AWARD NUMBER: W81XWH-18-1-0554

TITLE: Prevention of the Post-Traumatic Fibrotic Response in Joints: a Critical Preclinical Evaluation of an Antifibrotic Antibody

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REPORT DATE: October 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
Oct 2020	Annual	Sept 30th 2019 – Sept 29th 2020

4. TITLE AND SUBTITLE

Prevention of the Post-Traumatic Fibrotic Response in Joints: a Critical Preclinical Evaluation of an Antifibrotic Antibody

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0554

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Andrzej Fertala

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Thomas Jefferson University
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Philadelphia, PA 19107

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our research addresses the problem of posttraumatic joint stiffness, a pathology that reduces the range of motion (ROM) of injured joints and contributes to the development of osteoarthritis. The fundamental hypothesis that drives the current study is that unwanted consequences of the fibrotic response to joint injury may be prevented or limited by targeting the formation of collagen fibrils, a main component of the fibrotic mass. Key observations from our studies are: (i) in comparison to the non-treated control, deposition of newly-formed collagen fibrils in posterior capsules from injured knees of rabbits treated with the antifibrotic antibody is reduced significantly, (ii) treatment with the antibody-based therapeutic does not cause any side effects, (iii) the healing of injured joints is not affected by treatment with the therapeutic antibody, (iv) mechanism of action of the antibody indicates blocking collagen fibrillogenesis by blocking procollagen molecules and increasing their degradation. Conclusion of these experiments will explain the utility and safety of the anti-collagen I antibody applied to reduce to block excessive fibrosis associated with joint injury.

15. SUBJECT TERMS
Posttraumatic joint stiffness, antifibrotic therapy, collagen, therapeutic antibody, fibrosis, knee joint, animal model, range of motion.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	39	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION

Objectives and Specific Aims

The second Annual Report describes crucial activities and results associated with studies on an antibody-based approach to reduce posttraumatic joint contracture. These studies are the continuation of earlier research carried out by our team to establish the efficacy of antibody-based therapy to limit joint stiffness.

The fundamental, long-term objective of the present study is to reduce posttraumatic joint stiffness in military personnel by blocking fibrotic scarring. The central hypothesis is that limiting the excessive formation of collagen fibrils will reduce posttraumatic joint stiffness but will not impact the healing of injured joint tissues in any significant way.

We emphasized that testing this hypothesis is essential, mainly because of the clinical implications of our method to block excessive scarring. Specifically, we realize that applying any antifibrotic treatment, including the one proposed here, carries the risks of unwanted side effects. While some of the possible side effects may include tissue toxicity and off-target activity of the therapeutic compounds, others may limit the healing process that depends on collagen fibrillogenesis. As collagen fibrillogenesis is the direct target of our antibody, referred to as the anti-collagen antibody (ACA), studies of its impact on the healing of joint tissues are essential to define any unwanted side effects.

To test the central hypothesis, we defined two Specific Aims (SA):

SA1: To determine the safety of the ACA applied short-term and long-term to reduce posttraumatic joint stiffness.

SA2: To define the effects of the ACA on the healing of joint tissues.

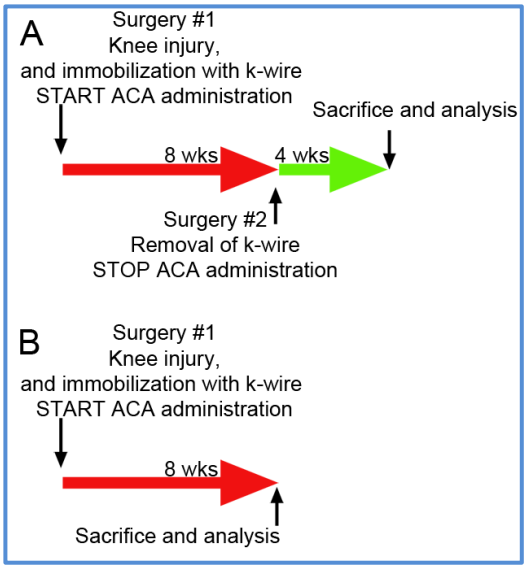


Figure 1. A schematic of experimental groups treated with the ACA and control antibody.

Experimental design and models

We pursue these aims by employing a clinically-relevant rabbit-based model of posttraumatic joint contracture. In this model, one of the knee joints undergoes injury surgically and then is immobilized using the Kirschner (k)-wire for eight weeks. During the first surgery, we also injure the patellar tendon by removing a defined segment of the tendon. Furthermore, we create an osteochondral defect to study the impact of the ACA on the healing of the subchondral tissue.

During the surgery, a programmable pump is implanted subcutaneously to allow delivery of the ACA directly into the articular space. The ACA is administered continuously for 8 weeks. As indicated in the original proposal, groups of rabbits receive three different concentrations of the ACA (referred to as concentrations A, <B, and <C).

There are two different sets of experimental groups:

(1). Eight weeks after the initial knee-injury surgery, the rabbits undergo the second surgery to remove the k-wires, and then, they are sacrificed four weeks later. During the four-week recovery period, the rabbits do not receive the ACA (Fig. 1A).

While studies of the first group provide information on the long-term impact of the ACA, due to the recovery period, they do not allow determining the potentially acute effects of the ACA that may occur during the administration of this antibody.

(2). Eight weeks after the initial surgery, the rabbits are sacrificed and analyzed (Fig. 1B).

As the rabbits are sacrificed while receiving the ACA, studies of the second group focus on potential acute effects of this antibody.

Assays to determine the impact of the aca on the healing process

after sacrificing the rabbits, we harvest selected tissues and organs and assay them for possible pathological changes that might occur due to extended exposure to the ACA. Additionally, we collect the hind legs to perform studies of the mechanical properties of joints healed in the presence or the absence of the ACA. These tests include measurements of the contracture of knee joints, mechanical properties of the patellar tendons, and mineralization of subchondral bone.

Besides studying the impact of the ACA on the healing of the joint tissues, we also analyze this antibody's mechanism of action. In brief, we first collect sera from the rabbits before injuring their knees, and then again at various stages of the healing process. Subsequently, we measure the changes in crucial markers of collagen-derived metabolites as the function of the presence or the absence of the ACA. We also utilize blood samples to analyze cellular and biochemical parameters whose changes provide clues about the overall impact of the ACA on the health of treated animals.

Completion of these experiments will determine the safety of the ACA and determine mechanisms of its action.

2. KEYWORDS

Posttraumatic joint stiffness, antifibrotic therapy, collagen, therapeutic antibody, fibrosis, knee joint, animal model, range of motion.

3. ACCOMPLISHMENTS

General note: There were no significant changes in the project's content or direction.

The major goals of the project

As indicated above, we formulated two Specific Aims that define our project. In the original Statement of Work (SOW), we defined the Major Tasks and set the timelines to complete them; these parameters serve as a blueprint for reaching the Specific Aims. The following paragraphs highlight the Major Tasks and indicate their current status.

Major Task 1: Production and purification of therapeutic antibodies (ACA) (Timeline: 1-25 months). In this task, we produce the ACA needed for animal studies. During our original study, we developed crucial methods to produce, purify, and characterize the ACA. We employ CHO cells and culture them in a bioreactor (Fig. 2).

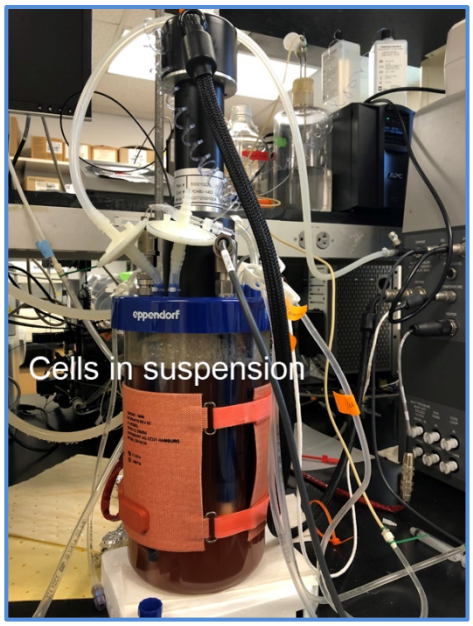


Figure 2. A new vessel of the bioreactor system we use to produce the ACA.

Subsequently, we isolate the ACA by affinity chromatography. Each batch of ACA is then concentrated and sterilized by filtration. Moreover, the target specificity and the affinity of each batch is defined using a biosensor.

Although we described the ACA production method in our earlier reports, a few points are worth noting here.

Initially, we cultured our cells in a disposable bioreactor vessel (Eppendorf, BioBLU 5p) filled with porous discs that allow growing cells attached to the discs' surfaces. One of the advantages of this method is that the cells are trapped within the porous discs, so that conditioned cell culture media can be readily replaced for the fresh batch without losing any cell mass.

Unfortunately, Eppendorf informed us that due to the COVID-19-related demand for this bioreactor vessel by biotech companies, they would not ship this product to us. Consequently, we had to develop a new method to grow cells for ACA production. In brief, we purchased a bioreactor BioBLU 3c vessels for growing cells in suspension. First, we had to "train" our CHO cells to grow in suspension and then develop conditions for culturing them for antibody production. This process included testing various media formulations and optimizing physicochemical parameters, such as gas flow, glucose concentration, mixing blade speed, and others. Consequently, we developed a new bioreactor system that allows us to continue the ACA production effectively. The new system delivers the antibody of expected quality and binding affinity.

Major Task 2: Concentration-dependent safety assays

Subtask 1 (Dose acceleration) and Subtask 2 (Safety studies). As indicated in our original proposal, we plan to test three different concentrations (referred to A, <B, <C) of the ACA and analyze the safety of this antibody and its impact on the quality of the healing of injured tissues that are a part of injured joints. Besides operating the rabbits from groups A and B, we operated group C on October 5th and October 7th. This group will be sacrificed at the beginning of December and then analyzed.

• Blood parameters. To determine the safety of the ACA, we analyzed crucial cellular and biochemical parameters of the blood samples collected at various time points. For the first ACA-concentration group (group A), the rabbits were placed into group #1 that included the recovery period (Fig. 1A). As indicated in our first annual report, we did not observe any tissue toxicity in the ACA-treated rabbits. Also, as presented here, we did not observe any alterations of blood parameters measured at the consecutive time points; all measured parameters stayed within limits established for healthy rabbits.

The second group of rabbits treated with increased ACA concentration (group B) was sacrificed 8 weeks after the knee injury while the rabbits were still receiving the ACA (Fig. 1B). Comprehensive assays of cellular and biochemical parameters of the blood showed no signs of any aberrations. Similar to group A, all parameters stayed within expected limits, regardless of some changes associated with injury and inflammation; supplementary Figures 1, 2, 3&4 (Appendix) summarize all measurements of blood parameters.

Crucial cellular blood parameters included:

(i) WBC (white blood cells count), (ii) RBC (red blood cell count), (iii) HGB (hemoglobin), (iv) HCT (hematocrit), (v) MCV (mean corpuscular volume), (vi) MCH (mean corpuscular hemoglobin), (vii) MCHC (mean corpuscular hemoglobin concentration), and (vii) PLT (platelet count).

A comprehensive biochemical panel included:

(i) Alb (albumin), (ii) Alp (alkaline phosphatase), (iii) Alt (alanine aminotransferase), (iv) Amy (amylase), , (v) Tbl (total bilirubin), (vi) BUN (blood urea nitrogen), (vii) Ca (calcium), (viii) Phos (phosphate), (ix) Cre (creatinine), (x) Glu (glucose), (xi) Tp (total protein), (xii) Glob (globulin), (xiii), Na^+, and (xiv) K^+.

• Histology. Histological assays of tissues from the rabbits did not reveal any pathological changes due to the ACA treatment (Appendix, Supplementary Figures 5 to 12).

In summary, comprehensive assays of the blood and tissue samples of rabbit groups treated with the ACA thus far, did not show any pathological changes. These results suggest that this antibody does not cause any systemic problems, despite continuous treatment for 2 months. Assays of the rabbits treated with the highest planned concentration (concentration C) will further determine the impact of the ACA on crucial systemic parameters, including blood and selected tissues and organs.

Subtask 3 (Assays of collagen metabolites). To define the impact of the ACA treatment on its main target, namely the assembly of collagen fibrils, we studied a few relevant parameters of dynamic collagen metabolism. In brief, in physiological conditions, the homeostasis of collagen-rich connective tissues is regulated by anabolic and catabolic processes. The anabolic processes include synthesis of procollagen molecules, their processing into collagen molecules, and assembly into insoluble fibrils. The catabolic processes include enzymatic degradation of collagen fibrils catalyzed mainly by matrix metalloproteinases (MMPs). During physiological conditions, in mature tissues, *e.g.,* ligaments and tendons, these processes remain in a dynamic balance, meaning that there is neither net gain nor loss of the collagen-rich architecture of these tissues.

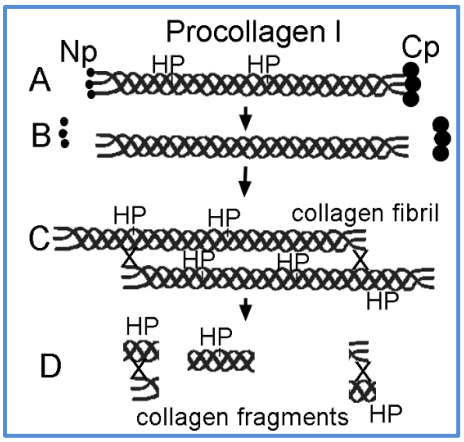


Figure 3. A schematic depicting the premises of assays of collagen fragments. A, A procollagen I molecule with intact N propeptides (Np) and C propeptides (Cp); hydroxyproline residues present in the triple-helical domain are also indicated (HP). B, collagen I molecule generated by extracellular cleavage of the Np and the Cp; here, we will analyze the Cp of procollagen I. C, A typical staggered arrangement of collagen molecules incorporated into a fibril. Collagen cross-links (XL) that link these collagen molecules are indicated (X). D, Collagen fragments formed due to the turnover/degradation of collagen fibrils. Note that all of these fragments include HP, and some will retain collagen cross-links; these fragments will be specifically analyzed.

In contrast, following tissue injury, processes of tissue repair alter the balance by increasing the synthesis of collagen and promoting tissue remodeling that includes both degeneration and formation of collagen fibrils.

As these interlacing processes have different dynamics during various stages of the wound healing process, analyzing them is challenging. Researchers can get a glimpse of these processes, however, by analyzing some collagen metabolites. In brief, as terminal N (Np) and C (Cp) propeptides of procollagen are enzymatically processed, they can be measured in serum with various methods, including ELISA. Similarly, products of

degradation of collagen fibrils, including collagen-specific hydroxyproline (HP) and fragments of chemical cross-links (XL) can also be detected in the serum samples. Figure 3 presents the premise of the study.

