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Ageing alters the severity of Sunitinib-induced cardiotoxicity: Investigating the mitogen activated kinase kinase 7 pathway association

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Abbreviations:

CF: coronary flow; DMSO: dimethyl sulphoxide; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HR: heart rate; KH: Krebs Henseleit; LVDP: left ventricular developed pressure; MKK7: mitogen activated kinase kinase 7; TTC: triphenyl-tetrazolium chloride

Abstract

Anti-cancer drug Sunitinib is linked to adverse cardiovascular events, which have shown to involve mitogen activated kinase kinase 7 (MKK7) pathway. Sunitinib-induced cardiotoxicity in 3, 12 and 24 months old male Sprague-Dawley rats and MKK7 expression and activation was investigated using the Langendorff perfused heart model followed by Western blot analysis. Cardiac function and infarct size were measured during/after 125 minutes of Sunitinib treatment. Left ventricular cardiac samples were analysed by qRT-PCR for expression of MKK7 mRNA and cardiac injury associated microRNAs. Infarct size was increased in all Sunitinib treated age groups. Haemodynamic alterations were observed following Sunitinib administration. Left ventricular developed pressure (LVDP) was decreased in all age groups, while heart rate (HR) was decreased in 3 and 12 months groups. Sunitinib treatment decreased the expression of miR-27a in all age groups, while miR-133a and miR-133b levels were increased in 3 months and decreased in 24 months groups. MKK7 mRNA and p-MKK7 levels were decreased in the 3 months group after Sunitinib treatment. MKK7 mRNA level was increased in 24 months group and p-MKK7 levels were increased in 12 months group following Sunitinib treatment. This study highlights the importance and impact of ageing and anti-cancer therapy-induced cardiotoxicity.

Keywords:

Cardiac aging; Anti-cancer therapy; Sunitinib-induced cardiotoxicity; mitogen activated kinase kinase 7; cardiotoxicity microRNAs.

1. Introduction

Life expectancy has increased substantially due to a combination of medical advances and improved quality of life. With the continuously growing elderly population, the number of elderly patients with cancer unfortunately increases. The median age at diagnosis of cancer is 65 years, and the rate of cancer diagnosis increases with age in both males and females (Miller et al. 2016). Due to ageing of the population and the cardiotoxic nature of cancer treatment, there is an increasing number of elderly patients with cancer and comorbid cardiovascular diseases. It is therefore vital to unravel the pathways linked to developing cardiovascular diseases during anti-cancer treatment in elderly cancer patients to stratify the anti-cancer treatment at the time of cancer diagnosis.

Ageing of the heart involves progressive deteriorations in its structure and function and is the leading risk factor for cardiovascular morbidity and mortality. Older people are significantly more likely to develop cardiovascular diseases (Lakatta 2003), such as left ventricular hypertrophy, diastolic dysfunction, valve degeneration, increased cardiac fibrosis, and decreased maximal exercise capacity (Dai et al. 2012). Furthermore, cardiac ageing is strongly associated with the development of heart failure (Ho et al. 1993). Needless to say, ageing of the heart causes it to become extremely vulnerable to external stress, such as cardiotoxic anti-cancer therapy.

The tyrosine kinase inhibitor Sunitinib is used in the treatment of renal cell carcinoma, gastro-intestinal stromal tumour and pancreatic nero-endocrine tumour (Le Tourneau et al. 2007). Sunitinib inhibits tyrosine kinases by competitively binding to the ATP-binding site domain of various receptor tyrosine kinases, notably: vascular endothelial growth factor 1-3 and platelet derived growth factor-α and -β (O'Farrell et al. 2003). Binding to the ATP-

binding domain causes the inhibition of dysregulated or over expressed tyrosine kinases involved in the regulation of angiogenesis cell proliferation and cell survival (Mendel et al. 2003). Unfortunately, Sunitinib is associated with severe cardiotoxic adverse effects due to the broad molecular targets and lack of kinase selectivity of this tyrosine kinase inhibitor. Sunitinib-induced cardiotoxicity causes adverse effects in cardiomyocytes, which can lead to cardiac ischaemia and produce arrhythmias (Cohen et al. 2011). Also, left ventricular hypertrophy, hypertension and heart failure development have been reported in response to Sunitinib treatment (Ewer et al. 2014; Gupta and Maitland 2011). Sunitinib-induced cardiotoxicity develops in approximately 2.7 % of the overall population (mean age 65 years) (Khakoo et al. 2008), while the overall incident of congestive heart failure has been reported to be 4.1 % (Richards et al. 2011). In the long-term follow-up study by Brunello *et al.* Sunitinib-induced cardiotoxicity in the elderly (≥ 70 years old) was recorded to be 13.3 %. The study followed 68 metastatic renal cell carcinoma patients treated with Sunitinib, out of which 9 developed cardiac events including asymptomatic decrease in LVEF, acute myocardial infarction, and congestive heart failure (Brunello et al. 2013). The rate of adverse cardiac event in the elderly treated with Sunitinib is increased dramatically in the study by Brunello *et al.* compared to the overall population, however it should be noted that the metastatic renal cell carcinoma patients in the study by Brunello *et al.* had a high prevalence of existing cardiovascular comorbidities prior to the Sunitinib treatment, which could explain the higher rate of cardiac events.

The stress activated protein MKK7 belongs to the mitogen activated kinase kinase superfamily, which allows the cell to respond to exogenous and endogenous stimuli (Foltz et al. 1998). MKK7 activation of the downstream c-Jun N-terminal kinases (Tournier et al. 2001) results in processes including: proliferation, differentiation, apoptosis and

tumorigenesis (Chang and Karin 2001). As MKK7 activity has been associated with cardiomyocyte damage (Liu et al. 2011), it would be interesting to assess changes in MKK7 expression levels in the presence of Sunitinib at both transcriptional and post-translational levels at various age stages. Establishing an age dependent involvement of the MKK7 pathway during Sunitinib-induced cardiotoxicity would lead to advance our understanding of the cardiac ageing pathways during stress stimuli and could lead to improve the anti-cancer treatment guidelines by implementing adjunct therapy options.

This study investigates for the first time the involvement of the MKK7 pathway during Sunitinib-induced cardiotoxicity at various age stages via the assessment of cardiac function and injury using a Langendorff perfused rat heart model in: *Adult* rats (3 months), *Middle-aged* rats (12 months) and *Elderly* rats (24 months). Furthermore, the differential expression patterns of cardiotoxicity-linked microRNAs miR-1, miR-27a, miR-133a and miR-133b is determined at the specific age groups.

2. Materials and methods

2.1. Materials

Sunitinib malate and triphenyl-tetrazolium chloride were purchased from Sigma Aldrich (UK) and dissolved in dimethyl sulphoxide (DMSO) and stored at -20 °C. Krebs perfusate salts were from VWR International (UK) or Fisher Scientific (UK). Ambion MicroPoly(A)Puris kit, Ambion *mir*Vana miRNA Isolation Kit, Reverse Transcription Kit, Applied Biosystems MicroRNA Reverse Transcription Kit, TaqMan Universal master mix II (no UNG), MKK7 mRNA primers, Applied Biosystems primers assays (U6, rno-miR-1, hsa-miR-27a, hsa-miR-133a, and hsa-miR-133b) were purchased from Life Technologies (USA). The iTaq Universal SYBR Green Supermix was purchased from BioRad (UK). Phospho-

MKK7 (Ser271/Thr275), Total MKK7 rabbit mAb antibodies and anti-rabbit IgG, HRP-linked antibody and anti-biotin, HRP-linked antibody were purchased from Cell signaling technologies (UK).

2.2. Animals and Ethics

Adult male Sprague-Dawley rats (12-weeks old and 300-350 g in body weight); were purchased from Charles River UK Ltd (UK) and housed suitably, received humane care and had free access to standard diet according to "The Guidance on the Operation of the Animals (scientific procedures) Act of 1986". As aged animals > 6 month of age are not supplied by any Laboratory animal supplier we kept 30 12-weeks old rats until they were 12 month old and additional 30 12-weeks old rats until 24 months old, therefore the experiments using 3, 12 and 24 month rat hearts at various intervals. Animals were selected at random for drug treatment groups and the collected tissue was blinded for infarct size assessment. The experiments were performed after approval of the protocol by the Coventry University Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments. A total of 79 animals were used for this study and the data from 63 rats were included (*3 month:* Tissue collection, Control n=6 and Sunitinib n=6, TTC collection, Control n=4 and Sunitinib n=5; *12 month:* Tissue collection, Control n=7 and Sunitinib n=7, TTC collection, Control n=6 and Sunitinib n=6, *24 month:* Tissue collection, Control n=5 and Sunitinib n=5, TTC collection, Control n=3 and Sunitinib n=3), while data from 15 rats were excluded from analysis due to the established haemodynamic exclusion criteria, which can be found in the "2.3. Langendorff perfusion model" section below.

2.3. Langendorff perfusion model

Rats were sacrificed by cervical dislocation (Schedule 1 Home Office procedure) and the hearts were rapidly excised and placed into ice-cold Krebs Henseleit (KH) buffer (118.5 mM NaCl, 25 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.7 mM $CaCl_2$, and 12 mM glucose, pH7.4). The hearts were mounted onto the constant flow Langendorff system and retrogradely perfused with KH buffer. The pH of the KH buffer was maintained at 7.4 by gassing continuously with 95 % O_2 and 5 % CO_2 and maintained at 37 ± 0.5 °C using a water-jacketed organ chamber. Oxygen content of the perfused buffer has been monitored in previous studies, showing that the oxygen content is constant throughout the Langendorff experiment. The left atrium was removed and a latex iso-volumic balloon was carefully introduced into the left ventricle and inflated up to 5-10 mmHg. Functional recordings (LVDP and HR) were taken via a physiological pressure transducer and data recorded using Powerlab, AD Instruments Ltd. (UK). Coronary flow (CF) was measured by collecting and measuring the volume of perfusate for 1 minute. All haemodynamic parameters were measured at 5 minute intervals for the first 35 minutes of drug treatment, and then at 15 minute intervals until the end of the experiment.

