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Biomechanical characterization of aortic valve tissue in humans and common animal models

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Abstract

Aortic valve disease develops in an escalating fashion in elderly patients. Current treatments including total valve replacement and valve repair techniques are still suboptimal. A thorough understanding of the animal and human valve tissue properties, particularly their differences, is crucial for the establishment of preclinical animal models and strategies for evaluating new valve treatment techniques, such as transcatheter valve intervention and tissue engineered valves. The goal of this study was to characterize and compare the biomechanical properties and histological structure of healthy ovine, porcine, and human aortic valve leaflets. The biaxial mechanical properties of the aortic valve leaflets of 10 ovine (~1 year), 10 porcine (6–9 months), and 10 aged human (80.6 ± 8.34) hearts were quantified. Tissue microstructure was analyzed via histological techniques. Aged human aortic valve leaflets were significantly less compliant than both ovine and porcine leaflets, with the ovine leaflets being the most compliant. Histological analysis revealed structural differences between the species: the human and porcine leaflets contained more collagen and elastin than the ovine leaflets. Significant mechanical and structural differences in the aortic valve tissues of 6- to 9-month-old porcine models and 1-year-old ovine models with respect to those of aged humans, suggest that these animal models may not be representative of the typical patient undergoing aortic valve replacement.

Keywords

aortic valve; mechanical properties; animal trials; transcatheter aortic valve

INTRODUCTION

The aortic valve is responsible for maintaining unidirectional flow of oxygenated blood from the heart to the rest of the body; however proper function may be compromised by valvular diseases such as aortic stenosis. Aortic valve disease progresses in elderly patients such that 48% of aortic valves are sclerotic by the age of 85 years old.^{1,2} The current preferred treatment for stenosis is complete aortic valve replacement with either a mechanical or bioprosthetic valve, yet there remains no perfect replacement device. Although mechanical heart valves have demonstrated superior durability, they require a regular intake of anticoagulants. Bioprosthetic valves, on the contrary, display superior blood compatibility and hemodynamics but are plagued with limited durability. More recently, transcatheter aortic valves (TAVs) have garnered interest as a minimally invasive treatment option. TAVs are comprised of similar materials to traditional surgical bioprosthetic valves and are delivered to the site of the diseased valve via catheter. Much

research is also being conducted to develop tissue engineered valves, which utilize biomaterials such as decellularized xenograft or allograft valves as scaffolds.^{3–6}

However, due to the limited availability of human valve tissues, animal models including pigs, sheep, calves, and dogs have played an important role in the preclinical evaluation of new valve treatment techniques. A review by Gallegos et al.⁷ indicated that the ovine (78%) and porcine (9.5%) models are most commonly used. The porcine model was initially more commonly used than the ovine model to evaluate heart valve performance due to concerns that the ovine model could not accurately predict valve-related thrombogenic complications in humans.^{7,8} Studies suggest that the ovine platelet activity is significantly lower than in humans, whereas porcine platelet activity is similar to humans.^{7,8} Therefore, the porcine model may be useful for blood compatibility studies. However, long-term evaluation of replacement valves has not been successful in porcine models due to a high incidence of hemorrhagic complications, and perivalvular leak resulting from normal somatic growth.^{7,8} For these reasons, the ovine model has become the preferred model for the evaluation of valve performance, especially for long-term durability studies due to its similar annulus size, equivalent heart rate, cardiac output, and rate of somatic growth to humans.^{7,8} Yet, little has been done to quantify the mechanical properties of ovine tissue. Instead, many groups have studied the mechanical properties of porcine aortic valve tissues under the assumption that they have similar mechanical properties as human tissues.^{9–12}

The goal of this study was to elicit and quantify the potential differences in the material properties of ovine, porcine, and human aortic valve leaflets by means of mechanical testing and structural analysis. A thorough understanding of the animal and human valve tissue properties, particularly their differences, is crucial for the establishment of preclinical animal models and strategies for evaluating new valve treatment techniques, such as transcatheter valve intervention and tissue engineered valves.

