Title:
Endothelial dysfunction and glycocalyx shedding in heart failure: insights from patients receiving cardiac resynchronisation therapy.

Brief Title: Glycocalyx shedding in systolic heart failure

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Abstract

Objectives:

To determine (a) whether chronic heart failure with reduced ejection fraction (HFrEF) is associated with increased glycocalyx shedding; (b) whether glycocalyx shedding in HFrEF with left ventricular dyssynchrony is related to inflammation, endothelial dysfunction and/or redox stress and is ameliorated by cardiac resynchronisation therapy.

Background:

Glycocalyx shedding has been reported to be increased in heart failure and is a marker of increased mortality. Its role in dyssynchronous systolic heart failure and the effects of cardiac resynchronisation therapy (CRT) are largely unknown.

Methods:

Twenty-six patients with dyssynchronous HFrEF were evaluated before and 6 months after CRT insertion. Echocardiographic septal to posterior wall delay (SPWD) assessed intra-ventricular mechanical dyssynchrony, and quality of life, integrity of nitric oxide (NO) signalling, inflammatory and redox-related biomarkers were measured. Glycocalyx shedding was quantitated via plasma levels of the glycocalyx component, syndecan-1.

Results:

Syndecan-1 levels pre-CRT were inversely correlated with LVEF (r=-0.45, p=0.02) and directly with SPWD (r=0.44, p=0.02), QOL (r=0.39, p=0.04), plasma NT-proBNP (r=0.43, p=0.02), and the inflammatory marker, symmetric dimethylarginine (SDMA) (r=0.54, p=0.003). On multivariate analysis, syndecan-1 levels were predicted by SPWD and SDMA (β=0.42, p=0.009 and β=0.54, p=0.001, respectively). No significant correlation was found
between syndecan-1 levels and other markers of endothelial dysfunction/inflammatory activation. Following CRT there was no significant change in syndecan-1 levels.

Conclusions:

In patients with dyssynchronous HFrEF, markers of glycocalyx shedding are associated with magnitude of mechanical dyssynchrony and elevation of SDMA levels and inversely with LVEF. However, CRT does not reverse this process.

**Keywords:** Glycocalyx shedding, cardiac failure, resynchronization therapy, endothelial function, symmetric dimethylarginine

**List of abbreviations:**

CRT: cardiac resynchronisation therapy
SPWD: Septal to posterior wall delay.
QOL : Quality of life
LVEF: Left ventricular ejection fraction
NT-proBNP: N-terminal pro brain natriuretic peptide
AIx : Augmentation index
NO: nitric oxide
SDMA: Symmetric dimethylarginine
TXNIP: thioreredoxin interacting protein.


**Introduction**

In spite of several ground-breaking advances in the understanding and management of heart failure over the last few decades, HFrEF remains common in most Western societies with substantial associated morbidity, mortality and economic burden [1]. This underscores the need for further understanding of the pathophysiological mechanisms of heart failure. It is known that chronic heart failure is associated with immune and inflammatory activation [2], as well as with vascular endothelial dysfunction [3] with its associated potential for disturbance in laminar blood flow [4]. There is increasing evidence that shedding of the vascular endothelial glycocalyx may represent a link between these two processes [5], largely via activation of matrix metalloproteinases [6] and resultant induction of endothelial dysfunction. However, the extent of endothelial glycocalyx shedding in patients with severe HFrEF and concomitant left ventricular dyssynchrony, and its putative association with nitric oxide signalling and inflammatory activation, remain largely unexplored. Importantly, a direct correlation between glycocalyx shedding and loss of laminar blood flow has been observed [7].

Over the last two decades, cardiac resynchronisation therapy (CRT) has emerged as the standard of care in patients with HFrEF associated with concomitant dyssynchronous ventricular contractions, especially with left bundle branch block. CRT offers symptomatic benefit to approximately two-thirds of recipients; several landmark trials have shown that CRT, with or without defibrillator insertion, not only improves ‘soft’ clinical end-points, but also reduces risk of mortality and reduces heart failure hospitalisations [8, 9]. The basis for this benefit of CRT remains uncertain but it is thought that reduction in dyssynchrony contributes to improvement in clinical status [10]. Nonetheless, large multi-centre prospective trials have suggested that no echocardiographic measure of dyssynchrony is sufficient by itself to predict response to CRT [11].
Recently, efforts to understand the mechanisms of benefit associated with CRT have begun to address factors beyond improvement in left ventricular contractile efficiency, turning to possible effects on peripheral vascular function [12-14]. It is now known that the integrity of the endothelial glycocalyx layer plays a prominent role in maintenance of vascular endothelial function by modulating vascular permeability, coagulation, vascular tone, and complement activation [15]. Increased glycocalyx shedding occurs in conditions of increased shear and oxidative stress [7]. Release into plasma of syndecan-1, a marker of glycocalyx shedding and thus of endothelial dysfunction, has been found to be increased in patients with heart failure [16]. This elevation of plasma syndecan-1 concentrations is also a correlate of increases in the combined end point of all-cause mortality and hospitalised heart failure in patients with heart failure with preserved ejection fraction [17]. The role of glycocalyx shedding in patients with systolic heart failure and concomitant left ventricular dyssynchrony remains unknown.

