**Dynamic Microenvironmental Stiffness and Serum Composition Modulate Sex-Specific Pulmonary Artery Adventitial Fibroblast Activation**

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**ABSTRACT**

Pulmonary arterial hypertension (PAH) is a non-reversable condition that causes stiffening and narrowing of the pulmonary arteries. Many factors have been correlated with the initiation of pulmonary vascular remodeling in PAH. Female patients are more susceptible to, but have increased survival for, PAH, which the underlying mechanisms are not fully understood. This study focuses on human pulmonary arterial adventitial fibroblast (hPAAF) activation, which increases production of extracellular matrix proteins and microenvironmental stiffness in the pulmonary arteries. Here, we employed soft hydrogels that were dynamically stiffened to observe how the age and sex of human serum influenced hPAAF activation in response to microenvironmental stiffening. Results showed female and male cells responded differently to increases in microenvironmental stiffness and serum composition. Female hPAAF activation was relatively high on both soft and stiffened hydrogels, with little difference in activation between the two conditions, unless cultured in younger (age < 50) female serum. Male hPAAFs were less activated than female cells on soft hydrogels and less responsive to increases in microenvironmental stiffness regardless of serum composition. Increased circulating sex-hormone concentrations decreased activation on soft hydrogels, while increasing activation on stiffened hydrogels. Male-cell activation was highly influenced by progesterone, which was measured to be high in the commercially available fetal bovine serum tested here. Collectively, these results suggest that it may be possible to model the estrogen paradox observed in PAH *in vitro* and that it is critical for researchers to report cell sex and serum source when conducting *in vitro* experimentation.

**KEYWORDS**

Pulmonary arterial hypertension, hydrogel, biomaterial, sex-differences, sex hormones

## 1. INTRODUCTION

Pulmonary arterial hypertension (PAH) is a chronic disease that causes stiffening and narrowing of the arteries in the lung, resulting through overactive pulmonary vasculature remodeling. This remodeling increases vascular resistance, causing the heart to work harder to pump blood through the lungs, and eventually leads to right ventricular heart failure. In the United States and Europe, there are 15 to 50 people per million who have PAH, with females four times as likely to develop PAH than males [1, 2]. Despite the increased prevalence of PAH in females, males tend to have worse right ventricle function and survival rates once they develop PAH. This disparity is due to the 'estrogen paradox' or 'estrogen puzzle', where studies have found evidence to demonstrate increased estrogen levels promote development of PAH but estrogen also has protective effects protecting against severe disease development [3-6]. It has been reported that the average age of diagnosis in females is approximately 36 ± 15 years [1-3] and although female predominance in PAH incidence and survival is well established, patients continue to receive the same treatments regardless of their sex [4]. There remains a need to engineer improved cell-culture models, which more accurately recapitulate these key aspects of PAH pathophysiology using cells and media additives derived from female or male patients, and better understand why females are more likely to develop PAH but survive longer than males. Accordingly, in this study, dynamic

hydrogels that mimic the increases in microenvironmental stiffness from healthy (E = 1-5 kPa) to hypertensive pulmonary arterial tissues (E>10 kPa) [7] and cell-culture media containing sex- and age-specific human sera were employed to investigate sex-specific adventitial fibroblast activation in response to microenvironmental stiffening.

There are three main cell types responsible for vascular remodeling, each residing in one of the three layers of the pulmonary artery wall: endothelial cells of the tunica intima, smooth muscle cells of the tunica media, and adventitial fibroblasts. This investigation focuses on adventitial fibroblast activation in the pulmonary arterial walls because fibroblasts are responsible for extracellular matrix (ECM) deposition, which significantly contributes to arterial remodeling [8]. Abnormal fibroblast activation increases ECM deposition, resulting in vessel stiffening and thickening. In PAH, vessel stiffening and thickening occurs through a buildup of collagens and matrix metalloproteinases (MMPs) and a concurrent decrease of their regulators, tissue inhibitors of metalloproteinases (TIMPs). A decrease of TIMPs allows for MMPs to stimulate more ECM remodeling [9]. This cycle leads to mechanistic downstream consequences for cells that interact with the ECM, such as increases in fibrotic pathways. Activated fibroblasts differentiate into myofibroblasts in response to increased microenvironmental stiffness through mechanotransduction. Myofibroblast differentiation can be characterized by expression of alpha-smooth muscle action (αSMA), cell-shape changes, and increased ECM production [8-10].

Fibroblast activation has been studied *in vitro* using a variety of cell sources and biomaterials. Aguado et al. showed that sex-specific valvular interstitial cells, which are aortic valve resident fibroblasts, had increased levels of activation on stiff

microenvironments than on soft microenvironments [11]. A study by Bertero et al. showed that human pulmonary arterial adventitial fibroblast (hPAAF) had increased fibrotic response when grown on stiffer substrates than when grown on soft substrates [12]. Both of these studies used fetal bovine serum (FBS), as FBS is commonly used as a supplement in cell culture media for *in vitro* models because it contains amino acids, fatty acids, growth factors, hormones, and vitamins [13]. Although FBS is widely used, it presents various biological challenges such as unexpected cell growth characteristics, cytotoxicity of uncharacterized factors, batch-to-batch variability, and undisclosed amounts of circulating sex hormones [14]. A few studies have moved to using human serum in cell culture medium as an FBS replacement to avoid these limitations [14]. Aguado et al. investigated the effect of patient serum obtained before and after aortic valve replacement on fibroblast activation using biomaterials with elastic modulus values matching values for healthy cardiac tissue *in vitro*. Patient serum from before aortic valve replacement tended to increase fibroblast activation while patient serum from after aortic valve replacement tended to decrease activation of fibroblasts. This differential activation was likely due to the increased immune response, cytokine signaling, and extracellular matrix proteins present before aortic valve replacement compared to the after aortic valve replacement serum [15]. One study collected and analyzed male and female human serum for 174 different analytes. The results showed that, in addition to circulating sex hormones, 77 of the analytes were significantly different between male and female sera, 40 analytes were elevated in female serum, and 37 different analytes were elevated in male serum [16]. Research on the effects of sex hormones on pulmonary artery cell types have investigated the influence of

estrogens, specifically estradiol (E2), on the increased prevalence of PAH in females [3]. These studies are especially important because estrogen and progesterone levels change over the lifespan in female patients, with measurable decreases directly correlated to aging, and both woman and men that develop PAH tend to have higher levels of circulating E2 [3].