Here, we studied the impact of the ACA that binds both intact and processed procollagen molecules on crucial indicators of the formation and degradation of collagen-based tissues. The goal of these studies was to determine mechanisms of reduction of collagen fibril formation by the ACA.

In the group of rabbits that included the recovery period (Fig. 1A) we analyzed the above parameters in serum samples collected at time 0, 2, 4, 8, and 12 weeks (wk). Please note that the ACA was administered from time 0 to 8 wk; from 8 wk to 12 wk, the rabbits did not receive the antibody.

In our previous annual report, using repeated-measures ANOVA with a Greenhouse-Geisser correction (IBM SPSS Statistics v. 26), we presented an analysis of changes in the concentration of measured parameters (HP, Cp, and XL) across various time points. Here, we present global differences between the parameters of the control (CTR) and the ACA-treated groups.

Note for all measurements presented here: All analyses used log-transformed data to account for the skewed distribution of many of the outcomes of interest. Therefore, the results are expressed as geometric means (GM) and geometric mean ratios (GMR), instead of arithmetic means and mean differences. Because measurements of the injured and the uninjured limb of each animal are correlated, as are the repeated serum measurements of each animal over time, we used mixed-effects linear regression.

• Serum parameters of collagen metabolism. The trajectory of GMs of the ACA group was compared to that of the CTR group. We used a 2-sided alpha 0.05, and significant p-values indicate an overall difference between ACA and CTR. If supported by the data, we also fit a linear trajectory curve and compared slopes between ACA and CTR groups.

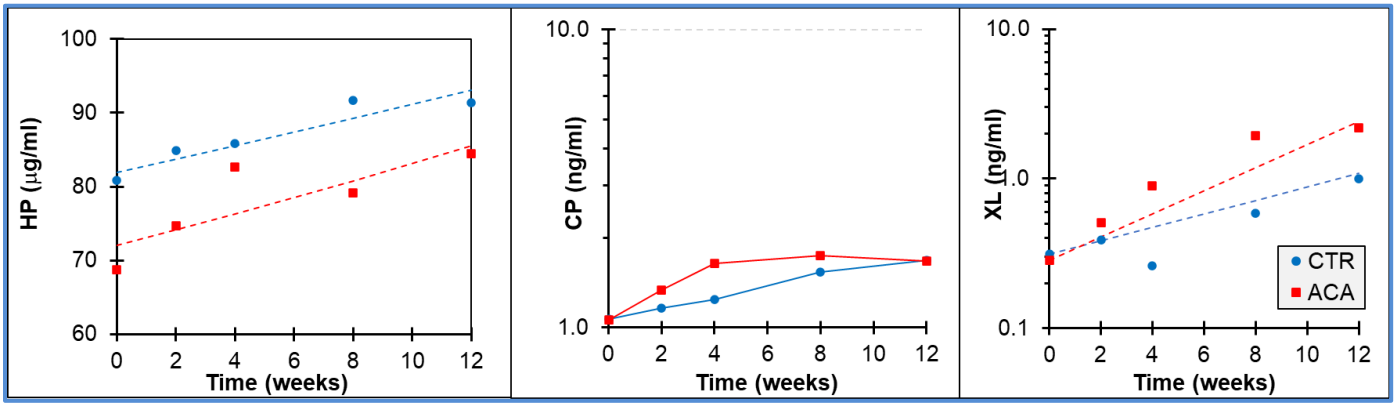


Figure 4. Graphic representations of trajectories of changes of serum hydroxyproline (HP), C propeptides (CP), and the C-terminal cross-links of collagen I (XL).

First, we compared the GM trajectories considering all data points, including 0, 2 wk, 4 wk, 8 wk, and 12 wk. (Fig. 4). As indicated earlier, the ACA was administered up to the 8 wk time point but not after, during the recovery period. Thus, while during the 0 to 8 wk time frame, the collagen metabolism was influenced by the ACA directly, during the 8 wk to 12 wk recovery period, it was influenced indirectly. Table 1 compares the GM trajectories of analyzed parameters measured during 0-to-8 wk and 0 to 12 wk periods.

Table 1. A summary of the comparison of trajectories of changes in analyzed parameters of the ACA and CTR groups.

	Parameter	HP		CP		XL	
	Treatment	CTR	ACA	CTR	ACA	CTR	ACA
0-12w	Linear-fit slopes;	1.01	1.01	1.04	1.04	1.11	1.19
	GMR[1]/week ACA/CTR		1.00		1.00		1.08
	95% CI[2]		(0.99, 1.02)		(0.96, 1.03)		(0.99, 1.17)
	P		0.61		0.83		0.07
0-8w	Linear-fit slopes;	1.02	1.02	1.04	1.06	1.08	1.27
	GMR/week ACA/CTR		1.00		1.03		1.18
	95% CI		(0.98, 1.02)		(0.99, 1.07)		(1.03, 1.34)
	P		0.82		0.20		0.02

[1]GMR; geometric mean ratios, [2]CI confidence interval.

Please note that GMR/week means the geometric mean ratio per week, *i.e.*, the estimated GM today divided by the estimated GM a week ago. For instance, for XL measured for the 0-12 wk period, that is 1.11 for CTR and 1.19 for the ACA group, *i.e.,* every week, XL increases on average by 11% and 19%, respectively. The effect of ACA treatment is 1.19/1.11 = 1.08, *i.e.*, the ACA-associated increase per week is ~8% larger than the increase per week in the CTR group.

Based on the above considerations, we provide the following summaries of results and their brief interpretation:

Assays of HP. We propose that changes in serum HP in uninjured animals indicate a turnover-related natural degradation of collagenous matrices that form various connective tissues.

In the context of our study on injured animals, however, changes in serum HP may also indicate degradation of collagen molecules newly synthesized in response to joint injury. We hypothesized that increased concentrations of HP in serum might indicate an accelerated degradation of new collagen molecules whose incorporation into stable fibrils is blocked by the ACA.

Previously reported results for changes in the serum concentration of HP across various time points indicated that in the CTR group, the HP concentration increased significantly between time 0 and 12 wk. In the ACA group, the concentration also increases significantly between time 0 and 4 wks. Here, the global analysis showed similar trajectories of GM changes over time in both the ACA and CTR groups.

We interpret the increase of serum HP during the first four weeks of the active scar formation process in the rabbits treated with the ACA as an indication of reduced incorporation of newly-synthesized collagen molecules into insoluble fibrils. We propose that this observation may be a result of the anti-fibrillogenesis activity of the ACA due to its binding to procollagen I molecules synthesized in response to injury.

Assays of CP. We propose that changes in serum CP in non-injured animals indicate a turnover-related natural biosynthesis of collagenous I-rich matrices that form various connective tissues.

It is essential, however, to comprehend that during collagen fibrillogenesis, procollagen I molecules, which include the C-terminal propeptides, associate transiently with collagen fibrils, and this association represents a mechanism that controls the fibril diameter. Thus, we expect that during normal fibrillogenesis, the bulk of the C-terminal propeptides remains bound transiently to the fibrillar matrices before being steadily released.

Here, however, we hypothesize that due to the ACA-procollagen I binding, the amount of collagen matrix-bound procollagen molecules is reduced, despite procollagen production in response to injury. Consequently, we predict that free procollagen molecules degrade, thereby increasing the serum concentration of the C-terminal propeptides.

Previously reported results for the changes in the serum concentration of CP across various time points indicated that in the control group the CP concentration did not differ significantly between the time points. In the ACA group, the concentration increased significantly between time 0 and 8 wks and between time 0 and 12 wks. The global analysis demonstrated here showed similar trajectories of GM changes over time in both the ACA and CTR groups. Due to somewhat strong non-linear curvature observed for the ACA plot, interpretation of this global analysis that relies on a log-linear fit is difficult.

Consequently, relying on earlier reported and current assays, we interpret the increase in serum CP during the period of active scar formation in the rabbits treated with the ACA as an indication of poor incorporation of newly-synthesized procollagen I molecules into insoluble fibrils. We suggest that this observation may be a result of the anti-fibrillogenesis activity of the ACA due to its binding to procollagen I molecules synthesized in response to injury.

Assays of XL. In connective tissues, chemical cross-links (XLs) stabilize the structural integrity of the collagen fibrils. These XLs are formed between defined lysine and hydroxylysine residues of adjoining collagen molecules. As indicated above, during the physiological remodeling of collagen-rich tissues, MMPs degrade collagen fibrils, thereby releasing collagen-derived peptides that include XLs, so that they may be detected in the serum. As the XLs are only present in collagen fibrils (i.e., not in newly-produced collagen molecules), assaying their serum concentration allows determining the extent of degradation and remodeling collagen-rich tissues.

Here, we analyzed the concentration of selected cross-linked C telopeptide in the sera of injured rabbits treated with the ACA or a control antibody. As indicated in Fig. 4 and Table 1 the rate of increase of the concentration of serum XLs was significantly higher in the ACA-treated group than that for control.

This observation is somewhat surprising considering that the ACA blocks fibrillogenesis, and the increase of the XL serum concentration indicates the degradation of existing fibrils. Trying to explain this observation, we speculate that our result on the dynamics of the XL changes in the sera of the ACA-treated rabbits may reflect a shift in the dynamics of scar formation and remodeling. In particular, following injury, the scar tissue is formed in a process driven by inflammation. Subsequently, the scar tissue undergoes remodeling during which it is replaced by a tissue that may resemble an uninjured (*i.e.,* original) one. Although the scar tissue provides temporary support for the injury site, its persistent presence alters vital functions of a tissue or organ. In the context of arthrofibrosis, studied here, excessive scarring of injured joints limits their motion. Consequently, reducing excessive scarring is an important element of preserving the function of injured joints.

We interpret that a relatively rapid increase in the serum concentration of the XLs in the ACA-treated rabbits resulted from the faster remodeling phase of the wound healing process. It is likely that due to the ACA-dependent reduction of the scar formation phase, the remodeling phase had an earlier onset. If confirmed with groups treated with higher ACA concentrations, the ACA-dependent acceleration of tissue remodeling would be an additional powerful mechanism by which this antibody improves the mobility of joints after traumatic injuries.

Major Task 3: To determine the impact of the ACA treatment on the healing of joint tissues

While scar formation is a natural mechanism of healing of injured tissues, excessive scarring indicates fibrosis, an unwanted process that alters tissue functions. In the orthopedic setting, scarring of collagen-rich tissues, including tendons, ligaments, joint capsules, muscles, cartilage, and others, affects the functions of joints.

While limiting excessive scar formation is a crucial goal of antifibrotic therapies, applying these therapies may hamper the healing processes that require balanced scarring to occur. As fibrotic scarring and normal wound healing rely on similar biological processes of the biosynthesis of elements needed to restore injured tissue, maintaining proper healing while reducing fibrosis is challenging.

Here, we studied crucial parameters of the healing process as the function of the ACA treatment. These parameters included: (i) histology-based quantification of collagen fibrils formed within the osteochondral defect created during knee injury, (ii) Fourier transform infrared spectroscopy (FTIR)-based assays of the osteochondral scar, (iii) micro-computer tomography (mCT) of the subchondral bone adjoining the site of the osteochondral defect, (iv) joint contracture, and (v) mechanical properties of the injured patellar tendon. Although we already presented the measurements of the contracture of injured joints from the ACA-treated and control groups in our previous report, here we apply additional statistical tests to analyze our results further.

Description of experiments

Although we presented crucial experimental methods in our earlier reports, the following paragraphs briefly highlight crucial experimental methods and summarize our findings:

Rabbit surgeries. Following the initial surgery, to damage the knee capsule, cartilage, the subchondral bone, and the patellar tendon, the rabbits are maintained for eight weeks with their operated knee immobilized with the k-wire. During this time, they receive the ACA delivered from a pump implanted subcutaneously during the initial surgery (Fig. 5). In one group (Fig. 1A), after eight weeks, the rabbits undergo the second surgery to remove the k-wires and the pumps. Subsequently, the rabbits recover for four weeks, and then they are sacrificed. In another group, the rabbits are sacrificed eight weeks after the first surgery, so they do not go through the recovery period.

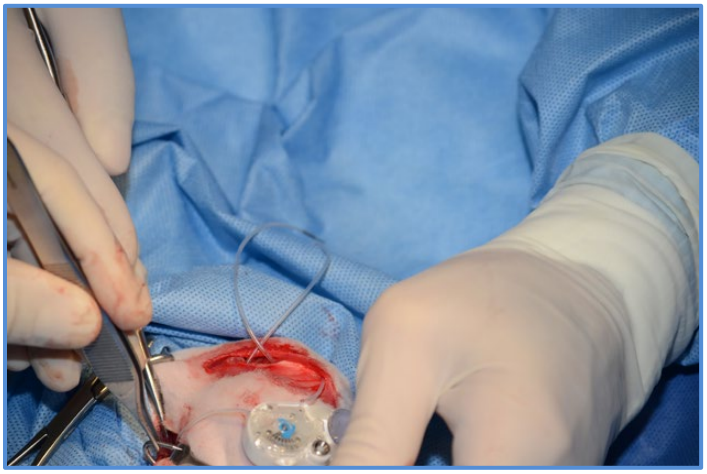
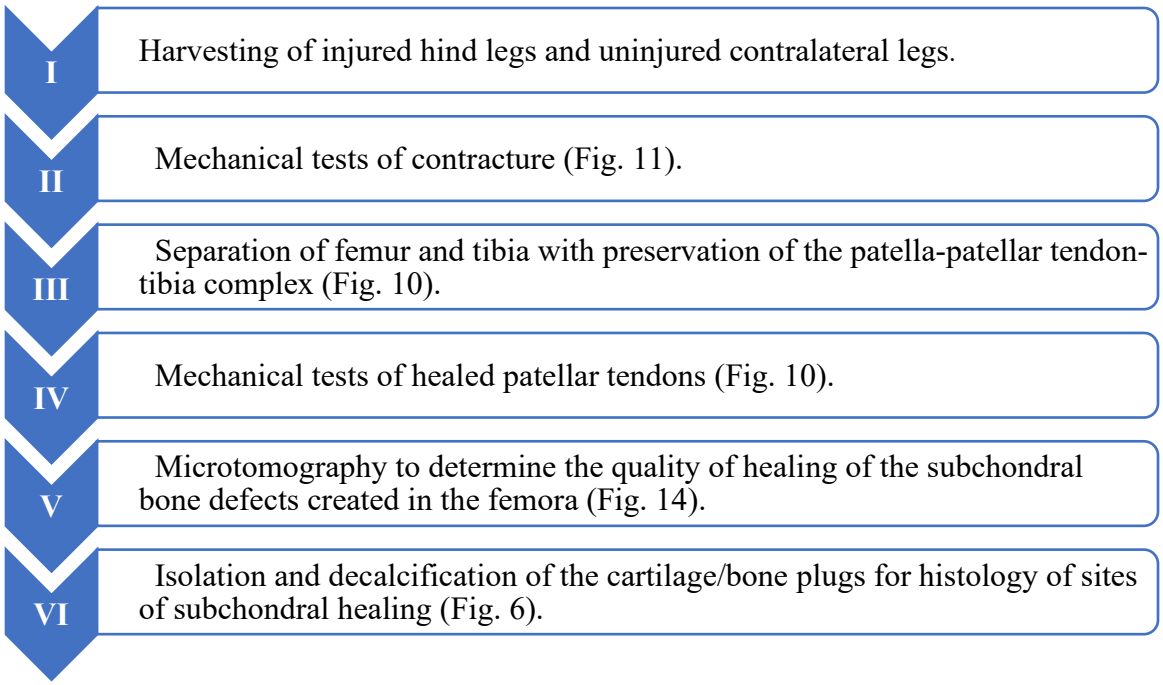


Figure 5. An illustration of an element of the rabbits' surgery: installing a subcutaneous, programmable pump for delivery of the ACA.

