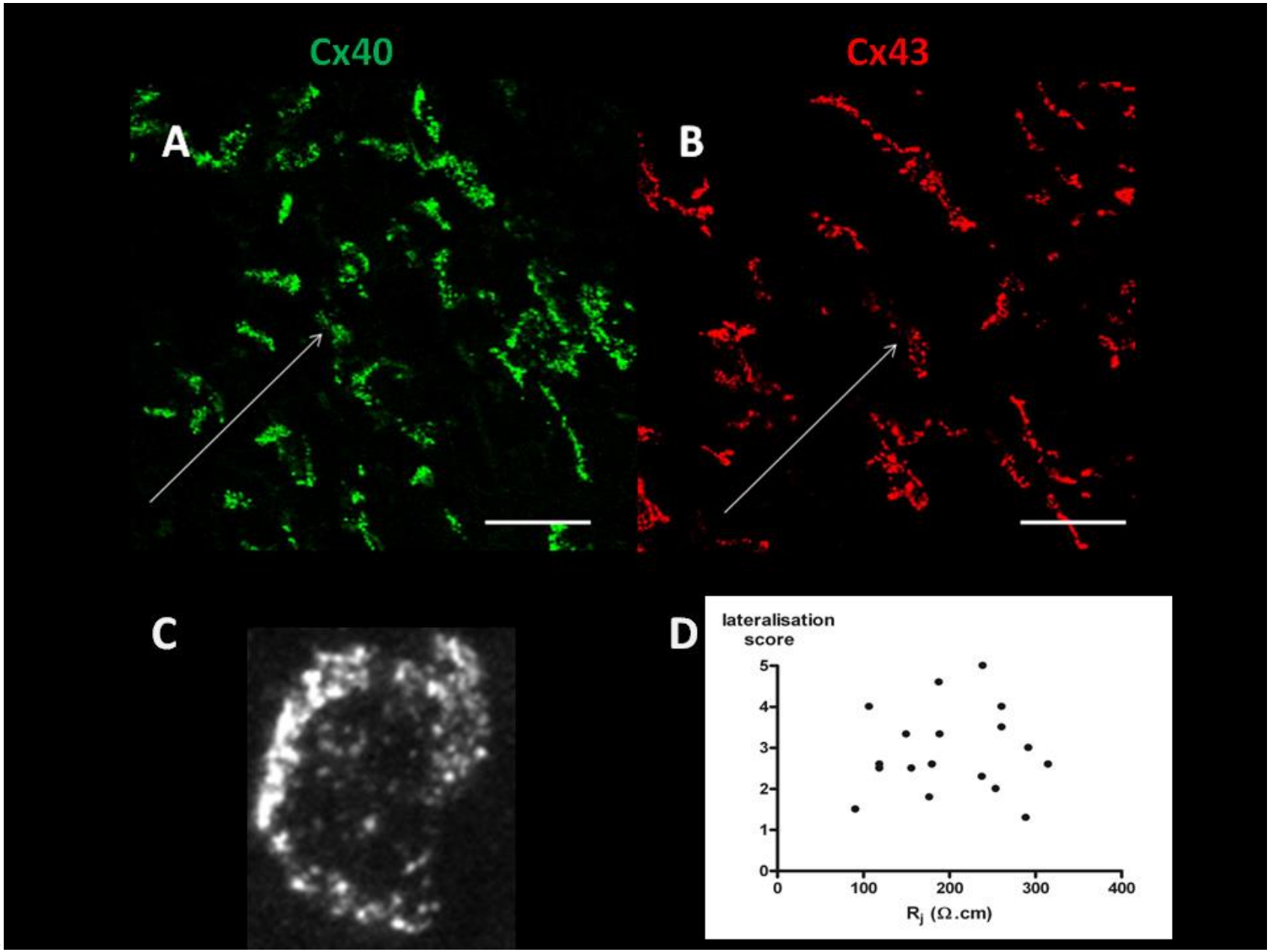

Cx40
A
Cx43
B
C
D
lateralisation score
5
4
3
2
1
0
0
100
200
300
400
Rj (Ω.cm)

