Studies on the Vitamin C transporter gene SLC23A2 and cardiac conduction

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Studies on the Vitamin C transporter gene SLC23A2 and cardiac conduction

Joyce Chi Man TANG

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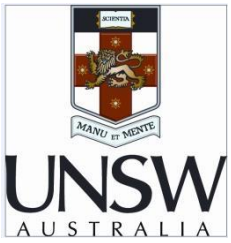
Master of Science (Research)



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This thesis considers the role of Vitamin C genes on cardiac conduction. Firstly we performed a systematic literature review to ascertain the protective effects of Vitamin C dosing in preventing in-hospital onset atrial fibrillation post cardiac surgery. We noted considerable heterogeneity across published studies on Vitamin C dosing and AF outcomes in cardiac surgery. We wondered if this heterogeneity may be due to genetic polymorphisms in the vitamin C transporter, responsible for Vitamin C uptake into cardiac tissues. There are no studies that we are aware of that have explored Vitamin C transporter polymorphisms and cardiac conduction.

It is known that 6 weeks dosing of Vitamin C reportedly decreases electrocardiogram (ECG) QRS duration in young healthy volunteers. Our aims were to explore genetic polymorphisms in the vitamin C transporter on cardiac ventricular conduction. We used a retrospective rural bio-bank where ECG derived QRS duration was available. Specifically we determined Vitamin C transporter rs1776964 genotyping using DNA (derived from blood samples) from 274 Australian rural subjects (with and without type 2 diabetes mellitus) aged 21 years and over. We demonstrated that the ECG QRS duration had no significant association across rs1776964 genotypes.

In conclusion further vitamin C transporter genotype studies may be required to understand associations between Vitamin C dosing and QRS duration. Vitamin C transporter polymorphisms may explain the heterogeneity in atrial fibrillation outcomes in post cardiac surgery, although we cannot demonstrate an association in our study with ventricular conduction.

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Abbreviation / Term	Definition
AA	Ascorbic acid
ACS	Acute coronary syndrome
AF	Atrial fibrillation
BMI	Body mass index
CABP	Coronary artery bypass graft
CASP	Central aortic systolic pressure
CRP	C-reactive protein
CTS	Cardiothoracic surgery
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DHAA	Dehydroascorbic acid
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
ERP	Effective refractory period
HT	Hypertension
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidases
OR	Odds ratio
PCR	Polymerase chain reaction
POAF	Post operational atrial fibrillation
ROS	Reactive oxygen species
SA	Sinoatrial
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SNP	Single nucleotide polymorphism
SVCT	Sodium dependent Vitamin C transporters
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor necrosis factorα
Vit C	Vitamin C
YO	Years old

ABSTRACT

This thesis considers the role of Vitamin C genes on cardiac conduction. Firstly we performed a systematic literature review to ascertain the protective effects of Vitamin C dosing in preventing in-hospital onset atrial fibrillation post cardiac surgery. We noted considerable heterogeneity across published studies on Vitamin C dosing and AF outcomes in cardiac surgery. We wondered if this heterogeneity may be due to genetic polymorphisms in the Vitamin C transporter, responsible for Vitamin C uptake into cardiac tissues. There are no studies that we are aware of that have explored Vitamin C transporter polymorphisms and cardiac conduction.

It is known that 6 week dosing of Vitamin C reportedly decreases electrocardiogram (ECG) QRS duration in young healthy volunteers. Our aims were to explore genetic polymorphisms in the Vitamin C transporter on cardiac ventricular conduction. We used a retrospective rural bio-bank where ECG derived QRS duration was available. Specifically we determined Vitamin C transporter *rs1776964* genotyping using DNA (derived from blood samples) from 274 Australian rural subjects (with and without type 2 diabetes mellitus) aged 21 years and over. We demonstrated that the ECG QRS duration had no significant association across *rs1776964* genotypes.

In conclusion further Vitamin C transporter genotype studies may be required to understand associations between Vitamin C dosing and QRS duration. Vitamin C transporter polymorphisms may explain the heterogeneity in atrial fibrillation outcomes in post cardiac surgery, although we cannot demonstrate an association in our study with ventricular conduction.

CHAPTER 1 – LITERATURE REVIEW

Cardiac conduction and remodelling

Cardiac conduction is often disturbed in structural heart disease. Ventricular chamber remodelling may alter tissue geometry. Hypertrophic signalling results in cardiac inflammation and oxidative stress. This can result in further structural and electrical remodelling (Nattel et al. 2007). Cardiac myocytes may respond to stretch (as a result of ventricular remodelling or fibrosis) via ion channel remodelling (Nattel et al. 2007). This can result in conduction changes and provide a substrate for potential arrhythmias. As an example, atrial fibrillation (AF) is often associated with atrial and or ventricular remodelling. Atrial fibrillation (AF) is a common cardiac arrhythmic condition, and presents as a significant public health problem, especially in the aging populations. The full underlying mechanisms of AF are still unclear. Previous studies demonstrated that oxidative stress and inflammation may contribute to the pathogenesis and perpetuation of AF (Carnes et al. 2001; Korantzopoulos et al. 2005; Korantzopoulos et al. 2007; Liu et al. 2012; Mihm et al. 2001; Tousoulis et al. 2009). In recent decades, antioxidant therapy has been proposed for AF prevention. Vitamin C, which consists of ascorbic acid, dehydroascorbic acid and its other associated forms, is a major dietary anti-oxidant (Da Costa et al. 2013; Korantzopoulos et al. 2007).

Vitamin C background and biochemistry

More specifically Vitamin C is a water soluble molecule that exhibits both anti-oxidant and anti-inflammatory effects (Da Costa et al. 2013; Naidu 2003). Unlike other animals that can synthesize ascorbic acid themselves, humans require intake of Vitamin C through food and/or supplements. The body pool of ascorbic acid in an adult is

approximately 1.2-2.0g, with a half-life of 10-20 days (Naidu 2003). Vitamin C is often referred to as ascorbic acid (AA), dehydroascorbic acid (DHAA) and its associated forms (see below).

It is known that both AA and DHAA have anti-oxidative properties and in a biological medium, AA is oxidized to DHAA. DHAA can be reversibly reduced to AA, or hydrolyzed and oxidized to 2,3-diketogulonic acid and oxalic acid. DHAA is suggested to have different biological roles compared to AA. For example, in cell culture systems, AA exhibit distinct UV absorption, fluorescence and electrochemical detection profiles. DHAA levels represent less than 5% of the total AA in human plasma samples. The determination of total AA is usually the sum of AA and DHAA content (Deutsch 2000; Fenoll 2011; Gazdik et al. 2008; Klimczak and Gliszcyńska-Świgło 2015; Liu et al. 2012; Loh et al. 2003; Loricchio et al. 2007; Lykkesfeldt 1995; Marzilli et al. 2012; Nováková et al. 2009; Sadowska-Bartosz and Bartosz 2015; Watson et al. 2004).

The rapid rise in plasma AA levels after Vitamin C intake is reflected by the metabolic turnover rate. The tissue AA concentration can be maintained at satisfactory levels despite depletion of plasma AA levels. That is tissue levels are somewhat independent of Vitamin C intake, implying that the tissue AA reserves may be replenished at a relatively slower rate (Omaye et al. 1987).

This review aims to explore AF and Vitamin C interactions on cardiac electrical stability. We show that there are gaps in the current research with respect to Vitamin C and cardiac electrical stability. The next chapter is an experimental chapter and explores

Vitamin C genetics and electrocardiogram (ECG) derived QRS duration. Increased QRS duration (beyond a normal reference range) is a risk factor for AF development and progression (Lin et al. 2009).