Tissue Collection. After sacrificing the rabbits, internal tissues and organs are collected for histology to determine the potential effects of the long-term application of the ACA. The following tissues and organs are collected: (i) brain, (ii) esophagus, (iii) stomach, (iv) intestines, (v) heart, (vi) aorta, lung, (viii) thymus, (ix) spleen, (x) liver, (xi) kidney, (xii) testis, (xiii) ovaries, (xiv) tendon, (xv) sciatic nerve, (xvi) posterior knee capsule, and (xvii) cartilage/bone plug.

Moreover, we collect five blood samples/rabbit: (i) before surgery, (ii) two weeks after surgery, (iii) four weeks after surgery, (iv) eight weeks after surgery, and (v) twelve weeks after surgery (only in the group that includes the recovery period).

Histological, spectroscopic, tomographic, and mechanical assays of joint tissues. The following diagram illustrates the flow of assays of joint tissues we perform to determine the impact of the ACA on their mechanical properties and healing:



Quantitative histological assays of collagen-based architecture of the scar tissue formed in the osteochondral defects

***Histology*.** We analyzed the potential effects of the ACA on the quality of a new tissue formed during the healing of the osteochondral defects created during the initial surgery (Fig. 6). First, an osteochondral plug encompassing the site of the defect was excised with a trephine bur (Fig. 6C). Subsequently, the plug was fixed, decalcified, and processed for histology.

In brief, 3-µm thick sections of paraffin-embedded tissue samples were stained with H&E and with collagen-specific picrosirius red (Polysciences, Inc., Warrington, PA). The latter staining technique combined with polarized-light microscopy makes it possible to describe the thickness, the organization, and to a certain degree, the collagen type-specific composition of the fibrils. As the thickness of fibers increases, their birefringence color changes from green to yellow to orange to red, *i.e.,* from shorter to longer wavelengths. Employing a polarizing microscope (Eclipse LV100POL, Nikon Inc., Melville, NY) and the NIS Elements software (Nikon Inc., Melville, NY), we determined the percentages of differently colored collagen fibrils (Fig. 7). Applying the built-in settings of the NIS Elements software, three main groups of colors were defined: (i) green, (ii) yellow, and (iii) orange-red. The entire image was analyzed, and then the areas occupied by pixels corresponding to the defined colors were determined. Finally, the percentage of each color group in the analyzed samples was calculated (Fig. 7).

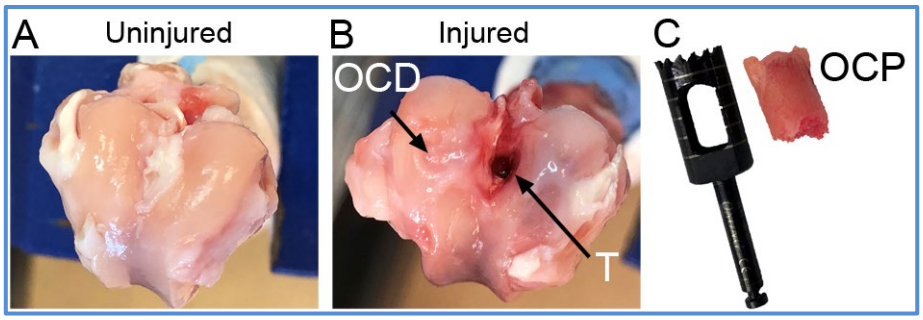


Figure 6. A, Uninjured femoral condyles. B, An osteochondral defect (OCD) healed for 12 wks. C, An osteochondral plug (OCP) that encompases the site of injury seen in panel B. T indicates a hole through whch the ACA was delivered from a pump via a silicon tube (see Fig. 5).

As indicated in Fig. 7, there were no significant differences between corresponding fractions of collagen fibrils formed in the presence of the ACA or control hIgG. This observation was confirmed in both the 8 wk group and the 12 wk group.

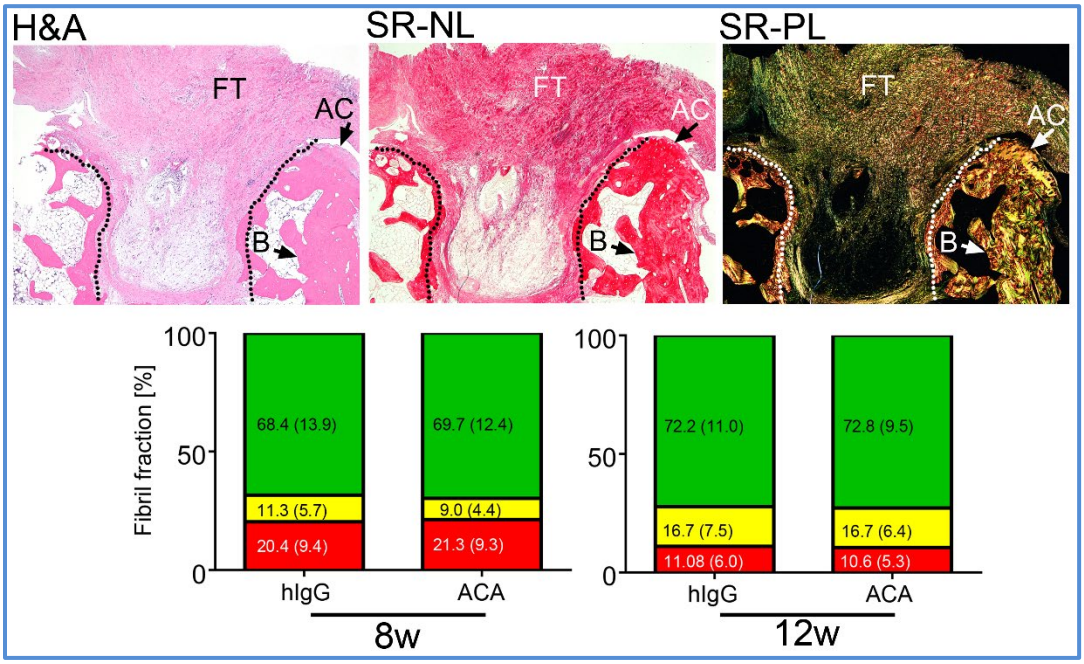


Figure 7. Histological quantification of various populations of collagen fibrils formed within osteochondral defects. The upper row shows a defect (delineated) in which a collagen-rich scar has been formed for 8 or 12 weeks. The site of injury was stained with hematoxylin and eosin (H&E) and with collagen-specific picrosirius red (SR). The SR-stain samples were observed in normal light (NL) and polarized light (PL). The lower panel shows graphic summaries of the measurement of the red-birefringence, yellow-birefringence, and green-birefringence fractions of collagen fibrils. Corresponding segments of the bars include the means and standard deviations (in parentheses).

FTIR. FTIR spectroscopy allows qualitative and quantitative assays of various molecules that form tissues without any need for histological staining. Here 3-μm thick longitudinal sections were prepared from the paraffin-embedded samples. These samples were then deposited on the MirrIR low-e microscope slides (Kevley Technologies, Chesterland, OH).

Next, an FTIR spectrometer (Spotlight 400, Perkin Elmer, Waltman, MA) was used to analyze samples of the fibrillar tissue formed within the osteochondral defects. The tissues were sampled in the trans-reflectance mode.

The measurements were done in the imaging mode in the 4000 to 748 cm-1 wavenumber spectral range, at a pixel resolution of 25 μm, with 32 scans per pixel, and a spectral resolution of 8 cm-1.

Following scanning of the multiple areas of the samples, co-added spectra (Fig. 8B) were generated with the Spectrum Image software (PerkinElmer, Inc., Waltman, MA). Subsequently, employing the Spectrum software (PerkinElmer, Inc., Waltman, MA), we calculated the ratios of the areas of the integrated collagen-specific peak (centered around 1338 cm-1; attributed to the CH_2 wagging vibration of proline side chains) and the glycosaminoglycan (GAG)-specific peak (centered around 1376 cm-1; associated with CH_3 symmetric vibration of GAGs).

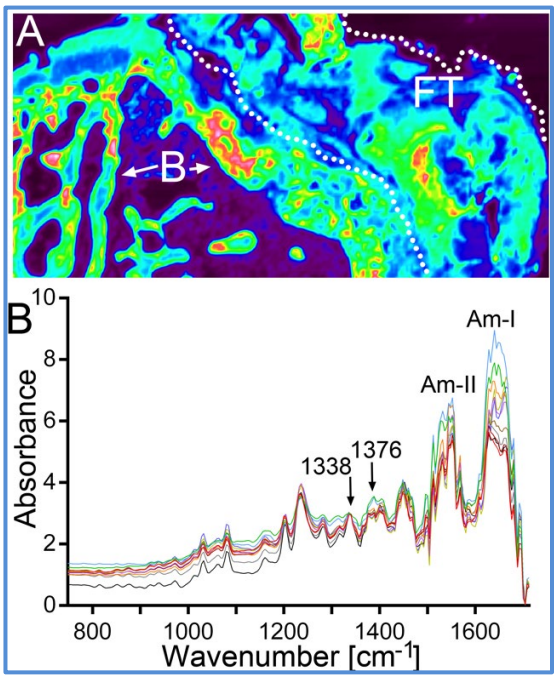


Figure 8. FTIR spectroscopy of the scar tissue formed within osteochondral defects. A, A map of the average intensity of the FTIR signals of a region of the osteochondral defect. Elements of bone (B) and fibrotic tissues (FT; delineated) are indicated. B, FTIR spectra of a few analyzed samples with the Amide I (Am-I), Amide II (Am-II) peaks indicated for reference. For calculations of the relative content of collagenous proteins, we analyzed the ratios of the peaks centered around wavenumbers 1338 cm-1 and 1376 cm-1.

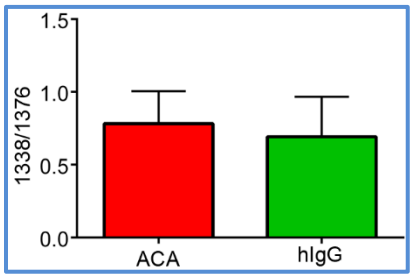


Figure 9. A graphic summary of the 1338/1376 cm-1 ratios measured within scar tissue formed in osteochondral defects.

As indicated in Figure 9, the collagen/GAG ratios were similar in the ACA-treated and control groups. This result indicates that the ACA did not affect the relative amount of collagen deposited in the healing osteochondral defect in any significant way.

Analysis of mechanical properties of joints. While analyzing histological and spectroscopic characteristics of the healing tissues provides information on the impact of the ACA on the relative quantity and quality of collagen-rich deposits, mechanical tests of the healing tissues provide information on the ability of these tissues to function properly. Besides, the results of mechanical tests reflect not only the quantity of collagen-based structures but also their organization and the quality of healing tissues as a whole.

As described in our original proposal, we measured the joint contracture and selected parameters of patellar tendons. Although we also planned to measure the mechanical properties of healed cartilage defects, we realized that these measurements are not feasible. The main reason for not doing these assays was that 12 weeks after the initial surgery, cartilage tissue did not form (Fig. 7). Instead, the osteochondral defect was filled with non-cartilaginous fibrotic tissue (we measured this tissue histologically and using FTIR; see above). Furthermore, the topography of the surfaces of the fibrotic tissue formed in the osteochondral defects was rough, which made it incompatible with micro indentation-based assays. This problem was further aggravated by the fact that

frequently, the entire surface of the cartilage of the injured joint was covered with a dense fibrotic membrane that blocked access to the sites of osteochondral defects.

Although we already presented some of the mechanical data, here we show additional analyses of the groups maintained for 12 wks (including the recovery period). Even though we already obtained some results for the groups maintained for 8 wks, we will present them together with analyses of the rabbits we operated recently; we plan to sacrifice these rabbits at the beginning of December.

Assays of the patellar tendon. During the initial surgery, we create a full-thickness, 3 mm x 10 mm defect in the patellar tendon to study its healing in the presence or the absence of the ACA. In contrast to the broad knee injury we create to cause joint contracture, the well-defined injury in the patellar tendon allows for more precise measurements of the impact of the ACA on the healing process.

As described in our proposal, the crucial measurements to determine this impact include assays of the mechanical properties of the healed tendons. To perform these measurements, we isolated the patella-patellar tendon-tibia complexes and analyzed them mechanically in a blinded fashion (Fig. 10).

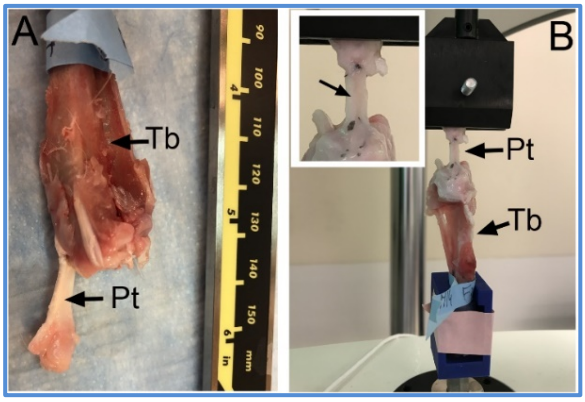


Figure 10. A. Isolation of the patellar tendon (Pt)-tibia (Tb) complex. B. A setup of the complex seen in A for the mechanical measurements. The insert depicts the Pt in which the arrow indicates the healed defect created during the initial surgery.

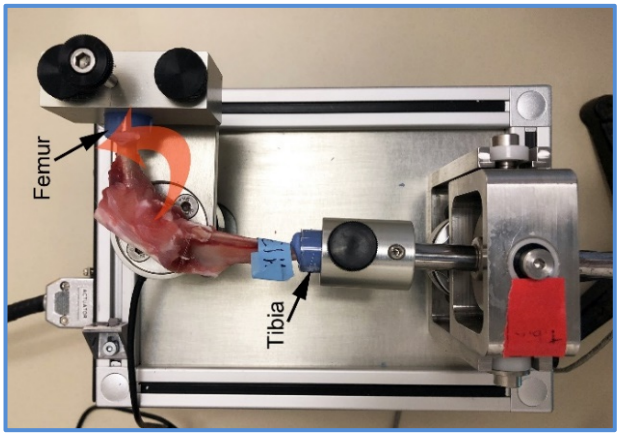


Figure 11. A depiction of a mechanical tester for measuring the joint contracture. The red arrow indicates the movement of the femur.