Figure 3

Figure 4

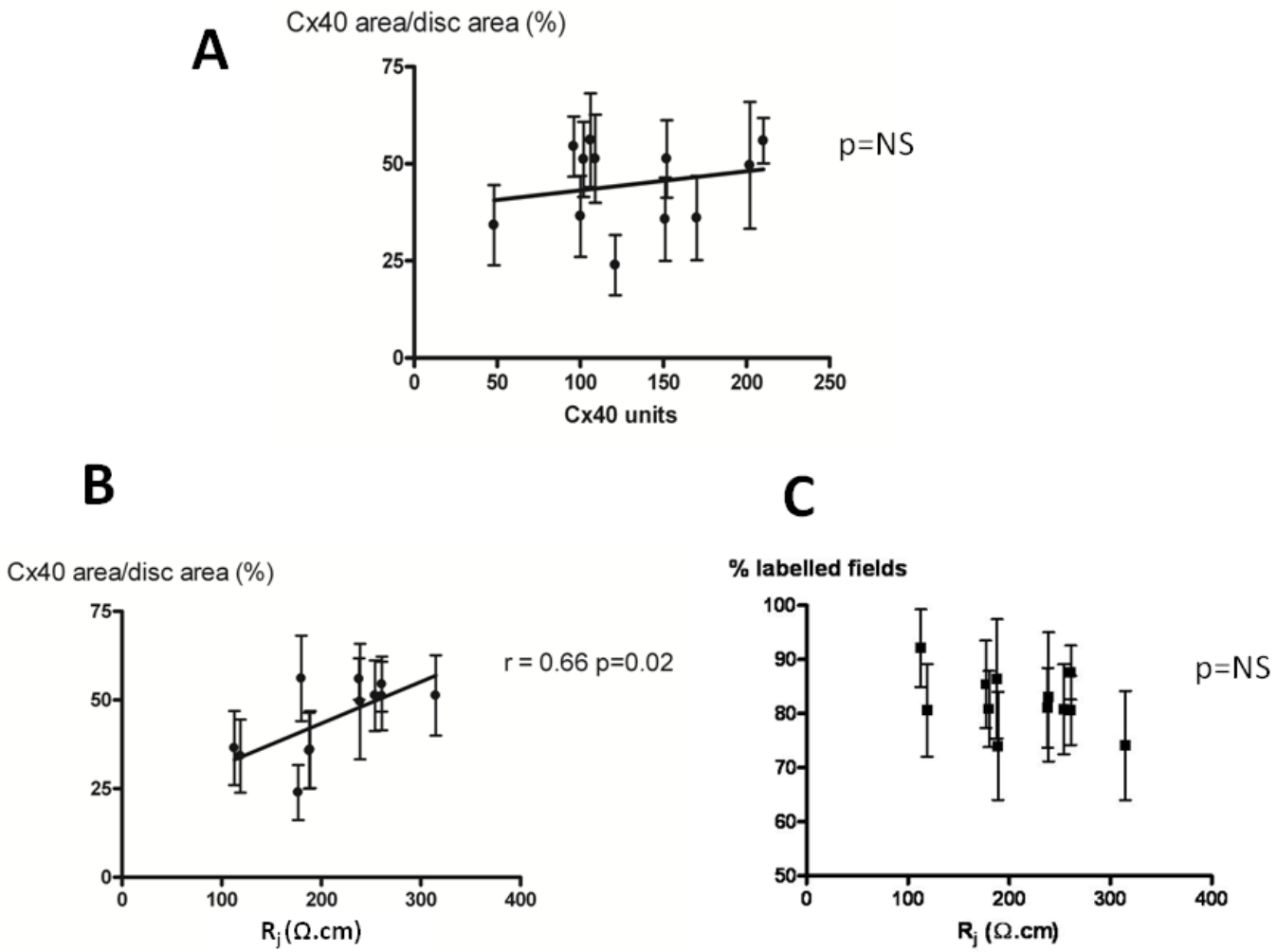
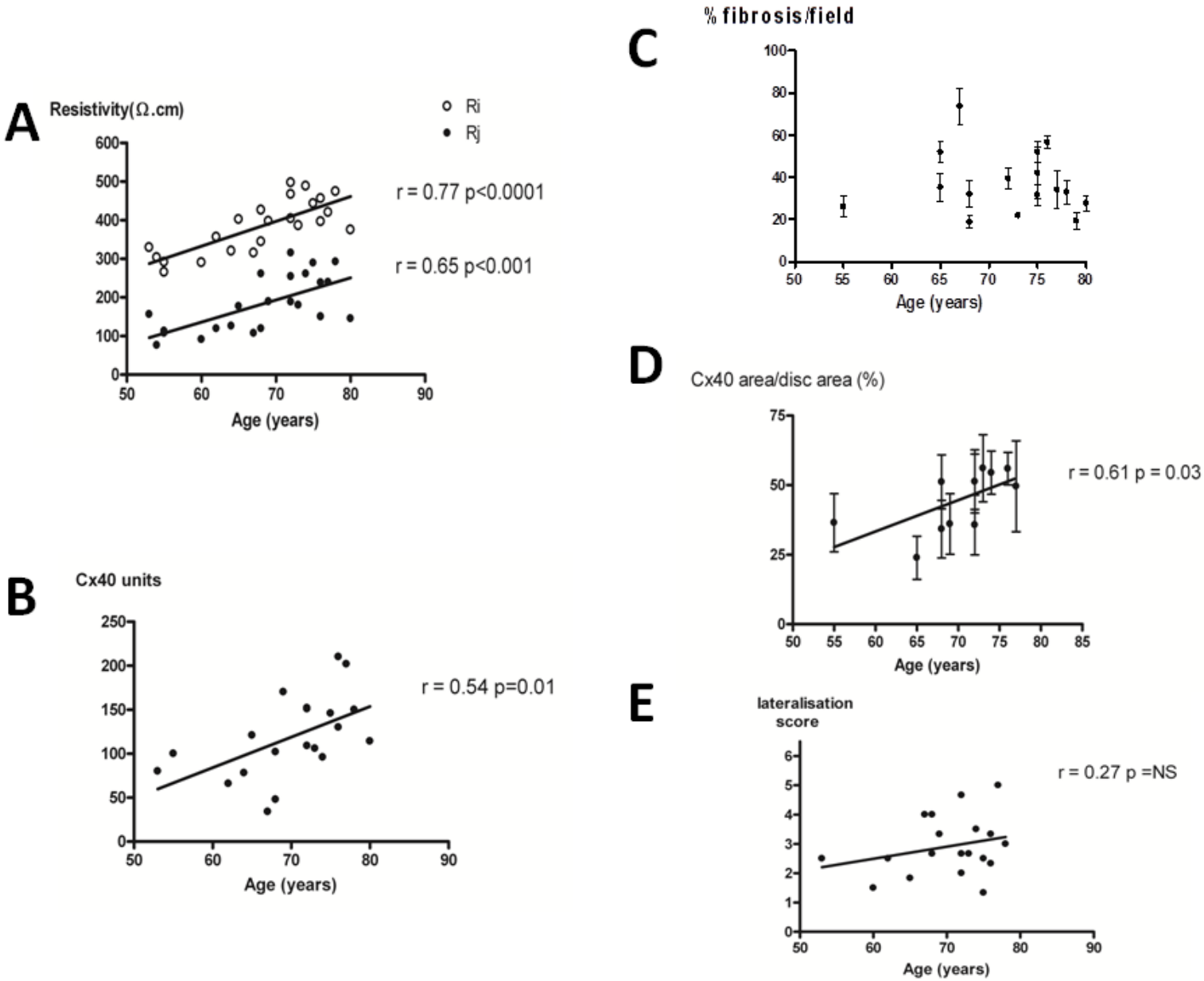

A
Cx40 area/disc area (%)
75
50
25
0
0
50
100
150
200
250
Cx40 units
p=NS
B
Cx40 area/disc area (%)
75
50
25
0
0
100
200
300
400
Rj (Ω.cm)
r = 0.66 p=0.02
C
% labelled fields
100
90
80
70
60
50
0
100
200
300
400
Rj (Ω.cm)
p=NS

Figure 5


A
Resistivity(Ω.cm)
Ri
Rj
r = 0.77 p<0.0001
r = 0.65 p<0.001
Age (years)
B
Cx40 units
r = 0.54 p=0.01
Age (years)
C
% fibrosis/field
Age (years)
D
Cx40 area/disc area (%)
r = 0.61 p = 0.03
Age (years)
E
lateralisation score
r = 0.27 p =NS
Age (years)