METHODS

Fresh frozen human cadaver hearts ($n = 10$) age 80.6 ± 8.34 years and weight 478.5 ± 135.9 g were obtained from National Disease Research Interchange (Philadelphia, PA). The human specimen characteristics are presented in Table I. The use of human tissues was approved by the Research Compliance Office of the University of Connecticut for this study. Fresh porcine hearts age 6–9 months and weight 431.0 ± 71.0 g were obtained from Animal Technologies Inc. (Tyler, TX), and fresh ovine hearts age ~1 year and weight 317.9 ± 78.9 g were obtained from a local abattoir, Brothers Quality Inc. (Stafford Springs, CT) ($n = 10$ for each species). The three aortic valve (AV) leaflets—the left coronary leaflet (LCL), the right coronary leaflet (RCL), and the noncoronary leaflet (NCL)—were excised from each heart and stored in cryopreservation solution (10% dimethyl sulfoxide) at -80°C until biaxial mechanical testing could be performed. Cryopreservation has been shown to retain the native tissue collagen and elastin structural integrity and thus the mechanical properties.¹³ Prior to testing, the leaflets were thawed via the published technique.¹⁴ The leaflets were then prepped for biaxial mechanical testing while taking care to align the edges of each specimen along the circumferential and radial directions of the aortic valve to determine the differences in the mechanical properties with respect to the anatomical orientation. The thickness of each sample was measured in five places along the leaflet edge and belly region with a Mitutoyo rotating thickness gauge (Model 7301) with an accuracy of ± 0.01 mm. Four graphite markers delimiting a square approximately $3 \text{ mm} \times 3 \text{ mm}$ in size were glued to the ventricular surface in the lower belly region for optical strain measurements. The lower belly region has been shown to have the most uniform stress and strain field, and the ventricular layer is the primary contributor to the planar orthogonal mechanical response of the leaflet during closure of the valve.²

Planar biaxial mechanical testing

Biaxial testing was carried out according to the methods presented in Sacks and Sun¹⁵ with several modifications: (1) the leaflets were not trimmed to a square shape (i.e., the semilunar shape was preserved throughout biaxial testing) due to the small size of the native leaflet and in an effort to preserve the natural fiber structure and orientation throughout testing, (2) the membrane tension rather than the three-dimensional stress was measured, which is common practice for the biaxial testing of valve leaflets,^{10–12,16} (3) the area tested was not directly in the center of the leaflet, rather a little below the center further away from the nodulus of Arantii because this region has a more homogeneous fiber structure,¹⁰ and (4) a preconditioning protocol of up to 80 cycles was adopted to achieve a nearly stable mechanical response for the leaflet tissues.

Briefly, tissue samples were submerged in aqueous 0.9% NaCl solution maintained at a temperature of 37°C by a circulating thermoregulation pump (Fisher Scientific model: 8001). Tension-controlled test protocols were utilized whereas the ratio of the membrane tension components $T_{11}:T_{22}$ was kept constant with $T_{12} = T_{21} = 0$, where T_{ij} is defined as the axial force per unit length over which the force is applied. Extensive preconditioning of up to 80 cycles, with a rest period of approximately 1 min between each set of 10 consecutive cycles, was performed to reduce tissue hysteresis and achieve a stable tissue response. Each sample was tested at approximately 80 N/m. At the maximum load, seven consecutive tension protocols were conducted at the following ratios: $T_{11}:T_{22} = 0.75:1, 0.5:1, 0.3:1, 1:1, 1:0.75, 1:0.5$, and $1:0.3$. Tissue samples were assumed to be incompressible and planar, and biaxial testing data were analyzed using the post-preconditioning state as the reference state. The three leaflets (LCL, RCL, and NCL) from each of the 10 hearts dissected for each species were tested, thus a total of 90 specimens were tested.