The role of Oxidative stress and Inflammation on AF

Atrial fibrillation is known to increase the risk of ischemic stroke and cardiac disease (Gaggin and Januzzi Jr. 2013; Hu et al. 2015; Liu et al. 2012). Structural remodelling of the atria and electrical remodelling have both been observed in AF models, as indicated by left atrial distension and elevated atrial fibrosis (Tousoulis et al. 2009). These mechanisms of electrical and structural remodelling in AF have been studied with respect to both oxidative stress and inflammation. Indeed both inflammation and oxidative stress have been suggested to play an important role in AF initiation and progression (Carnes et al. 2001; Hu et al. 2015; Korantzopoulos et al. 2005; Korantzopoulos et al. 2007; Liu et al. 2012; Mihm et al. 2001; Tousoulis et al. 2009). Oxidative stress is exerted by the excessive production of reactive oxygen species (ROS).

Oxidative stress has been shown in various translational models to be associated with the structural development of AF (Huang et al. 2009; Korantzopoulos et al. 2007). In a study by Mihm and colleagues (2001), oxidative stress in the atrial myocardium, may alter myofibrillar energetics and contribute to atrial contractile dysfunction, leading to the development of pro-arrhythmic atrial circuits that initiate or prolong AF. In these cases fibrosis is also a common factor associated with atrial fibrillation. It has been demonstrated that AF is associated with increased interstitial fibrosis and myocyte hypertrophy. For example, in surgically removed atrial appendages of AF patients

undergoing a MAZE procedure (arrhythmia surgery) significant hypertrophy was present compared with control groups (normal sinus rhythm and undergoing cardiac surgery for non cardiac arrhythmia procedures).

Nitration and carbonyl formation was found to be increased in myofibrillar proteins among AF patients (Mihm et al. 2001). An increase in protein nitration was also observed in a dog model with atrial tachycardia pacing, suggesting that oxidative stress was enhanced in this model (Carnes et al. 2001).

The gene expression patterns in myocardial tissues from AF patients have been examined by Kim and colleagues (2003) using microarrays. Gene expression profiles showed that 30 genes were up-regulated and 25 were down regulated, of these five genes were related to promoting ROS, while two genes were related to anti-oxidation pathways. The results suggested an imbalance in gene pathways for ROS regulation may be associated with AF (Kim et al. 2003). In a recent study, an animal model was used to demonstrate the reduction in AF susceptibility by genetically inhibiting the mitochondrial ROS production. This association between AF and myocardial oxidative stress was reported by Xie and associates (2015).

Other studies have also reported that oxidative stress-mediated calcium overload induces atrial remodelling and electromechanical dysfunction in the atrium (Abraham et al. 2003; Dhalla et al. 2000; Loh et al. 2003). The L-type calcium channel is the main route for calcium influx into cardiac myocytes. Cysteines on the alpha sub-component for the L-type calcium channel are targets for redox modification and glutathionylation and modulate channel dynamics. The cysteine responsible for modification of L-type

calcium channel function has now been identified. These L-type calcium channel modifications are responsible for calcium overload. Shiroshita-Takeshita and colleagues (2004) have demonstrated that simvastatin restores calcium channels. That is during tachycardia pacing in the dog the L-type Ca^{2+} channel alpha subunit expression is down-regulated. Atorvastatin has positive effects on calcium regulatory proteins, atorvastatin administration has also demonstrated positive effect on prophylaxis of postoperative atrial fibrillation (POAF) after heart valve surgery (Boos et al. 2006; Patti et al. 2006; Samadikhah et al. 2014).

Shiroshita-Takeshita and associates (2007) demonstrated that in a dog congestive heart failure model that induced atrial structural remodelling was associated with AF promotion, however, attenuated by simvastatin. These studies suggest that statins having a positive effect on cardiac remodelling. It is interesting that simvastatin restores the up-regulation of the alpha sub-unit of the L-type calcium channel that is prone to oxidative stress. Whether the beneficial effects of atorvastatin are L-type ion channel related or due to its anti-inflammatory anti-oxidative properties require further investigation.

Statins are known to have a number of off label secondary anti-inflammatory and anti-oxidant effects (as mentioned above). It is known that a low-grade inflammation is usually observed in many systemic diseases including AF (Harrison et al. 2011; Marzilli et al. 2012). An association between inflammation and AF development has been explored (Hu et al. 2015; Korantzopoulos et al. 2007; Tousoulis et al. 2009). Cardiopulmonary bypass surgery results in significant systematic inflammation. For example, C-reactive protein (CRP), an inflammation marker present in the systemic

circulation (released from the liver), has been measured in large population-based studies describing AF development or burden (Aviles et al. 2003; Bruins et al. 1997). CRP levels associated with AF recurrence relate to electrical or pharmacological cardioversion while noted in some studies, show a weak to no association in pooled meta analysis (Loricchio et al. 2007).

Myeloperoxidase (MPO) is a heme enzyme secreted from activated neutrophils, monocytes and macrophages, which is suspected to play a role in structural remodelling of the atria (via apoptosis of cardiac myocytes) and hence may be associated with AF (Rudolph et al. 2010; Zhang et al. 2001). MPO plasma levels are notably higher in AF patients than controls (Rudolph et al. 2010). Tumor necrosis factor α (TNF-α) is another inflammatory biomarker that is elevated in patients with paroxysmal AF (Sata et al. 2004; Tousoulis et al. 2009).

MPO has been found to have direct correlation with symmetric dimethylarginine, which increases production of TNF-α (Procter et al. 2015). This may imply a correlation between MPO and TNF-α. Despite these studies supporting a role of MPO in AF, an association or an absence of an association between MPO levels with and without AF has not been demonstrated, suggesting a localized MPO cardiac tissue response is more likely responsible (Schnabel et al. 2014). The inflammatory enzyme MPO also induces oxidative stress and is likewise associated with AF in animal models (Boos et al. 2006; Hu et al. 2015; Procter et al. 2015; Rudolph et al. 2010).

The famous expression ‘AF begets AF’ has been coined; recognizing subsequent inflammatory responses that AF induces. For example further inflammation will

stimulate further electrical remodelling in the atria which then perpetuates further AF. Meanwhile, ROS can provoke signal transduction which elevates pro-inflammatory cytokine production and contributes to the AF-associated inflammation (Hu et al. 2015; Korantzopoulos et al. 2007). The relationship between oxidative stress, inflammation and atrial fibrillation are possibly inter-linked, forming a vicious cycle.

Animal studies investigating Vitamin C's therapeutic effects on AF

Antioxidant therapy for prevention of AF continues to be proposed and explored across clinical and pre-clinical studies (Carnes et al. 2001; Colby et al. 2011; Dehghani et al. 2014; Eslami et al. 2007; Korantzopoulos et al. 2005; Papoulidis et al. 2011; Sadeghpour et al. 2015). In a dog model Carnes and colleagues (2001), demonstrated that pre-treatment of Vitamin C reduced depletion of Vitamin C in atrial tissue. Noting that tissue and plasma levels are different – see above. The atrial effective refractory period (ERP) was measured and atrial tissue was collected from dogs subjected to rapid atrial pacing. The pacing-induced reduction in ERP was diminished in the ascorbate treated group, it was noted that there was no change in the non-pacing ERP. The ascorbate and 3-nitrotyrosine content in atrial tissue was evaluated by capillary electrophoresis assay and dot-blot analysis respectively. Results showed that ascorbate supplementation prevented the 3-nitrotyrosine increase and ascorbate decrease in atrial tissue. As 3-nitrotyrosine is a biomarker of peroxynitrite formation, the study suggested that the ascorbate treatment prevented atrial electrical remodelling (Carnes et al. 2001). In contrast, a study by Shiroshita-Takeshita and associates (2004) whom induced atrial rapid pacing in dogs, Vitamin C dosing was not associated with a reduction in AF incidence. Carnes and colleagues (2001) measured the ERP in conscious dogs sedated with butorphanol tartrate and acepromazine maleate while the

dogs in the Shiroshita-Takeshita and associates' study (2004) were anesthetized with ketamine/diazepam/isoflurane and ventilated mechanically. These sedative/anaesthetic agents are known to have different cardiopulmonary effect profiles, which may influence cardiac conduction differently (Fayyaz et al. 2009; Kerr et al. 2009; Trim 1983). It is noted that the length of time of pacing employed was different between the two dog models. Finally, one study explored AF induction under anaesthesia using programmed electrical stimulation and the measuring ERP.