Assays of joint contracture. We analyzed the impact of the ACA on the contracture of the injured joints. In brief, the tibia and the femur are transected about 6 cm from the knee joint. Subsequently, the ends of the bones are potted in polycarbonate cylinders with the use of polymethyl methacrylate and acrylic copolymer (Fig. 11, arrows). After securing the limb in the grips, the femur and tibia are positioned at a right angle, and then the instrument is set to 0°. Subsequently, applying the rate of loading set to 40°/min, an extension torque is applied to 0.2 Nm, and the joint extension is recorded.

Figures 12 and 13, as well as Tables 2, 3, and 4 and summarize the mechanical data.

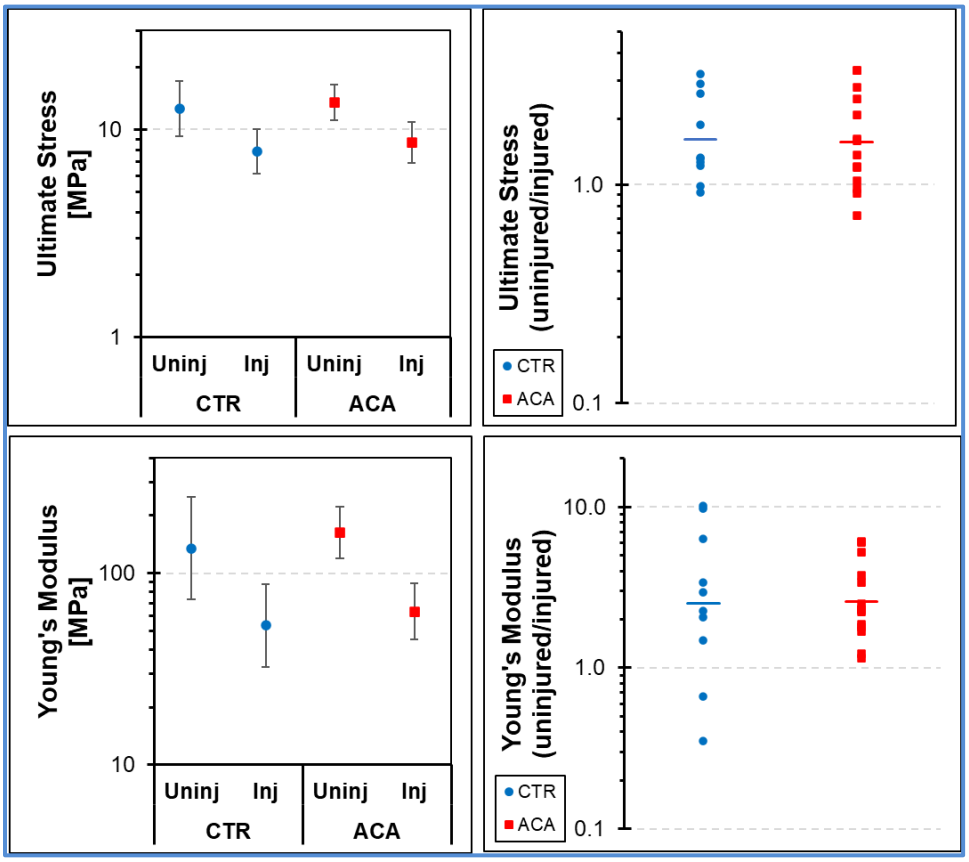


Figure 12. A graphic representation of differences in mechanical parameters of patellar tendons in rabbits treated with the ACA and control antibody.

Table 2&3. Summaries of outcomes of non-inferiority tests performed for indicated parameters of patellar tendons.

Table 2	**Ultimate stress [MPa]**			
	N	GM		GMR
		Unin	Inj	Unin/Inj
CTR	11	12.6	7.9	1.61
ACA	14	13.5	8.6	1.57
ACA/CTR				**0.98**
95% UCL[1]				**1.35**
P				**0.109**

[1]UCL, upper confidence limit

Table 3	**Young's Modulus [MPa]**			
	N	GM		GMR
		Unin	Inj	Unin/Inj
CTR	11	135	53	2.52
ACA	14	163	63	2.58
ACA/CTR				**1.02**
95% UCL				**1.99**
P				**0.301**

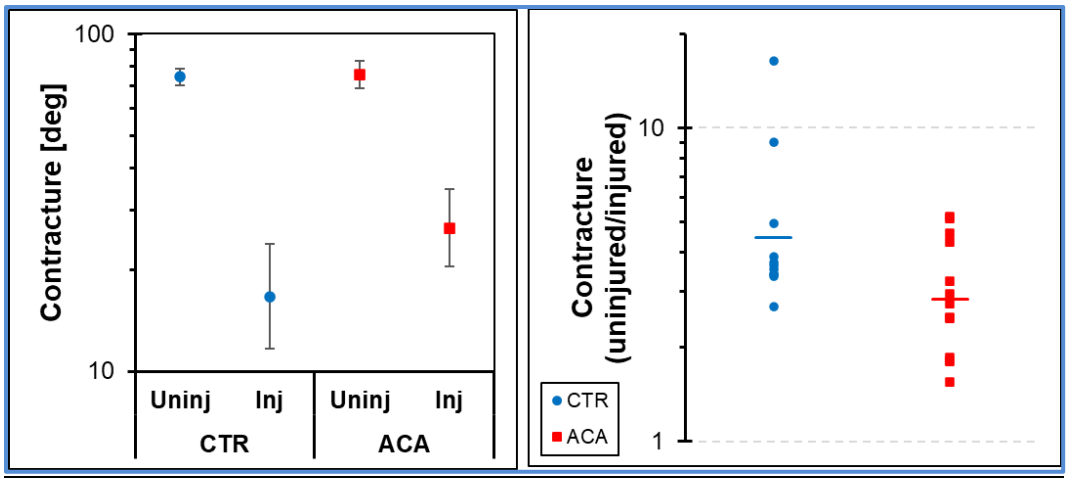


Figure 13. A graphic representation of the differences in joint contracture in rabbits treated with the ACA and control antibody.

Table 4. A summary of outcomes of the superiority test performed for joint contracture.

Table 4	**Contracture [deg]**			
	N	GM		GMR
		Unin	Inj	Unin/Inj
CTR	11	74	17	4.47
ACA	14	76	27	2.84
ACA/CTR				**0.64**
95% CI				**(0.42, 0.96)**
P				**0.035**

In the following paragraphs, we briefly explain the results of the mechanical properties, focusing on the ultimate stresses of the patellar tendons as an example. Geometric means (GM) for uninjured tendons of analyzed groups, one treated with control antibody (CTR), and another one with the ACA are ~13 MPa, indicate proper randomization (Fig. 12) GMs for injured patellar tendons in both groups are lower. The drop in CTR is from 12.6 to 7.9 and from 13.5 to 8.6 in the ACA. Corresponding GM ratios (GMR; see Fig. 12) are 12.6/7.9 = 1.61 (CTR) and 13.5/8.6 = 1.57 (ACA).

Considering the above ratios, 1.61 (CTR) and 1.57 (ACA), on average, in the CTR group, the GM of the uninjured tendon was 61% higher than the GM of the injured one, and in the ACA group, the GM of the injured tendon was 57% higher than the GM of the injured one. Thus, it seems that the ACA treatment reduced the "gap" between uninjured and injured tendons from 1.61 to 1.57. That is the formal treatment effect: 1.57/1.61 = 0.98 (the smaller, value the bigger effect).

According to our hypothesis presented in our original plan, however, we are willing to accept up to 1.25 (25% increase of the "gap"). To formally test this non-inferiority hypothesis, we put a 95% one-sided (upper) confidence limit to the 0.98 value; in this case, we get 1.35. So, even though our best estimate is that the ACA slightly reduces

the uninjured-to-inured difference, we cannot exclude the possibility that it increases it up to 35%. Therefore, we have not proven non-inferiority (the associated p-value is 0.109; Tab. 2).

A similar conclusion is for measurements of the Young's modulus of the patellar tendons (Fig. 12 and Table 3).

According to the hypotheses stated in our original proposal, we also performed the superiority test to analyze the contracture (Fig. 13 and Table 4). There, the effect of the ACA is: 2.84/4.47 = 0.64 and the 95% CL is 0.42, 0.90. Thus, we conclude that ACA reduces the uninjured/injured ratio, compared to CTR (p-value of 0.035).

Effect of the aca on the subchondral bone. Following the collection of the injured and control femorae, we performed microtomography (mCT) to measure crucial parameters that define subchondral trabecular bone. Our primary focus was on the Bv/Tv (percent of bone volume to the total analyzed volume) and the BMD (bone mineral density). We analyzed specific values of the Bv/Tv and the BMD obtained from the injured regions and the matching regions of uninjured bones. Figure 14 depicts the analyzed regions.

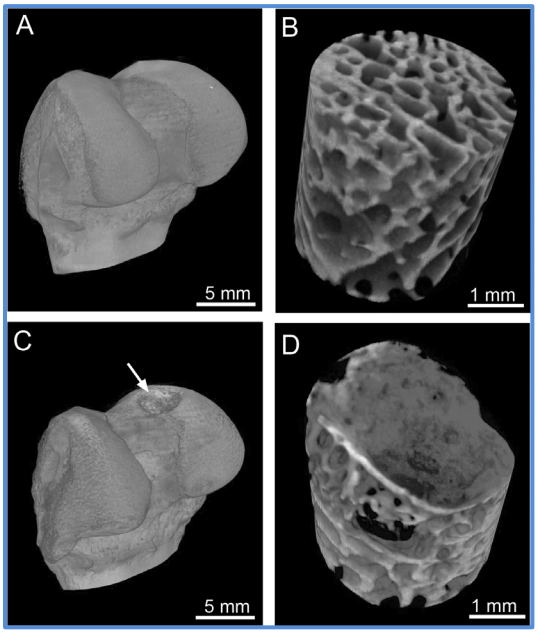


Figure 14. Representative mCT images of the uninjured (A) and injured (C, arrow) bones. Panels B (uninjured bone) and D (injured bone) indicate subchondral bone regions analyzed here to determine the extent of bone formation in the injured area (C&D).

Although we already presented some results of our assays, here, we present our non-inferiority tests done in a way described for the mechanical studies of the patellar tendons. Figure 15 and Tables 5 and 6 summarize the results.

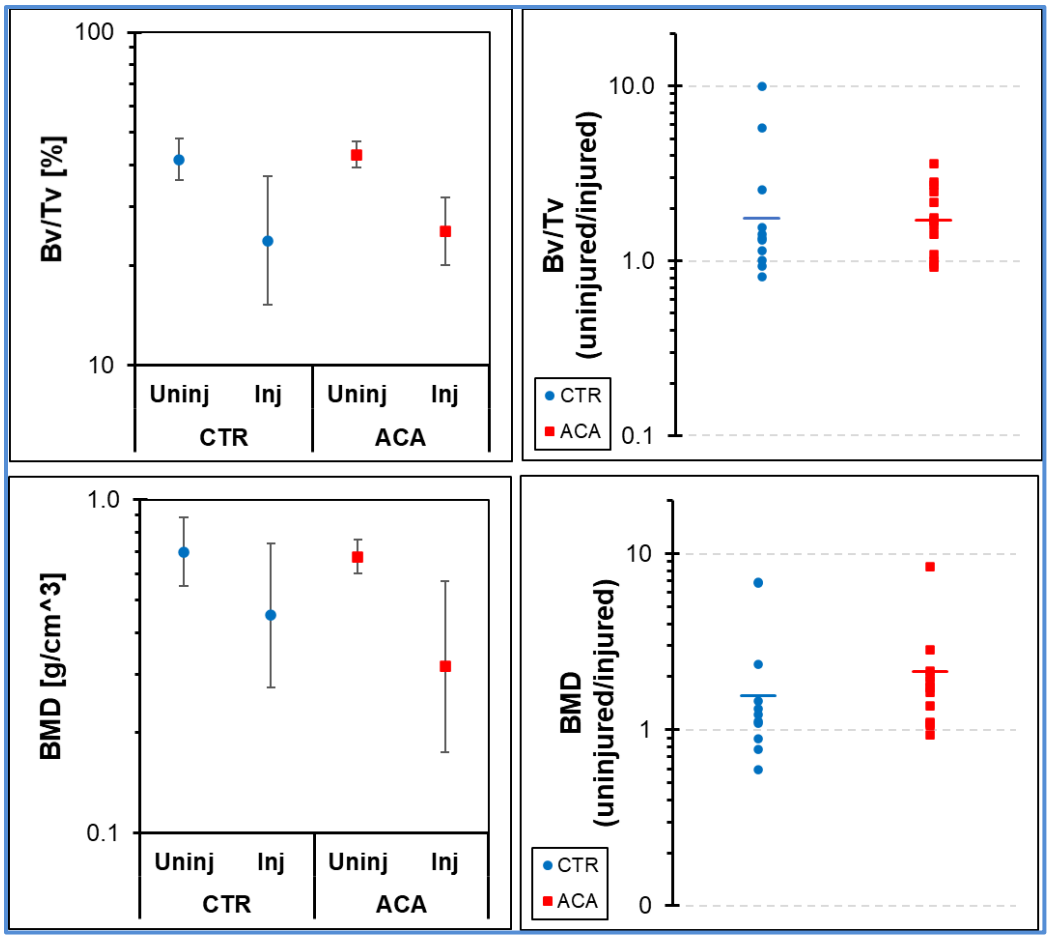


Figure 15. A graphic representation of differences in bone mineralization parameters in rabbits treated with the ACA and control antibody.

Table 5 & 6. Summaries of the outcomes of the non-inferiority tests performed for the bone mineralization parameters.

Table 5

	Bv/Tv [%]			
	N	GM		GMR
		Unin	Inj	Unin/Inj
CTR	11	41.6	23.7	1.76
ACA	14	43	25.3	1.70
ACA/CTR				**0.97**
95% UCL				**1.54**
P				**0.175**

Table 6

	BMD [g/cm^3]			
	N	GM		GMR
		Unin	Inj	Unin/Inj
CTR	11	0.7	0.45	1.55
ACA	14	0.68	0.32	2.14
ACA/CTR				**1.38**
95% UCL				**2.49**
P				**0.61**

Opportunities for training and professional development.

Nothing to report.

Dissemination of the results.

We presented our initial results at the Keystone Conference: Fibrosis and Tissue Repair: From Molecules and Mechanics to Therapeutic Approaches (February 2020)

"Consequences of Blocking Excessive Scarring with a Novel Anti-fibrotic Antibody in a Rabbit Model of Arthrofibrosis." A. Steplewski, J. Fertala, P. Beredjiklian, J. Abboud, M. Wang, M. Rivlin, and A. Fertala

Plans for the next reporting period.