Here, we build on this work by presenting a strategy to model pulmonary artery adventitial fibroblast activation, using poly(ethylene glycol)-alpha-methacrylate (PEGαMA) hydrogels that accurately replicate the stiffness of healthy distal pulmonary artery tissue (1-5 kPa) and can undergo a secondary photopolymerization step to stiffen the hydrogel (>10 kPa) to replicate vessel stiffening [7]. Female or male hPAAFs were grown on these hydrogels in cell-culture medium supplemented with FBS to observe the inherent differences in fibroblast activation in response to matrix stiffening, where fibroblast activation was measured by αSMA expression. Sex-specific human sera from donors, grouped by age (< 50 or ≥ 50 years old) were then used to supplement the cells, examining how sex-matched or mis-matched serum affected fibroblast activation in response to matrix stiffening. A design of experiment analysis determined the magnitude of the influence of each variable (e.g., stiffness, cell sex, serum sex, and serum age) on fibroblast activation.

## 2. MATERIALS AND METHODS

### 2.1 PEGαMA Synthesis

Poly(ethylene glycol)-hydroxyl (PEG-OH; 8-arm, 10 kg $mol^{-1}$; JenKem Technology) was dissolved in anhydrous tetrahydrofuran (THF; Sigma-Aldrich) in a

flame-dried Schlenk flask and purged with argon. Sodium hydride (NaH; Sigma-Aldrich) was added with a funnel into the reaction at 3× molar excess to PEG-hydroxyl groups and reacted for 45 minutes at room temperature. Ethyl 2-(bromomethyl)acrylate (EBrMA; Ambeed, Inc.) was added drop-wise at a 6× molar ratio to PEG-OH groups, and the reaction was stirred at room temperature for 48 hours protected from light. The mixture was neutralized with 1 N acetic acid until gas evolution ceased and filtered through Celite 545 (EMD Millipore). The solution was concentrated by rotary evaporation at 50 °C and precipitated in cold diethyl ether (Fisher Scientific) at 4 °C overnight. The solid product was then dried under vacuum overnight at room temperature. The product was purified using dialysis against deionized water (1 kg $mol^{-1}$ MWCO, ThermoFisher) for four days, then flash frozen at -80 °C, and lyophilized to give the final product. The functionalization of the product was verified by 1H NMR. 1H NMR (300 MHz, CDCl3): δ (ppm) 1.23 (t, 6H, CH3–), 3.62 (s, 114H, PEG backbone), 4.17–4.21 (t, s, 8H, –CH2–C(O)–O–O, –O–CH2–C([double bond, length as m-dash]CH2)–), 5.90 (s, 1H, –C = CH2), 6.31 (s, 1H, –C[double bond, length as m-dash]CH2) [17]. PEGαMA with functionalization over 90% as measured by comparing the αMA vinyl end group peak to that of the PEG backbone was used in subsequent experiments (Figure S1).

**2.2 Hydrogel Formation**

PEGαMA hydrogels were made by dissolving the PEGαMA in pH 8.4 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Life Technologies) at 12.5-15.5 weight percent (wt%). 1-4,Dithiothreitol (DTT; Sigma-Aldrich) and KCGPQGIWGQCK (MMP-2 degradable peptide; GenScript) were used as crosslinkers in a 70:30 molar

ratio respectively and were dissolved in 15 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich). Fibronectin mimetic peptide (CGRGDS; GL Biochem) was used at a 2 mM concentration to facilitate cell adhesion to hydrogels and was dissolved in TCEP.

Hydrogels for rheological evaluation were formed by placing 40-μL drops of precursor solution between two glass slides covered in parafilm, and allowing 30 min for polymerization. Hydrogels for stiffening experiments were swelled in phosphate buffered saline (PBS; HyClone) with 2.2 mM lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; Sigma-Aldrich) photoinitiator overnight. The hydrogels were stiffened by exposure to ultraviolet (UV) light at 365 nm, 10 mW $cm^{-2}$ (Omnicure, Lumen Dynamics) for 5 min. Previous studies have shown no significant differences in fibroblast viability and transcriptome when the cells were exposed to 365 nm light [18].

Hydrogels for cell-culture experiments were fabricated by placing 90-μL drops of precursor solution between a 3-(trimethoxysilyl)propyl acrylate (Sigma-Aldrich) silanated 18-mm glass coverslip and a glass slide treated with SigmaCote (Sigma-Aldrich), and allowing 30 min for polymerization. These hydrogels were soaked in growth media (basal medium with 0.1% insulin, 0.1% gentamicin sulfate – amphotericin, 0.1% human epidermal growth factor, 0.2% human fibroblast growth factor-beta, and 5% fetal bovine serum (FBS); Lonza) overnight at 37 °C prior to cell seeding.

**2.3 Hydrogel Characterization**

Rheology was performed as previously described [19]. Mechanical properties of soft and stiffened hydrogels were assessed on a Discovery HR2 rheometer (TA Instruments) using an 8 mm parallel plate geometry and the Peltier plate set at 37 °C.

The geometry was lowered until 0.03 N axial force was read, and the gap distance was recorded. The gap distance was adjusted starting at 25% compression until the storage modulus (G') stabilizes under compression, and the percent compression at which G' plateaued was used for measurement samples [20]. The samples were exposed to frequency oscillatory strain with a frequency range of 0.1 to 100 rad $s^{-1}$ at 1% strain. The elastic modulus (E) was calculated using rubber elastic theory, assuming a Poisson's ratio of 0.5 for bulk measurements of elastic hydrogel polymer networks.

**2.4 hPAAF Activation**

Female and male hPAAFs (Creative Bioarray (female; donor age = 11)); AccelGen (male; donor age = 2)) were expanded at 37°C and 5% $CO_2$ on tissue-culture-treated polystyrene in growth media (basal medium with 0.1% insulin, 0.1% gentamicin sulfate – amphotericin, 0.1% human epidermal growth factor, 0.2% human fibroblast growth factor-beta, and 5% FBS; Lonza). Female and male hPAAFs were seeded at 20,000 cells $cm^{-2}$ onto the hydrogels in growth media. The next day the media was changed to activation media (growth media with 1% serum). Sera used in these experiments included FBS (Gibco) or pooled human sera from healthy donors purchased from Innovative Research. Human sera were pooled together for experiments, age < 50 (younger) sex-specific sera were pooled together in equal parts and age ≥ 50 (older) sex-specific sera were pooled together in equal parts. Human sera information can be found in Table 1. All hPAAFs were cultured on soft hydrogels for 7 days with 2.2 mM LAP added to the activation media on day 6. Half of the hydrogels were exposed to UV light at 365 nm, 10 mW $cm^{-2}$ (Omnicure, Lumen Dynamics) for 5 min on day 7 to stiffen these samples and media was changed to ensure the LAP was

rinsed out. Soft hydrogels were collected for analysis on day 7 and stiffened hydrogels were collected on day 9, as previously described [19].