Clinical Studies investigating Vitamin C effect on AF

Atrial fibrillation is an arrhythmia and is commonly observed in patients after cardiac surgery. This complication is usually termed as "Post-operative atrial fibrillation" (POAF). In cardiac surgery, cardioplegic arrest and cardiopulmonary bypass are the main triggers of ROS generation (Suleiman et al. 2011), that may likely cause post operative AF.

Carnes and colleagues (2001) in post coronary artery by-pass patients, also examined the effect of oral ascorbic acid in preventing post operative atrial arrhythmias. In this study there was a significant reduction in the incidence of postoperative AF with ascorbate supplementation. The early recurrence rates were also found to be reduced in patients who had Vitamin C treatment after cardioversion for persistent AF (Korantzopoulos et al. 2005). Da Costa and associates (2013) demonstrated an anti-inflammatory role for Vitamin C in AF. It is noted that the majority of human studies in Vitamin C, have been based on the assumption for increased oxidative stress in chronic or permanent AF.

Systematic review aims

To date there has not been a systematic review on the AF and Vitamin C associations across published human studies. We are interested if there is a consistent effect with Vitamin C dosing and atrial fibrillation outcomes in surgical and non-surgical populations. The methodology below is described for these studies.

Methodology

To assess the Vitamin C protective effects against cardiac surgery induced AF literature, two searches were performed in SCOPUS on all articles up to the search date of the 19th of April 2016 using the key search terms {"Ascorbic acid" or "Vitamin C" and "Atrial fibrillation"}. Searches included by "Article Title, Abstract, Keywords". A total of 213 articles were identified, these articles were screened and 204 articles were excluded from this review based on the following exclusion criteria:

- Review article, Letter, Editorial, Conference Paper, Note
- Case Report or study with no Vitamin C intervention
- Studies not on human subjects
- Articles not available in English
- Studies not related to effect of Vitamin C on Atrial Fibrillation occurrence
- Studies used a combination of Vitamin C and E for treatment which no data was available for Vitamin C effect alone

Nine studies were documented in Table 1 below for comparison. All studies investigated the effects of Vitamin C on atrial fibrillation (AF) recurrences after cardiovascular surgery. All studies had variable interventions of giving patients pre-treatment of 1-2g Vitamin C either orally or intravenously and or a post-operative

treatment of Vitamin C for 4-7 days. It is interesting to note that a meta-analysis conducted by Hemiliä and Suonsyrjä (2017) has found that Vitamin C administrated orally resulted in less POAF incidents than administrated intravenously.

Seven of the reviewed Vitamin C studies were effective in reducing AF recurrence after surgery, while 2 studies showed no beneficial effects in comparison with the control group. For the studies showing beneficial effects of Vitamin C on AF, 5 out of 6 studies were giving patients a total of 1g Vitamin C per day postoperatively, while the other study was giving 2g per day. The on-pump and off-pump procedures were not specified in most studies – it is presumed that on pump procedures would be more inflammatory. It is therefore a possibility that the on-pump and off-pump procedures may differentially influence Vitamin C AF risk post cardiac surgery.

Moreover, there were studies which suggested that age (<60 years old), sex and race may contribute to the difference of Vitamin C outcomes in the prevention of atrial fibrillation (Bjordahl et al. 2012; Pfister et al. 2014; Rodrigo et al. 2012). Across studies, men were mainly recruited. The mean age of participants ranged from 18-90 years old, across studies the average population was above 60 years old. Only one study considered race as a confounder on the therapeutic effectiveness of Vitamin C. Bjordahl and colleagues (2012) showed no beneficial effects of Vitamin C in the prevention of postoperative AF, however the investigators noted a significant difference in ethnic background frequencies between the intervention group and the control group. We note ethnic background at baseline was not mentioned in most studies we reviewed.

Because there are inherent differences in plasma Vitamin C levels and ultimately

cellular and tissue levels, genetic variations of Vitamin C transporters may need to be considered. Particularly where there is variability across outcomes for Vitamin C dosing and prevention of post-operative atrial fibrillation (as noted above).

Vitamin C transporters

Vitamin C is known to be transported from the gut into blood stream and end organ tissue cells via sodium dependent Vitamin C transporters (SVCTs), these transporters are genetically encoded by the SLC23 family. The transcriptional regulations of these genes determine Vitamin-C homeostasis. SVCT1, encoded by SLC23A1 and mapped to 5q31.2, and expressed in many parts of the body. These genes as suggested above regulate the uptake and excretion of Vitamin C while SLC23A3 is an orphan transporter. Its main function remains to be determined (Dalgård et al. 2013; Duell et al. 2013; Michels et al. 2013; Senthikumari et al. 2014). Studies of Bürzle and associates (2013) showed that the SVCT1 is up-regulated during ascorbic acid depletion while SVCT3 is unchanged. SVCT2 (specifically SLC23A2), is the principle transporter determining Vitamin C cellular levels. Thus the SVCT2 is responsible for bioaccumulation of Vitamin C in end organ cells such as the heart. There is no evidence for SVCT2 having a significant role on circulating plasma ascorbate levels. The Vitamin C transport protein is expressed in both leukocytes and platelets, but not in erythrocytes (Babaev et al. 2010; Bürzle et al. 2013; Eck et al. 2007; Santiago et al. 2014; Skibola et al. 2008; Wright et al. 2009; Yang et al. 2014). Michels and colleagues (2013) suggested that intracellular ascorbic acid levels regulate the expression of SVCT2 in many tissues, except brain. To maintain intracellular ascorbic acid concentrations during oxidative stress, expression of SVCT2 is up-regulated via oxidized low-density lipoprotein. In mice, it was found that a severe Vitamin C deficiency accelerates atherosclerosis and also increases lipid peroxidation. This results in a doubling of the

size of atherosclerotic plaques (Babaev et al. 2010). Michels and colleagues (2013) concluded that the heart and vascular endothelium express SVCT2.

Low plasma levels of Vitamin C level have been shown to be associated with a higher risk of cardiovascular diseases (Naidu 2003; Okamoto 2002). On the other hand, a single dose of oral Vitamin C may vary between individuals, in terms of plasma levels and also tissue distribution. These differences may be due to genetic polymorphisms in SVCT genes or possibly via environmental methylation of a particular SVCT gene. Genotyping of the human SLC23 transporters has resulted in more than 2200 single nucleotides polymorphisms (SNPs) having been found. There are only limited studies on select SNPs of these Vitamin C transporters exploring associations with cardiovascular risk factors (Bürzle et al. 2013; Corpe et al. 2010; De Jong et al. 2014; Duell et al. 2013; Eck et al. 2004; Eck et al. 2007; Michels et al. 2013; Santiago et al. 2014; Savini et al. 2008; Senthikumari et al. 2014; Skibola et al. 2008; Wright et al. 2009; Zanon-Moreno et al. 2011; Zanon-Moreno et al. 2014). Dalgård and colleagues (2013) demonstrated that polymorphisms *rs6139591* and *rs1776964* in SLC23A2 are associated with acute coronary syndrome among women, but a similar association was not found in men.