Each Langendorff heart was perfused for 125 minutes with drug or vehicle in normoxic conditions after a 20 minutes stabilisation period to recover from the Langendorff cannulation procedure (Cooper et al. 2018; Sandhu et al. 2017). Generally, the hearts became stable after 10 minutes, therefore all haemodynamic parameters were normalised to the last 10 minutes of the stabilisation period to take into account the variations between individual starting HR, LVDP and CF levels. Hearts were excluded in the study with: a LVDP below 80 mmHg or above 150 mmHg, a HR below 225 beats per minute or above 325 beats per minute, and a CF below 3.5 ml/g heart weigh or above 12.0 ml/g heart weight during the stabilisation period.

Haemodynamic effects are presented as a percentage of the last 10 minutes of the mean stabilisation period for each parameter to allow clear comparison across drug groups. The maximal change in LVDP, HR, and CF were calculated by calculating mean ± S.E.M. at the specific time points in Control and Sunitinib treated hearts in all three age groups (ie. 3, 12, and 24 month).

Sunitinib malate (1 µM) was administered throughout the perfusion period. The dose of 1 µM Sunitinib was chosen in line with previous studies (Henderson et al. 2013). Langendorff perfused hearts treated with vehicle (i.e. DMSO) were recorded as Control group. The hearts were then weighed and either stored at -20 °C for triphenyl-tetrazolium chloride (TTC) staining or the left ventricular tissue was dissected free and immersed in RNAlater from Ambion (USA) for qRT-PCR or snap frozen with liquid nitrogen for Western blot analysis.

2.4. Infarct size analysis

Infarct size analysis was performed as described in our previous paper (Cooper et al. 2018). The mean of infarct to risk ratio for each treatment group and the mean ± S.E.M was plotted as a bar chart. The infarct size determination was randomized and blinded.

2.5. Analysis of microRNA expression profiles

MicroRNA was isolated from left ventricular tissue and the expression of housekeeping reference RNA U6 snRNA and target microRNAs rno-miR-1, hsa-miR-27a, hsa-miR-133a, and hsa-miR-133b was performed as described in our previous paper (Cooper et al. 2018). Analysis of qRT-PCR data of microRNAs was performed using the Ct values for U6 snRNA as reference for the comparison of the relative amount of microRNAs (rno-miR-1, hsa-miR-27a, hsa-miR-133a and hsa-miR-133b). The values of each of the microRNA was calculated

to compare their ratios. The formula used was $X_0/R_0=2^{(CTR-CTX)}$, where X_0 is the original amount of target microRNA, R_0 is the original amount of U6 snRNA, CTR is the Ct value for U6 snRNA, and CTX is the Ct value of the specific target microRNA. Averages of the Ct values for each sample group (Control and Sunitinib treated hearts) and each individual primer set were calculated and bar charts were plotted with mean ± S.E.M data. The mean of the Control group was set as 1 for all microRNAs.

2.6. Measurement of MKK7 mRNA expression

Total mRNA was extracted from left ventricular tissue and expression of MKK7 and GAPDH was performed as described in our previous paper (Cooper et al. 2018). Analysis of qRT-PCR data of MKK7 mRNA were performed using the Ct values for GAPDH mRNA as reference for the comparison of the relative amount MKK7 mRNA. The formula used was $X_0/R_0=2^{(CTR-CTX)}$, where X_0 is the original amount of MKK7 mRNA, R_0 is the original amount of GAPDH mRNA, CTR is the CT value for GAPDH mRNA, and CTX is the CT value for MKK7 mRNA. Averages of the Ct values for each sample group (Control and Sunitinib treated hearts) and MKK7 was calculated and bar charts were plotted with mean ± S.E.M. The mean of the Control group was set as 1 for the MKK7 mRNA study.

2.7. Western blot detection of phosphorylated MKK7

Protein was isolated form left ventricular tissue and Western blot analysis using Phosphorylated (Ser^{271}/Thr^{275})-MKK7 (p-MKK7) and total MKK7 was performed as described in our previous paper (Cooper et al. 2018). The relative changes in the p-MKK7 protein levels were measured and corrected for differences in protein loading as established by probing for total MKK7. Results were expressed as a percentage of the density of

phosphorylated protein relative to the density of total protein using Image Lab 4.1 from BioRad (UK).

2.8. Data analysis and statistics

Results are presented as mean ± S.E.M. For haemodynamics data comparison of Control and Sunitinib groups the statistical analysis was done by Two-way repeated measures ANOVA test with the Bonferroni post hoc test. When comparing Control and Sunitinib within an age group significance of data sets was measured by 2-tailed Student's t-test. When age groups were compared (i.e. 3 month vs 12 month, 3 month vs 24 month and 12 month vs 24 month) One-way ANOVA analysis with the LSD post hoc test was applied. We used the GraphPad Prism program version 5 and the IBM SPSS Statistics version 22 software for statistical analysis and p-values <0.05 were considered statistically significant. When comparing Control versus Sunitinib treatment statistical significance is shown as "*" on top or bottom (depending on increase or decrease) of the Sunitinib group. And when the Sunitinib treated age groups are compared, statistical significance is shown with "$" highlighted with a capped line linking the relevant age groups together.

3. Results

3.1. Haemodynamic parameters

In this study, we recorded LVDP, HR, and CF to determine whether 1 µM Sunitinib induces signs of cardiac dysfunction during a 125 minute Langendorff perfusion in 3, 12 and 24 month old rat hearts.

3.1.1. Sunitinib adversely affects LVDP in all age groups

When treatment groups were normalised to the stabilisation period, Sunitinib treatment significantly decreased normalised LVDP in 3 and 24 month groups compared to their group Controls (Figures 1A-C). However, the onset of LVDP decline varied between the age groups. The maximal drop in LVDP from Control to Sunitinib treated hearts was observed at 125 minutes in the 3 month group (Control: 85 ± 4 %; Sunitinib: 70 ± 4 %, $P<0.05$) and at 95 minutes for the 24 month group (Control: 77 ± 1 %; Sunitinib: 56 ± 2 %, $P<0.05$) (Figures 1-3). The largest decline in LVDP decline for the 12 month group at 125 minutes (Control: 80 ± 2 %; Sunitinib: 69 ± 1 %), however, this was without statistical significance.

3.1.2. Sunitinib alters HR in 3 and 12 month animals

When the HR were normalised to the stabilisation period, HR was significantly decreased in the 3 month and 12 month groups when Control hearts were compared to hearts treated with Sunitinib. The HR for 24 month group remained stable during the Sunitinib treatment. Maximal drop in HR from Control to Sunitinib treated hearts was observed at 125 minutes in the 3 month group (Control: 100 ± 2 %; Sunitinib: 80 ± 5 %, $P<0.001$), at 125 minutes for the 12 months group (Control: 98 ± 3 %; Sunitinib: 80 ± 3 %, $P<0.01$), and at a much earlier stage at 30 minutes for the 24 months group (Control: 98 ± 4 %; Sunitinib: 88 ± 6 %). At an early stage (5 and 10 minutes) HR in the 3 month group was increased significantly by Sunitinib treatment when compared to control hearts ($p<0.05$) (Figures 2A-C).

3.1.3. Sunitinib treatment does not alter CF

When the CF was normalised to the stabilisation period, CF was slightly increased in the three age groups where hearts were perfused with Sunitinib when compared to Control hearts. Maximal increase in CF from Control to Sunitinib treated hearts was observed at 10 min in

the 3 months group (Control: 89 ± 3 %; Sunitinib: 107 ± 4 %), at 5 min for the 12 months group (Control: 103 ± 2 %; Sunitinib: 119 ± 10 %), and at 50 min for the 24 month group (Control: 90 ± 2 %; Sunitinib: 93 ± 4 %) (Figures 3A-C).

3.2. Infarct size assessment

Sunitinib treatment produced significant increases in infarct size (normalised to heart weight) ratio in all age groups in an age dependent trend when compared to Control hearts (Figure 4A-B). The infarct size to heart weight ratio was increased 5.1-fold in the 3 months group (Control: 3.29 ± 0.55 %; Sunitinib: 17.14 ± 0.39 %, $P<0.001$), increased 3.3-fold in the 12 months group (Control: 3.06 ± 0.09 %; Sunitinib: 10.19 ± 0.84 %, $P<0.001$), and increased 2.5-fold in the 24 months group (Control: 2.25 ± 0.21 %; Sunitinib: 5.53 ± 0.15 %, $P<0.05$) (Figure 4A-B). When the infarct size was normalised to that age group's specific Control heart infarct size, we observed that the Sunitinib treatment resulted in a significantly higher infarct sizes in the 3 month group when compared to the 24 month group ($P<0.01$) (Figure 4B). The infarct size in the Control hearts did not alter in the 3 age groups.