**Table 1.** Human sera information.

| Sex | Age | Pooled |
|---|---|---|
| Female | 63 | Age ≥ 50 |
| Female | 50 | |
| Female | 43 | Age < 50 |
| Female | 36 | |
| Female | 29 | |
| Male | 64 | Age ≥ 50 |
| Male | 56 | |
| Male | 47 | Age < 50 |
| Male | 20 | |
| Male | 20 | |

Samples were fixed with 4% (vol/vol) paraformaldehyde (PFA, Electron Microscopy Sciences) and permeabilized with 0.5% Triton X-100 (Thermo Scientific) solution for 20 minutes, quenched with 100 mM glycine (Fisher Scientific) for 15 minutes, and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 hour. All steps for immunostaining were performed at room temperature. Primary alpha smooth muscle actin (αSMA) antibody (mouse anti-human αSMA monoclonal antibody (MA5-11547); Invitrogen) was diluted 1:250 in 3% BSA and 0.1% Tween 20 (Thermo Scientific) in PBS (IF solution), and incubated for 1 hour. Samples were washed 3 times with IF solution. Goat anti-mouse Alexa Fluor-555 (Invitrogen) secondary antibody was diluted 1:250 in IF solution and Actin 488 ReadyProbes (AlexaFluor 488 phalloidin; Invitrogen) were added to the solution according to the manufacturer's instructions.

Samples were incubated with this solution for 1 hour and washed 3 times with IF solution. 4′,6-diamidino-2-phenylindole (DAPI; BioLegend) was diluted 1:10,000 from a 5 mg $mL^{-1}$ stock solution in deionized water, incubated for 10 minutes, and washed 1 time with deionized water, 1 time in PBS, and 1 time in IF solution. Samples were mounted using Prolong Gold Antifade (Invitrogen) for imaging and sealed with nail polish for storage.

Six images were taken at random fields of view on each sample on an Olympus upright epifluorescence microscope (BX-63) and the images were processed with ImageJ software (NIH). Fibroblast activation was defined as the percentage of cells with αSMA present over the total number of cells counted by DAPI-stained nuclei ((cells positive for αSMA/total # cells) *100%).

**2.5 Sex-Hormone Analysis**

Testosterone (Abcam ab174569) concentrations in sera were measured with an ELISA following the manufacture's protocol.

Estradiol and Progesterone concentrations in sera were measured by the Clinical and Translational Research Center at the University of Colorado, Anschutz Medical Campus using a chemiluminescent immunoassay (Beckman Coulter) following the manufacturer's protocol.

**2.6 Statistical Methods**

Data were analyzed using two-way analysis of variance (ANOVA) with Tukey's honest statistical difference (HSD) test for multiple comparisons. Values were reported as mean ± 95% confidence interval (95% CI) and considered significantly different if the

p-value < 0.05, with each data point representing a different sample. Statistical analysis was performed using GraphPad Prism software.

A custom design of experiments generated with JMP software (SAS, Cary, NC) analyzed the impact of each variable on fibroblast activation. The level of each variable (e.g., stiffness, cell sex, serum sex, and serum age) and the associated hPAAF activation results were entered into the custom design and analyzed with a least-squares regression model to plot response curves and determine the most significant factors.

## 3. RESULTS

### 3.1 Hydrogel Synthesis and Characterization

Soft hydrogels were designed to mimic the stiffness of healthy pulmonary artery tissue and stiffened into the range of hypertensive pulmonary artery tissue. Soft hydrogels were polymerized by an off-stoichiometry (r-ratio: 0.375) Michael addition reaction under slightly basic conditions with two crosslinkers (DTT and an MMP2-degradable crosslinker), along with a cell adhesion peptide (CGRGDS), which mimics binding sites on the ECM protein fibronectin (Figure 1A). Stiffened hydrogels were formed by soaking the soft hydrogels in a photoinitiator (LAP) overnight then applying UV light to the hydrogels to initiate homopolymerization between the unreacted moieties on the PEGαMA (Figure 1A). An average elastic modulus value of 4.6 ± 0.9 kPa was achieved for the soft hydrogels matching the range for healthy pulmonary artery (Figure 1B). Likewise, an elastic modulus of 19.7 ± 1.1 kPa was measured for the stiffened hydrogels, matching the range for hypertensive arterial tissue (Figure 1B).

The female and male hPAAFs were grown in 2D on the dynamic hydrogels in two different conditions, soft and stiffened. hPAAFs were cultured on soft hydrogels for 7 days before the samples were collected and analyzed. On day 6 the LAP photoinitiator was added to the cell-culture medium for all samples and allowed to soak into the hydrogels overnight. For the stiffened condition, UV light was applied to the hydrogels on day 7 to initiate stiffening. Media was changed the next day to remove any unreacted photoinitiator and the cells were grown for two more days on the stiffened hydrogel before the samples were collected and analyzed (Figure 1C).