We also looked at reported studies on SLC23A2 polymorphisms in cardiac patients. A review of *pubmed* and *scopus* using "SLC23A2" and "heart" (or "myocardium" or "cardiac") revealed only 2 published studies. One study demonstrated that SLC23A2 polymorphisms are associated with systolic blood pressure responses to the cold pressor test. Previous studies have demonstrated that blood pressure responses to the cold pressor test are associated with future adverse cardiovascular events. For example,

Cassey and associates (2008) have shown that a greater increase in systolic central aortic pressure likely contributes to increased left ventricular myocardial oxygen demand during CPT-induced hypertension in individuals at risk of future higher systolic blood pressure.

The other study explored a relationship between risk of acute coronary syndrome (ACS) and polymorphisms in SLC23A2 gene. A positive association was found in women but not men. Interestingly, the *rs1776964* polymorphism, had a higher risk among TT-homozygous women, despite higher median Vitamin C dosing. This compared with the *rs1776964* CC genotype and lower Vitamin C intake where there was less risk for a coronary event (Dalgård et al. 2013). This suggests that increased Vitamin C intake is unlikely to compensate for *rs1776964* TT polymorphism in the SLC23A2 gene.

There have been no studies exploring SLC23A2 genotypes on cardiac conduction *per se*. The subject of the next experimental chapter is on cardiac conduction, electrocardiogram QRS duration and SLC23A2 genotypes. Hence we have identified a significant gap in the literature regarding the association between SLC23A2 and cardiac conduction. We have shown reviewing the literature that more work is still required to understand whether there are benefits of using Vitamin C clinically to improve AF outcomes in cardiac surgery. It is not known if the presence of genetic polymorphisms in the Vitamin C transporters influences Vitamin C dosing outcomes in preventing AF.

Table 1. Summary of Vitamin C in Cardiac Surgery for AF prevention in Clinical Trials

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Studies showed positive results – Vitamin C attenuated postoperative atrial fibrillation in patients								
Sadeghpour et al. 2015	CABG or simple congenital valvular disease surgery	Both On pump and Off pump were measured	57.28±14.09yo	80 men (70.8%)	Vitamin C	113	2g Vitamin C intravenously before surgery and 1g oral tablet per day for 4 postoperative days	Vit C 40 patients (35.5%) vs Placebo 99 patients (55.9%) with AF occurred (P=0.001)
			54.22±14.39yo	111 men (62.7%)	Placebo	177	2g Placebo ampoule intravenously before surgery and 1g oral tablet per day for 4 postoperative days	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Samadikhah et al. 2014	CABG	On pump and Off pump procedures were not specified	61.0±11.5yo	43 men (71.7%)	Vitamin C + Atorvastatin	60	2g oral Vitamin C tablets in operation day and 1g oral tablet for 4 postoperative days	Vit C 6 patients (10%) vs Placebo 15 patients (25%) with AF occurred (P=0.03)
			60.5±11.3yo	39 men (65%)	Placebo + Atorvastatin	60	2g oral Placebo tablets in operation day and 1g oral tablet for 4 postoperative days	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Dehghani et al. 2014	CABG	On pump operation only	60.52±5.83yo	38 men (76%)	Vitamin C + beta-blocker	50	2g Vitamin C tablet 1 week before surgery and 500mg tablets twice a day for 5 days postoperative	Vit C 4 patients (8%) vs Control 16 patients (32%) with AF occurred (P=0.003)
			62.12±6.93yo	36 men (72%)	Beta-blocker Only	50	None	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Papoulidis et al. 2011	CABG	On pump operation only	73.1yo±7.2 SD	57 men (67.1%)	Vitamin C	85	2g Vitamin C intravenously for 3 hours before surgery and 500mg twice a day for 6 postoperative days	Vit C 38 patients (44.7%) vs Placebo 52 patients (61%) with AF occurred (P=0.041)
			71.3yo±6.8 SD	63 men (74.1%)	Placebo	85	0.9% Normal Saline intravenously twice a day for 6 postoperative days	Significant in both men and women.

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Eslami et al. 2007	CABG	On pump and Off pump procedures were not specified	60.78yo±6.8 SD	36 men (72%)	Ascorbic Acid + β-blocker	50	2g ascorbic acid tablet orally the night before surgery and 1g tablet twice daily for 5 postoperative days Treated with β-blocker	AS 2 patients (4%) vs Control 13 patients (26%) with AF occurred (P=0.002)
			59.6yo±7.5 SD	31 men (62%)	Control - β-blocker only	50	Treated with β-blocker	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Korantzopoulos et al. 2005	Cardioversion treatment for persisting AF	On pump and Off pump procedures were not specified	68yo±10 SD	13 men (59.1%)	Vitamin C	22	2g oral Vitamin C 12 hours before cardioversion and 500mg twice orally daily for 7 postoperative days	Vit C 1 patients (4.5%) vs Control 8 patients (36.3%) with AF relapsed (P=0.024)
			68yo±6 SD	13 men (59.1%)	No intervention	22	None	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Carnes et al. 2001	CABG	On pump and Off pump procedures were not specified	68yo±10.1 SD	36 men (83.7%)	Ascorbic acid	43	2g the night before surgery and 500mg twice daily for 5 postoperative days	AS 7 patients (16.3%) vs Control 15 patients (34.9%) with AF occurred (P=0.048)
			59.5yo±11.4 SD	36 men (83.7%)	No intervention	43	Performed 6-week after ascorbic acid group study No intervention	

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Studies showed negative results – Vitamin C did not attenuate postoperative atrial fibrillation in patients								
Bjordahk et al. 2012	CABG	Both On pump and Off pump were measured	63yo±12.4 SD	61 men (68.5%)	Ascorbic acid	89 (81 on-pump)	2 x 1g ascorbic acid orally at the evening before surgery and 1g twice daily orally or enteral tube for 5 days postoperative	Vit C 27 patients (30.3%) vs Placebo 29 patients (30.2%) with AF occurred (P=0.985)
			63yo±12.4 SD	63 men (65.6%)	Placebo	96 (87 on-pump)	2 x 1g ascorbic acid orally at the evening before surgery and 1g twice daily orally or enteral tube for 5 days postoperative	Races difference in group were significant (more White in Placebo, more black & Hispanic in Vit C)

Study	Procedures	On/Off Pump	Age	Sex (Male)	Group	No. of Patients	Dose of Intervention	Results
Colby et al. 2011	Cardiothoracic surgery (CTS)	Both On pump and Off pump were measured	68.4±9.4 SD	9 men (69%)	Ascorbic acid	13 (7 on-pump)	2g tablets were given the night before surgery and 500mg oral twice daily for 4 postoperative days	AS 4 patients (31%) vs Placebo 5 patients (45%) with AF occurred (P=0.498)
			62.1±6.9 SD	10 men (91%)	Placebo	11 (5 on-pump)	2g tablets were given the night before surgery and 500mg oral twice daily for 4 postoperative days	Sampling size too small

CHAPTER 2 – EXPERIMENTAL

Introduction

The dietary sources of Vitamin C are Ascorbic acid and dehydroascorbic acid (DHAA (an oxidized form of Vitamin C)). There is current interest in how Vitamin C transport is affected by aging, genetics and also disease (Wilson 2005). In humans, Vitamin C is known to be co-transported from the blood into cells via membrane bound sodium dependent Vitamin C transporters (SVCTs). These transporters are genetically encoded by the SLC23 family (Brüzle et al. 2013; Duell et al. 2013). The transcriptional regulation of these genes is associated with Vitamin C cellular specific homeostasis (Michels et al. 2013; Savini et al. 2008). For example, SVCT1 is encoded by SLC23A1 and mapped to 5q31.2, where it regulates the uptake and excretion of Vitamin C (Corpe et al. 2010; De Jong et al. 2014). SVCT2 is encoded by SLC23A2 and mapped to 20p13 (Eck et al. 2004; Zanon-Moreo et al. 2014). SVCT2 has been identified as the main transporter for Vitamin C into cardiovascular tissues (Brüzle et al. 2013; Dalgård et al. 2013). Michels and colleagues (2013) suggested that intracellular ascorbic acid levels provide feedback pathways to regulate the expression of SVCT2.