3.3. microRNA expression profiles

Here, we investigate the altered expression profiles of microRNAs miR-1, miR-27a, miR-133a and miR-133b after Sunitinib-induced cardiotoxicity (Figure 5). The expression of miR-1 is not altered significantly in any of the age groups in response to Sunitinib treatment, compared to specific age group Controls. A significant reduction in miR-27a expression was observed in all 3 age groups response to Sunitinib treatment, compared to specific age group Controls (0.4 fold decrease in 3 months group, $P<0.001$; 0.43 fold decrease in 12 months group, $P<0.05$; 0.69 fold decrease in 24 months group, $P<0.05$). The expression of miR-133a and miR-133b followed the same pattern in each age group; Sunitinib treatment showed a

300 significant increase in miR-133a and miR-133b levels, in the 3 months group (miR-133a:
301 2.70 fold increase, P<0.001; miR-133b: 3.79 fold increase, P<0.01) and a significant decrease
302 in the 24 months group (miR-133a: 0.70 fold decrease, P<0.001; miR-133b: 0.64 fold
303 decrease, P<0.01) compared to specific age group Controls (Figure 5).
304
305 **3.4. MKK7 mRNA expression**
306 MKK7 mRNA profiling in response to Sunitinib treatment revealed a significant decrease in
307 MKK7 mRNA in 3 month old rats (~ 3 fold decrease, P<0.05) and an increase in MKK7
308 mRNA in 24 month old rats (~ 12 fold increase, P<0.01) (Figure 6). When the Sunitinib-
309 induced alteration of MKK7 mRNA was normalised to Control for each age group, we
310 observed an age dependent increase in MKK7 mRNA in 12 month and 24 month groups
311 when compared to the 3 month group (P<0.001) (Figure 6).
312
313 **3.5. Phosphorylated MKK7 protein expression profile**
314 We investigated the effect of Sunitinib induced cardiac injury on MKK7 phosphorylation
315 levels in different age groups. In the 3 month group p-MKK7 levels were significantly
316 decreased after Sunitinib treatment, compared to Control (Control: 79 ± 8 %; Sunitinib: 46 ±
317 2 %, P<0.05). Contrarily, in 12 month group there was a significant increase in p-MKK7
318 levels after Sunitinib treatment, compared to Control (Control: 56 ± 3 %; Sunitinib: 71 ± 5 %,
319 P<0.05). There were no changes in p-MKK7 levels in 24 month group following Sunitinib
320 treatment compared to Control hearts (Figure 7A). There was a significant increase in p-
321 MKK7 levels in the 12 month group compared to 3 month group when the Sunitinib
322 treatment was normalised to specific age group Controls (~2 fold increase, P<0.01), and a
323 significant decrease in the 24 month group compared to the 12 month group (~ 2/3 fold
324 decrease, P<0.05) (Figure 7B).

4. Discussion

In the clinic, the level of cardiotoxicity generated by Sunitinib treatment is largely underestimated (Schmidinger et al. 2008). This is highlighted by the increasing number of cancer survivors, developing acute and delayed toxicities later in life (Lipshultz et al. 2013). Age is a well-established risk factor which may predispose a patient to cardiotoxicity. As Sunitinib treatment is administered to patients at a variety of ages (from paediatrics to elderly patients), it is important to identify the level of Sunitinib-induced cardiotoxicity produced in various age groups (Hutson et al. 2014; Janeway et al. 2009).

Cardiovascular events have been reported during and after Sunitinib treatment in the clinic and in *in vitro* settings (Di Lorenzo et al. 2009; Sandhu et al. 2017). In the clinic, doctors are advised to monitor patients who display cardiac risk factors and/or history of coronary artery disease, the left ventricular ejection fraction and also for hypertension development in patients undertaking oral Sunitinib treatment, however, most patients are not monitored for adverse cardiovascular reactions to Sunitinib treatment (Kollmannsberger et al. 2007). This study shows that ageing strengthens the hearts ability to resist Sunitinib-induced heart tissue damage, however, the cardiac function is dramatically impaired (i.e. haemodynamic parameter LVDP). Younger animals appear to be more sensitive to toxicities in early stages of treatment, however, all age groups were found to have Sunitinib-induced cardiotoxicity. Furthermore, the stress activated MKK7 has shown to have a role in the level of Sunitinib-induced cardiotoxicity (Cooper et al. 2018).

4.1. Sunitinib is cardiotoxic in all three age groups

Here we investigated age-associated differences in Sunitinib-induced cardiotoxicity, by measuring changes in haemodynamic parameters (i.e. LVDP, HR, and CF) and the level of

heart tissue infarction in 3, 12, and 24 month rats. The concentration of 1 μM Sunitinib was chosen in line with the clinically relevant study by Goodman *et al.* 2007, where patients suffering from Imatinib refractory or intolerant gastrointestinal stromal tumour, and patients with metastatic renal cell carcinoma where treated with Sunitinib. The steady state blood concentrations of Sunitinib was reported to be in the range of 0.1 – 1.0 μM (Goodman et al. 2007; Henderson et al. 2013).

It should be noted that our study shows the direct and acute cardiac effect of Sunitinib perfusion of rat hearts. As the isolated Langendorff model detects the acute effect of Sunitinib on the myocardium, systemic vasculature and neurohormonal effects of Sunitinib are thus excluded. The study by Mooney *et al.,* showed the link between acute Sunitinib-induced cardiotoxicity and calcium/calmodulin-dependent protein kinase II (CaMKII) activity. CaMKII is a key regulator of cardiac contractile function and dysfunction. Their study showed that acute administration of Sunitinib resulted in decreased LVDP and systolic and diastolic blood pressure, and this was correlated with decreased CaMKII activity. Thus, Sunitinib's lack of kinase selectivity could result in impacting CaMKII at an acute treatment stage (Mooney et al. 2015). The present study found significant declines in LVDP in all 3 age groups following treatment with Sunitinib. LVDP is a measurement of cardiac function (i.e. force of contraction) in terms of pressure in the left ventricle, where LV diastolic pressure is subtracted from LV systolic pressure (i.e. LVDP = left ventricular systolic pressure – left ventricular diastolic pressure) (Kolwicz and Tian 2010). On the other hand left ventricular ejection fraction (LVEF) represents the heart's pumping efficiency, and is calculated by taking the fraction of chamber volume ejected in systole (i.e. stroke volume (SV = end-diastolic volume – end-systolic volume)) in relation to the volume of the blood in the ventricle at the end of diastole (i.e. end-diastolic volume) (LVEF = SV/end-diastolic volume

x 100) (Kosaraju and Makaryus 2018). Although LVDP and LVEF are calculated using different parameters, they will be correlated as both represent the cardiac function depending on similar inputs. Interestingly, the 24 month group produced the largest maximum drop in LVDP (Figure 1C). Left ventricular dysfunction is one of the main adverse cardiac side-effects of Sunitinib, after hypertension (Chu et al. 2007). During a 3 year study following 48 patients treated with Sunitinib, Telli *et al.* reported significant declines in left ventricular ejection fraction in 21 % of patients, and 15 % of patients developed symptoms of heart failure (Telli et al. 2008). Chu et al. also demonstrated deterioration of myocardial contractility in response to Sunitinib treatment (Chu et al. 2007). Left ventricular dysfunction and a decline in cardiac contractility have been linked to mitochondrial dysfunction, which occurs as a result of the inhibition of ribosomal S6 kinase and AMP-activated protein kinase by Sunitinib (Hasinoff et al. 2008). The heart has a high energy demand and through ageing, essential cellular processes - including autophagy - become dysfunctional (Peart et al. 2014). This results in an accumulation of impaired cellular machinery, such as mitochondria. In turn, this reduces the level of ATP available for cardiomyocytes and reduces heart function, both of which have been linked to age associated heart failure (Moyzis et al. 2015). The younger hearts may facilitate a more efficient process of autophagy and cell death pathways, which prevent the accumulation of dysfunctional mitochondrial and protein signalling (Zhao et al. 2010). It is likely that Sunitinib caused a further depletion in ATP levels in aged hearts through the inhibition of AMPK, which lead to a decline in left ventricular function (Force et al. 2007). However, this needs to be investigated in further detail.

In response to Sunitinib treatment, an initial early significant increase in HR at 5 and 10 minutes was found in the 3 months group (Figure 2A). In patients, initial atrial fibrillation with rapid ventricular responses as well as tachycardia have been identified in the first cycle

of Sunitinib treatment (Grossmann et al. 2008). This highlights the instant cardiovascular effects Sunitinib can generate. However, after 15 minutes of Sunitinib perfusion both, the 3 month and 12 month group demonstrated significant declines in HR (Figure 2A-B). Henderson *et al.* showed a dose-dependent decline in HR under ischemic conditions in Langendorff studies (Henderson et al. 2013). In the clinic declines in HR are a common side effect of Sunitinib treatment (Azizi et al. 2008). Bello *et al.* demonstrated that Sunitinib induces QT-interval prolongation in patients and there is a dose-dependent increased risk of ventricular arrhythmias with Sunitinib treatment (Bello et al. 2009). At a cellular level Sunitinib had been shown to block the cardiac human ether-a-go-go related gene (ERG) channel which is associated with long QT syndrome (Doherty et al. 2013). Interestingly, Sunitinib treatment did not produce significant declines in HR in the 24 month group (Figure 2C). Ageing can produce an accumulation of compensatory cardiomyocyte remodelling in the heart (Gosse 2005). This could be due the 24 months control group having a much lower HR at baseline (Table 3). Over time, the heart enlarges in response to increase in haemodynamic load, neuro-hormonal and pro-hypertrophic signalling (Gosse 2005). Remodelling fundamentally begins with molecular changes, such as altered cell growth regulation and protein expression. This results in impairment of myocardial performance and causes a lower heart rate (Lupon et al. 2015).