**Objectives of the current study**

1. To confirm activation of glycocalyx shedding in chronic dyssynchronous HFrEF and to identify the clinical determinants of the extent of this putative change with particular regard to severity of heart failure and extent of left ventricular dyssynchrony.

2. To evaluate the potential interactions of glycocalyx shedding with concomitant inflammation, endothelial dysfunction and redox stress, and in particular to determine whether glycocalyx shedding is associated with:
   a. Impaired function of the NO signalling cascade
b. Increased expression of the inflammatory activator, thioredoxin-interacting protein (TXNIP), the expression of which is increased by non-laminar flow [18].

3. To determine whether CRT, possibly by ameliorating non-laminar flow, reduces the extent of glycocalyx shedding.

**Methods**

Patients with conventional indications for CRT (n=26) were prospectively evaluated before and six months after CRT implant. Patients’ quality of life was evaluated using the Minnesota Living with Heart Failure Questionnaire, with higher scores indicating more impaired quality of life.

**Echocardiographic measurements**

All echocardiographic measurements were performed according to the American Society of Echocardiography and European Association of Cardiovascular Imaging guidelines [19]. A Phillips echocardiogram machine model iE33, 2009, Bothell WA, 98041 USA was used for image acquisition and analyses were performed using Echopac Software Only BT 11 Version 113, 2013 General Electric Co. M-mode echocardiographic analysis was used to assess left ventricular intra-ventricular dyssynchrony. Septal to posterior wall delay (SPWD) was calculated as the time difference between the onset of the QRS to the peak of deformation of the inter-ventricular septum and the left ventricular posterior wall. Although extent of dyssynchrony was analysed as a continuum, SPWD of 130ms or more was considered diagnostic of clinically significant intra-ventricular mechanical dyssynchrony [20]. Left ventricular volumes including end-diastolic, end-systolic and stroke volumes were measured in two dimensions and ejection fraction calculated by the modified Simpson’s method in biplane using 2D images.
Radial artery applanation tonometry: determination of augmentation index.

Vascular endothelial function was assessed by changes in augmentation index (AI$_X$) using radial artery applanation tonometry as previously described [21]. Briefly, patients were first rested in a supine position for 30 minutes. Using a commercially available pulse waveform analyser, the SphygmoCor system (AtCor Medical, Sydney, Australia, model CvMS V9) baseline AI$_X$ was computed as the average of three readings. Sublingual nitroglycerin (NTG 300µg) was administered and AI$_X$ re-measured every 5 minutes for twenty minutes. The difference between the lowest value of AI$_X$ with NTG and the baseline AI$_X$ (that is, the maximum change in AI$_X$ with NTG) was utilised as a measure of endothelium-independent NO signalling. Subsequently, 400µg of inhaled salbutamol was administered and measurements were repeated every 5 minutes for twenty minutes. The difference between the lowest AI$_X$ with salbutamol and the baseline (the maximum change in AI$_X$ with salbutamol) represents a measure of endothelium-dependent NO signalling [22]. Using the acquired radial artery waveform, a validated, generalized transfer function was used to generate the corresponding central aortic pressure waveform from which AI$_X$ values were calculated. All measurements were indexed to a heart rate of 75 beats per minute and only high fidelity tracings were used.

Platelet expression of thioredoxin-interacting protein (TXNIP)

Platelet expression of TXNIP was quantitated by immunofluorescence as previously described [23].

Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA)
For estimation of asymmetric dimethylarginine and symmetric dimethylarginine concentrations in plasma, 10ml of blood was collected in heparinised vacutainer tubes and immediately put in ice, before centrifugation at 4°C at 1800g for 15 minutes. Plasma was collected in Eppendorf tubes and stored at -70°C until analysed. Subsequent determination was performed via high performance liquid chromatography as described by Heresztyn et al [24].