Oxidative stress is associated with abnormal cardiac conduction. In excitable myocardial cellular tissues, reactive oxygen species regulate both cellular metabolism and ion homeostasis. Elevated cardiac myocyte ROS can cause alterations of the cardiac sodium channel ($Na_{v1.5}$), abnormal Ca^{2+} handling, changes of mitochondrial function, and gap junction remodelling, leading to conduction disturbances and arrhythmias (Barrington el at. 1997; Caouette et al. 2003; Hemilä and Suonsyrjä 2017; Panth et al. 2016; Wolin and Gupte 2005). Feedback loops regulate Vitamin C's

cellular uptake via expression of transporter genes. It is noted that background levels of oxidative stress will influence Vitamin C uptake and expression of transporters (Carnes et al. 2001; Wilson 2005). As suggested in the previous chapter, Vitamin C has been investigated in a number of studies and shown to have a positive effect on reducing atrial fibrillation in post cardiac surgery (Carnes et al. 2001; Eslami et al. 2007; Korantzopoulos et al. 2005 – also see introductory Chapter 1). This reduction in atrial fibrillation burden is presumably because of ascorbic acids anti-oxidant effects (Violi et al. 2014).

On the other hand, many of Vitamin C's effects would also be theoretically influencing ventricular conduction, particularly passive conduction mechanisms. An electrocardiogram (ECG) with increased QRS duration (a marker of slowed cardiac conduction) is associated with increased cardiac mortality in population studies (Badheka et al. 2013). Obesity, and other environmental factors such as systolic hypertension can influence QRS duration (Devi et al. 2013; Sun et al. 2013). Typically QRS duration increases as a function of increased cardiac mass or as a result of impaired ventricular contractility (e.g. heart failure, aortic stiffening) and or due to passive conduction alterations, such as a reduction in cellular coupling via connexins or connexin phosphorylation changes (Akar et al. 2004; Leng et al. 2015; Wiegerinck et al. 2006). Interestingly, ascorbic acid and or its synthetic analogue (L-Ascorbic acid 6-palmitate (AAP)) may cause inhibition of gap-junctional intercellular communication via phosphorylation of connexin 43 (via activation of a MEK-ERK pathway).

In 2008, the first evidence emerged that Vitamin C may affect QRS duration, where the

effects of a chronic, low dose (300 mg/day for 6 weeks) Vitamin C supplementation demonstrated a decrease in QRS duration at the end of 6 weeks in 20 healthy males (Jaja et al. 2008b). These studies were repeated in a mixed population of 10 sickle cell anaemia and 12 healthy individuals where a reduction in QRS duration from 0.08±0.002mV to 007±0.02 mV (p<0.001) was observed in healthy individuals (Jaja et al. 2008a).

We wondered in the absence of known Vitamin C plasma concentrations, whether gene polymorphisms in the Vitamin C transporter relevant to cardiac tissues would be important. The question may be asked if polymorphisms in the Vitamin C transporter genes SLC23A2 (*rs1776964*) may influence cardiac electrocardiogram QRS duration? Additionally, whether an inflammatory state such as diabetes may be associated with both the Vitamin C transporter gene SLC23A2 and any of the following ECG parameters, QRS duration, QTc or PQ intervals?

The association between Vitamin C gene polymorphisms and QRS duration has not been previously studied in rural community populations. Our aims are to explore whether *rs1776964* polymorphisms are associated with QRS duration in a mixed rural population of diabetes and non-diabetics. We will also adjust for known cardiovascular and metabolic risk factors and explore other ECG parameters of interest.

Methods

Study Population and Design

Ethics: The research protocol for data collection and bio-banking was approved by institutional review board of Charles Sturt University. Each subject provided written

and informed consent.

A retrospective cross-sectional population data set was obtained on 274 participants from the CSU Diabetes Screening Research Initiative database. Participants were drawn from Albury-Wodonga and surrounding districts on the New South Wales-Victoria border (Southeast Australia). Inclusion criteria included being over 21 years of age and above and having both ECG-derived QRS duration data and bio-banked DNA to permit analysis of the Vitamin C transporter SLA23A2 polymorphisms.

Demographic information included age, gender, height, weight (for BMI calculation), waist circumference, blood pressure family history of Type 2 Diabetes and Cardiovascular Disease. Subjects' own status of Type 2 diabetes, Hypertension and Cardiovascular Disease were also recorded. BMI was calculated using the formula body mass divided by square of height (kg/m2). Blood pressure was recorded using a standard mercury sphygmomanometer (Welsh Allyn Australia P/L), the average of three measurements were obtained. All blood pressure measurements were obtained in a seated position after 5 minutes of rest. Glucose, Cholesterol, Triglyceride and HbA1c level were measured at South West Pathology Albury.

Electrocardiogram measures of QRS duration

A Welch Allyn PC-Based ECG system was used to record participants 12-lead ECG. From which an automated averaged QRS duration, QTc and PQ calculation from a 10s epoch of recording was obtained.

DNA Genotyping

QIAamp DNA Blood Mini Kit (Cat No.: 51106, QIAGEN, Venlo, Netherlands) was used to extract genomic DNA from whole blood samples. The extracted DNA was dissolved in 100μL DNase-free water and its concentration was tested using a Nanodrop 1000 (Thermo Scientific, MA, U.S.). DNA was then stored at -30°C until usage. The SNP of SLA23A2 was genotyped: *rs1776964* (Chromosome 20, 4899662 (Assembly GRCh38.p7)). The SLA23A2 SNP *rs1776964* was genotyped on genomic DNA extracted from 274 peripheral blood samples using Taqman SNP Genotyping Assay (*rs1776964*: C_505153_1_, Life Technologies, CA, U.S.), performed on a LightCycler 480 (Roche, Penzberg, Germany). The standard 5μL PCR reaction system (including 15ng of genomic DNA template) was prepared using TaqMan Universal PCR Master Mix reagent kits under the guidelines provided (Thermo Fisher Scientific Inc., U.S.). One percent (1%) of available sample genotyping was replicated to ensure quality control and the results maintained 100% concordance.

Statistical Analysis

Data values are presented as mean ± standard error (SE) for numeric variables, or for categorical variables as percentages. T-test or the chi-square test was applied to calculate the P value. A P value ≤ 0.05 was considered statistically significant. Logistic regression models were used to analyse the association of variables in relation to SLA23a2 SNP genotypes. All data analysis was performed using SPSS 22.0 (IBM, Armonk, New York, U.S.). The relationship between Type 2 diabetes status and ECG parameters and/or SNPs was evaluated using a General Linear Model.

Results

Summary data for the non-diabetic and Type 2 Diabetes Mellitus (T2DM) sub-groups including age, gender, blood pressure, and ECG parameters are presented in Table 2. Biochemical values for cholesterol and blood sugar differed significantly between Type 2 diabetes mellitus and non-diabetes (Table 2). There were a higher percentage of participants identified as obese (BMI≥30) in the T2DM sub-group compared to the non-diabetic group. More participants had hypertension in the T2DM sub-group compared to the non-diabetic sub-group (Table 2).