It should be noted that the maximal decrease in LVDP and HR of hearts treated with Sunitinib when compared to Control, for the 24 month group did not occur at the end time point of 125 mins as observed for the 3 and 12 months groups, instead LVDP maximal decrease for 24 month group occurred at 95 mins, while HR maximal decrease occurred at 30 mins. This inconsistency in time is most like due to biological variability of the aged hearts.

Furthermore, we investigated the level of Sunitinib-induced infarct size of treated hearts in 3, 12, and 24 month rats. All of the age groups demonstrated significant increases in infarct size after 1 µM Sunitinib treatment, compared to control hearts (Figures 4A-B). Henderson *et al.* measured troponin levels as a marker for myocyte injury, and the group demonstrated significant increases in troponin levels release from an isolated rat heart model treated with 1 µM Sunitinib (Henderson et al. 2013). In a study using induced pluripotent stem cell-derived cardiomyocytes, Cohen *et al.* demonstrated that Sunitinib treatment resulted in a loss of ATP and increased oxidized glutathione, which was thought to induce apoptosis (Cohen et al. 2011). In addition to this, 1 µM Sunitinib treatment on isolated human myocardium tissue and isolated mouse left ventricular myocytes, was shown to produce a significant decline in intracellular Ca^{2+} levels and an increase in levels of reactive oxygen species generation, which can cause apoptosis (Rainer et al. 2012). Interestingly, in the present study, the 3 months group produced a much larger infarct size than both the 12 and 24 month groups (Figures 4A-B). It has been established that younger patients (< 20 years) are more susceptible to cardiac injury during and after cancer therapy (Hancock et al. 1993). QT-interval prolongation and a decrease in ejection fraction has been reported in children during the first cycle of Sunitinib treatment (Dubois et al. 2011). This suggests that younger hearts have an initial increase in sensitivity to Sunitinib-induced cardiotoxicity. The 12 and 24 month groups had a smaller infarct sizes than 3 month old rats (Figure 4B). Capitanio *et al.* demonstrated that elements of heart protection could be present in disease-free ageing of Sprague-Dawely rats. There was an activation of cellular protective mechanisms such as a reduction in reactive oxygen species generation, resistance to apoptosis and inhibition of mitochondrial permeability transition pore opening (Capitanio 2016). Therefore, the 12 and 24 months Sunitinib treated groups could have existing cardioprotective resistance to cell

death and tissue injury through ageing, and thus would not be greatly affected by 2 hours of Sunitinib therapy.

However, the huge decline in LVDP of the 24 month group is indicative of cardiovascular dysfunction. Ageing lowers the threshold for development of cardiovascular diseases. The cardiac defence mechanisms protecting the heart from injuries and the injury repair pathways become defective. Furthermore, the cardiac structure alters with ageing, leading to vascular stiffening, enlargement of left ventricular wall thickness, and fibrosis. In addition to this, there are some key functional changes in the ageing heart that lead to a decline in the reserve capacity, which impairs the heart's capacity to function properly during the strained workload (Strait and Lakatta 2012). With this in mind the steep decline in LVDP observed in Sunitinib treated aged animals (i.e. 27 % in 24 months) compared to younger ones (i.e. 17 % in 3 months) could be a result of dysfunctional structural and functional changes in aged animals. These dysfunctional structural and functional changes could also explain why the infarct size was more predominant in the Sunitinib treated younger animals (5.1 fold increase) compared to aged animals (2.5 fold increase), as Sunitinib exposure for 2 hours might not have been adequate to induce infarct in stiff and enlarged cardiac tissue. Further investigation into the structural and functional properties of aged heart tissue in response to Sunitinib treatment is required to establish why Sunitinib treatment caused the hearts of 24 month rats to produce reductions in function, yet produced smaller infarct sizes than younger animals when compared to untreated hearts.

4.2. Key cardiac injury linked microRNAs are altered by Sunitinib treatment

Short non-coding RNA microRNAs carry out the negative regulation of mRNA transcripts by repressing translation (Bartel 2004). Specific microRNAs expression patterns have been

linked to cardiomyocyte differentiation and in response to stress (Babiarz et al. 2012) and have also been shown to be differentially expressed during the development of heart failure (Thum et al. 2007). Furthermore, microRNAs are critical regulators in the expression and function of eukaryotic genomes. Changes in the expression of certain microRNAs could be indicative of specific diseases or medical conditions (Lu et al. 2008). The expression profiles of miR-1, miR-27a, miR-133a and miR-133b tend to be altered during cardiac injury and during the progression of heart failure (Akat et al. 2014; Tijsen et al. 2012). We show Sunitinib induced changes in expression profiles of miR-27a, miR-133a and miR-133b in the 3 age groups investigated.

In response to Sunitinib, miR-27a was reduced in all age groups (Figure 5). miR-27a has been shown to down-regulate FOXO-1 protein, a transcription factor which regulates genes involved in the apoptotic response, cell cycle, and cellular metabolism (Guttilla and White 2009). It has also been observed that over expression of FOXO1 resulted in decreased cell viability because of inhibition of cell cycle and induction of apoptosis. A down regulation of miR-27a has been linked to an increased sensitivity to Adriamycin induced apoptosis (Zhang et al. 2010). This suggests that miR-27a is an effective regulator of apoptosis. In coronary sinus samples miR-27a is significantly downregulated in heart failure patients (Marques et al. 2016). The significant decrease in miR-27a expression during Sunitinib treatment during the current study follows the same trend in expression as patients with heart failure and apoptosis at a cellular level, which could suggest that a down-regulation of miR-27a predicts an increase in apoptosis or heart tissue damage within the heart, as we have shown an increase in infarct size in all age groups.

Interestingly, miR-133a and miR133b are both significantly upregulated in 3 months group, but downregulated in 24 months in response to Sunitinib treatment (Figure 5). In our previous Sunitinib studies have seen similar findings with either significant or a strong tendency towards an increased expression of miR-133a and miR-133b after Sunitinib administrating during Langendorff perfused hearts compared to vehicle treated hearts (Cooper et al. 2018; Sandhu et al. 2017). miR-133a has a partial complimentary target site in the 3'-untranslated region of the human ERG potassium channel transcripts, implying that miR-133a overexpression inhibits the ERG potassium channel expression (Xiao et al. 2007). A reduction in ERG potassium channel expression results in delayed myocyte repolarization, which is attributed to a long QT interval (Xiao et al. 2007). Therefore, the increase in miR-133a found in the 3 months group suggests an increase in ERG inhibition, which could be responsible for a slower heart rate. Sunitinib treatment of both 12 months and 24 months groups demonstrated significant reductions in miR-133a. This could suggest that Sunitinib causes attenuation of the ERG potassium channel expression by miR-133a may have taken place (Bello et al. 2009). However, Sunitinib also induced significant reductions in HR in the 12 month group. This suggests that alternate mechanisms to Sunitinib-induced ERG inhibition could be occurring in older animals. This highlights the complexity of Sunitinib-induced HR reductions at different ages.

In addition, miR-133a has been shown to be upregulated during oxidative stress (Izarra et al. 2014). In cardiomyocytes miR-133b has been shown to be upregulated during apoptosis, but downregulated during hypertrophy (Ramasamy et al. 2015). This could suggest that Sunitinib treatment resulted in increased levels of oxidative stress and cell death in the 3 month group, which resulted in a larger infarct size compared to 12 month and 24 month groups. MicroRNAs have previously been shown to be differentially expressed when young rodent

hearts are compared to aged rodent hearts (Zhang et al. 2012). Perhaps, ageing provides alternate signalling mechanisms which reduce levels of Sunitinib-induced cell death or heart tissue damage. This needs to be investigated further.

4.3. The level of MKK7 transcription and protein phosphorylation is greatly affected by Sunitinib treatment and the age of rat hearts treated

MKK7 is a stress signalling protein with a vital role in cellular stress response and is fundamental in regulating cell survival, proliferation and cell death (Foltz et al. 1998). MKK7 has previously been shown have an important role in protecting the heart from heart failure (Liu et al. 2011), and furthermore MKK7 has been shown to be involved in the development of reductions in haemodynamic parameters and an increase in infarct size in 3 month old rats (Cooper et al. 2018). Alterations in the level of MKK7 protein activation have been shown to be age specific (Jiang et al. 1993). As Sunitinib produces adverse effects in the heart (Ewer et al. 2014; Gupta and Maitland 2011), it would be important to establish whether MKK7 levels are altered in response to Sunitinib treatment in ageing animals. Here we show the level of MKK7 transcription and MKK7 protein phosphorylation is affected by ageing and Sunitinib treatment.

Firstly we investigated whether MKK7 mRNA levels are altered in response to Sunitinib-induced cardiac injury. In the 3 months group Sunitinib treatment leads to a significant reduction in both MKK7 mRNA and phosphorylated MKK7, compared to Control (Figures 6 and 7A). Sunitinib treatment significantly increased the levels of MKK7 mRNA in the 12 month group and there was a tendency for an increased in MKK7 mRNA levels in the 24 month group (Figure 6).