**Matrix metalloproteinase-2 and matrix metalloproteinase-9**

Matrix metalloproteinase-2 was estimated from blood samples collected in EDTA tubes while matrix metalloproteinase-9 was estimated from blood samples collected in heparinised tubes. For both, collected blood was immediately put on ice and was centrifuged for 15 minutes at 1800g at 4°C within 30 minutes of collection. Plasma was collected and stored at -70°C until analysed. Assays were performed with RnD Quantikine quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kits (Minneapolis USA) according to the manufacturer’s instructions.

**Platelet aggregometry test; responsiveness to NO**

Platelet response to NO was assessed in vitro by whole blood impedance aggregometry according to a previously described protocol [25]. Therapy with P2Y12 receptor antagonists was temporarily interrupted prior to carrying out the tests in order not to interfere with ADP responses.

**N-Terminal proBNP levels**

Samples were collected in heparinised tubes and analysed for NT-proBNP levels with the Elecsys proBNP system (Roche diagnostics GmbH, Sandhofer Strasse 116, D-68305)
Mannheim, Germany). The Sandwich principle was used according to manufacturer’s instructions.

**Plasma metanephrine and normetanephrine**

Analyses for catecholamine metabolites were performed with fresh samples collected in K<sub>3</sub>EDTA bottles and analysed utilizing liquid chromatography-tandem mass spectrometry. The upper limits of the normal range for plasma concentrations were 500 pmol/L for free metanephrine and 900 pmol/L for free normetanephrine.

**Measurement of plasma levels of syndecan-1.**

For syndecan-1 assay, blood was collected in EDTA tubes and immediately put on ice and centrifuged for 15 minutes at 1800g at 4°C within 30 minutes of collection. Plasma was collected and stored at -70°C until analysed. Analysis was performed utilizing Human sCD138 ELISA kit (Diaclone SAS, France version 7, 2015) according to manufacturer’s instructions.

**Ethics approval.**

The study complied with the Declaration of Helsinki and approval for the study was granted by the Ethics and Human Research Committee of The Queen Elizabeth Hospital and all participants provided written informed consent.

**Statistical analyses**

All data are expressed as mean ± SD unless stated otherwise. Univariate correlations between syndecan-1 levels and endothelial/NO- signalling, echocardiographic parameters and
neurohumoral activation were performed using Pearson correlation coefficients for normally distributed data and Spearman correlation for non-parametric data. Backward stepwise multiple regression analysis was performed utilising parameters with significant univariate correlations with baseline syndecan-1 levels. The interaction of CRT with glycocalyx shedding was assessed by Wilcoxon matched-pairs signed rank test. Apart from the multiple regression analyses performed using SPSS 23 version 11.8.2 (2013 Citrix systems), all other analyses were performed using Prism 6 for Mac OS X version 6.0h October 2015. A p-value < 0.05 was considered statistically significant.

Results

The baseline characteristics of patients are listed in Table 1. Half of the patients had an ischaemic basis for heart failure, the majority had class III-IV symptomatic status and all had substantial evidence of dyssynchrony both electrically and mechanically. All patients were also extensively treated medically for systolic heart failure prior to CRT implantation. The biochemical and physiological data for this group are summarized in Table 2.

In general, renal function was well preserved, as was platelet response to the NO donor, sodium nitroprusside. Median syndecan-1 levels were clearly elevated beyond the previously reported range for normal subjects (34.1 ± 8.0 ng/ml [26].

Univariate correlates of Syndecan-1 at baseline:

Syndecan-1 levels correlated negatively and significantly with left ventricular ejection fraction (r=-0.45, p=0.02). There was also a statistically significant positive correlation between syndecan-1 levels and septal to posterior wall delay (r=0.44, p=0.02) and a statistically significant positive correlation between syndecan-1 and NT-proBNP (r=0.43, p=0.02). There was a trend towards a significant positive correlation with plasma
normetanephrine ($r=0.37$, $p=0.09$). A direct correlation between syndecan-1 levels and degree of impairment of quality of life measured with the Minnesota Living with Heart Failure Questionnaire (with higher values indicating greater impairment) was also observed ($r=0.39$, $p=0.04$). These relationships are depicted in Figure 1.