In the next reporting period, we will continue our studies with groups of rabbits that receive the highest dose of the ACA.

We plan to disseminate our findings in the form of publications that will present comprehensive results of our studies.

4. IMPACT

The impact on the development of the principal discipline(s)

The studies we carried out in the second reporting period will continue to have a significant positive impact on the principal disciplines, including fibrosis and orthopedics. By validating the antifibrotic target, namely collagen fibrillogenesis, we create a new paradigm for how to treat a large group of fibrotic diseases, including lung fibrosis, kidney fibrosis, ocular fibrosis, abdominal adhesions, and others. Moreover, based on our preliminary results, we expect that our antifibrotic approach is safe, and it does not impact the overall healing process in any significant way. Thus, our studies offer a novel approach to limit fibrotic diseases not only effectively but also in a safe manner.

As our research aims to reduce posttraumatic joint stiffness, its outcomes will have a positive impact in the area of orthopedics. As fibrotic scarring is a common problem in the area of orthopedics, including due to accidental trauma and surgery, our studies will provide a new therapeutic tool to limit the formation of unwanted scar tissue. We believe that our study will not only help to limit excessive scar formation around joints, but also around other vital elements of the musculoskeletal system, including in the spine, around peripheral nerves, within muscles, and elsewhere. Recently, our team has published a paper on the scarring of peripheral nerves, thereby indicating our commitment to targeting a wide range of fibrotic responses to injury of orthopaedically-relevant tissues ("Collagen-rich deposit formation in the sciatic nerve after injury and surgical repair: A study of collagen-producing cells in a rabbit model". J. Fertala, M. Rivlin, M.L. Wang, P. Beredjiklian, A. Steplewski, A. Fertala. Brain Behav 2020)

The impact on other disciplines

As antibody-based therapies represent one of the most attractive approaches to treat a broad spectrum of diseases, including cancer, immune diseases, and others, the results of our studies contribute to the area of novel biologics significantly. Because our research raised the interest of the scientific community, we plan collaborations in the area of biomaterials, drug delivery, protein structure, to name a few. Recently, we established a collaboration with ophthalmologists from the Wills Eye Hospital in Philadelphia to consider using the antibody-based technology to limit excessive scarring of the injured cornea and to improve outcomes of glaucoma surgery.

Impact on technology transfer

We established a collaboration with a commercial partner to develop a humanized version of our antibody. Based on in vitro tests, a few humanized variants prepared by our partner show satisfactory characteristics, including expected specificity and affinity. A fully humanized version will offer a clinically attractive antifibrotic therapeutic readily applicable for clinical tests.

The impact on society beyond science and technology.

With the direct clinical implications of our project, its impact on the main stakeholders is high. If successful, our project will benefit the following groups of stakeholders:

- **Military personnel**: effective recovery and improved readiness.
- **Patients**: higher patient satisfaction and quality of life due to the reduced number of aggressive physiotherapy, hospital admissions, and revision surgeries, as well as fewer cases of severe disability.
- **Families**: positive outcomes of patients' recovery will have a positive impact on their families as a whole.
- **Health care system**: a significant cut in the immense costs of revision surgeries (by 50 %) and severe disabilities.
- **Health care professionals**: effective preventive treatment option, less burden in revision surgeries.
- **Employers**: less sick leave days, earlier return to work and increased work longevity.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

No changes.

Actual or anticipated problems or delays and actions or plans to resolve them

As indicated above, due to the COVID-19 problem, we encountered the shortage of a crucial element of our bioreactor we use to produce the antibody we apply in this study. We managed to circumvent this problem by changing the method of production of the antibody.

Due to the high demand for experimental rabbits imposed by the commercial sector, we also encountered some problems purchasing these animals. We found, however, another supplier so that we can continue our research. We hope that we will be able to purchase the rabbits if we still need them.

Similar to other universities, our school also experienced a lockdown. Although our initial timeline had shifted, we will be able to reach our original goals.

Changes that had a significant impact on expenditures.

None, thus far.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS

Nothing to report.

Technologies or techniques

We developed an efficient technology for the pilot-scale production of the ACA in suspension and batch characterization of this antibody.

Inventions, patent applications, and/or licenses

Nothing to report.

Other Products

We generated and preserved a vast collection of tissues relevant to the excessive scarring of joint tissues. These samples will provide opportunities to study mechanisms of posttraumatic joint contracture.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Andrzej Fertala
Project Role	PI
Research Identifier (ORCID ID)	https://orcid.org/0000-0001-9153-1912
Nearest person month worked	20% = 2.4 calendar months
Contribution to Project	Dr. Fertala is an expert in the structure and function of extracellular fibrous proteins with a particular focus on collagenous proteins in health and disease. He developed the concept, and then demonstrated its feasibility, that the pathological process of excessive deposition of collagenous material may be inhibited by blocking collagen fibril formation. He generated a clinically-relevant antibody characterized by the ability to block the formation of collagen-rich fibrotic deposits. Dr. Fertala oversees all aspects of the planned project and closely interacts with other members of the team. Specifically, Dr. Fertala monitors procedures for the preparative-scale production of the antifibrotic antibody. Dr. Fertala collaborates closely with participating clinicians and Dr. Tomlinson. Moreover, Dr. Fertala leads all analyses that are done in order to establish the safety and efficacy of the approach proposed here to limit posttraumatic joint stiffness. Besides, Dr. Fertala is responsible for coordinating analyses of all data obtained from the proposed study. In these analyses, Dr. Fertala communicates with Dr.

	Daskalakis, the biostatistician assigned to this project. Moreover, Dr. Fertala is responsible for communicating with the DoD.
Funding Support	Not applicable

Name	Andrzej Steplewski
Project Role	Research Associate
Research Identifier (ORCID ID)	https://orcid.org/0000-0001-6869-3999
Nearest person month worked	50% = 6 calendar months
Contribution to Project	Dr. Steplewski is an expert in connective tissue research and has extensive experience in protein engineering and preparative-scale protein production. Under the guidance of Dr. Fertala, he oversees the production and characterization of recombinant antibodies needed for the proposed study. He also participates in animal surgeries by assisting orthopaedic surgeons. In collaboration with Dr. Tomlinson, Dr. Steplewski also participates in biomechanical assays associated with Specific Aim 2. Moreover, Dr. Steplewski runs microCT assays, FTIR-based assays.
Funding Support	Not applicable

Name	Jolanta Fertala
Project Role	Research Associate
Research Identifier (ORCID ID)	https://orcid.org/0000-0001-9027-0917
Nearest person month worked	50% = 6 calendar months
Contribution to Project	Dr. J. Fertala has extensive experience in the physiology and pathology of skeletal connective tissues. She performs the histological assays and microscopic quantification of collagen-rich deposits in tissues obtained from animal groups. Moreover, she studies the architecture of scar tissue formed in the presence of the therapeutic antibody. She is also responsible for the biochemical assays of fibrotic deposits. These assays include processing crude samples, extracting collagenous proteins, determining the concentration of collagen-specific hydroxyproline, and determining the composition of collagen-rich deposits. She closely interacts with clinical partners to discuss the clinical relevance of biochemical and morphological data. She also collaborates with the analytical team that performs biochemical and histopathological tests of blood and collected organs and tissues. Moreover, she performs assays of collected sera for changes in hydroxyproline, C-terminal propeptides, and cross-linked telopeptides.
Funding Support	Not applicable

Name	Ryan Tomlinson
Project Role	Collaborator
Research Identifier (ORCID ID)	https://orcid.org/0000-0002-6713-6047
Nearest person month worked	9% = 1.08 calendar months
Contribution to Project	Dr. Tomlinson has an extensive experience in biomechanics of orthopaedic tissues. He is responsible for mechanical assays of joint tissues collected following sacrificing the rabbits.
Funding Support	Osteo Science Foundation; The Role of NSAIDs in Osseointegration of Dental Implants Pfizer, Inc.; Effect of Celecoxib and muMab911 on Stress Fracture Risk and Repair in Mice NIH/NIDCR; NGF-TrkA Signaling in Dental Implant Osseointegration and Osseoperception NIH/NIAMS; NGF-TrkA Signaling in Load-Induced Bone Formation

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards

Not applicable.

Quad Charts

See Appendix

9. APPENDICES

Quad Chart.

Results on assays of blood parameters and histology of tissues and organs.

APPENDIX

Prevention of the Posttraumatic Fibrotic Response in Joints

Insert ERMS/Log Number: OR170347
Award Number: W81XWH-18-1-0554

PI: Andrzej Fertala, Ph.D. **Org:** Thomas Jefferson University **Award Amount:** $993,637.00

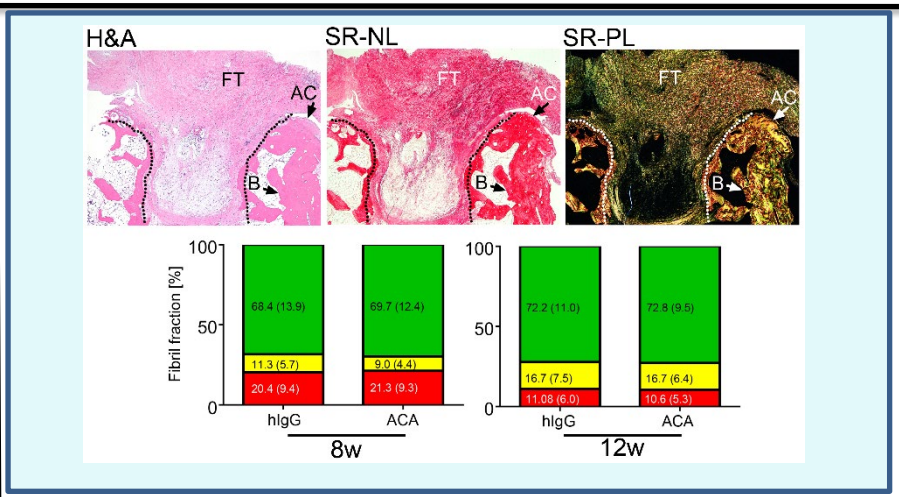
Study/Product Aim(s)

Two Specific Aims will be implemented to test antibody-mediated reduction of post-traumatic joint stiffness:

- Specific Aim 1: "To determine the safety of the ACA applied short-term and long-term to reduce post-traumatic joint stiffness"
- Specific Aim 2 "To define the effects of the ACA on the healing of joint tissues"

Approach

The research strategy includes three key technical elements: (i) production of the inhibitory anti-collagen antibody (ACA), (ii) creation and utilization of a rabbit model of post-traumatic joint contracture to test the efficacy of the inhibitory ACA to decrease the fibrotic response and to preserve ROM, and (iii) microscopic, biochemical, and biomechanical tests of joints to determine the organ-level, tissue-level, cell-level, and molecular-level outcomes of the novel therapeutic approach in the context of relevant controls.



Histological quantification of scarring in the osteochondral defect.

Timeline and Cost

Activities / CY	18	19	20	21
Implement/test the model system.				
Execution of Specific Aim 1				
Execution of Specific Aim 2				
Final tests/Data analysis				
Estimated Budget ($K)	$77	$319	$344	$253

Updated: October 29, 2020

Goals/Milestones

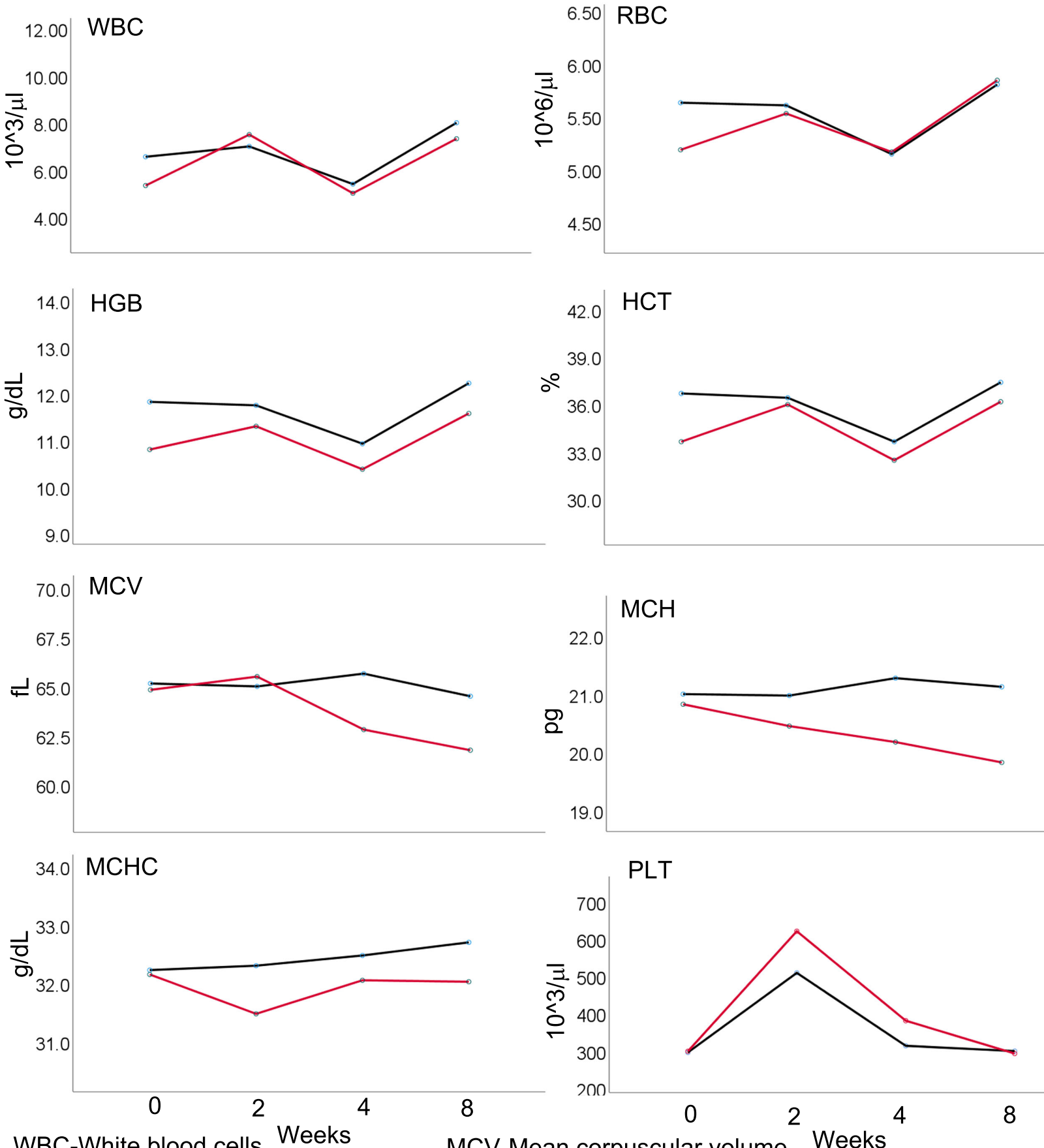
CY18 Goal – Implementation of model systems.
☑ Launch production of ACA and create relevant animal model.
CY19 Goals-Model validation and execution of Specific Aim 1.
☑ Carry out tests of the safety of ACA and the selected animal model.
☐Study the effects of varying amounts of inhibitory IgG on knee joints.
CY20 Goal – Execute and achieve Specific Aim 2.
☑ Analyze the biological and biomechanical characteristics of injured joints treated with anti-fibrotic ACA.
CY21 Goal – Determine the safety of the ACA.
☐ Data analysis and statistical evaluation of results.