The genotyping frequencies across sub-groups (T2DM and non-diabetics) are in Hardy Weinberg equilibrium for *rs1776964* genotypes (Table 3). The association of *rs1776964* ECG cardiac conduction changes for QRS duration, QTc, QTd, and PQ interval were determined (Table 4). Logistic regression models adjusted for age and gender (across the entire cohort) demonstrated no significant changes in ECG parameters of QRS duration, QTc or PQ interval, when comparing the Vitamin C transporter for *rs1776964* GG compared to *rs1776964* GA or AA.

We next explored the effect of *rs1776964* genotypes across T2DM and non-diabetic subgroups. We found that the *rs1776964* AA genotype approached statistical significance with respect to changes in the PQ interval (p=0.51). There were no other statistical changes with ECG parameters (QTd, QRSd, QTc) across non-diabetic and T2DM sub-groups with respect to being stratified by *rs1776964* genotypes (Table 5).

It is understood that pre-existing cardiovascular disease is prevalent in T2DM and may influence PQ interval conduction. We therefore re-explored PQ intervals after

removing subjects with any self-reported pre-existing cardiovascular disease. In this model genotypes were not considered, although age was corrected in linear models. In linear models (with pre-existing cardiovascular disease subjects removed) PQ interval duration was significantly increased in males without T2DM when compared to females without T2DM (Table 6). It was noted that the presence of diabetes in either sex had no effect on PQ intervals when compared to females with no T2DM (Table 6).

Table 2. Demographic information

	Controls (N=194)	T2DM (N=80)	P value
Age, years	66.93 ± 0.77	69.76 ± 0.94	0.021
Male	39.18%	48.75%	0.178
Waist, cm	95.15 ± 0.90	105.65 ± 1.49	<0.001
BMI	27.42 ± 0.36	30.27 ± 0.58	<0.001
Obesity (BMI ≥ 30)	23.56%	46.25%	<0.001
SBP, mmHg	129.57 ± 1.27	133.53 ± 2.00	0.094
DBP, mmHg	76.44 ± 0.61	76.41 ± 0.99	0.981
Screening glucose, mM	5.44 ± 0.07	8.29 ± 0.40	<0.001
HbA1c, %	5.65 ± 0.03	6.94 ± 0.15	<0.001
Total cholesterol, mM	5.23 ± 0.08	5.54 ± 1.34	<0.001
Triglyceride, mM	1.23 ± 0.05	1.66 ± 0.11	0.001
Total cholesterol/HDL	3.45 ± 0.09	3.42 ± 0.14	0.816
PQ, ms	176.55 ± 2.28	178.05 ± 2.88	0.682
QRS, ms	102.32 ± 1.23	103.95 ± 1.86	0.470
QTc, ms	428.92 ± 1.54	427.92 ± 2.66	0.734
QTd, ms	60.56 ± 2.53	60.72 ± 3.47	0.972
CVD	16.06%	26.92%	0.060
HT	45.88%	82.28%	<0.001
Family history T2DM	27.84%	53.75%	<0.001
Family history CVD	0.3918	0.35	0.585

*T-test or the chi-square test was applied to calculate the P value

Table 3. Hardy Weinberg equilibrium of *rs1776964*

	Control (N=194)	T2DM (N=80)
GG	65	18
GA	89	42
AA	40	20
P for HWE	0.351	0.650

Table 4. Logistic regression models of *rs1776964* susceptibility across genotypes, using the total cohort (diabetes and non-diabetes)

Disease	GG (N=83)	GA (N=131)	AA (N=60)
T2DM			
OR (95% CI)*	1.00	1.67 (0.88, 3.19)	2.00 (0.93, 4.29)
P value*	/	0.119	0.077
CVD			
OR (95% CI)	1.00	0.78 (0.38, 1.60)	0.80 (0.33, 1.96)
P value	/	0.500	0.624
HT			
OR (95% CI)	1.00	1.19 (0.66, 2.13)	0.99 (0.49, 2.01)
P value	/	0.567	0.986
Family DM			
OR (95% CI)	1.00	0.89 (0.50, 1.59)	0.89 (0.44, 1.80)
P value	/	0.690	0.752
Family CVD			
OR (95% CI)	1.00	0.85 (0.48, 1.50)	1.27 (0.64, 2.50)
P value	/	0.569	0.492
Obesity			
OR (95% CI)	1.00	1.21 (0.65, 2.24)	1.31 (0.63, 2.72)
P value	/	0.550	0.468
PQ > 200 ms			
OR (95% CI)	1.00	0.90 (0.40, 2.04)	1.31 (0.50, 3.41)
P value	/	0.807	0.586
QRS > 120 ms			
OR (95% CI)	1.00	1.00 (0.38, 2.59)	0.79 (0.22, 2.88)
P value	/	0.993	0.722
QTc > 440 ms			
OR (95% CI)	1.00	0.75 (0.39, 1.44)	0.81 (0.36, 1.80)
P value	/	0.384	0.600
QTd > 80 ms			
OR (95% CI)	1.00	1.15 (0.57, 2.30)	0.79 (0.32, 1.97)
P value	/	0.697	0.620

*Odds Ratio (OR) and P value are investigated using logistic regression with adjustment of age and gender.

Table 5. Effect of *rs1776964* genotypes on Sub-group parameters: General linear regression models adjusted for age and gender

	Non-diabetics			T2DM		
	GG (N=65)	GA (N=89)	AA (N=40)	GG (N=18)	GA (N=42)	AA (N=20)
PQ						
coefficient	/	0.63 (-9.08, 10.35)	2.22 (-9.44, 13.87)	/	3.15 (-10.98, 17.27)	16.35 (-0.05, 32.75)
P value	/	0.898	0.708	/	0.658	**0.051**
QRS						
coefficient	/	0.62 (-4.68, 5.92)	1.53 (-4.93, 7.98)	/	-1.93 (-10.83, 6.96)	0.69 (-9.76, 11.15)
P value	/	0.819	0.624	/	0.666	0.895
QTc						
coefficient	/	0.88 (-5.92, 7.68)	-3.26 (-11.55, 5.03)	/	1.79 (-11.73, 15.30)	9.12 (-6.77, 25.01)
P value	/	0.800	0.439	/	0.793	0.256
QTd						
coefficient	/	-1.85 (-12.91, 9.21)	-7.18 (-20.65, 6.29)	/	10.67 (-6.68, 28.01)	3.50 (-16.88, 23.89)
P value	/	0.742	0.294	/	0.224	0.733
Waist circumference						
coefficient	/	-0.83 (-4.61, 2.95)	-1.43 (-6.10, 3.25)	/	2.12 (-4.99, 9.23)	3.57 (-4.58, 11.72)
P value	/	0.666	0.548	/	0.555	0.386
BMI						
coefficient	/	-0.41 (-2.04, 1.22)	-0.29 (-2.31, 1.73)	/	-0.51 (-3.39, 2.36)	0.79 (-2.51, 4.08)
P value	/	0.624	0.777	/	0.723	0.635

	Non-diabetics			T2DM		
	GG (N=65)	GA (N=89)	AA (N=40)	GG (N=18)	GA (N=42)	AA (N=20)
SBP						
coefficient	/	-2.41 (-7.87, 3.04)	-2.67 (-9.51, 4.18)	/	-1.81 (-11.56, 7.94)	3.92 (-7.26, 15.10)
P value	/	0.384	0.443	/	0.713	0.487
DBP						
coefficient	/	-0.23 (-3.00, 2.54)	0.95 (-2.53, 4.43)	/	-3.15 (-8.17, 1.87)	-3.29 (-9.15, 2.47)
P value	/	0.871	0.592	/	0.215	0.259
HbA1c						
coefficient	/	-0.017 (-0.17, 0.14)	-0.05 (-0.23, 0.13)	/	0.52 (-0.16, 1.20)	0.35 (-0.46, 1.16)
P value	/	0.824	0.557	/	0.129	0.387
Total cholesterol						
coefficient	/	0.16 (-0.20, 0.52)	0.28 (-0.16, 0.72)	/	2.61 (-3.97, 9.19)	-0.17 (-4.53, 7.20)
P value	/	0.374	0.212	/	0.431	0.964
Triglyceride						
coefficient	/	-0.07 (-0.29, 0.16)	0.03 (-0.25, 0.31)	/	0.35 (-0.21, 0.91)	0.24 (-0.39, 0.87)
P value	/	0.570	0.820	/	0.216	0.450
Total cholesterol/HDL						
coefficient	/	-0.04 (-0.44, 0.35)	0.01 (-0.46, 0.48)	/	0.26 (-0.45, 0.97)	0.31 (-0.49, 1.11)
P value	/	0.829	0.966	/	0.470	0.435

Table 6. Linear regression models exploring associations with PQ intervals in participants reporting no pre-existing CVD and and stratified on the basis of T2DM and gender.