With regard to vascular endothelial function as assessed with applanation tonometry and NO signalling, there was no significant correlation between syndecan-1 levels and baseline augmentation index ($r=-0.23$, $p=\text{NS}$), nor with change in augmentation index in response to salbutamol ($r=-0.12$, $p=\text{NS}$). There was also no significant correlation between levels of syndecan-1 and those of asymmetric dimethylarginine (ADMA) ($r=0.28$, $p=0.15$), inhibition of ADP-induced platelet aggregation with sodium nitroprusside ($r=-0.33$, $p=0.12$), nor with platelet thioredoxin-interacting protein (TXNIP) levels ($r=0.22$, $p=\text{NS}$). On the other hand, there was a strong and significant positive correlation between syndecan-1 and symmetric dimethylarginine (SDMA), ($r=0.54$, $p=0.003$).

**Multivariate determinants of syndecan-1 at baseline**

Backwards stepwise multiple linear regression analysis was performed utilising parameters that exhibited significant univariate correlations with syndecan-1 levels (ejection fraction, SPWD, SDMA concentrations and NT-pro BNP concentrations, together with baseline eGFR ($p=0.10$ on univariate analysis).

The results, summarized in Table 3, revealed that baseline SPWD and SDMA concentrations were both directly and significantly related to syndecan-1 levels. Importantly, eGFR was not significantly related to syndecan-1 levels.

**Effects of CRT**
There was no significant change in syndecan-1 levels between baseline and 6 months post-CRT median (IQR) 55 ng/ml (39.2-75.2) vs 59 ng/ml (31.7-79.6) respectively, p=0.45. There was also no significant correlation between changes in LVEF post-CRT and those in syndecan-1 levels at 6 months, (r=0.31, p=0.13). Similarly, no correlations were found between changes in syndecan-1 levels and other measures of vascular biology and NO signalling including change in baseline augmentation index (r=−0.16, p=NS), change in AIx with salbutamol (r=−0.35, p=NS), platelet ADP-aggregation inhibition with sodium nitroprusside (r=−0.01, p=NS), ADMA (r=−0.14, p=NS), SDMA (r=0.13, p=NS), nor platelet TXNIP content, (r=0.05, p=NS). In addition, there were no significant correlations between changes in syndecan -1 and NT-proBNP (r=0.04, p=NS), nor with changes in SPWD (r=0.06, p=NS).

**Discussion**

In this study, we sought to determine whether glycocalyx shedding, as measured by release of syndecan-1 into plasma, plays a major part in the pathophysiology of severe systolic heart failure associated with extensive left ventricular dyssynchrony. The bases for these evaluations were: (1) endothelial glycocalyx shedding may be triggered by inflammatory activation, particularly involving matrix metalloproteinases [27], and (2) in turn may precipitate endothelial dysfunction and abnormal responsiveness of blood vessels to shear stress: all of which have been reported in systolic heart failure [4].

To date, there have been 2 reported clinical studies relevant to the current observations. Tromp et al [17] performed an evaluation of syndecan-1 levels in a large cohort of patients with chronic heart failure, many of whom had preserved ejection fractions. Levels of syndecan-1 were not elevated beyond the normal range (median 20.1ng/ml), and these exhibited correlations on multivariable regression analyses with markers of fibrosis and
remodelling, but not with extent of LV systolic dysfunction. No correlations with extent of
dyssynchrony were sought. A Brazilian study [16] of patients with acutely decompensated
heart failure (with mean LVEF of 41.5 ±14.4%) revealed far greater mean syndecan-1 levels
(approximately 130ng/ml), which were correlated with extent of acute kidney injury and with
in-hospital mortality rate. The current study is therefore the first to indicate that chronic
systolic heart failure is associated with evidence of increased glycocalyx shedding.

In the current study, univariate analyses suggested that the extent of syndecan-1 elevation was
directly related both to the degree of impairment of LVEF and also to the degree of
mechanical dyssynchrony. As regards correlations with biochemical/physiological markers, to
our surprise there was no correlation between syndecan-1 levels any parameters of integrity of
NO signalling, given that in canine models, the integrity of the endothelial glycocalyx layer
had been found to flow-mediated NO synthesis[28]. To the best of our knowledge, no
previous study had evaluated this putative association in human studies. Therefore it appeared
that variability in NO signalling in such patients may have primarily reflected factors such as
treatment modalities [29, 30]. In support of this possibility, syndecan-1 levels noted in the
Brazilian cohort of patients with acutely decompensated heart failure[16] were much higher
than in the currently evaluated patients. In our centre, we have also observed that in patients
with acute takotsubo cardiomyopathy, plasma syndecan -1 levels were much higher (97 ±
65ng/ml) [31] than in our cohort of patients with chronic HFrEF, who were already receiving
optimal medical treatment.