Comments/Challenges/Issues/Concerns

- If timelines change, comment here: N/A at current stage of the project
- If off by more than one quarter in spending, comment here.

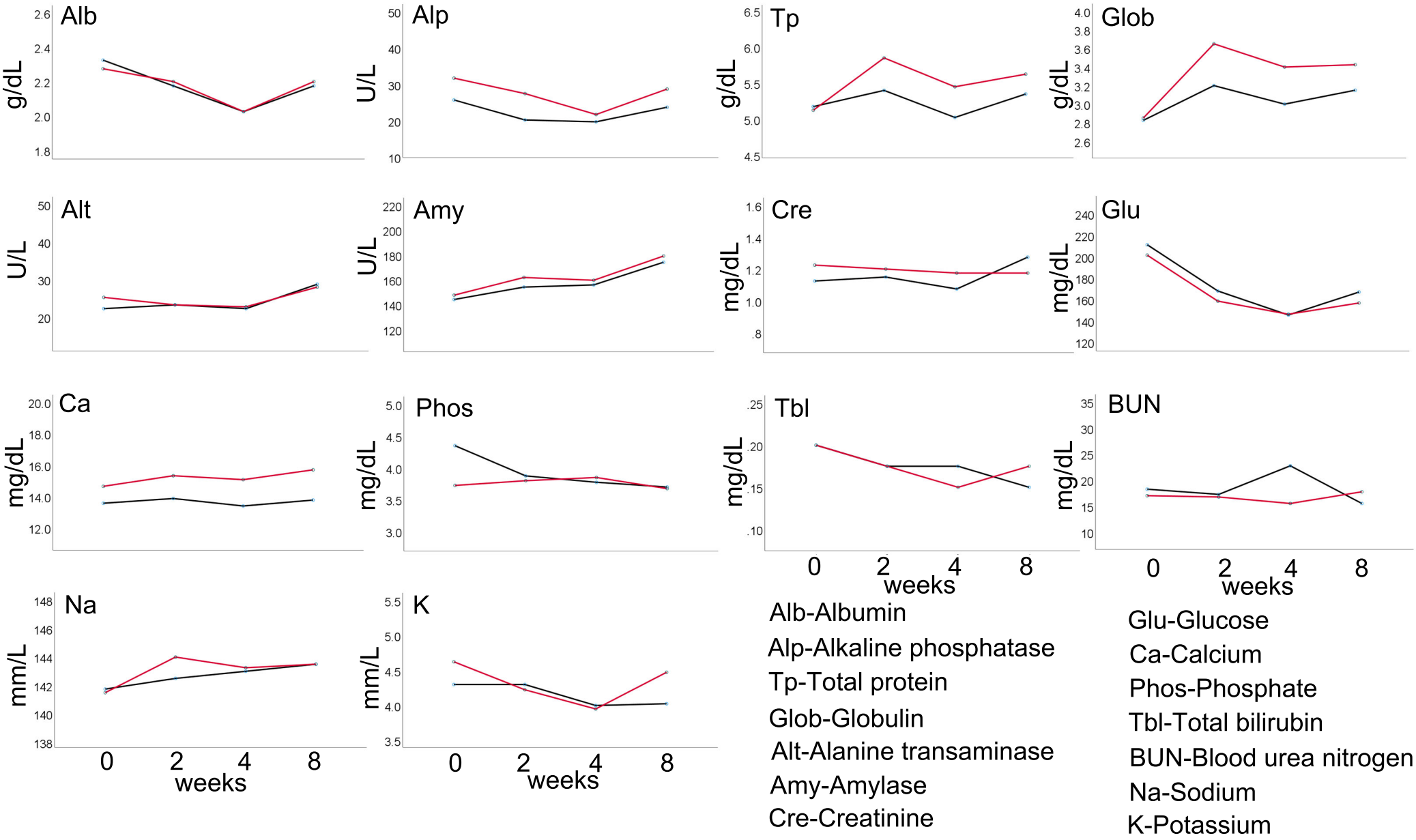
Budget Expenditure to Date

Projected Expenditure: $656,040
Actual Expenditure: $672,545

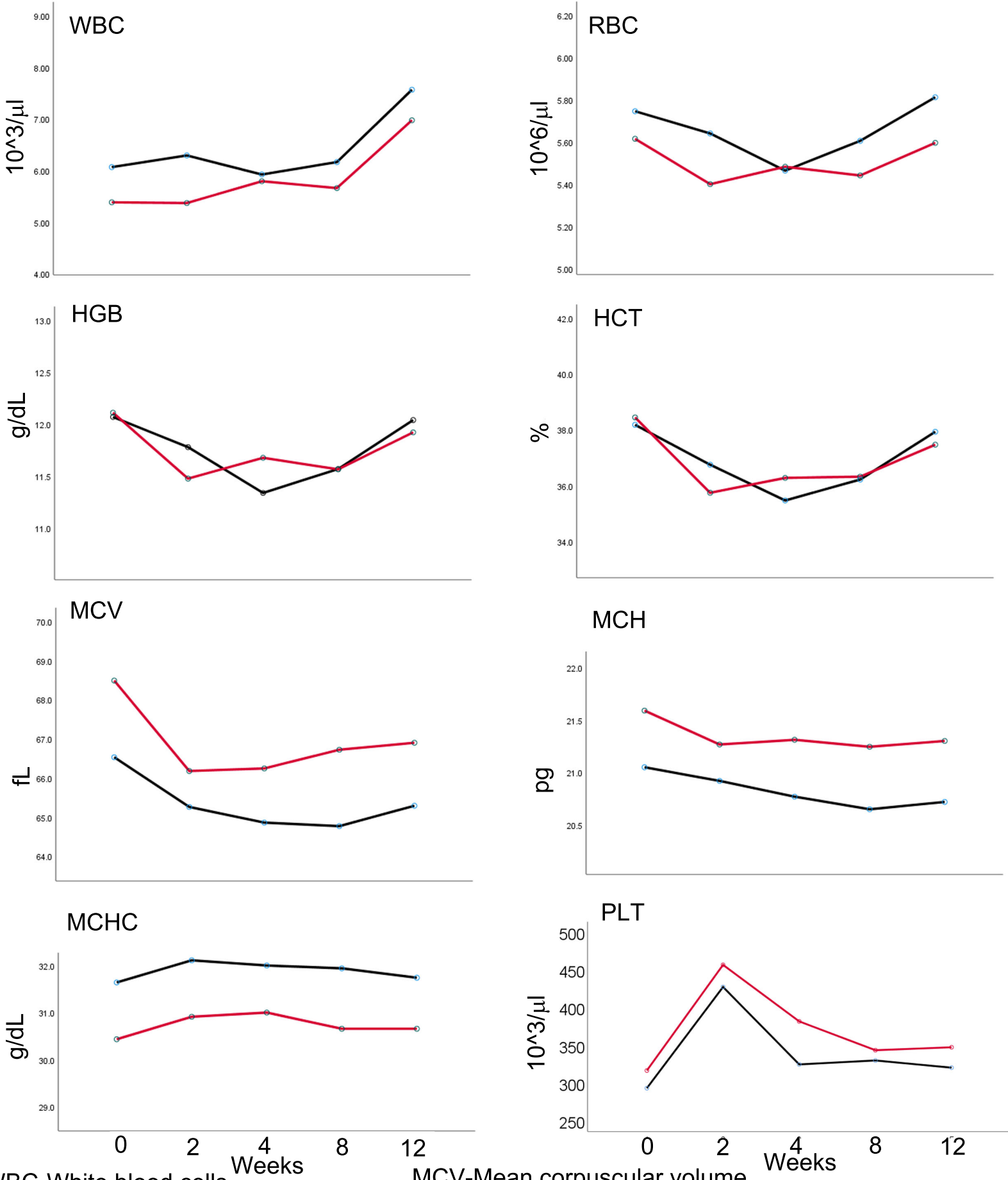


WBC-White blood cells
RBC-Red blood cells
HGB-Hemoglobin
HCT-Hematocrit
MCV-Mean corpuscular volume
MCH-Mean corpuscular hemoglobin
MCHC-Mean corpuscular hemoglobin concentration
PLT-platelets

Supplementary Figure 1. Cell parameters of the blood samples collected at various time points from rabbits maintained for 8 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.



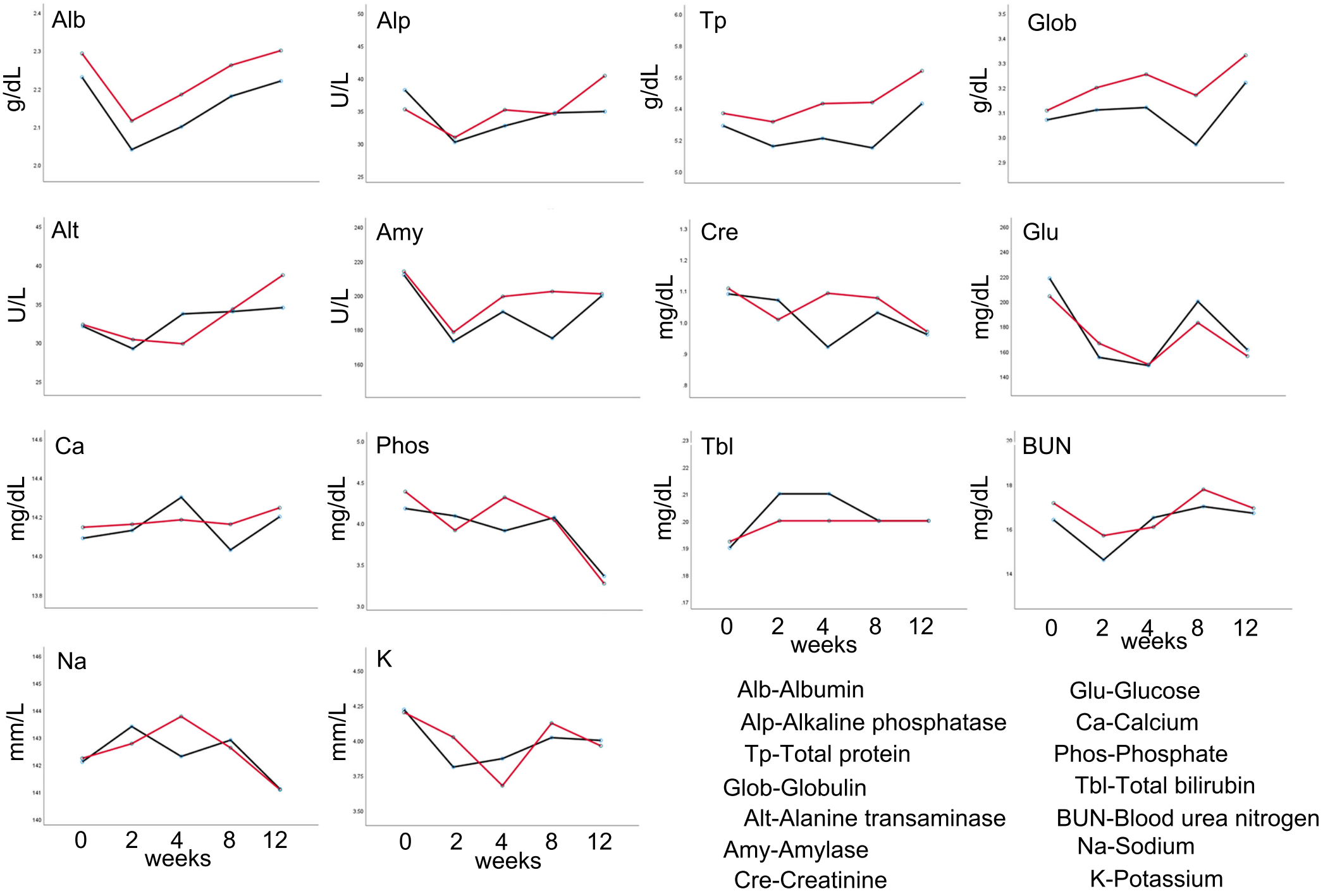
Supplementary Figure 2. Biochemical parameters of the blood samples collected at various time points from rabbits maintained for 8 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.



WBC-White blood cells
RBC-Red blood cells
HGB-Hemoglobin
HCT-Hematocrit

MCV-Mean corpuscular volume
MCH-Mean corpuscular hemoglobin
MCHC-Mean corpuscular hemoglobin concentration
PLT-platelets

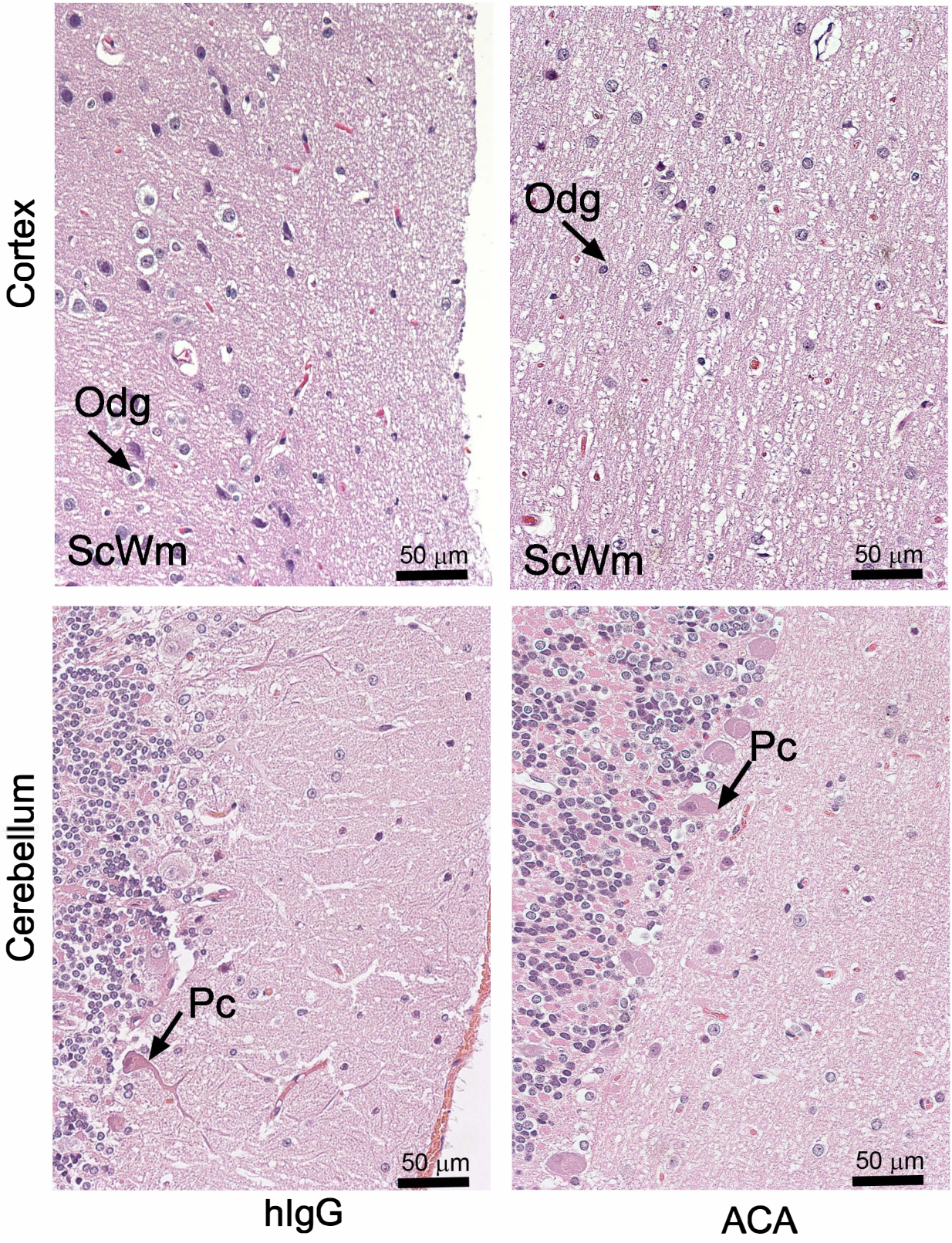
Supplementary Figure 3. Cell parameters of the blood samples collected at various time points from rabbits maintained for 12 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.



