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Circulating biomarkers of fibrosis and cardioversion of atrial fibrillation: A prospective, controlled cohort study

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Abbreviations

AAD	Anti-arrhythmia drug
AF	Atrial fibrillation
CVA	Cerebrovascular accident
DCCV	Direct current cardioversion
ECG	Electrocardiogram
EDV	End-diastolic volume
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
EP	Electrophysiology
FGF-23	Fibroblast growth factor 23
Gal-3	Galectin-3
ICTP	Type I collagen carboxyl telopeptide
LA	Left atrium
LV	Left ventricle
MRI	Magnetic resonance imaging
NT pro-BNP	N-terminal pro-brain natriuretic peptide
PIIINP	Procollagen type III N terminal peptide
TIA	Transient ischaemic attack

Abstract

Introduction. External direct current cardioversion (DCCV) is an established treatment for AF but is associated with procedural risk and high AF recurrence rates. Cardiac fibrosis has been associated with AF, and circulating biomarkers have been suggested as a method of its assessment, but which biomarkers are suitable is yet to be determined. This study examines the differences between levels of procollagen type III N terminal peptide (PIIINP), type I collagen carboxyl telopeptide (ICTP), galectin-3 (gal-3) and fibroblast growth factor 23 (FGF-23) in DCCV patients, and disease-and-age-matched controls. Their predictive value for AF recurrence was analysed.

Methods. 79 patients undergoing DCCV and 40 age-and-disease-matched controls were included. Biomarkers were analysed using ELISA. Linear regression was used to examine relationships between biomarker levels and baseline characteristics, including echocardiographic measurements. Cox regression was used to assess relationships between baseline characteristics, including biomarker levels, and AF recurrence.

Results. There was no statistically significant difference between biomarker levels in the DCCV and control groups. Diabetes mellitus was related to higher FGF-23 ($p=0.007$) and PIIINP ($p=0.027$). Female sex ($p=0.014$), hypertension ($p=0.001$), and higher body mass index ($p=<0.001$) were related to higher gal-3 levels. FGF-23 was weakly predictive of AF recurrence (HR 1.003 $p=0.012$).

Conclusion. PIIINP, ICTP, and Gal-3 are not predictive of AF recurrence after DCCV. FGF-23 may be associated with arrhythmia recurrence, but further work is required to clarify this. The presence of AF has no effect on levels of these biomarkers when compared to age and disease-matched controls.

Highlights

- Cardiac fibrosis is associated with AF, and biomarkers may detect this
- Improvement in patient selection for cardioversion is desirable
- Four biomarkers were studied (ICTP, galectin-3, PIIINP, and FGF-23)
- A weak association between FGF-23 and AF recurrence was found
- There were no biomarker differences between AF patients and controls

Introduction

Elective direct current cardioversion (DCCV) remains an established treatment for symptomatic persistent atrial fibrillation (AF).(1) It is immediately successful in up to 90% of cases, however more than half of patients will have experienced recurrence of AF after one year.(2,3) Even in anticoagulated patients, DCCV carries a small risk (approximately 1%) of arterial thromboembolism and stroke.(4) Improved assessment of the likelihood of recurrence may aid better patient selection for rhythm control with DCCV, reducing the number of patients unnecessarily exposed to this risk.

Left atrial (LA) fibrosis has been associated with AF,(5) and recurrence of AF after rhythm control intervention. Clinical methods of assessment of this fibrosis include late gadolinium enhancement MRI, and invasive electrophysiological mapping. Both of these methods have been shown to have predictive value for arrhythmia recurrence after percutaneous ablation.(6,7) However, there is limited access to MRI, and EP mapping is an invasive test requiring direct access to the left atrium. Neither is therefore suitable for assessing the risk

of AF recurrence after cardioversion in usual clinical practice, and more practical methods are desirable. Such a method of assessment for DCCV patients may include the use of circulating biomarkers. Studies have assessed markers of inflammation, myocardial injury, and atrial stretch with varying levels of success.(8-13) A number of substances involved in fibrosis can be measured in peripheral blood and therefore have the potential to act as biomarkers. Evidence is conflicting regarding their use in rhythm control in general, and is sparse in the context of DCCV.(14-19) In this study, we examined type III procollagen N terminal peptide (PIIINP) and type I collagen carboxyl telopeptide (ICTP) - two direct markers of collagen turnover. We also included galectin-3 (gal-3), a more recently identified biomarker of fibrosis that is involved with a number of steps in the inflammatory-fibrotic process, and finally fibroblast growth factor 23 (FGF-23), a hormone principally studied in the context of phosphate homeostasis, but with recent evidence suggestive of a role in AF and possibly AF-related fibrosis.