Constitutive modeling

The aortic leaflets were all assumed to be anisotropic, nonlinear hyperelastic materials. Therefore, the membrane tension (T), analogous to the Second Piola-Kirchhoff stress, can be computed by Eq. (1), where E represents the Green-Lagrangian strain tensor, W is a strain energy function, and H is the initial membrane thickness.

$$T = H \frac{\partial W}{\partial E} \quad (1)$$

The membrane tension was utilized to quantify the AV biomechanics rather than the Second Piola Kirchhoff stress to avoid having to use ambiguous thickness measurements in the subsequent calculations.^{10–12,16} In this study, experimental tension-strain data from human, porcine, and ovine aortic valve leaflets was fitted with the generalized Fung-type strain energy function for the planar biaxial response of soft biological tissues given by the following equations¹⁷:

$$W = \frac{c}{2} (e^Q - 1) \quad (2)$$

$$Q = A_1 E_{11}^2 + A_2 E_{22}^2 + 2A_3 E_{11} E_{22} + A_4 E_{12}^2 + 2A_5 E_{11} E_{12} + 2A_6 E_{22} E_{12} \quad (3)$$

where c and A_i are material parameters. The experimental data from stress-controlled protocols was fitted simultaneously to reduce the effect of multiple colinearities, and material parameters were obtained using the Marquardt-Levenberg nonlinear regression algorithm with MYSTAT 12 software (Systat Software, Inc. Chicago, Illinois).

Histological analysis

The fibrous structure of human, porcine, and ovine aortic valve leaflets was examined in both the circumferential and radial directions via histological analysis. Tissue specimens were cryopreserved after biaxial tests. After thawing, tissues were fixed in formalin for 24 hours. The fixed tissue specimens were then dehydrated through a process of varied alcohol concentrations, embedded in paraffin, and serially sectioned at 5 μm through the thickness. Tissue sections were mounted on microscope slides and dried. After deparaffinization, the slides were stained with Verhoeff Van Gieson to identify the fibrous components of interest: collagen and elastin. Digital images of each section were obtained utilizing an Olympus U-TVO.5xC digital camera coupled with an Olympus BX40 light microscope. The relative content of elastin, collagen, and cell nuclei in each specimen was assessed qualitatively.

Data analysis

The peak Green strain was quantified and compared among tissues at a membrane tension of 60 N/m.^{10,18–21} Paired Student's *t*-tests were utilized to compare the biaxial mechanical properties for each tissue type (LCL, RCL, and NCL) among each species sample set (human, porcine, and ovine). Independent samples Student's *t*-tests were used to compare the compliance for each tissue type between species. Nonparametric tests including Wilcoxon signed rank tests and Mann-Whitney rank sum tests, were used in place of paired Students' *t*-tests and independent samples Students' *t*-tests respectively in cases where the data sets were not approximately normally distributed. For all statistical considerations, a *p*-value <0.05 was deemed to indicate a significant difference between the sample means.

RESULTS

Anatomic differences were analyzed among the AV leaflet specimens of each type. In general, the ovine AV leaflets appeared to be the thinnest, whereas the human leaflets were the thickest. The LCL and NCL were thicker in human AVs compared to ovine with *p*-values of 0.04 and 0.009, respectively. The porcine NCL was also thicker than the ovine NCL with a *p*-value of 0.031, but thinner than the human NCL with a *p*-value of 0.038. No additional significant differences were found between the mean thicknesses. However, when comparing only the thickness of the testing region in the lower belly of each leaflet, significant differences were seen among each of the species: the ovine leaflets were significantly thinner than both the porcine and human leaflets with *p*-values of 0.014 and <0.001, respectively, and the porcine leaflets were also thinner than the human leaflets with a *p*-value <0.001 (Fig. 1). One factor contributing to the greater thickness of the human leaflets were the crystalline calcium deposits found along the edges of some samples, and in rare cases, throughout the testing area of the specimen (Fig. 2). Calcium deposition may indicate the onset of sclerosis. None of the porcine or ovine specimens exhibited any calcification.