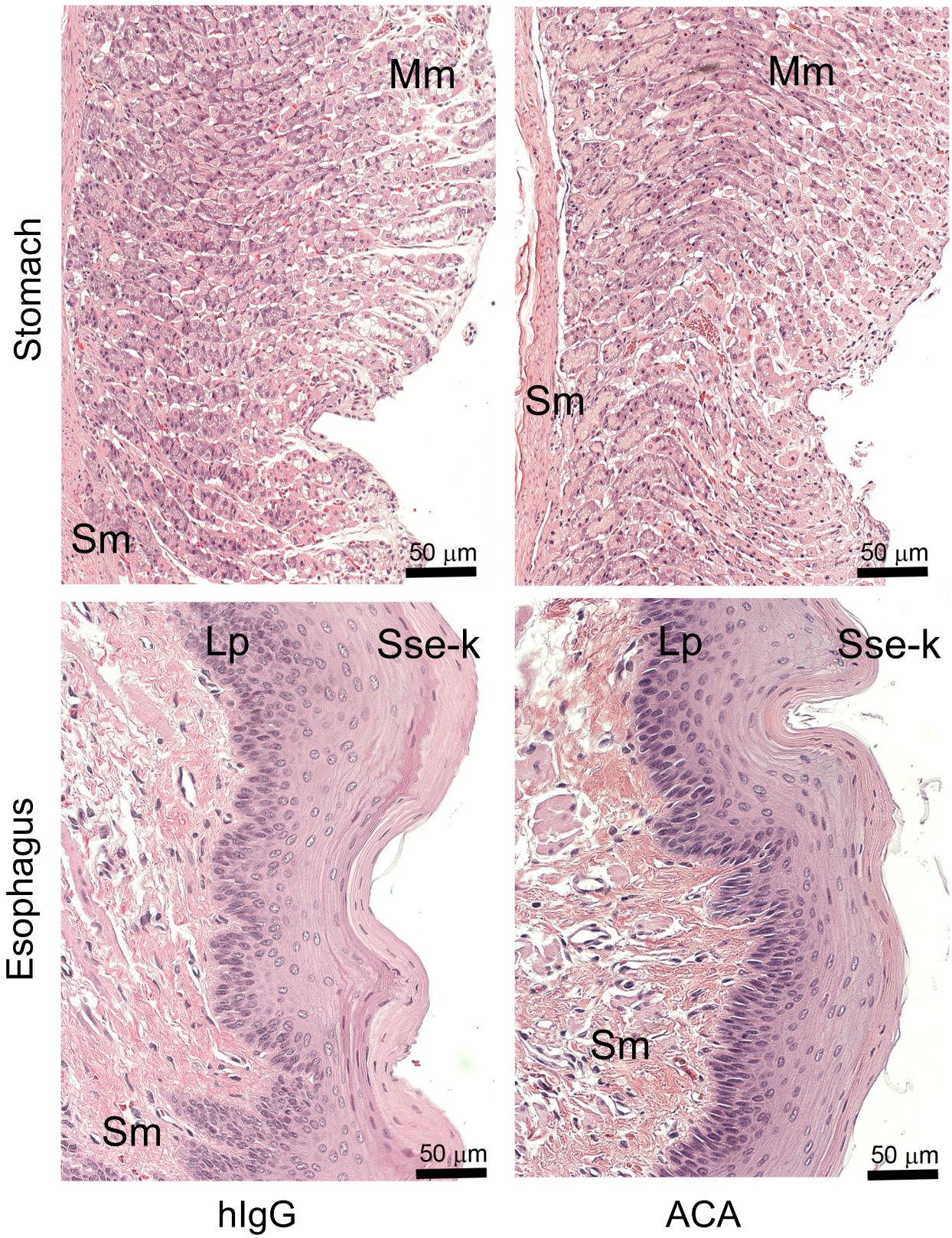
Supplementary Figure 4. Biochemical parameters of the blood samples collected at various time points from rabbits maintained for 12 weeks after surgery in the presence (red lines) or the absence (black lines) of the ACA.

Supplementary Figures 5 to 12:

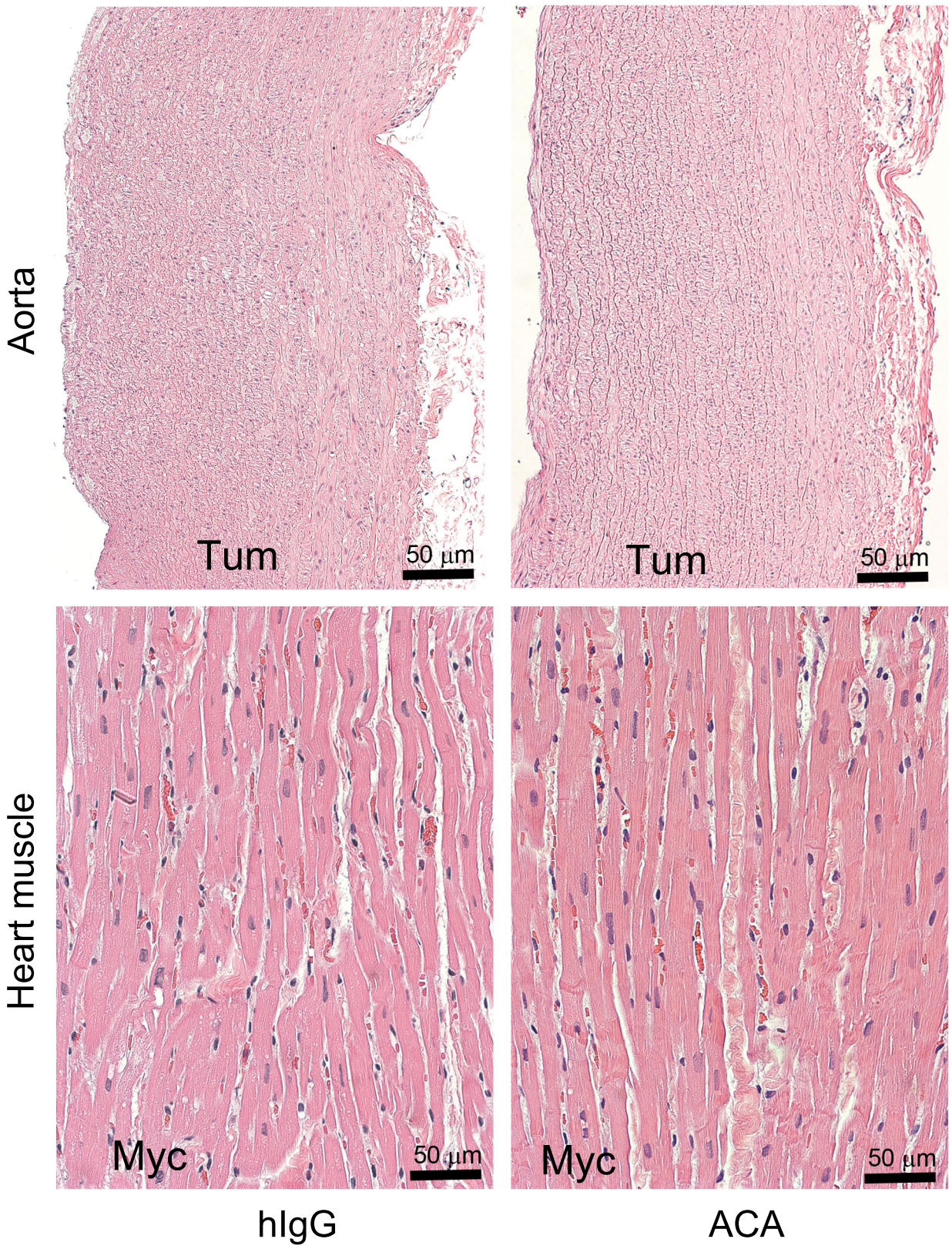
Histology of tissues and organs collected from rabbits treated with the ACA or control antibody for 8 weeks.



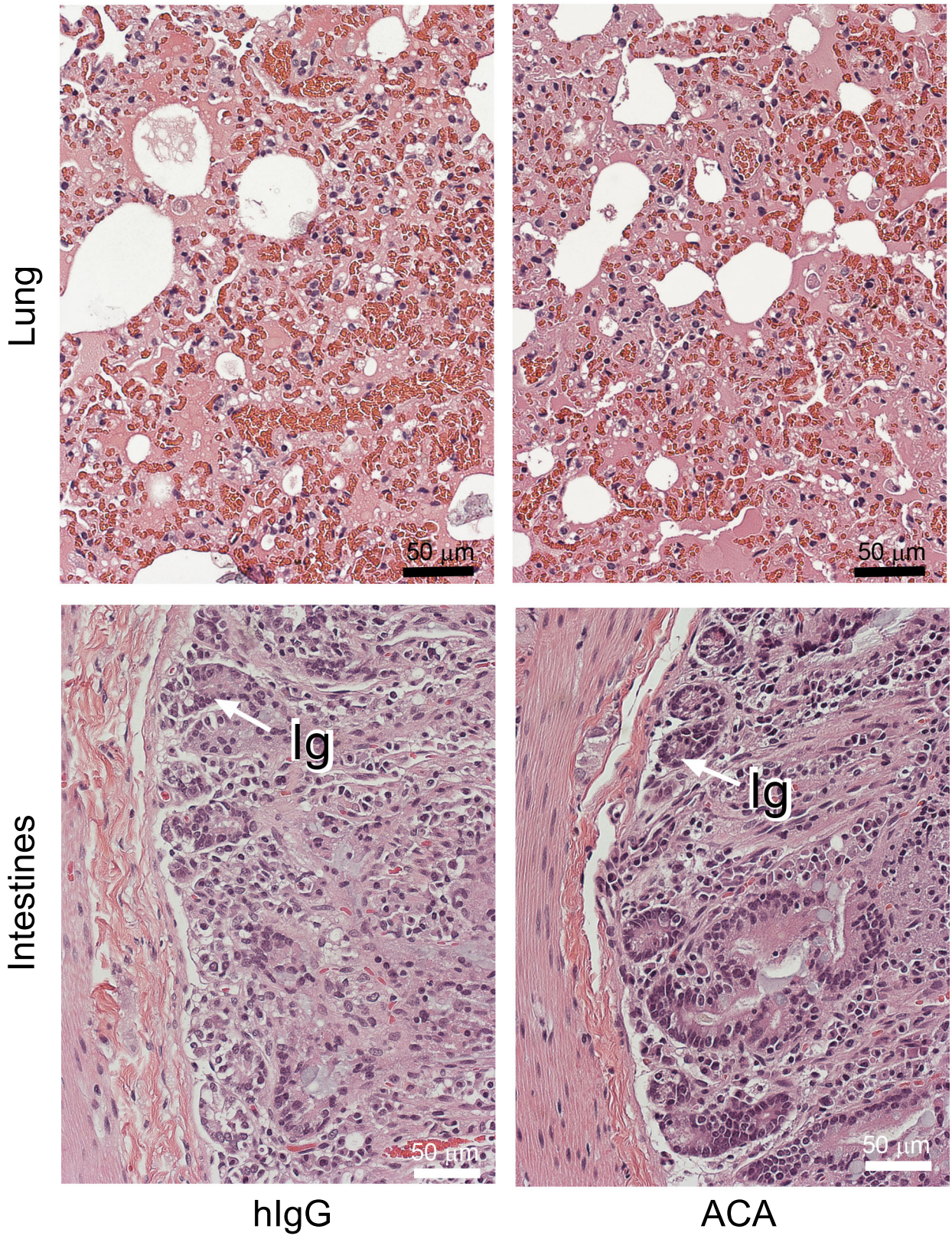
Supplementary Figure 5. Histology of brain tissue. Symbols: ScWm; subcortical white matter, Odg; oligodendroglia, Pc; Purkinje cells, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