We designed a study to test the hypothesis that these four fibrosis biomarkers would predict AF recurrence after DCCV. We also hypothesised that these markers would be related to parameters of left atrial remodeling assessed by standard echocardiographic measurements, and would be higher in AF patients when compared to disease and age-matched controls.

Keywords

Fibrosis; atrial fibrillation; cardioversion; biomarkers

Methods

The study was approved by the National Research Ethics Service Committee – Leeds West (ref. 13/YH/0349). All patients recruited gave written informed consent. At a single institution, patients due for DCCV for atrial fibrillation were screened between October 2014 and August 2015. Main inclusion criteria were the presence of persistent symptomatic AF requiring cardioversion and the ability to give informed consent. Main exclusion criteria were designed to avoid confounding by non-cardiac fibrotic pathology and included the presence of chronic kidney disease, malignancy, connective tissue disease or systemic inflammatory disease (e.g. interstitial lung disease or inflammatory bowel disease). Patients with a history of AF ablation were also excluded.

Participants in the control group were selected from patients attending general cardiology clinic for non-arrhythmia related conditions or symptoms. Participants were selectively recruited to create a control cohort matched for age, gender and comorbidity with the DCCV cohort. Similar exclusion criteria were applied, with the presence of any history of AF, other sustained atrial arrhythmia, or undiagnosed palpitations additionally.

External electrical cardioversions were carried out under general anaesthetic according to standard local clinical protocol. Patients were fasted for a minimum of six hours prior to induction of anaesthesia. Biphasic discharges, synchronized to the ECG R wave, were delivered up to a maximum of three times, using an escalating energy protocol of 100J, 150J, 200J. Defibrillator patches were applied to the chest wall in antero-posterior

orientation. If sinus rhythm was not restored after three discharges, no further attempts at sinus rhythm restoration were made.

Blood was taken from a peripheral vein prior to cardioversion and transferred to serum separator tubes for a minimum of 30 minutes. After coagulation, the tubes were centrifuged for 15 mins at 1600g. The separated serum was then aliquoted into sterile non-pyrogenic Eppendorf tubes and frozen at -70^{o}C. Serum was thawed once, just prior to ELISA analysis. Samples were analysed using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Pro-collagen type III N-terminal peptide (PIIINP) and galectin-3 (Gal-3) were analysed using kits produced by Elabscience (Beijing, China). Type I collagen C-terminal telopeptide (ICTP) was analysed using kits produced by Cusabio Life Science (Wuhan, China). Kits were processed according to the manufacturer's instructions, and serial dilutions of serum were used to determine the appropriate dilution factor for each biomarker. Standards of known concentration and serum samples were tested in duplicate. Serum concentrations were extrapolated from optical density readings using a 4-parameter logistic curve derived from the standards, with background correction using blank wells. Inter- and intra-assay coefficients of variation were <15%. Minimum limits of detection were; ICTP = 25 ng/mL, Gal-3 = 0.156 ng/mL, FGF-23 = 15.625 pg/mL, PIIINP = 23.438 pg/mL.

All participants underwent trans-thoracic echocardiography prior to ablation, performed by a single operator with over 5 years' experience. Images were obtained according to British Society of Echocardiography guidelines.(20)

Normally distributed data are expressed as ‘mean ± standard deviation’. Non-parametric data are expressed as ‘median (interquartile range)’. Categorical data are expressed as ‘frequency (percentage)’. Normality of data was assessed using the Shapiro-Wilks test. Comparison between two groups was carried out using Student’s t test, for normal data. Where non-parametric data could not be transformed successfully, Mann-Whitney U test was used. Univariate linear regression was used to assess the effect of baseline characteristics on biomarker levels. Variables with significant effects in univariate analysis were then examined in multivariate linear regression analysis. Univariate Cox regression analysis was used to assess predictors of arrhythmia recurrence. A two-sided p value of 0.05 was used to determine statistical significance.