Syndecan-1 levels also exhibited direct univariate correlations with NT-proBNP and SDMA
and this is consistent with greater cardiomyocyte distension and increased inflammation
respectively[32, 33].
On multivariate analyses, there were persistent significant associations with degree of mechanical dyssynchrony and with SDMA levels; the latter remained significant after taking eGFR values into account, which is important given the substantial influence of renal function on SDMA clearance [34]. On the other hand, TXNIP expression, which might have been expected to be a direct correlate, given its association with non-laminar flow [18], exhibited no significant association with syndecan-1 levels. Again this may well reflect suppression of TXNIP expression by ACE inhibitor therapy [35].

CRT did not reduce syndecan-1 levels 6 months post procedure in spite of the fact the cohort as a whole exhibited significant improvement in LVEF as well as in clinical symptoms. Thus the lack of effect on syndecan -1 levels may indicate that CRT is relatively ineffective in restoring laminar flow, and/or that the deposition of fibrous tissue leads to ongoing inflammatory activation, which perpetuates glycocalyx shedding

As regards the two significant correlates of syndecan-1 levels on multivariate analyses, (that is, with dyssynchrony and SDMA levels), only association, rather than causation, can be identified definitely at this stage. However, syndecan-1 release appears to engender fibrotic change in tissues [17], and indeed conducting system fibrosis is likely to be critical to development of mechanical dyssynchrony [36]. As regards SDMA, this is generated by protein catabolism and cleared largely, but not entirely, unchanged in the urine. Since it has also been suggested that SDMA may represent a surrogate for glomerular function [34], it is important that its association with syndecan-1 levels was independent of eGFR. Since it has recently emerged that SDMA contributes to inflammatory activation [37], the association with syndecan-1 may reflect the inflammatory origin of glycocalyx shedding, but SDMA may
also contribute to development of myocardial inflammation and fibrosis in this group of patients.

Study Limitations

The study was limited mainly by its small size, with possible risk of Type 2 error, for example, regarding effect of CRT insertion, which might have expected to lower syndecan-1 levels, and also relationship with extent of systolic dysfunction. Importantly, the patients were extensively treated medically and this may have affected findings. Finally, as mentioned earlier, we are unable to draw any definite conclusions regarding cause- and- effect relationships, but it does appear that reducing mechanical dyssynchrony by CRT does not reverse glycocalyx shedding.

Conclusions:

In this study, we demonstrated for the first time that in patients with severe left ventricular systolic dysfunction and concomitant mechanical dyssynchrony, the activation of glycocalyx shedding and that the extent of this process correlated directly with the degree of left ventricular mechanical dyssynchrony. Furthermore, independent of renal function, glycocalyx shedding is also associated with significantly higher plasma levels of the inflammatory marker, SDMA. Larger studies and perhaps with patients with less advanced stages of heart failure are required to shed more light on the possible effects of CRT in patients with systolic heart failure and concomitant left ventricular dyssynchrony.
Clinical Perspectives:

Patients under consideration for cardiac resynchronisation therapy (CRT) usually constitute a group with severe systolic heart failure refractory to conventional pharmacotherapy. In the current study, we provide the first clinical evidence that such CRT candidates suffer from acquired damage to the glycocalyx, or outer layer of endothelial cells. This will cause increased capillary permeability, predisposing towards development of congestion irrespective of severity of hemodynamic changes. Interestingly, glycocalyx damage increased directly with the extent of mechanical dyssynchrony, but was not reversed within 6 months of CRT insertion.

Translational outlook:

Glycocalyx ‘shedding’ is a well-described process activated by pro-inflammatory enzymes such as matrix metalloproteinases. In animal models, inhibition of these enzymes, for example by low dose doxycycline, reverses damage to the glycocalyx, and ameliorates circulatory disturbances. The results of the current study should therefore increase therapeutic focus on peripheral circulatory hemostasis in severe heart failure, and potential for institution of treatments to protect the glycocalyx in such patients.
Conflicts of interest

The authors declare that there was no conflict of interest

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References


Figure Legends:

Figure 1: Univariate correlates of clinical, echocardiographic and biochemical plasma concentrations of syndecan-1 at baseline:
A: with LVEF
B: with SPWD
C: with NT-proBNP
D: with normetanephrine
E: with QOL score

Figure 2: NO and redox-signalling correlates of plasma syndecan-1 levels at baseline
A: Baseline AIX
B: AIX change with salbutamol
C: Platelet aggregation inhibition with sodium nitroprusside(SNP)
D: with TXNIP
E: with SDMA