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	N	Mean ± standard error	P*
Male with T2DM	23	173.48 ± 5.13	0.785
Male without T2DM	53	186.70 ± 5.00	0.001
Female with T2DM	33	178.52 ± 4.38	0.134
Female without T2DM	99	168.63 ± 2.45	/

*General linear regression is applied with adjustment of age

Discussion

We have demonstrated that in a rural cross-sectional population that *rs1776964* polymorphisms for the Vitamin C transporter SVCT2 are not associated with changes in cardiac conduction. Specifically we found an absence of *rs1776964* gene associations with QRS duration, and QTc. Our finding for a lack of a gene association for QRS duration was consistent for both non-diabetics and T2DM sub-groups. Furthermore these findings are surprising given that Vitamin C dosing has been shown to reduce QRS duration in healthy individuals and in individuals with sickle cell anaemia (Jaja et al. 2008). This requires some theoretical discussion for why this may be the case.

Vitamin C is a water soluble organic compound and plays a cardiac protective role via anti-oxidant and anti-atherogenic actions (Babaev et al. 2010; Colby et al. 2011). To maintain intracellular ascorbic acid concentrations during oxidative stress in cardiac tissues, expression of the SVCT2 is up-regulated by oxidized low-density lipoproteins (Babaev et al. 2010; Muskiet et al. 1991). If oxidative stress increases in the myocardium this is likely to interfere with cellular functioning and impair cardiac conduction.

Interestingly, a translational rat model with aortic banding, that increases cardiac mass, does not influence plasma Vitamin C levels, despite cardiac remodelling due to pressure overload (Rohrbach et al. 2008). On the other hand ventricular Vitamin C levels are elevated in cardiac tissues during both development of pathological cardiac hypertrophy and chronic ongoing ventricular remodelling (Rohrbach et al. 2008). This suggests that an increase in expression of the Vitamin C transporter SVCT2 is a response to cardiac overload, facilitating storage of myocardial tissue Vitamin C (Rohrbach et al.

2008).

We have not measured oxidative plasma mediators and their respective levels in our studies. This may be perceived as a limitation using our retrospective cohort data. That is oxidative stress may blunt any observable effects on QRS duration with a functional Vitamin C transporter polymorphism. On the other hand in sickle cell anaemia which can result in a significant increase in background oxidative stress, there was still a documented improvement in QRS duration (although blunted compared to healthy individuals) with Vitamin C dosing (Jaja et al. 2008a). Sickle cell disease may promote higher oxidative stress levels via mechanisms leading to increased non-physiological levels of free hemoglobin that catalyse the Fenton reaction (Chirico and Pialoux 2012). Because of the increased coagulation state in sickle cell disease micro-vascular clotting and reperfusion may create areas of increased systematic inflammation and oxidative stress, for example via activation of the xanthine-xanthine oxidase system (Osarogiagbon et al. 2000). Alternatively, higher autoxidation of Hb S may be responsible for an increase in hydrogen peroxide (Aslan et al. 2000). It is also suggested that any increased in background ROS induces a chronic inflammation state with increased recruitment of neutrophils and monocytes to end organ tissues. This is particularly so for the formation of inflammatory red cell neutrophil aggregates (Dominical et al. 2014). It is therefore appreciated that sickle cell anaemia may induce cardiac remodelling changes. In a study of 1700 subjects with sickle cell anaemia, where median age was 31, and median haemoglobin (Hb) was 87 (80-95) g/L both left ventricular dilation and left atrial dilation was noted on echocardiography in 35 and 78 percent of subjects respectively (Damy et al. 2016). This suggests in sickle cell disease that cardiac remodelling is prevalent. If cardiac remodelling is present that

influences cardiac conduction such as QRS duration, one wonders how responsive the heart will be to Vitamin C dosing, because of pre-existing structural changes. As suggested by Queiroz and Lima (2013), several studies have explored the levels of carotenoids, flavonoids, Vitamins C and E and zinc in sickle cell anemia patients (Kiefmann et al. 2008), none have had a therapeutic effect clinically to reduce the effects of sickle cell disease (Muskiet et al. 1991). On the other hand a response (as suggested above) on QRS duration has been noted in the single study on sickle cell anaemia with Vitamin C dosing for only 6 weeks (Jaja et al. 2008a).

Vitamin C's mechanistic effects on QRS duration may be independent of cellular uptake. We suggest that Vitamin C – QRS duration interactions may possibly be related also to fast sodium ion channels. For example, QRS duration is sensitive to cardiac myocyte expression and function of fast sodium ion channels. The few studies on ascorbic acid and cardiac ion channels have explored the pharmacological role of Vitamin C in preventing fast sodium channel changes, for example during ischemia. Specifically ascorbic can prevent Na^+ current (I_LNa) changes with lysophosphatidylcholine (Pan et al. 2003). In the absence of Vitamin C – computer modelling of human ventricular myocyte action potentials suggest prolonged action potential duration as a consequence of lysophosphatidylcholine on peak and late Na^+ channel currents (Pan et al. 2003). Additionally Zhou and colleagues (2006) has demonstrated that Vitamin C pre-dosing in guinea pig ventricular myocytes can in part prevent some of the effects of hypoxia on Na^+ currents (I_{NaT} and I_{NaP}). Suggesting Vitamin C may preserve Na^+ channel function during physiological stresses and suppress the enhanced function of recombinant I_LNa (Gautier et al. 2008) that would increase QRS duration. As Gintant and associates (2011) suggested in their review, small reductions in cardiac Na^+ current will promote

increased QRS duration. For example Heath and colleagues (2011) demonstrated that QRS interval prolongation (~10–20%) in either preclinical or clinical studies with flecainide. They noted the free plasma concentrations to influence QRS duration were 6- to 30-fold below IC_{50} values required to block $hNa_{V1.5}$.

Our study was premised on a number of assumptions: i) that plasma levels of Vitamin C are not linearly associated with Vitamin C cellular stores; ii) cellular Vitamin C stores are required to be increased in cardiac pathology; iii) Vitamin C stores are associated with QRS duration and iv) intracellular Vitamin C may affect cardiac conduction. However, we cannot rule out Vitamin C functions via an extracellular ion channel interaction.

A prolongation of the PQ interval (AV nodal conduction delay) is a risk for atrial fibrillation. The human ECG derived PQ interval is relatively stable and not subject to variable circadian changes (Malik et al. 2008). Interestingly Malik and associates (2008) noted no differences in the ratio of heart rate to PQ interval between males and females. In our study we observed in subjects without pre-existing CVD, a significant increase in PQ interval in males without diabetes compared to women without diabetes. It is also interesting that the ILSA project (Italian Longitudinal Study of Aging) noted increasing PQ interval frequencies in aging populations, greater than would be normally expected in cross-sectional population studies (Bressan et al. 1998). In our linear models ECG derived PQ interval approached statistical significance with the *rs1776964* AA genotype for the entire T2DM cohort. Our diabetic population was older than the non-diabetic population (mean age with T2DM 69 years). Increasing age is also a risk factor for AF and according to Bressan et al (1998) also for PQ interval prolongation.