To calculate power, we hypothesized that there would be an increase of 0.5 standard deviations in the levels of biomarkers in AF patients compared to controls. In order to achieve this variance using criteria of $1-\beta = 0.8$ and $p<0.05$, 35 patients per group would be required for the comparison part of the study. In order to achieve 80% statistical power to detect the difference in outcome based on biomarker levels, using $p=0.05$ to indicate significance, at least 20 AF recurrence events would be required. Therefore, target recruitment was set at 80 for the DCCV group and 40 for the control group.

Results

80 DCCV patients and 40 control patients were recruited. One patient was diagnosed with multiple myeloma after DCCV and died before follow up was complete. This patient’s data was excluded from analysis. Two further patients were lost to follow up. These patients were excluded from outcome analysis, but were included in baseline analysis.

DCCV patients vs. controls

Valid biomarker results were obtained as follows: for the AF group, ICTP n= 76, Gal-3 n=74, PIIINP n=38, FGF-23 n= 27. For the control group, ICTP n = 35, Gal-3 = 36, PIIINP n = 31, FGF-23 n = 12. The patient characteristics are summarised in table 1. LA volume and diameter were significantly higher in the DCCV group, but there were no other differences. Biomarker levels were not significantly different between the groups, although PIIINP levels were approaching significance for being higher in AF patients (p = 0.068).

Table 1. Baseline characteristics and comparison between DCCV and control groups

Characteristic	DCCV Group n=80	Control Group n=40	P Value
Age (years)	63.1 ± 9.9	62.2 ± 10.3	0.627
BMI (kg/m^2)	30.7 ± 7.2	29.0 ± 5.2	0.172
Female sex	24 (30.0)	13 (32.5)	0.780
Hypertension	42 (52.5)	21 (52.5)	1.000
Diabetes Mellitus	9 (11.3)	6 (15)	0.558
Ischaemic heart disease	14 (17.5)	6 (15)	0.729
Stroke or TIA	6 (7.5)	5 (12.5)	0.282
Time since 1st AF diagnosis (months)	6.6 (15.1)	-	-
CHA_2DS_2VASc		-	-
0	14 (17.5)		
1	25 (31.3)		
2	17 (21.3)		
3	13 (16.3)		
4	7 (8.8)		
5	3 (3.8)		
6	1 (1.3)		
AAD	10 (12.5)	-	-

Rate – limiting drugs 0 1 2 3	 8 (10.0) 58 (72.5) 12 (15.0) 2 (2.5)	-	-
LA Volume (mL)	85.3 ± 25.8	56.4 ± 19.3	**<0.001**
LA Diameter (mm)	44.4 ± 5.6	39.7 ± 5.9	**<0.001**
LV EDV (mL)	105.3 ± 35.6	104.7 ± 32.5	0.941
LV EF (%)	54.1 ± 11.9	59.0 ± 12.8	0.731
Gal-3 (ng/mL)	25.2 (28.8)	25.9 (23.7)	0.194
PIIINP (pg/mL)	53.5 (108.5)	42.7 (19.2)	0.068
ICTP (ng/mL)	252.3 (190.4)	195.9 (315.2)	0.369
FGF-23 (pg/mL)	31.5 (102.3)	91.8 (90.3)	0.916

Baseline characteristics and relationship to biomarkers

For each of the four biomarkers, regression analysis was carried out to show relationships with patient characteristics across the DCCV cohort. Full results of this analysis are shown in table 2. In summary, statistically significant results after multivariate analysis were: Diabetes was related to higher PIIINP and FGF-23 levels; higher BMI, female sex, and hypertension were related to higher gal-3 levels.

Table 2. Regression analysis of biomarkers in relation to baseline characteristics, DCCV cohort.				
Characteristic	PIIINP	Gal-3	ICTP	FGF-23