Biaxial mechanical response

Representative experimental data generated from the biaxial mechanical testing of human, porcine, and ovine AV leaflets are presented in Figure 3 to illustrate the loading portion of the equibiaxial nonlinear tension-strain behavior in the circumferential and radial directions of each corresponding specimen. In general, all of the tested tissues exhibited anisotropic behavior, being much less extensible in the circumferential direction. In several cases the circumferential tension-strain response of the human leaflets under equibiaxial loading was negative indicating a high degree of tissue anisotropy. The human tissues were stiffer than the corresponding porcine and ovine tissues with essentially no low toe-region. Porcine samples exhibited a similar strain range to human samples, but had a more pronounced

transition zone from the low toe region to the deeper tension-strain curve. The ovine leaflets were the most extensible of the three species and exhibited an even more pronounced transition between the toe and high stiffness regions. The complete biaxial data set is presented in Figure 4.

To further illustrate the differences in AV mechanical properties between the species, the maximum strain at a membrane tension of 60 N/m was compared for each species (Fig. 5). The strain for each leaflet was significantly larger in the radial direction than in the circumferential direction for the ovine model ($p = 0.002$ for each leaflet), the porcine model ($p = 0.002$ for each leaflet), and for human patients with p -values of 0.002, 0.006, and 0.002 for the left-, right-, and noncoronary leaflets respectively, as one would expect. There was no statistically significant difference in compliance between the left, right and noncoronary leaflets within any species. Overall, the human AV leaflets achieved the lowest strains, whereas the ovine AV leaflets were the most compliant.

Constitutive modeling

The Fung strain-energy function was able to capture the biaxial mechanical properties of the ovine, porcine, and human aortic tissues with reasonable accuracy (Fig. 6). The mean material parameters obtained from fitting the experimental data to the model are listed in Table II.

Histology

Histological analysis revealed structural differences in the aortic valve tissue between the species (Fig. 7). There was a noticeably greater amount of collagen in the porcine and human AV leaflets than in the ovine leaflets. In the ovine leaflets the spongiosa layer appeared to be the thickest of the three layers, while in the porcine and human leaflets the fibrosa was much thicker than both the spongiosa and ventricularis. It was difficult to discern the spongiosa in many of the human leaflets [Fig. 7(b,c)]. Furthermore, the collagen fibers in the human leaflet were highly aligned along the circumferential direction [Fig. 7(c)]. It is also note-worthy that although there were cell nuclei present in the ovine and porcine leaflets, little to no cell nuclei were observed in the human leaflets.

DISCUSSION

The leaflets from the three different species displayed different properties and even appeared visually different. The human leaflets were significantly thicker within the biaxial testing region, than both the porcine and ovine leaflets. The most obvious difference between the mechanical properties of the human leaflets compared to the porcine and ovine leaflets was a marked decrease in the circumferential extensibility. The human leaflets essentially did not stretch at all within the experimental loading range. This phenomenon suggests highly aligned and straightened collagen fibers in the circumferential direction, which was confirmed by histological analysis.

There were also clear AV structural differences between the species. In the ovine and porcine leaflets, the three leaflet layers: the ventricularis, the spongiosa, and the fibrosa, were clearly identifiable, whereas in the human leaflets it was difficult to discern the spongiosa layer. While all the leaflets appeared to primarily consist of collagen, the ovine AV leaflets contained a relatively small amount of elastin compared to the porcine and human, and both the ovine and porcine leaflets contained a large number of cell nuclei, in contrast to the human AV leaflets which contained little to no nuclei. It is important to note that the elastin fibers in the human leaflets appeared fragmented [Fig. 7(f)]; therefore,

although elastin was present, collagen was the main load-bearing constituent, which may explain the high stiffness of the tissue.