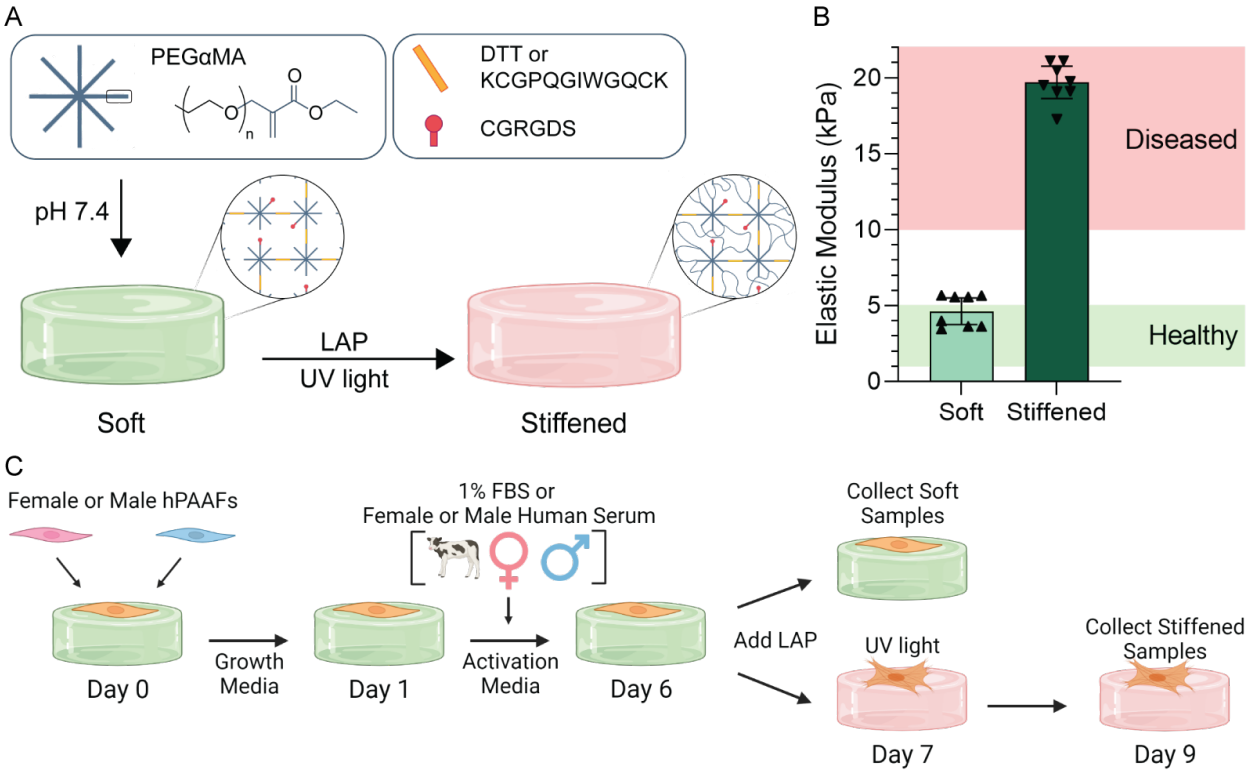


**Figure 1.** Characterization of PEGαMA hydrogels. A) Schematic of the hydrogel formulation containing PEGαMA, DTT, MMP-2 degradable crosslinker, and CGRGDS were reacted under basic conditions to form soft hydrogels. Incubation in LAP and exposure to UV light initiated photopolymerization to stiffen the hydrogels. B)

Rheological measurements showed the average elastic modulus (E) of soft hydrogels (E = 4.63 ± 0.88 kPa) were within the range of healthy pulmonary arteries (1-5 kPa) and the average elastic modulus of stiffened hydrogels (E = 19.70 ± 1.06 kPa) increased into the range of pathologic pulmonary arteries (>10 kPa). Columns represent mean ± 95% CI, n = 8. C) Schematic representation of the timeline used for cell experiments. Created with BioRender.com.

### 3.2 Sex-Specific hPAAF Activation in Response to Increases in Microenvironment Stiffness

Female or male cells were cultured in medium containing 1% FBS on soft or stiffened hydrogel conditions to measure the differences in hPAAF activation related to cell origin in response to increases in microenvironment stiffness. Samples were immunostained for αSMA, a myofibroblast marker, to measure the percent of activated hPAAFs on the two hydrogel conditions at the conclusion of the experiment. F-actin and nuclei (DAPI) were also stained to visualize cell morphology and quantify the total number of cells, respectively (Figure 2A and B). Upon quantification, female hPAAFs were 62.6% ± 17.1% activated on soft hydrogels and 67.9% ± 15.8% activated on stiffened hydrogels (Figure 2C). Male hPAAFs were 44.6% ± 10.4% activated on soft hydrogels and 81.8% ± 7.2% activated on stiffened hydrogels (Figure 2C). Image analysis quantification of αSMA expression was verified by western blot analysis (Figure S2). Female hPAAFs demonstrated a non-significant 1.08-fold change in activation of between stiffened and soft conditions, while male hPAAFs had a significant change of 1.83-fold in activation.

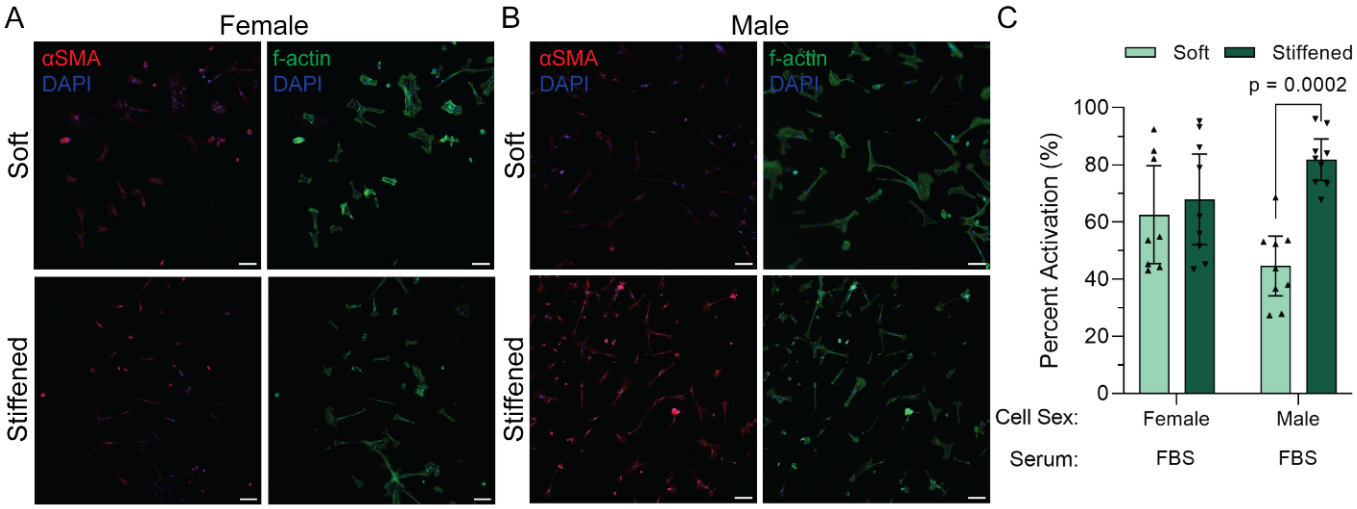


**Figure 2.** Female and male hPAAF activation in response to increases in microenvironmental stiffness. Representative fluorescence microscopy images of A) female and B) male hPAAFs grown on soft and stiffened conditions in FBS that were stained for αSMA (red), f-actin (green), and DAPI (blue). C) Quantification of the percent of activated fibroblasts showed no significant differences in female hPAAF activation on stiffened hydrogels compared to soft controls. A significant increase in male hPAAF activation was measured ($p$ = 0.0002) indicating that female cells grown in FBS were not as sensitive to increases in microenvironmental stiffness. Columns represent mean ± 95% CI, n = 3, two-way ANOVA, Tukey HSD. Scale bar: 100 μm.