	Beta	P Value	Beta	P Value	Beta	P Value	Beta	P Value
Age	0.306 *0.444*	0.061 *0.088*	-0.025	0.832	-0.063	0.588	-0.050	0.806
BMI	-0.079	0.639	0.538 *0.424*	<0.001 ***<0.001***	0.073	0.528	-0.114	0.572
Female sex	1.396	0.171	0.312 *0.273*	0.007 ***0.014***	-0.260 *-0.194*	0.023 *0.116*	-0.178	0.374
Time since AF diagnosis	0.351 *0.173*	0.033 *0.296*	-0.112	0.353	-0.081	0.494	-0.082	0.689
Hypertension	0.117	0.483	0.387 *0.398*	0.001 ***0.001***	-0.132	0.254	-0.479 *-0.355*	0.012 *0.055*
DM	0.273 *0.411*	0.098 ***0.027***	0.174	0.139	-0.120	0.303	0.463 *0.455*	0.015 ***0.007***
IHD	0.323 *0.058*	0.048 *0.770*	-0.010	0.932	-0.052	0.654	-0.148	0.461
CVA or TIA	-0.031	0.855	0.022	0.852	-0.186	0.107	-0.156	0.437
CHA_2DS_2VASc	0.365 *-0.279*	0.024 *0.365*	0.263 -0.410	0.024 0.736	-0.244 *-0.166*	0.034 *0.176*	-0.388 *-0.219*	0.046 *0.226*
LA volume	-0.244	0.146	-0.008	0.945	0.136	0.892	-0.044	0.837
LA diameter	-0.331 *-0.205*	0.042 *0.245*	0.013	0.915	0.127 *0.144*	0.283	0.053	0.801
LV EDV	-0.299 *-0.106*	0.097 *0.556*	-0.033	0.796	-0.002	0.985	-0.002	0.993
LV EF	-0.044	0.800	0.227 *0.138*	0.066 *0.169*	-0.006	0.961	-0.077	0.735
Values in italics = multivariate analysis. Bold = statisitically significant after multivariate analysis.								

Duration of follow up was 383 ± 54 days. 49 patients (61.3%) experienced AF recurrence within the duration of follow up. Median AF-free survival was 170 days.

There were no significant differences between distribution of biomarker levels in patients with AF recurrence and those without.

In univariate Cox regression analysis of all baseline characteristics (table 3), only FGF-23 was found to be weakly associated with arrhythmia recurrence (HR 1.003, p=0.012).

Table 3. Univariate Cox regression analysis, AF recurrence		
Characteristic	Hazard ratio	P value
Age	1.004	0.802
BMI	1.013	0.506
Female sex	0.624	0.124
Time since first AF diagnosis	0.999	0.657
Hypertension	0.831	0.519
Diabetes mellitus	0.876	0.799
IHD	0.681	0.351
CVA or TIA	2.384	0.101
CHA_2DS_2VASc	1.087	0.431
LA volume	1.000	0.992
LA diameter	1.000	0.994
LV EDV	0.991	0.074
LV EF	1.015	0.297
PIIINP	1.002	0.292

Gal-3	1.002	0.778
ICTP	1.001	0.382
FGF-23	1.003	**0.012**
ICTP / PIIINP ratio	1.022	0.607

Discussion

Selection of biomarkers

The hallmark of fibrosis is an increase in volume and turnover of extracellular matrix (ECM). In the heart, ECM is comprised predominantly of type I, and to a lesser extent type III, collagen. PIIINP is cleaved from the pro-peptide of type III collagen in the extracellular space and thereby enters the bloodstream, so serves as a marker of the synthesis of type III collagen. It has been shown to be present in higher concentrations in LA tissue of AF patients.(21) ICTP is a product of the breakdown of type I collagen by matrix metalloproteinase 1.(22) By including both of these substances in the study, the aim was to assess turnover of ECM, so the ratio of type I collagen catabolism to type III collagen synthesis was examined by ICTP:PIIINP ratio, however this value was not related to baseline characteristics or outcome.

Gal-3, a member of the lectin family, has been found to have diverse direct and indirect actions associated with fibrosis in multiple tissues. It is associated with the presence of myofibroblasts and macrophages, which have been shown to proliferate in cardiac fibrosis. It appears to have activity related to cell adhesion, growth and differentiation, apoptosis and chemo-attraction, and has been associated with fibrogenesis, inflammation and

ventricular remodeling.(23-25) It has been associated with risk of developing AF, both in the general population, and after myocardial infarction.(26,27) Studies assessing its association with arrhythmia recurrence after ablation have shown mixed results. (28,29) It has not been studied in the context of cardioversion previously. Higher levels have been associated with hypertension, female gender and higher BMI, and our findings agree.(30-32)

FGF-23 has come to light in recent years in the context of its endocrine role in phosphate homeostasis. It has been studied principally in heart failure and kidney disease, and does appear to be related to cardiac outcomes in the latter, as well as independently of renal function.(33,34) In the fibrotic atrial myocardium found in AF, cardiomyocytes have been found to de-differentiate into a more primitive cell type.(35) FGF-23 has been shown *in vitro* to be required for cardiomyocyte proliferation and differentiation in early embryonic stages, and, speculatively, may have a role in the cardiomyocyte changes found in the fibrotic atria of AF patients, including calcium handling.(36) It has been associated with increased atrial wall tension manifest by NT-proBNP, but results of studies assessing its association with incident AF are mixed.(37,38) It has not been assessed in the context cardioversion previously.