Many of the differences seen between the species with respect to both the tissue structure and mechanics may be effects of aging. Stephens et al. show an increase in leaflet thickness in the porcine AV leaflet from an age of 6 weeks to 6 months old,¹² while McDonald et al. show a significant increase in leaflet area and thickness in the aging human AV.²² Therefore, the increased thickness in the human leaflets may be an effect of the advanced age (80.6 ± 8.3 years) of the patients in this study. Leaflet tissue stiffness has been shown to increase with age as well.² Reduction of collagen crimp magnitude consistent with aging may also explain the significantly smaller circumferential strains in human valve leaflets compared to porcine and ovine leaflets. It is suggested that the number of crimps in leaflet collagen fibers remain constant with growth; however with increasing age, the crimp amplitude decreases and the period increases, resulting in longer length but reduced extensibility.¹⁶

An increase of collagen content as well as thickening of the fibrosa and collagen marbling with the GAGs throughout the spongiosa has been observed in the aging porcine valve,¹² while an increase of GAGs and elastin content with a decreased amount of collagen has been reported for aged human valves.²² It has been suggested that there is a high proportion of GAGs in the valve tissue of children under 10 years old, and this quantity decreases between the ages of 30 and 50 years, before increasing again beyond the age of 50,²² which may indicate valvular degeneration. McDonald et al. report a striking increase in the percentage of elastin present in the aging human valve as well, particularly in individuals at or above the age of 50 years.²² They suggest that the increase in elastin occurs in response to a decrease in collagen content,²² which may explain the thick layer of elastin observed in the human leaflets in this study [Fig. 7(f)].

Another factor to consider is the calcification present on some of the human AV leaflets. The calcification was found primarily along the commissures of the leaflets which are subjected to significant flexural deformation,²³ and often also in the center of the leaflet. Calcium deposition will increase tissue stiffness and the bending stresses experienced by the leaflets, which further accelerates tissue degeneration.²⁴

Upon first glance, the biaxial response of the porcine leaflets tested in this study appears different from that measured in previous studies,^{10,11} with smaller strains especially in the radial direction. This may be explained by two factors: the marker reference state and the sample preparation. The strain data in this study was calculated by referencing the four tissue marker positions after rigorous preconditioning. Even after 20 cycles of preconditioning, the tissue exhibited significant residual strains upon unloading (Fig. 8); therefore, the tissue response was evaluated after 40–80 cycles of preconditioning. Carew et al.²⁵ demonstrated that the most reproducible mechanical testing data of the porcine aortic valve leaflet can be obtained by 50 cycles of preconditioning with a 24-hour recovery period between the first and second sets of 25 consecutive cycles. In this study, 1 min was assumed to be an adequate rest period between each set of 10 consecutive preconditioning cycles. Moreover, we chose to reference the post preconditioning state because it may be more physiologically relevant than the previously used free float conditions.^{10,11} The smaller radial strains observed in this study are also likely due in part from the fact that in previous studies, the leaflet edges were trimmed which allows for rotation of the circumferentially-aligned collagen fibers and greater radial stretch, whereas in this study the entire intact leaflet was tested in order to preserve the native fiber structure. To facilitate the comparison of these results with those presented in the literature, the tension-strain behavior of a porcine AV leaflet was analyzed throughout preconditioning (Fig. 8). The equibiaxial response of

the porcine AV leaflet referenced to the free-float state is similar to that presented by Billiar and Sacks.¹⁰ Throughout preconditioning, the tension-strain response begins to stabilize after 50 cycles of consecutive loading and is repeatable after 80 cycles. The toe-region of the tension-strain curve is essentially lost. For the sample featured in Figure 8, we chose to analyze the 90th cycle as the tissue response was equivalent to that of the 80th cycle. The degree of anisotropy for porcine leaflets was calculated using the method of Billiar and Sacks.¹⁰ The degree of anisotropy in our study was 0.254 ± 0.029 compared to 0.247 ± 0.023 in Billiar and Sacks' study, both presented as a mean $\pm$ standard error. Thus, our results are consistent with Billiar and Sacks' results when analyzed using the free-float state.