**Concentration of Sex Hormones in Human Serum Samples**

The concentrations of the sex hormones estradiol, progesterone, and testosterone were analyzed in serum from each donor (Table 2). The concentration of progesterone was comparable between both age ≥50 and age < 50 female sera, and with age ≥ 50 male sera. Age < 50 male sera had higher levels of progesterone present than the other human sera, while FBS has the highest concentration of progesterone. Estradiol

concentrations were similar in both age ≥ 50 and age < 50 male sera, while estradiol was the highest in age < 50 female sera followed by age ≥ 50 female sera. FBS had the lowest concentration of estradiol present. Testosterone was comparable between FBS, age ≥50 and age < 50 female sera. Age ≥50 and age < 50 male sera had similar levels of testosterone, but more testosterone than the female sera or FBS.

**Table 2.** Concentrations of estradiol, progesterone, and testosterone present in each of the sera used.

| Sex | Age | Estradiol (pg/mL) | | Progesterone (ng/mL) | | Testosterone (ng/mL) | |
|---|---|---|---|---|---|---|---|
| | | | Average | | Average | | Average |
| Fetal Bovine Serum | | 25 | | 12.0 | | 0.401 | |
| Female | 63 | 27 | 57.5 | 0.1 | 0.2 | 0.761 | 0.657 |
| Female | 50 | 88 | | 0.3 | | 0.552 | |
| Female | 43 | 64 | | 0.1 | | 0.339 | |
| Female | 36 | 54 | 115.7 | 0.1 | 0.17 | 0.402 | 0.353 |
| Female | 29 | 229 | | 0.3 | | 0.319 | |
| Male | 64 | 47 | 38 | 0.3 | 0.2 | 7.029 | 5.867 |
| Male | 56 | 29 | | 0.1 | | 4.704 | |
| Male | 47 | 32 | | 1.3 | | 5.391 | |
| Male | 20 | 29 | 38.3 | 1.5 | 1.0 | 3.013 | 4.428 |
| Male | 20 | 54 | | 0.3 | | 4.881 | |

### 3.3 Sex-Matched Human Serum and Microenvironmental Stiffness Modulate hPAAF Activation

hPAAFs were cultured in medium supplemented with 1% sex-matched human serum, pooled by age, on soft and stiffened hydrogels to investigate the effects of

serum composition and age-specific changes in circulating sex-hormone concentration on hPAAF activation in response to increases in microenvironment stiffness. Serum from individual donors were pooled together into four different groups separated by sex and then by age (Table 1). Cells were cultured in < 50 or ≥ 50 sex-matched sera and samples were immunostained for αSMA, f-actin, and DAPI (Figure 3A-E). Female hPAAFs grown on soft conditions were 50.5% ± 5.7% activated when grown in age < 50 female sera and 72.9% ± 7.2% on stiffened conditions representing a 1.44 fold-change in activation. Male hPAAFs demonstrated an average activation of 45.1% ± 4.4% on soft conditions and 64.6% ± 9.6% on stiffened conditions when grown in age < 50 male serum representing a fold-change in activation of 1.43 (Figure 3C). The average percent female hPAAF activation was 64.3% ± 7.4% on soft conditions and 55.1% ± 9.4% on stiffened conditions when grown in age ≥ 50 female serum, showing a fold-change in activation of 0.86, whereas the average percent activation of male hPAAFs was 62.2% ± 5.6% on soft conditions and 86.7% ± 5.7% on stiffened conditions with a fold-change in activation of 1.39 when grown in age ≥ 50 male serum. That is, over 60% of female and male cells cultured on soft hydrogels in sex-matched, age ≥ 50 serum were activated and there was no statistically significant increase in female cell activation upon stiffening. In fact, the average percent male cell activation was significantly higher on stiffened surfaces compared to female cell activation, indicating that circulating sex hormone concentrations may influence female cell activation levels.