Younger animals were shown to have a marked reduction of active MKK7 during Sunitinib treatment when compared to older animals. This could be a result of the younger animals not being fully developed for stress activated cellular signalling pathways, which could have resulted in activation of cell death pathways, which led to the huge increase in infarct size compared to the older animals. In a study by Zhang *et al.*, it was shown that ageing resulted in selective upregulation of stress protein genes and transcripts involved in cell growth, death, and signalling, namely extracellular signal-regulated kinase 2/3, c-Jun N-terminal kinase 2, caspase 6, cyclin-dependent kinase 4, proliferating cell nuclear antigen, and heterogeneous nuclear ribonucleoprotein K in Fischer 344 rats. Furthermore, they detected a downregulation of genes involved in antioxidant defences and drug metabolism, including glutathione transferase subunit P, cytochrome P-450 VII, nicotinamide adenine dinucleotide phosphate cytochrome P-450 reductase, bleomycin hydrolase, N-oxide forming dimethylaniline monooxygenase 1, and serum paraoxonase (Zhang et al. 2002).

We have observed a decrease in Sunitinib-induced infarct size in aged animals when compared to young animals (Figure 4A). The MKK expression after Sunitinib treatment at transcriptional level however increases in aged animals when compared to young animals (Figure 6), and the phosphorylated level of MKK7 after Sunitinib treatment is also increased from 3 to 12 months rats, while the phosphorylated MKK7 level is not altered by Sunitinib in the 24 month rats (Figure 7A). Liu *et al.*, demonstrated that pressure overload in MKK7 knockout mice was associated with elevated cardiomyocyte apoptosis and enhanced deterioration of ventricular function (Liu et al. 2011). The study by Liu *et al.* supports our current and previous findings, as the reduction in MKK7 transcription is associated with increased sensitivity to Sunitinib-induced cardiotoxicity in 3 month animals, while an

increase in MKK7 transcription is associated with decreased sensitivity to Sunitinib-induced cardiotoxicity in older animals (Figures 4A and 7A) (Cooper et al. 2018).

Interestingly, Hsieh *et al.* 2003, also demonstrated an increased level of MKK7 activation in response to reactive oxygen species generation in 24 month old mice compared to 3 month old mice (Hsieh et al. 2003). Previously, over-expression of MKK7 has also been shown to produce characteristic features of myocardial hypertrophy, which may have contributed to the loss of contractile function and cardiomyocyte viability following ischaemia/reperfusion injury (Wang et al. 1998). In turn, studies investigating cardiac hypertrophy have shown activated MKK7 levels to be significantly higher than in controls (Wang et al. 2008). This could suggest that the increase in MKK7 mRNA in the 12 month and 24 month groups and phosphorylated MKK7 in the 12 month group could indicant a hypertrophic response to Sunitinib treatment. However, Sunitinib treatment in the 24 month group did not alter p-MKK7 levels, but significantly increased MKK7 mRNA levels (Figures 6 and 7A). Perhaps overtime cells adapt cellular processes, including MKK7 signalling, to increase resistance to initiation of cell death pathways (Gosse 2005).

4.4. Conclusion

Ageing induces changes to the morphology, protein signalling and effective functioning of the heart. It is therefore important to investigate the potential cardiovascular effects of drugs in models of ageing. Here we have shown that ageing is associated with a more robust defence against Sunitinib-induced cardiac infarct, as younger animals display a significantly higher Sunitinib-induced cardiac infarct sizes compared to the older animals. Interestingly, we also showed that aged animals had a much more profound decrease in LVDP compared to younger animals, thus emphasising that ageing does alter the anti-cancer therapy-induced

cardiotoxicity in a defined and complex pattern, which must be investigated further at an intracellular level in order to pinpoint key pathways involved. Discovering the role of these key pathways involved in the development of Sunitinib-induced cardiotoxicity in elderly cancer patients could lead to the identification of novel and impactful adjunct therapy regimes to be implemented along with anti-cancer treatment, which will increase the outcome rate and quality of life in the elderly cancer patients.

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Conflict of interest

All authors have no conflict of interest to declare.

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Figures and legends

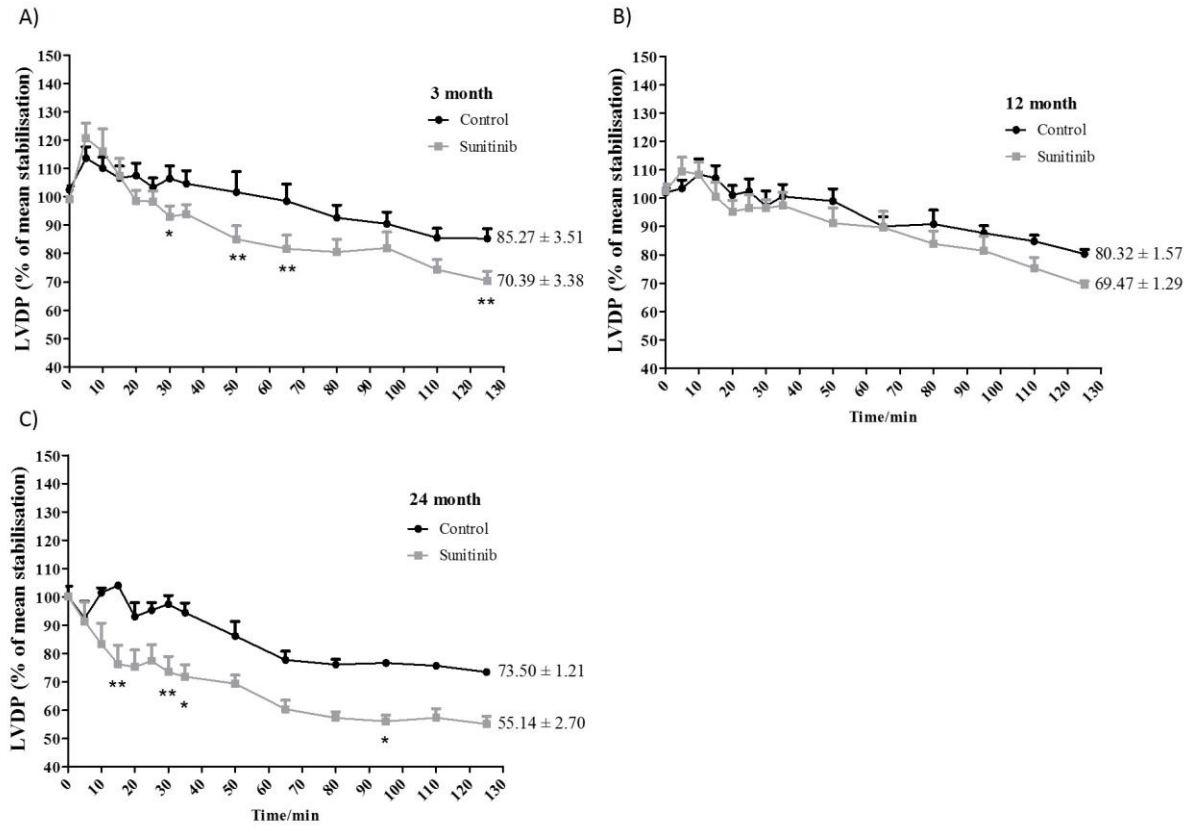

Figure 1: Representation of changes in LVDP measured during Langendorff experiments over time relative to the stabilisation period in Control and 1 µM Sunitinib treated hearts. A) 3 month (3m) (n=9 per group), B) 12 month (12m) (n=6 per group), and C) 24 month (24m) (n=3-5 per group). Data expressed as mean ± S.E.M. Statistics: Two-way repeated measures ANOVA test with the Bonferroni post hoc test comparing Control and Sunitinib treated hearts: * = $P<0.05$ and ** = $P<0.01$.