Overall, the significant differences between ovine, porcine, and human AV tissue properties presented here suggest that the ovine and porcine models may not be suitable to assess AV mechanics in the aged human. However, it is unclear whether these differences between the ovine, porcine, and human AV leaflets are representative of differences between the species, or whether they are primarily due to aging effects. Stephens and Grande-Allen suggest that the collagen matrix organization and turnover in a 6-month-old pig is comparable to that of a 17-year-old female and a 19-year-old male human, whereas a 6-year-old pig corresponds to an aged human.¹² Therefore, it is possible that the structural and mechanical properties of a 6-year-old pig are more similar to those of an aged human, although this has not been assessed. Likewise, it is possible that an older ovine model may be a more appropriate model of the aged human for AV studies.

Study limitations

The sample size for each species and tissue type was relatively small: a larger sample size would provide more conclusive results. Possible regional property differences within each leaflet selected for study were not considered in order to simplify the experimental procedure. Finally, considering the effects that age and disease state have on the mechanical properties of aortic tissues, the results presented here can only offer insight in the comparison between aortic tissues from 1 year old ovine models, 6- to 9-month-old porcine models, and elderly humans.

CONCLUSIONS

With the advent of new valve treatment techniques, such as transcatheter delivered and tissue engineered heart valves, there is an increasingly dire need to establish appropriate preclinical models for the evaluation of novel valve design and development strategies. The present study indicated that the 1-year-old ovine model and the 6- 9-month-old porcine model may not be representative of the typical aged human patient. The aged human aortic valve has stiffer mechanical properties and different histological structure than these two models. The future use of these animal models should be considered with caution.

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FIGURE 1.

AV leaflet thickness within the biaxial testing region compared between ovine ($n = 30$), porcine ($n = 30$), and human ($n = 30$).

FIGURE 2.

(a) A human aortic valve leaflet with calcification deposits (light yellow) covering the fibrosa surface, as well as an (b) ovine and (c) porcine leaflet without calcification. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 3. Representative equibiaxial data for one (a) ovine, (b) porcine, and (c) human aortic valve test specimen.

FIGURE 4.

Complete equibiaxial test data presented as a mean $\pm$ standard error for clarity, with $n = 10$ for each species and leaflet type. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Green strain presented as a mean $\pm$ standard deviation at a membrane tension of 60 N/m in the (a) circumferential and (b) radial directions with all statistically significant differences indicated by the corresponding p -value, with $n = 10$ for each species and leaflet type.

FIGURE 6.

Representative biaxial data (open circles) for one (a&b) ovine, (c&d) porcine, and (e&f) human AV leaflet with the Fung fitted tissue response (solid red line). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 7.

Five-micrometer AV leaflet sections stained with Verhoeff Van Giesson stain rendering collagen pink-red, and elastin and cell nuclei purple-black. Sections made through the leaflet thickness in the circumferential direction are given for the (a) ovine, (b) porcine, and (c) human AV leaflet. Sections made in the radial direction are also given for the (d) ovine, (e) porcine, and (f) human AV leaflet. Each image is oriented so that the ventricular layer is on the left side and the fibrosa layer is on the right side of the image. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 8.