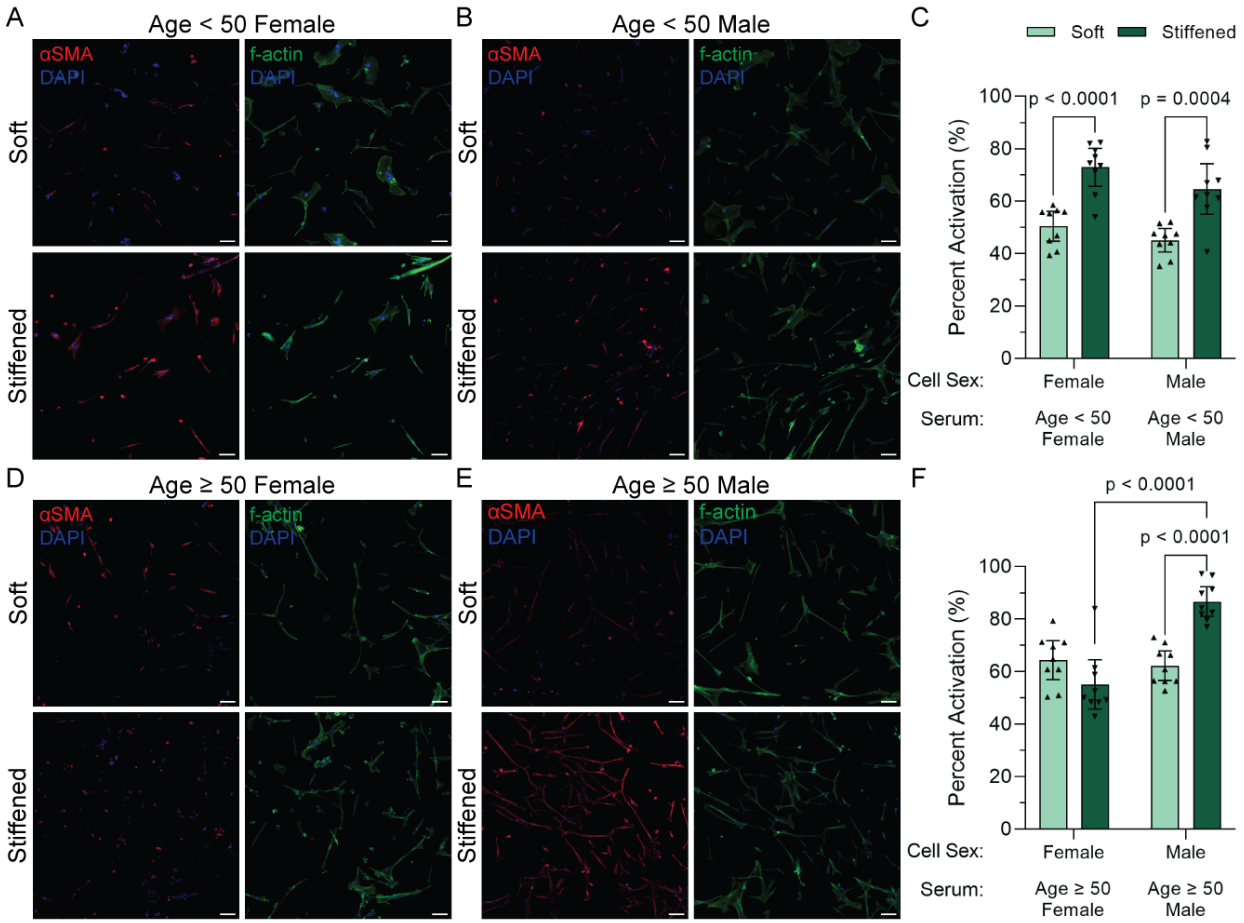


**Figure 3.** Female and male hPAAF activation in sex-matched human sera. Representative fluorescence microscopy images of A) female hPAAFs in age < 50 female sera and B) male hPAAFs grown in age < 50 male sera on soft and stiffened conditions were stained for αSMA (red), f-actin (green), and DAPI (blue). C) Quantification of the percent of activated fibroblasts of female and male hPAAFs grown in age < 50 female or male human serum, respectively, showed a significant increase in female and male hPAAF activation ($p$ = <0.0001 and 0.0004, respectively) in age < 50 sera on stiffened hydrogels compared to soft controls. Similar increases in activation were observed in female and male hPAAF activation in age < 50 serum. Columns represent mean ± 95% CI, n = 3, two-way ANOVA, Tukey HSD. Scale bar: 100 μm.

Representative fluorescence microscopy images of D) female hPAAFs in age ≥ 50 female serum and E) male hPAAFs grown in age ≥ 50 male serum on soft and stiffened conditions were stained for αSMA (red), f-actin (green), and DAPI (blue). F) Quantification of the percent of activated fibroblasts of female and male hPAAFs grown in age ≥ 50 female or male human serum, respectively, showed only a significant increase in male hPAAF activation in age ≥ 50 sera was measured ($p$ = <0.0001) on stiffened hydrogels compared to soft controls. There was not a significant increase in activation of the female cells grown in age ≥ 50 female serum. Columns represent mean ± 95% CI, n = 3, two-way ANOVA, Tukey HSD. Scale bar: 100 μm

### 3.4 Sex-Mis-Matched Human Serum and Microenvironmental Stiffness Modulate hPAAF Activation

To further explore the interplay between cell sex and circulating sex hormones, 1% human serum was added to the cell-culture media for cells of the opposite sex. Specifically, male serum cultured female cells and female serum cultured male cells. Samples were immunostained for αSMA, f-actin, and DAPI (Figure 4A-E). Male hPAAF activation in age < 50 female serum on the soft condition was 50.3% ± 6.1% and 78.9% ± 4.6% on the stiffened condition, while the female hPAAF activation in age < 50 male serum was 76.2% ± 10.5% on the soft condition and 88.0% ± 5.9% on the stiffened condition (Figure 4C). Male hPAAFs in female serum showed a 1.57-fold change in activation, while female hPAAFs in male serum resulted in a 1.15-fold change in activation. Female hPAAF activation when grown in age ≥ 50 male serum was 75.4% ± 4.4% on soft conditions and 81.6% ± 6.4% on stiffened conditions with a fold change in

activation of 1.08, whereas the activation of male hPAAFs grown in age ≥ 50 female serum was 87.8% ± 7.4% on soft conditions and 94.7% ± 3.9% on stiffened conditions with a fold change in activation of 1.08 (Figure 4F). Female and male cells cultured in sex mis-matched serum age<50 showed similar trends to cells cultured in FBS, while cells cultured in age ≥ 50 sex mis-matched serum all showed relatively high levels of activation.