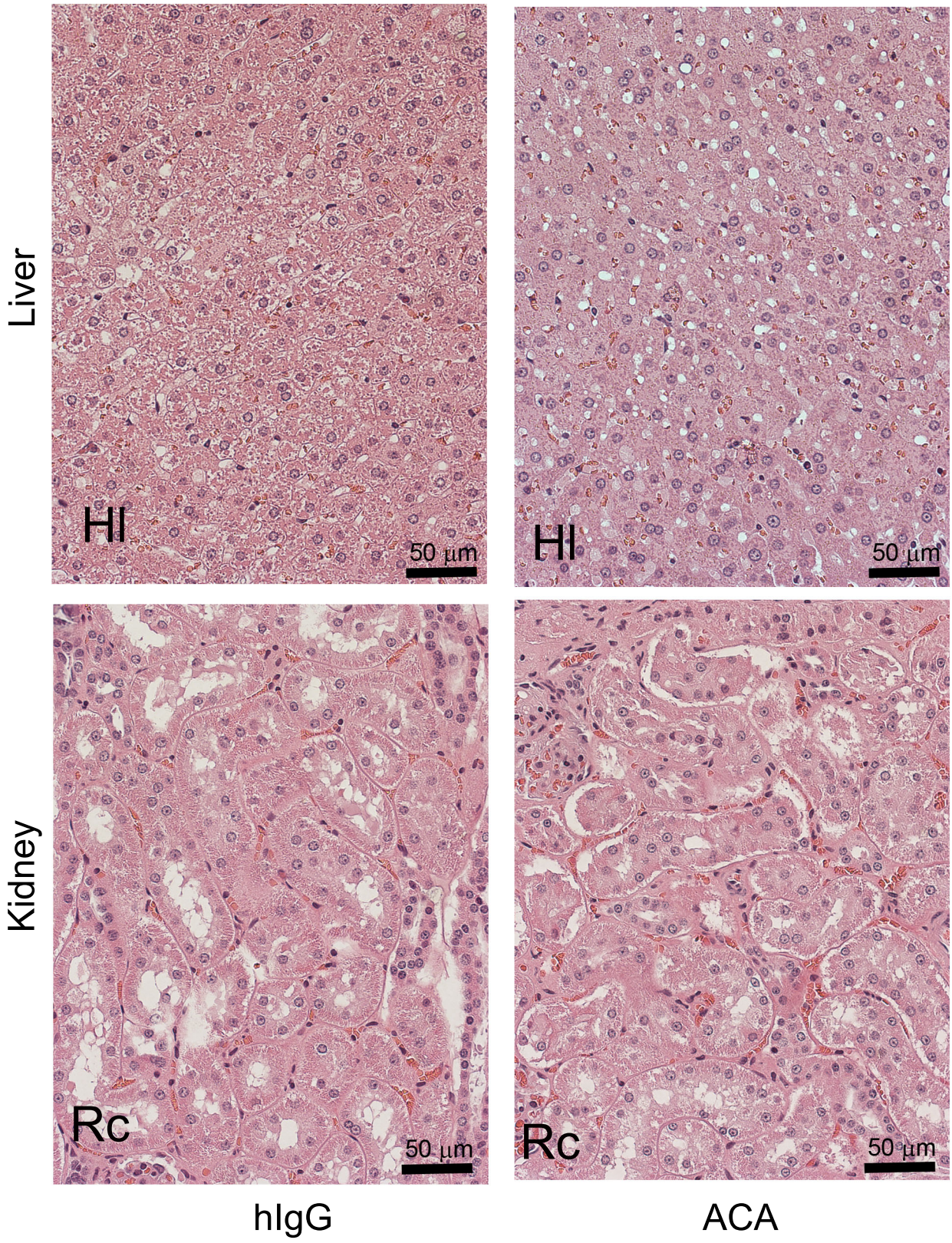
Supplementary Figure 6. Histology of stomach and esophagus. Symbols: Mm; mucous membrane, Sm; submucosa, Lp; lamia propia, Sse-k; stratified squamous epithelium-keratinized, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



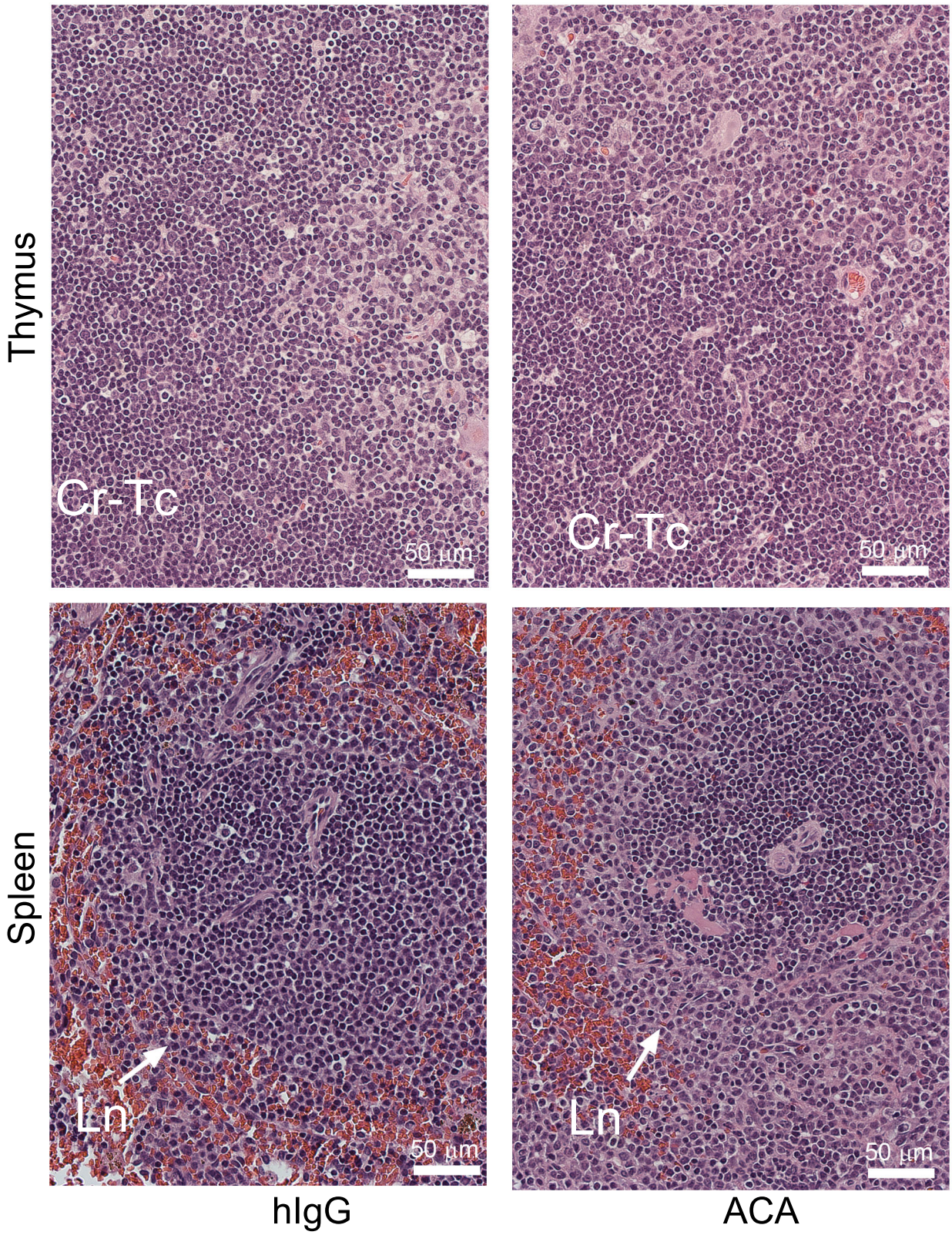
Supplementary Figure 7. Histology of aorta and heart muscle. Symbols: Tum; tunica media, Myc; myocardium, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



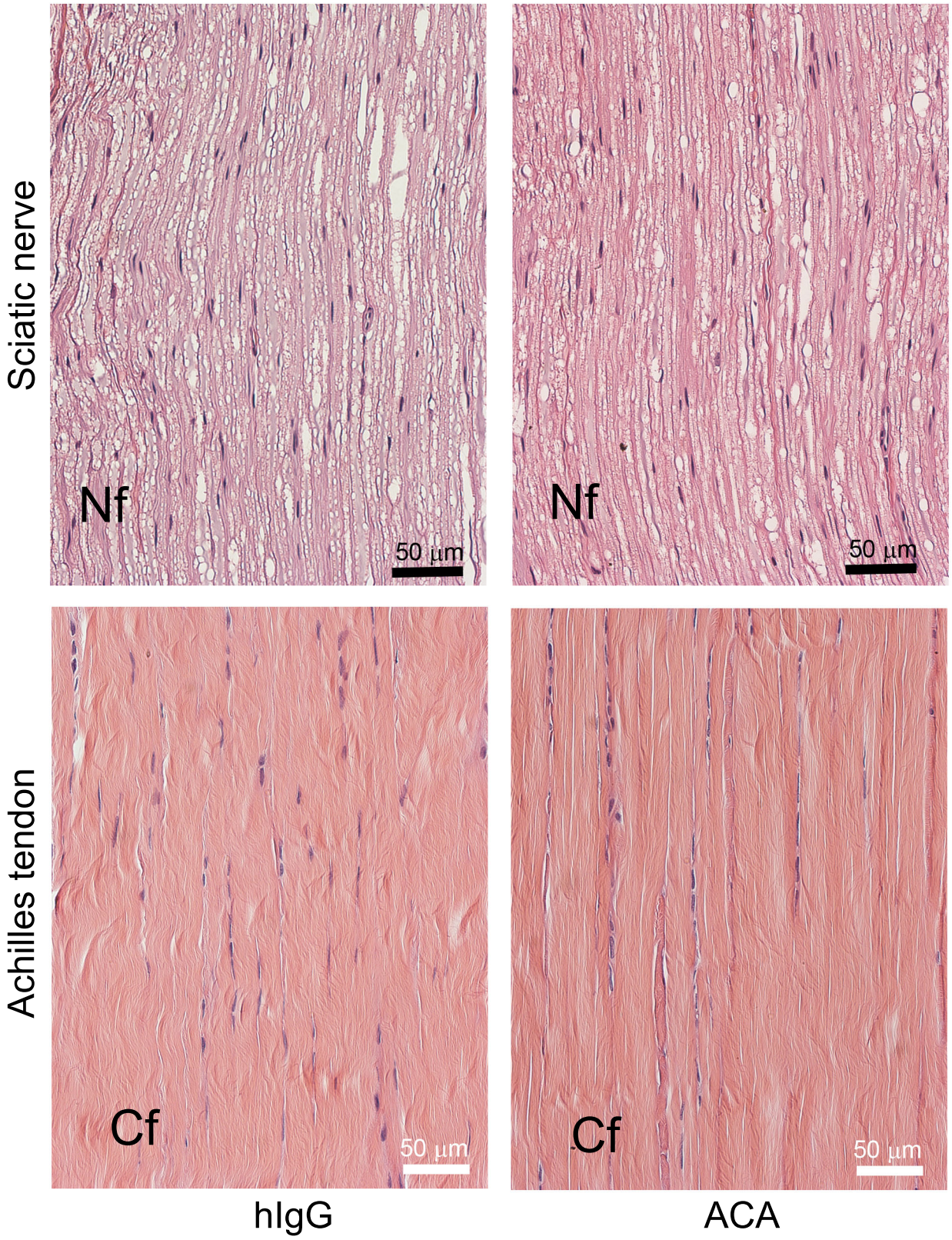
Supplementary Figure 8. Histology of the lung tissue and intestines. Symbols: Ig; intestinal glands, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA. Note: Because the lungs were not inflated prior to the fixation, the lung tissue appears compact.



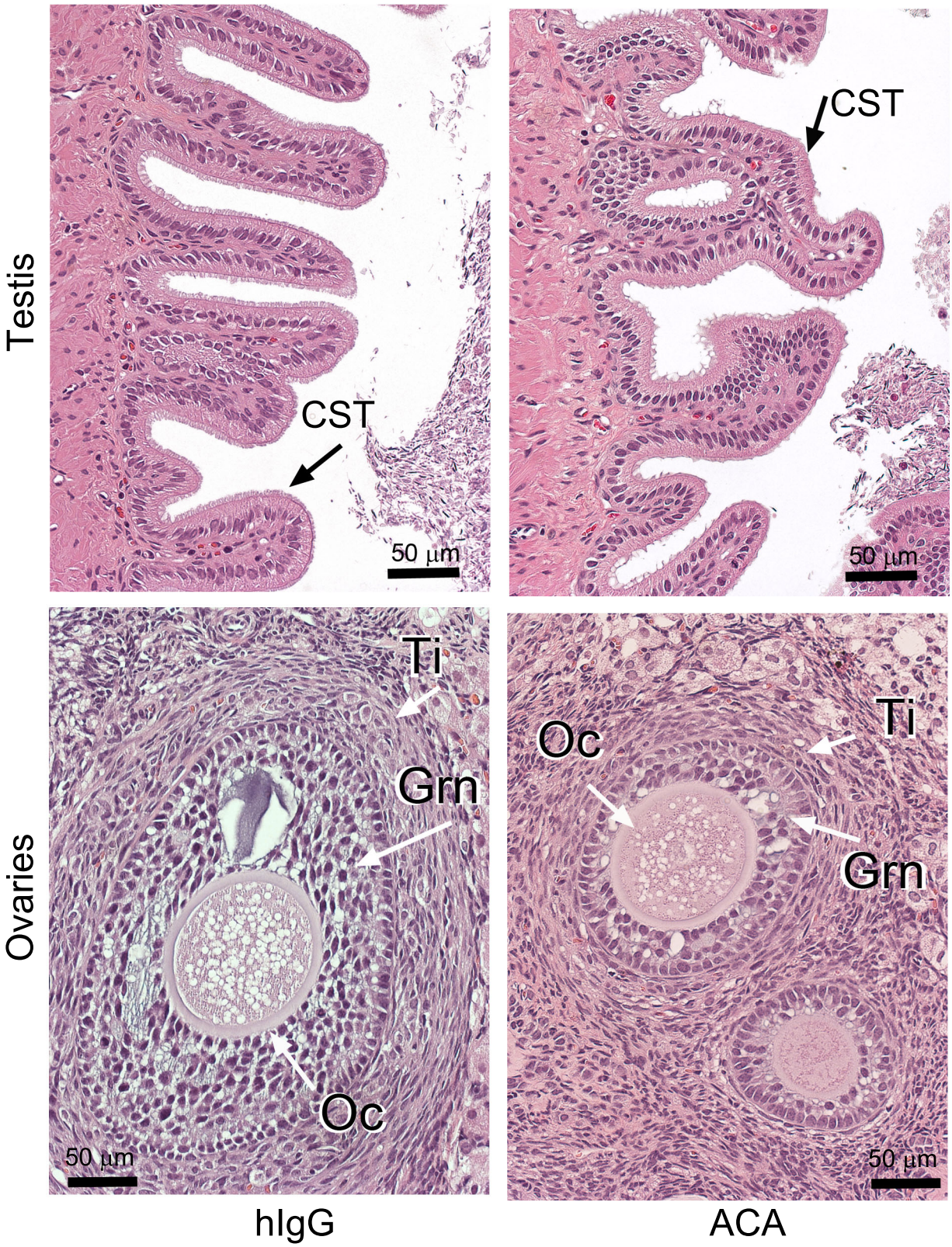
Supplementary Figure 9. Histology of liver and kidney. Symbols: Hl; hepatic lobule, Rc; renal cortex, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



Supplementary Figure 10. Histology of spleen and thymus. Symbols: Cr-Tc; cortex thymic cells, Ln; lymphoid nodule, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



Supplementary Figure 11. Histology of the sciatic nerve and the Achilles tendon. Symbols: Nf; neural filaments, Cf; collagen fibers, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.



Supplementary Figure 12. Histology of testis and ovaries. Symbols: CST; convoluted seminiferous tubules, Grn; granulosa cells, Ti; theca interna, Oc; oocyte, hIgG; rabbits treated with control IgG, ACA; rabbits treated with ACA.