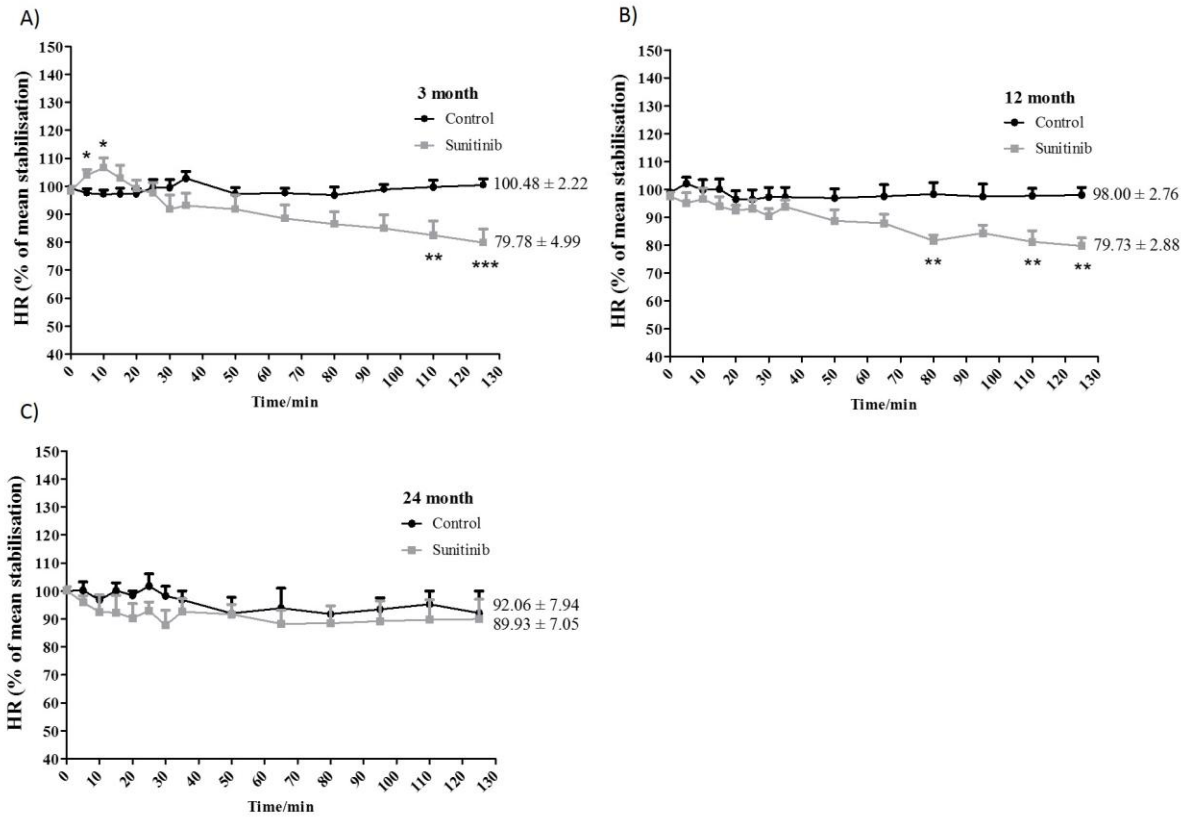

Figure 2: Representation of changes in HR measured during Langendorff experiments over time relative to the stabilisation period in Control and 1 µM Sunitinib treated hearts. A) 3 month (3m) (n=9 per group), B) 12 month (12m) (n=6 per group), and C) 24 month (24m) (n=3-5 per group). Data expressed as mean ± S.E.M. Statistics: Two-way repeated measures ANOVA test with the Bonferroni post hoc test comparing Control and Sunitinib treated hearts: ** = P<0.01 and *** = P<0.001.

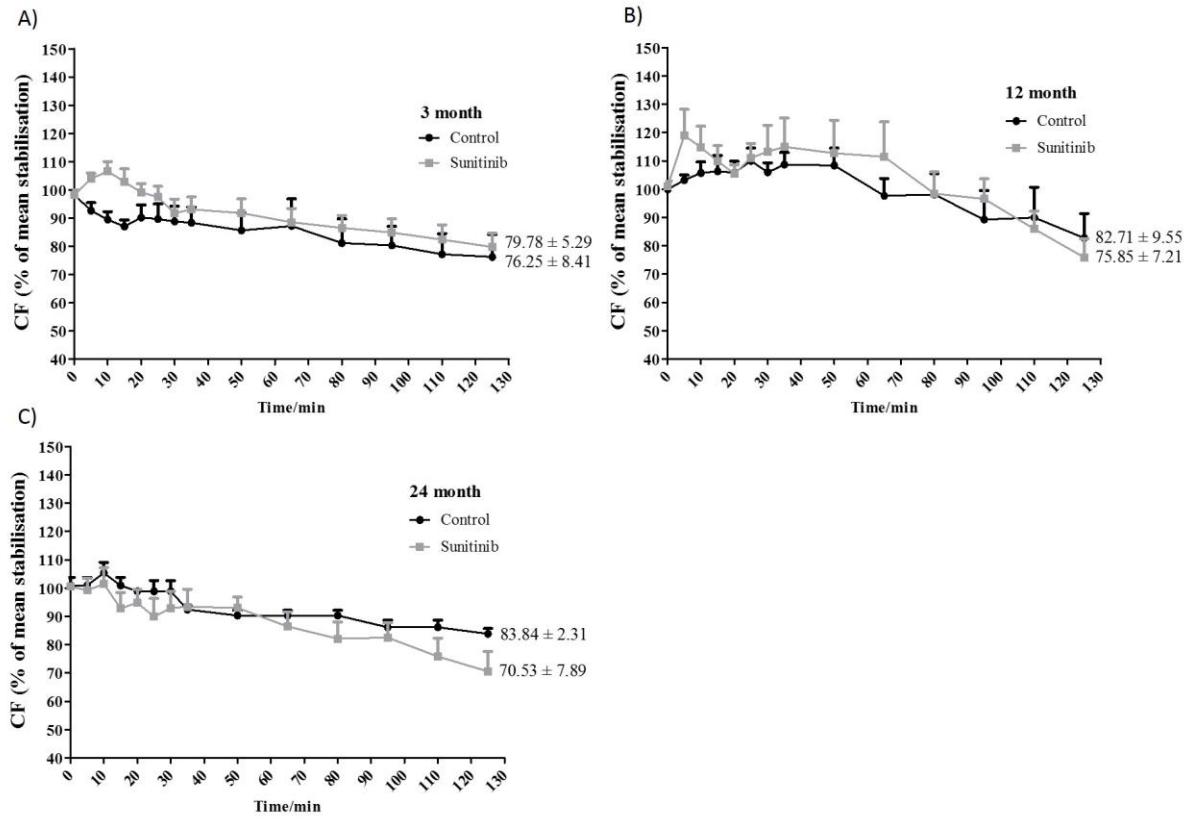

Figure 3: Representation of changes in CF measured during Langendorff experiments over time relative to the stabilisation period in Control and 1 µM Sunitinib treated hearts. A) 3 month (3m) (n=9 per group), B) 12 month (12m) (n=6 per group), and C) 24 month (24m) (n=3-5 per group). Data expressed as mean ± S.E.M.

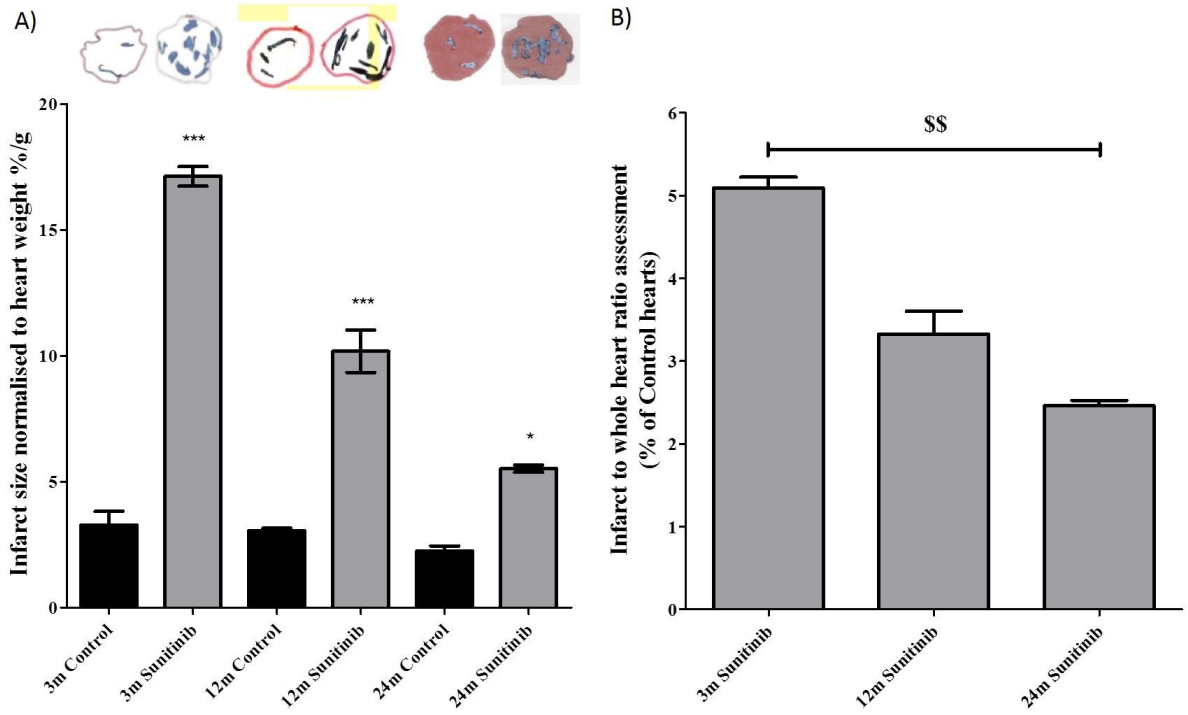

Figure 4: Infarct to whole heart ration assessment. Acetate sheet traces of infarct (blue/black) versus whole heart (red) shown above the graph. The hearts were drug perfused with 1 µM Sunitinib for 125 minutes in an isolated Langendorff heart model with the following groups: Control and 1 µM Sunitinib in 3 month (3m) (n=4-5 per group), 12 month (12m) (n=6 per group), and 24 month (24m) (n=3 per group). A) Infarct to whole heart ratio assessment for all three individual age groups for both Control and Sunitinib treated hearts, B) Infarct to whole heart ratio assessment as a percentage of Control hearts. Data expressed as mean ± S.E.M. Statistics: A) 2-tailed Student's t-test comparing Control and Sunitinib treated hearts (* = $P<0.05$ and *** = $P<0.001$). B) One-way ANOVA using LSD post hoc test (comparing 3 month versus 12 month, 3 month versus 24, and 12 month versus 24 month month): $$ = $P<0.01$.