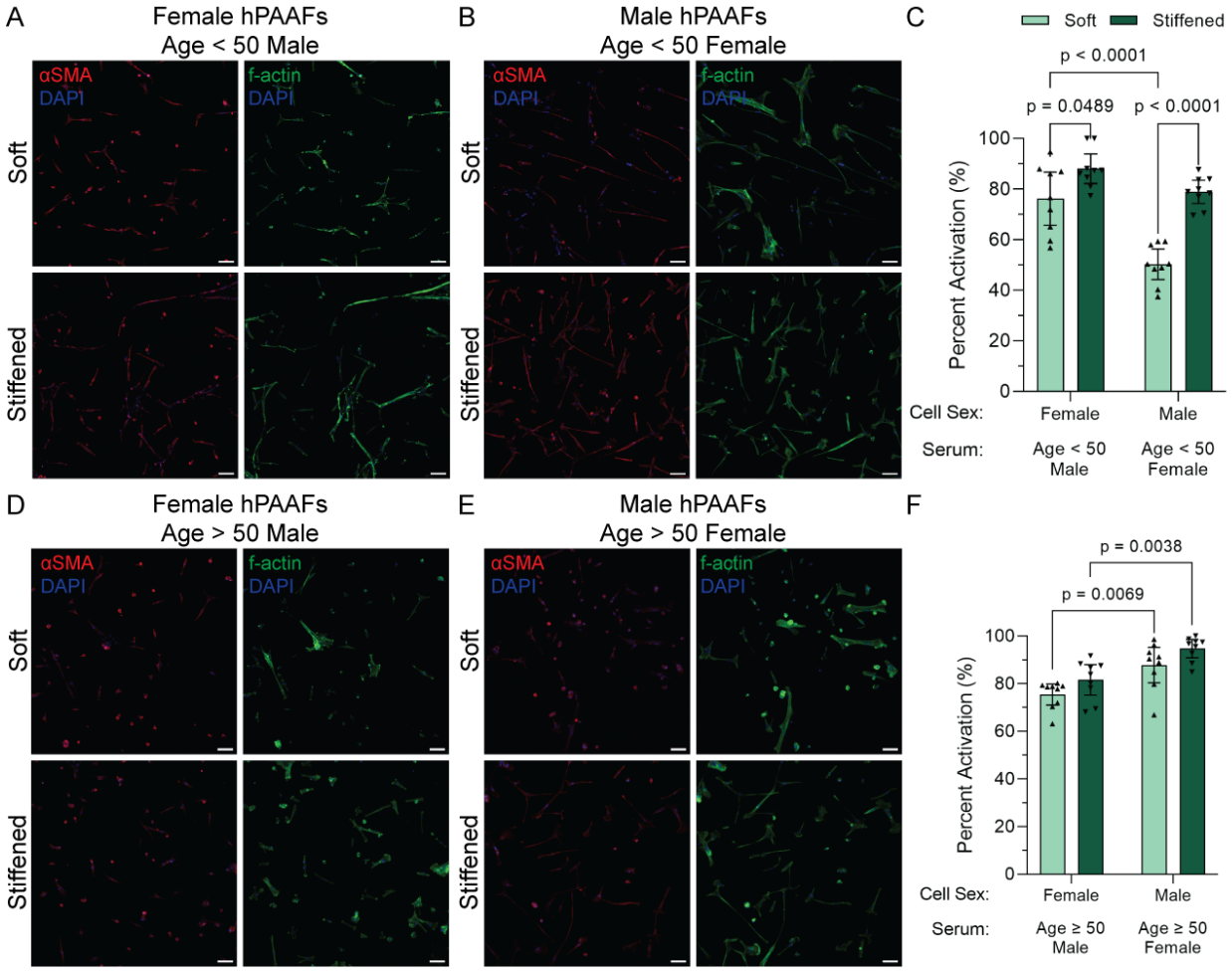


**Figure 4.** Female and male hPAAF activation in sex-mis-matched human serum. Representative fluorescence images of A) male hPAAFs in age < 50 female serum and B) female hPAAFs age < 50 male serum grown on soft and stiffened conditions were stained for αSMA (red), f-actin (green), and DAPI (blue). C) Quantification of activated

fibroblasts showed significant increases ($p$ = <0.0001 and 0.0489, respectively) in both male and female hPAAF activation in sex-mis-matched sera on stiffened hydrogels compared to soft controls, though the difference in activation of female hPAAFs in age < 50 male sera was minimal. A significant increase in activation was measured ($p$ = <0.0001) in female hPAAFs on soft hydrogels compared to male hPAAFs on soft hydrogels. Columns represent mean ± 95% CI, n = 3, two-way ANOVA, Tukey HSD. Scale bar: 100 μm. Representative fluorescence microscopy images of D) female hPAAFs in age ≥ 50 male serum and E) male hPAAFs grown in age ≥ 50 female serum on soft and stiffened conditions were stained for αSMA (red), f-actin (green), and DAPI (blue). F) Quantification of the percent of activated fibroblasts of female and male hPAAFs grown in age ≥ 50 male or female human serum, respectively, showed there was no significant increase in activation of either female or male hPAAFs in sex-mis-matched age ≥ 50 sera on stiffened hydrogels compared to soft controls. The activation seen in female hPAAFs was significantly lower in age ≥ 50 male serum than male hPAAFs were in age ≥ 50 female sera on both soft and stiffened conditions. Columns represent mean ± 95% CI, n = 3, two-way ANOVA, Tukey HSD. Scale bar: 100 μm.

### 3.5 Microenvironmental Stiffness and Circulating Sex Hormone Concentrations Interact to Increase hPAAF Activation

Next, a design of experiment approach further analyzed how each variable (microenvironmental stiffness, cell sex, serum sex, and serum age) and interactions among the variables tested influenced hPAAF activation. Microenvironmental stiffness had the greatest impact on hPAAF activation with a linear correlation between stiffness

and activation (Figure 5A). Male hPAAFs on average were more responsive than female hPAAFs to increases in microenvironmental stiffness. There was a greater difference in activation of male hPAAFs between soft and stiffened conditions compared to female hPAAFs (Figure 5B). Age was also significantly correlated with hPAAF activation. Age ≥ 50 sera showed greater levels of activation compared to age < 50 sera even on soft conditions (Figure 5C). There was also a larger difference in activation between soft and stiffened conditions in age < 50 sera than in age ≥ 50 sera. We further investigated these differences by evaluating serum pooled by sex and age using a human cytokine antibody array with 42 targets (Figure S3). Results showed increases in some biochemical cues that could increase cellular activation, such as interleukin 1β (IL-1β), chemokine (C-X-C motif) ligand 1 (CXCL1), monocyte chemoattractant protein-1 (MCP-1), CC chemokine ligand 17 (CCL17), CCL5, platelet-derived growth factor-BB (PDGF-BB), and transforming growth factor β-1 (TGFβ-1) in older male serum. Younger male serum exhibited increased levels of IL-15 and IL-13. No increases in the 42 cytokines evaluated here were detected in older female serum compared to young. In fact, younger female serum exhibited increases in IL-3, CXCL-1, CXCL-12, and PDGF-BB compared to older (Figure S3). Next, hPAAF activation was correlated with individual sex hormone concentrations (estradiol, progesterone, and testosterone) quantified in each individual serum. Increased levels of each sex hormone resulted in increases in activation on stiffened conditions. Conversely, on soft conditions, increased levels of all three sex hormones were correlated with decreases in activation for both female and male hPAAFs (Figure 5D-F). Female cells showed higher levels of activation on soft microenvironments than on stiffened microenvironments with low

estradiol concentration. At low concentrations of progesterone or testosterone, differences in female hPAAFs activation in response to microenvironmental stiffening were negligible; however, at higher concentration, stiffening resulted in increased activation. Similarly, differences in male hPAAFs activation levels between soft and stiffened conditions were smaller at lower sex hormone concentrations and increased with increasing concentrations of sex hormones. The combinatorial analysis of microenvironmental stiffness, estradiol concentration, progesterone concentration, and testosterone concentration effects on female or male hPAAF activation showed that microenvironmental stiffness had the biggest impact on activation for both female and male hPAAFs (Figure 5G-H). The magnitude of the effect of stiffness on male hPAAFs was 2.1 times greater than the magnitude of the stiffness effect on female hPAAFs. Neither female nor male hPAAF activation was highly influenced by the hormone concentrations alone, but when the interaction of each of the hormones was paired with stiffness, the effect of each hormone can be seen. The effect of estradiol concentration with stiffness had the biggest influence in female hPAAF activation after stiffness alone. Progesterone concentration combined with stiffness had the biggest influence on male hPAAF activation after stiffness alone.