The rational explanation for why the PQ interval may be increased in the T2DM subgroup with an *rs1776964* AA genotype is an increase in cardiac fibrosis or atrial enlargement affecting AV nodal conduction. We further demonstrated in T2DM subjects without self- reported cardiovascular disease, an association with PQ interval was no longer evident. Removal of diabetic cardiac risk factors suggests that the combination of Diabetes and cardiac disease may be required to influence the PQ interval.

In our cohort we had an aging population that would be at higher risk of suffering age related changes in oxidative stress and background cardiac age related inflammation (Lee et al. 2016; Ng et al. 2013). A polymorphism in the Vitamin C transporter may be best explored in subjects without pre-existing sub-clinical cardiovascular disease and in younger populations. The limitations of our study – is a cross sectional cohort, hence causation cannot be inferred from statistically significant associations. The sample size is limited, so we are unable to correct for multiple interactions in complex models, hence we have been cautious not to over interpret any interactions that we have observed.

In summary we have been unable to demonstrate an interaction between the SVCT2 Vitamin C transporter polymorphism and QRS duration in our rural cohort. Further studies are required to understand whether QRS duration is modified with acute or chronic Vitamin C dosing in healthy individuals if a SLC23A2 *rs1776964* AA polymorphism is present. Additionally given the suggestion that Vitamin C may also interact with the fast Na^+ channel, polymorphisms in the fast Na^+ channel may be also interesting to explore with respect to Vitamin C dosing and QRS duration changes.

CONCLUSION

This thesis topic was developed in an attempt to understand whether Vitamin C transporter genetic polymorphisms may explain why published Vitamin C atrial fibrillation study outcomes in cardiac surgery have considerable heterogeneity (see chapter 1). Atrial fibrillation risk is markedly increased as a response to cardiac surgery. Atrial fibrillation pathological mechanisms are likely to be the resultant of oxidative stress and inflammation (Hu et al. 2015; Huang et al. 2009; Korantzopoulos et al. 2007). Hence a number of studies have attempted to ascertain whether anti-oxidant dosing of Vitamin C would be beneficial in preventing atrial fibrillation as a result of an enhanced myocardial ROS *milieu* caused by cardiac surgery. The role of Vitamin C is somewhat complex as intracellular levels of Vitamin C are dependent on Vitamin C transport systems. Also intracellular concentrations do not correlate with plasma Vitamin C levels or urinary excreted metabolites of Vitamin C. Underlying cellular oxidative stress may increase uptake of Vitamin C via increased expression of Vitamin C transporters. Responses to background inflammation and oxidative stress determining Vitamin C cellular update will thus depend on the genetic functionality of cellular transporters.

In chapter 1 we explored atrial fibrillation prevention in cardiac surgery with Vitamin C. We noted a paucity of studies on Vitamin C and ECG parameters (apart from using the ECG to document the presence of AF). We are aware of two small studies on the effects of acute dosing of Vitamin C on QRS duration changes in Nigerian young adults whom are healthy or have sickle cell disease (Jaja et al 2008). In Chapter 2 of this thesis we failed to observe any association of Vitamin C *rs1776964* AA genotype with QRS duration. QRS duration is typically increased on the ECG because of underlying

slowing of ventricular and or septal conduction speed. Increased QRS duration has also been demonstrated to be a risk factor for atrial fibrillation (El-Chami et al. 2010).

QRS duration, while not a clinical end point *per se* (apart from ventricular resynchronization device applications) – QRS duration does have utility in population studies and is gaining theoretical acceptance as an independent marker of cardiac risk. Pharmacological agents that have a positive effect on reducing QRS duration may reduce long term cardiovascular risk. Further studies are needed to "tease out" interactions between QRS duration and cardiac remodelling and compounds that may directly modulate QRS duration. In any case we know that there is not a linear association between increased QRS duration and cardiac mass and other molecular mechanisms or local cardiac conduction remodelling is likely important.

In summary we have shown that QRS duration is not increased when associated with the Vitamin C *rs1776964* AA genotype. We have also determined that the Vitamin C *rs1776964* AA genotype may interact with PQ interval, although further studies and a larger sample size are required to verify this finding and demonstrate statistical significance. We have reviewed the atrial fibrillation literature with respect to the protective effects of Vitamin C – and have hinted at the limitations of present study designs (see below) and also suggested that a study of Vitamin C transporter genotyping is required to better understand the genetics of Vitamin C in AF risk in cardiac surgery.

LIMITATIONS OF THIS THESIS

1) A potential limitation of our studies is that we did not measure Vitamin C in the plasma. This was a retrospective data set and measures of Vitamin C had not been previously performed and plasma was not available. We would question measuring plasma Vitamin C with long term storage. Additionally, the relationship between Vitamin C in plasma and intra-cellular levels in cardiac tissues are known to be non-linear (as suggested in previous sections of the thesis). What would have been theoretically useful is to understand the Vitamin C concentration in cardiac cells. Particularly how this may influence cardiac conduction. And how Vitamin C concentrations are associated with underlying "local" cardiac tissue inflammatory or oxidative stress states. Unfortunately, the present study is limited by the absence of measurements of cardiac intra-cellular Indeed it is appreciated that cardiac tissue sampling is impractical in community population studies and would not be permitted ethically. Hence, it is not possible to predict the influence of myocardial intracellular levels of Vitamin C on QRS duration as a result of SLC23A2 *rs1776964* AA genotypes. On the other hand it would be practically easy to sample cardiac tissues during open heart cardiac surgery to determine tissue Vitamin C intracellular concentrations, and explore associations across *rs1776964* genotypes and cardiac conduction.
2) In chapter 1 exploring published studies on Vitamin C and AF prevention in cardiac surgery we noted some limitations. For example, a number of published studies failed to measure Vitamin C levels in the blood both pre and post operative dosing. Where Vitamin C levels were measured, the metabolite of Vitamin C measured was not described. Additionally length of storage and

type of freezer storage temperatures of bloods for Vitamin C measurement are important. Hence temperature and length of storage should ideally be described by investigators. The processing of blood tubes is important for Vitamin C, as Vitamin C is somewhat subject to rapid degradation. Hence a description of analytical preparation of blood tubes for Vitamin C stability and measurements is likely required. We could not find any studies contrasting Vitamin C plasma levels and tissue uptake and adjusting outcomes for atrial fibrillation risk.

3) In chapter 2 we did not perform an acute or short-term chronic Vitamin C dosing study to replicate the findings of the Nigerian studies on QRS duration. We did not have access to a population (we were using a bio-bank and retrospective data set). We suggest it may not have been possible to observe a Vitamin C - QRS duration effect in our population. The reasons may be pre-existing cardiovascular disease or sub-clinical disease in our population that may mask the effects of Vitamin C dosing on QRS duration. As discussed in Chapter 2 we noted from the literature that there may be direct effects of Vitamin C on extracellular Na^+ ion channels.
4) Ancestral genetic differences between populations may exist for Vitamin C effects. The response to Vitamin C may be important across different race and environmental backgrounds. Further population studies are required to validate our negative interaction with the Vitamin C transporter and QRS duration. It would also be interesting to explore the effect of Vitamin C transporter polymorphisms and changes in QRS duration over time.
5) Our sample size was modest. We noted that statistical significance was approached for an association between PQ duration and *rs1776964* AA

genotypes in our T2DM sub-population. A larger sample size may lead to statistical significance.

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