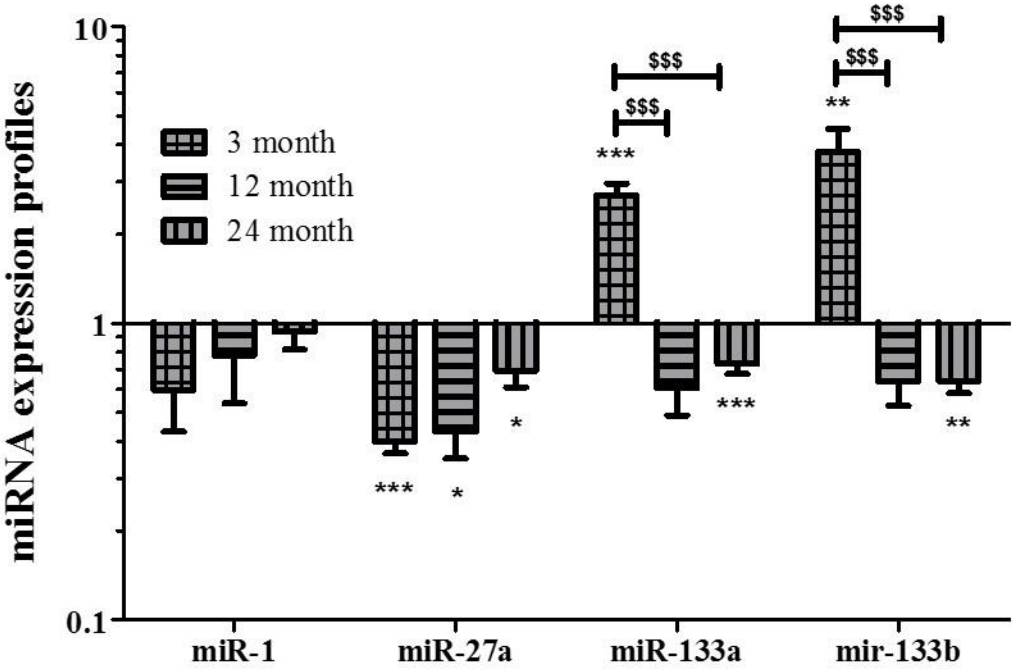

Figure 5: The effect of 1 µM Sunitinib on expression of cardiac damage specific microRNAs following 125 minute drug perfusion in an isolated Langendorff heart model. The qRT-PCR results are shown as a ratio of target microRNA normalised to U6 with Control group microRNA ratio set as 1 of microRNAs miR-1, miR-27a, miR-133a, and miR-133b presented on a log scale. Groups: 3 month (3m) (n=6 per group), 12 month (12m) (n=7 per group), 24 month (24m) (n=5 per group). Data expressed as mean ± S.E.M. Statistics: 2-tailed Student's t-test comparing Control and Sunitinib treated hearts in the specific age group (* = $P<0.05$, ** = $P<0.01$, and *** = $P<0.001$). One-way ANOVA using LSD post hoc test (comparing 3 month versus 12 month, 3 month versus 24, and 12 month versus 24 month month): $$$ = $P<0.001$.

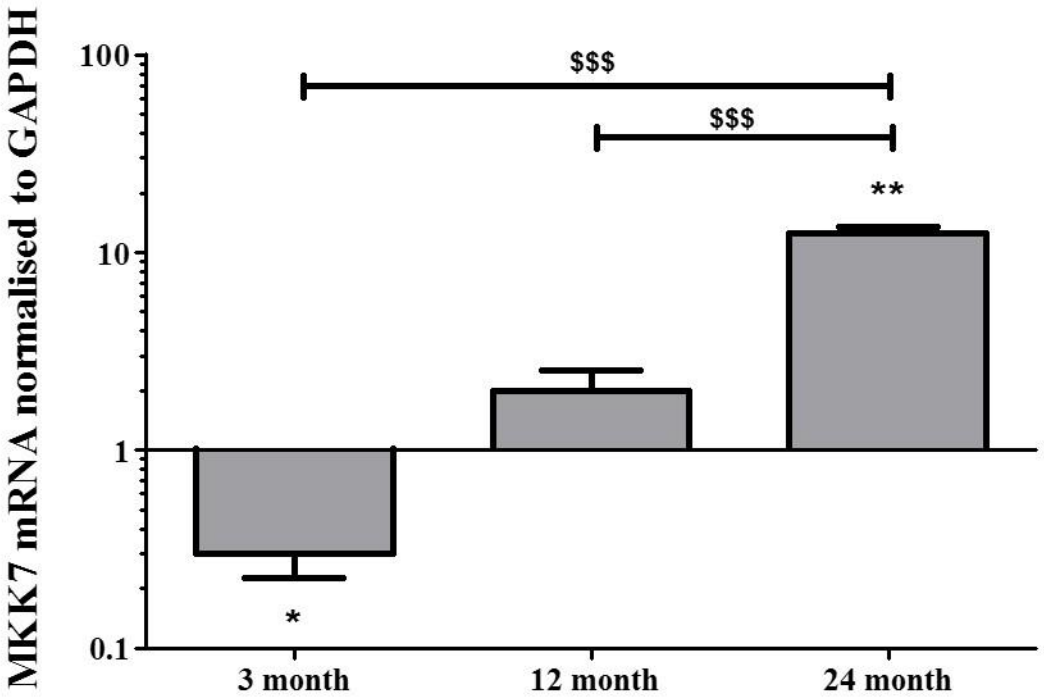

Figure 6: The qRT-PCR assessment of MKK7 mRNA expression levels in an isolated Langendorff heart model after 1 µM Sunitinib treatment. The qRT-PCR results are shown as a ratio of MKK& mRNA in Sunitinib treatment normalised to GAPDH mRNA with Control group ratio set as 1 presented on a log scale. Groups: 3 month (3m) (n=6 per group), 12 month (12m) (n=7 per group), 24 month (24m) (n=5 per group). Data expressed as mean ± S.E.M. Statistics: 2-tailed Student's t-test comparing Control and Sunitinib treated hearts in the specific age group (* = P<0.05 and ** = P<0.01). One-way ANOVA using LSD post hoc test (comparing 3 month versus 12 month, 3 month versus 24, and 12 month versus 24 month month): $$$ = P<0.001.

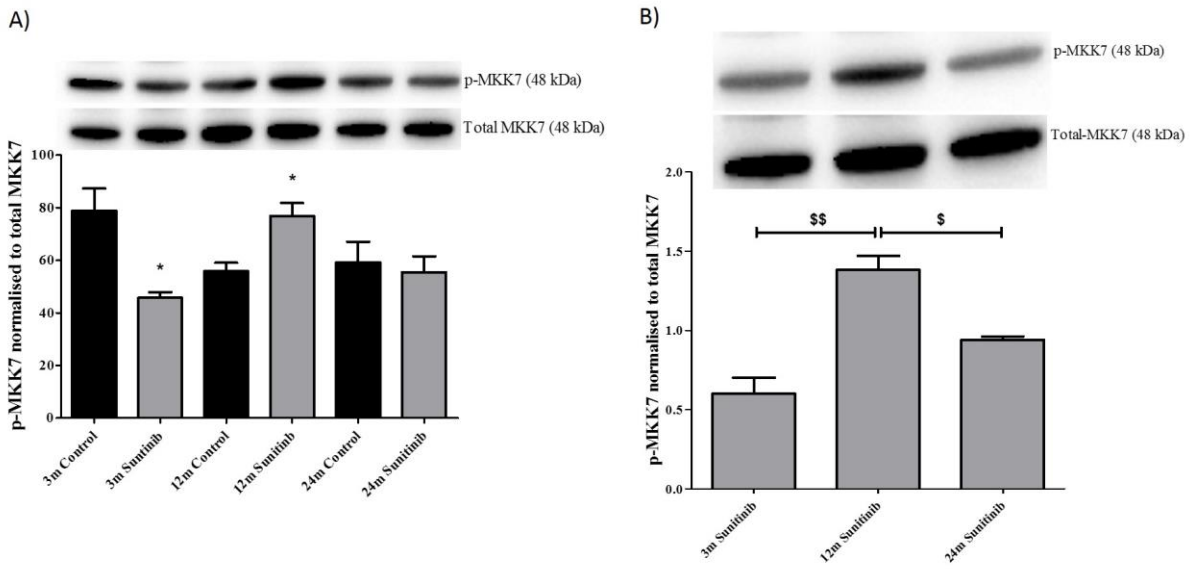

Figure 7: Western blot assessment of MKK7 phosphorylation levels of in an isolated Langendorff heart model after 125 minutes of 1 µM Sunitinib perfusion. Groups: 3 month (3m) (n=3 per group), 12 month (12m) (n=3 per group), 24 month (24m) (n=3 per group). A) p-MKK7 levels represented as a percentage of total-MKK7 found in the Control and Sunitinib treated heart tissue. B) The Sunitinib treatment groups normalised to the Control of the respective age groups to allow for comparison of p-MKK7levels within the three age groups. Data expressed as mean ± S.E.M. Statistics: A) 2-tailed Student's t-test comparing Control and Sunitinib treated hearts in the specific age group (* = $P<0.05$). B) One-way ANOVA using LSD post hoc test (comparing 3 month versus 12 month, 3 month versus 24, and 12 month versus 24 month month): $ = $P<0.05$ and $$ = $P<0.01$.

Tables for Supplementary data:

Table 1 - Raw data values of left ventricular developed pressure (LVDP) in mmHg obtained during 125 minutes of Langendorff perfused Control or Sunitinib (1 µM) hearts. Groups: 3 month (n=9), 12 month (n=6), and 24 month (Control: n=3; Sunitinib: n=5). Data expressed at mean ± S.E.M. Two-way ANOVA statistical analysis with Tukey post hoc test: 12 month and 24 month versus 3 month control: a ($p<0.05$) and aa ($p<0.01$); 24 month versus 12 month control: b ($p<0.05$); 12 month and 24 month versus 3 month Sunitinib: A ($p<0.05$); 24 month versus 12 month Sunitinib: B ($p<0.05$), BB ($p<0.01$), and BBB ($p<0.001$).