(a) Tension-strain behavior of a porcine AV leaflet throughout preconditioning referenced to the pre-preconditioning, zero load state, and (b) the 90th loading cycle tension-strain behavior referenced to the post preconditioning state. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Human Specimen Characteristics

TABLE I

Specimen	Age	Gender	Heart Weight (g)	Cause of Death	Heart-Related Disease(s)
1	79	F	545	RF	CHF
2	81	F	330	unk	CVA
3	96	F	545	CPA	N/A
4	82	M	714	COPD	Hypertension, CHF, coronary stents
5	75	M	425	CA	None
6	63	M	369	unk	HTN
7	87	F	295	unk	None(PKD)
8	80	F	397	unk	None
9	82	M	544	AP	None
10	81	F	621	Alz	None

Alz, Alzheimer's; AP, Aspiration Pneumonia; CA, cardiac arrest; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disorder; CPA, Chronic pulmonary aspergillosis; CVA, cerebrovascular accident; HTN, hypertension; RA, respiratory arrest; RF, respiratory failure; PKD, Polycystic kidney disease; unk, unknown.

TABLE II

Fung Model Coefficients for (a) Ovine, (b) Porcine, and (c) Human Aortic Valve Leaflets Presented as a Mean $\pm$ Standard Deviation

Parameter	LCL	RCL	NCL
a.			
C	4.10 $\pm$ 3.98	4.16 $\pm$ 2.95	2.71 $\pm$ 2.21
A1	58.26 $\pm$ 16.60	75.62 $\pm$ 39.23	59.22 $\pm$ 32.47
A2	40.30 $\pm$ 23.91	31.08 $\pm$ 11.68	35.17 $\pm$ 25.61
A3	25.40 $\pm$ 15.18	22.98 $\pm$ 10.33	25.31 $\pm$ 17.42
A4	17.14 $\pm$ 7.23	37.00 $\pm$ 48.09	23.29 $\pm$ 21.05
A5	0.93 $\pm$ 4.94	-3.31 $\pm$ 16.30	-1.22 $\pm$ 5.44
A6	1.72 $\pm$ 5.02	1.00 $\pm$ 2.68	-2.22 $\pm$ 5.91
R ²	0.93 $\pm$ 0.04	0.92 $\pm$ 0.02	0.91 $\pm$ 0.07
b.			
C	4.10 $\pm$ 3.10	4.51 $\pm$ 1.99	8.42 $\pm$ 8.04
A1	92.98 $\pm$ 84.60	81.51 $\pm$ 27.02	58.39 $\pm$ 25.71
A2	62.58 $\pm$ 62.11	41.34 $\pm$ 22.29	49.73 $\pm$ 41.84
A3	24.90 $\pm$ 15.91	25.22 $\pm$ 17.57	22.98 $\pm$ 14.30
A4	16.00 $\pm$ 12.43	18.84 $\pm$ 11.25	18.86 $\pm$ 17.08
A5	-1.40 $\pm$ 2.99	-0.65 $\pm$ 5.75	2.52 $\pm$ 5.42
A6	-0.91 $\pm$ 3.07	-1.80 $\pm$ 2.03	0.63 $\pm$ 4.57
R ²	0.91 $\pm$ 0.08	0.91 $\pm$ 0.05	0.92 $\pm$ 0.04
c.			
C	5.42 $\pm$ 3.70	6.79 $\pm$ 8.51	4.26 $\pm$ 2.66
A1	198.23 $\pm$ 209.51	140.68 $\pm$ 88.50	192.86 $\pm$ 156.26
A2	101.02 $\pm$ 104.40	96.59 $\pm$ 90.16	175.64 $\pm$ 195.47
A3	81.18 $\pm$ 65.08	55.71 $\pm$ 27.80	107.77 $\pm$ 123.99
A4	47.48 $\pm$ 59.65	47.78 $\pm$ 40.71	163.31 $\pm$ 256.98
A5	1.25 $\pm$ 54.22	-3.28 $\pm$ 37.05	81.51 $\pm$ 207.54
A6	-10.40 $\pm$ 42.74	-1.32 $\pm$ 16.78	47.65 $\pm$ 90.28
R ²	0.88 $\pm$ 0.04	0.89 $\pm$ 0.05	0.85 $\pm$ 0.05