DCCV patients vs controls

This study is, to our knowledge, unique in comparing AF patients selected for DCCV to age and disease-matched controls when assessing biomarkers of fibrosis. In this study, there was no difference between levels of the four biomarkers between the DCCV patients and controls, which were well-matched for age and comorbidities. PIIINP levels approached

significance for being higher in AF patients, suggesting that either there is no true difference, or perhaps that any true difference is so slight as to be undetectable in a study of this size. Overall these findings are suggestive that either the DCCV patients had similar levels of fibrosis to the controls, or any differences in fibrosis were not detectable using these biomarkers peripherally. This may be accounted for by the fact that the controls were not disease-free, and comorbidity in the control group may have accounted for a rise in biomarker levels, therefore masking any specific contribution of AF to biomarker levels in the DCCV cohort. It may be the comorbidities present in AF patients that cause the increase in fibrosis biomarkers noted in other studies. It should be noted that one of the limitations of this study is the impossibility of excluding non-symptomatic paroxysmal AF in the control group, however it is unlikely that this would have been a significant problem.

Previously, Sonmez et al. showed higher levels of gal-3 and PIIINP in AF patients compared to sinus rhythm age-matched controls, in a slightly smaller study.(39) While controls in that study were matched for most baseline characteristics, there was significantly higher heart failure in the AF group. This may have had an important confounding influence on their results, as both PIIINP and, more convincingly, gal-3 have been associated with reduced LV systolic function.(28) Gurses et. al. did find significantly higher levels of gal-3 in AF patients, although in their study they excluded patients with reduced ejection fraction, which again makes comparison difficult.(40) Zakeri et al. found no gal-3 or PIIINP differences, and lower ICTP levels in controls.(41) However 23% of their sinus rhythm patients had a previous history of AF. They were also younger, with better LV function. In an AF ablation cohort, Kornej et al. also showed no difference between gal-3 in AF patients and controls after multivariate analysis.(28) If nothing else, these studies highlight that work in this field involves heterogenous patient groups between studies and it can therefore be difficult to

draw firm conclusions regarding the overall AF patient population. In our study, only left atrial dimensions were different between the groups. As left atrial remodeling is a known effect of AF, this is to be expected. As the controls appear to be more well-matched than in other studies, the results are more robust.

Predictive value of the biomarkers

PIIINP, ICTP and Gal-3 had no predictive value for recurrence of AF. FGF-23, however, was found to be significantly, but weakly associated with recurrence, with a hazard ratio of 1.003 per 1 pg/mL increase in FGF-23 level. This result has not previously been reported, but must be interpreted with caution due to the low number of valid results for this biomarker compared to the overall number of patients, due to the limitations of the ELISA assay. It is possible that a more sensitive test may be able to explore this finding further.

Gal-3 has not been studied in the context of DCCV, however in ablation results have been mixed. Kornej et. al. found no association between AF recurrence and gal-3 levels in their ablation cohort.(28) Conversely Wu et. al. did show that gal-3 was associated with AF recurrence after ablation, however this finding is in the context of a younger cohort with lone AF so, again, is not comparable with this study.(42)

PIIINP was found to be independently associated with outcome by Kawamura et al. in their study of 142 patients undergoing pharmacological cardioversion, with DCCV if this failed.(17) Patients were followed up for 24 months. The baseline characteristics of patients in their study were similar to ours, with the notable exceptions of LV ejection fraction, which was lower in our cohort, and duration of AF which was much higher in our cohort. This may explain why this study did not reproduce their results, and we feel that our cohort more closely resembles ‘typical’ patients selected for DCCV, at least in our experience.

Finally Kallergis et. al. showed an association between recurrence and ICTP in their study of 164 DCCV patients. Notably however, they excluded patients with a significant number of comorbidities which may make their findings difficult to generalize to the AF population.

Conclusion

PIIINP, ICTP, and Gal-3 are not predictive of AF recurrence after DCCV. FGF-23 may be associated with arrhythmia recurrence, but further work is required to clarify this. The presence of AF has no effect on levels of these biomarkers when compared to age and disease-matched controls.

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