LVDP	**Control**			**Sunitinib**		
Time	3 month	12 month	24 month	3 month	12 month	24 month
0	112.15 ± 3.09	138.50 ± 8.22	118.77 ± 14.43	113.21 ± 3.13	143.82 ± 4.96^{A}	125.82 ± 12.92
5	123.77 ± 2.96	136.55 ± 5.66	109.83 ± 16.70	138.17 ± 8.01	142.95 ± 5.84	112.06 ± 5.28
10	119.93 ± 2.90	142.85 ± 16.26	119.97 ± 11.63	131.19 ± 7.22	141.67 ± 6.34	104.18 ± 13.19^{B}

15	116.01 ± 3.03	140.88 ± 14.27	122.55 ± 8.38	122.76 ± 8.44	140.18 ± 6.15	95.21 ± 11.56^{BB}
20	116.98 ± 3.46	144.30 ± 11.30	110.01 ± 12.90	112.67 ± 5.98	133.40 ± 5.98	94.77 ± 13.00^{BB}
25	112.87 ± 3.58	145.90 ± 14.50^{a}	112.78 ± 12.22	112.12 ± 4.99	134.52 ± 4.56	97.86 ± 13.85^{B}
30	115.84 ± 3.50	145.35 ± 14.71^{a}	115.38 ± 13.53	106.30 ± 5.56	135.07 ± 4.08^{A}	92.37 ± 11.87^{BB}
35	113.97 ± 4.49	142.13 ± 13.07	111.74 ± 12.62	106.92 ± 3.87	135.93 ± 5.02^{A}	90.16 ± 10.83^{BBB}
50	110.06 ± 6.35	142.68 ± 9.65^{a}	101.84 ± 12.23^{b}	96.97 ± 5.89	127.00 ± 5.34^{A}	86.77 ± 9.05^{BB}
65	107.63 ± 7.18	136.48 ± 10.89^{a}	92.16 ± 11.38^{a,b}	92.67 ± 4.65	124.89 ± 6.41^{A}	74.53 ± 4.21^{BBB}
80	101.16 ± 5.24	134.70 ± 14.11^{aa}	90.00 ± 9.41^{b}	91.29 ± 4.23	116.96 ± 4.93	71.43 ± 6.19^{BB}
95	98.19 ± 2.23	132.35 ± 8.40^{aa}	90.45 ± 7.44^{b}	92.84 ± 5.48	113.52 ± 6.04	69.83 ± 6.02^{BB}
110	93.20 ± 2.76	128.00 ± 9.44^{aa}	89.25 ± 7.40^{b}	84.55 ± 4.01	105.05 ± 3.70	72.47 ± 10.48^{B}
125	92.71 ± 2.31	123.65 ± 6.67^{a}	86.70 ± 7.46	80.16 ± 3.97	97.35 ± 2.86	69.44 ± 8.92

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Table 2 - Raw data values of heart rate (HR) in beats/minute obtained during 125 minutes of Langendorff perfused Control or Sunitinib (1 µM) hearts. Groups: 3 month (n=9), 12 month (n=6), and 24 month (Control: n=3; Sunitinib: n=5). Data expressed at mean ± S.E.M. Two-way ANOVA statistical analysis with Tukey post hoc test: 12 month and 24 month versus 3 month control: a ($p<0.05$) and aa ($p<0.01$); 24 month versus 12 month control: b ($p<0.05$) and bb ($p<0.01$); 12 month and 24 month versus 3 month Sunitinib: A ($p<0.05$); 24 month versus 12 month Sunitinib: BB ($p<0.01$).

HR	**Control**			**Sunitinib**		
Time	3 month	12 month	24 month	3 month	12 month	24 month
0	262.22 ± 6.80	262.00 ± 19.17	196.67 ± 10.80[a]	275.56 ± 7.93	242.00 ± 13.42	224.00 ± 19.56
5	257.78 ± 6.56	270.00 ± 19.04	196.67 ± 8.16[b]	271.11 ± 9.91	238.00 ± 13.87	220.00 ± 23.18
10	256.67 ± 7.29	262.00 ± 22.75	190.00 ± 7.07[a,b]	262.22 ± 8.06	240.00 ± 13.23	222.00 ± 24.60
15	256.67 ± 6.85	262.00 ± 19.81	196.67 ± 10.80[a]	255.56 ± 7.31	234.00 ± 9.08	220.00 ± 21.51
20	256.67 ± 6.85	252.00 ± 18.17	193.33 ± 8.16[a]	250.00 ± 7.50	228.00 ± 8.94	214.00 ± 23.87
25	262.22 ± 7.86	252.00 ± 20.43	200.00 ± 14.14[a]	256.67 ± 9.35	234.00 ± 5.70	212.00 ± 20.74

30	262.22 ± 8.06	254.00 ± 23.08	193.33 ± 14.72[a]	248.89 ± 7.59	226.00 ± 4.47	208.00 ± 21.62
35	271.11 ± 7.80	254.00 ± 23.08	190.00 ± 7.07[aa]	255.56 ± 7.31	234.00 ± 8.37	214.00 ± 19.56
50	256.67 ± 7.71	252.00 ± 18.17	180.00 ± 7.07[aa,b]	250.00 ± 7.91	218.00 ± 5.48	216.00 ± 21.10
65	257.78 ± 8.62	256.00 ± 23.61	183.33 ± 8.16[aa,b]	254.44 ± 8.86	218.00 ± 5.48	214.00 ± 17.89
80	255.56 ± 9.86	256.00 ± 20.80	180.00 ± 7.07[aa,b]	250.00 ± 6.61	200.00 ± 6.12[A]	212.00 ± 17.82[A]
95	261.11 ± 7.99	256.00 ± 25.88	183.33 ± 10.80[aa,b]	248.89 ± 7.80	208.00 ± 8.22	214.00 ± 14.83
110	263.33 ± 9.68	258.00 ± 21.62	186.67 ± 8.16[aa,b]	252.22 ± 7.45	200.00 ± 6.12	216.00 ± 11.51
125	265.56 ± 9.20	258.00 ± 23.02	180.00 ± 14.14[aa,bb]	245.56 ± 6.15	194.00 ± 4.47[A]	216.00 ± 17.89[BB]

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Table 3 - Raw data values of coronary flow (CF) in ml/minute/gram heart weight obtained during 125 minutes of Langendorff perfused Control or Sunitinib (1 µM) hearts. Groups: 3 month (n=9), 12 month (n=6), and 24 month (Control: n=3; Sunitinib: n=5). Data expressed at mean ± S.E.M. Two-way ANOVA statistical analysis with Tukey post hoc test: 12 month and 24 month versus 3 month control: a ($p<0.05$); 24 month versus 12 month control: b ($p<0.05$); 12 month and 24 month versus 3 month Sunitinib: A ($p<0.05$) and AA ($p<0.01$); 24 month versus 12 month Sunitinib: B ($p<0.05$).

CF	**Control**			**Sunitinib**		
Time	3 month	12 month	24 month	3 month	12 month	24 month
0	7.29 ± 0.42	6.94 ± 0.22	3.81 ± 0.17^{a}	6.10 ± 0.80	7.44 ± 0.91	5.57 ± 0.90
5	7.68 ± 0.38	7.17 ± 0.28	3.81 ± 0.17^{a}	6.18 ± 0.82	8.58 ± 0.79	5.47 ± 0.83
10	7.93 ± 0.59	7.36 ± 0.42	3.98 ± 0.14^{a}	5.91 ± 0.87	8.32 ± 0.82	5.48 ± 0.57
15	7.67 ± 0.65	7.40 ± 0.56	3.81 ± 0.12^{a}	5.75 ± 0.75	7.96 ± 0.67	5.09 ± 0.74
20	7.36 ± 0.50	7.35 ± 0.39	3.73 ± 0.08^{a}	5.85 ± 0.83	7.70 ± 0.76	5.24 ± 0.88
25	7.21 ± 0.47	7.65 ± 0.45	3.73 ± 0.14^{a,b}	5.87 ± 0.88	8.05 ± 0.71	5.02 ± 1.04
30	6.79 ± 0.52	7.37 ± 0.38	3.73 ± 0.14	5.58 ± 0.85	8.12 ± 0.70	5.16 ± 0.94
35	6.91 ± 0.53	7.55 ± 0.42	3.49 ± 0.08^{b}	5.45 ± 0.90	8.24 ± 0.73^{A}	5.10 ± 0.75^{AA}
50	6.81 ± 0.56	7.55 ± 0.59	3.41 ± 0.05^{b}	4.84 ± 0.93	8.04 ± 0.78^{A}	5.08 ± 0.68^{AA}
65	6.59 ± 0.58	6.82 ± 0.61	3.41 ± 0.05	4.51 ± 0.85	7.93 ± 0.79^{AA}	4.68 ± 0.58^{AA,B}
80	6.43 ± 0.51	6.85 ± 0.70	3.41 ± 0.05	4.35 ± 0.85	7.07 ± 0.62	4.54 ± 0.82
95	6.31 ± 0.57	6.24 ± 0.89	3.25 ± 0.09	4.06 ± 0.62	6.92 ± 0.51^{A}	4.54 ± 0.74

110	6.12 ± 0.57	6.29 ± 0.93	3.25 ± 0.09	3.89 ± 0.61	6.19 ± 0.55	4.22 ± 0.84
125	5.93 ± 0.56	5.78 ± 0.76	3.17 ± 0.06	3.89 ± 0.62	5.55 ± 0.72	3.87 ± 0.69

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