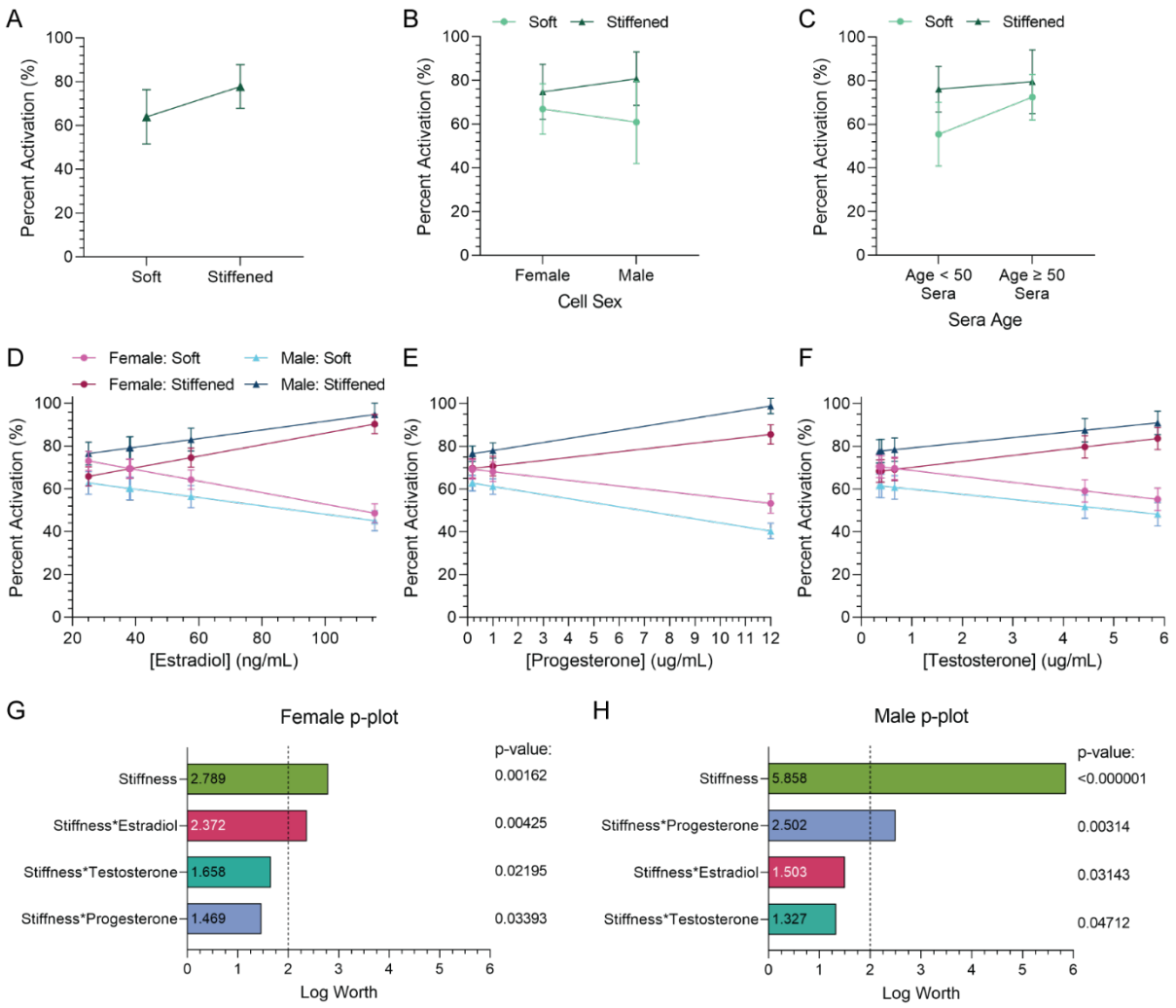


**Figure 5.** Interaction effect plots. Main effects plots showing how variables A) Microenvironmental stiffness, B) cell sex and microenvironmental stiffness, and C) sera age and microenvironmental stiffness influence hPAAF activation. Male hPAAFs tend to be more sensitive to changes in microenvironmental stiffness than female hPAAFs. Age < 50 human sera allowed for bigger changes in activation based on changes in microenvironmental stiffness, while age ≥ 50 sera tended to have higher levels of activation regardless of microenvironmental stiffness. Interaction effect plots of D) estradiol concentration, E) progesterone concentration, and F) testosterone concentration in the sera with cell sex and stiffness. As sex hormone concentrations

increased, activation on soft surfaces decreased, while activation of stiffened surfaces increased. Plots show mean ± 95% CI. P-plot of interaction effects on G) female hPAAFs and H) male hPAAFs. Female hPAAF activation was influenced the most by stiffness, followed by the compound effect of estradiol concentration and stiffness, while male hPAAF activation was greatly influenced by stiffness followed by the compound effect of progesterone concentration and stiffness.

## 4. DISCUSSION

Here, we quantified the differences in sex-specific hPAAF activation in response to increases in microenvironment stiffness while cultured in FBS, sex-matched or mis-matched, age-separated human donor sera. Increased stiffness combined with older serum age led to the highest levels of fibroblast activation. Interaction analysis revealed increased stiffness and higher levels of estradiol or progesterone together were key drivers of fibroblast activation in female and male cells, respectively. Fibroblast activation and vessel stiffening are hallmarks of PAH pathogenesis [9, 21]. Prior studies have demonstrated the impact of substrate stiffness on all three of the pulmonary artery cell types, but despite the known sexual dimorphism in PAH [1-6], these studies did not distinguish between male and female cells, and FBS (with undocumented hormonal milieu) was used as a supplement in the culture medium. Only a few studies have characterized the sex differences in pulmonary artery cells grown *in vitro*.

In this work, we found intrinsic sex-based differences in hPAAF activation in response to increases in microenvironmental stiffness as well as differences related to the species, sex, and age of the serum used to supplement the cell-culture medium. When female and male hPAAFs were grown on dynamically stiffened hydrogels using FBS-supplemented media, female hPAAFs were less sensitive to increases in microenvironmental stiffness than male hPAAFs, with a significant increase in activation of male hPAAFs on stiffened surfaces compared to soft controls, but no change in activation of female hPAAFs with stiffening. However, female hPAAFs were more activated, even on soft conditions, than male hPAAFs. While we hypothesized that female hPAAF activation would likely be higher than activation of male hPAAF (since

PAH predominately affects females over males [2-4]), the finding that female hPAAF were not responsive to stiffened conditions in FBS was not expected. A previous study investigating sex-specific human valvular interstitial cells, aortic valve leaflet fibroblasts, that were grown on soft and stiff substrates found female cells were more sensitive to microenvironmental stiffness than male cells. In that study, female cells showed significant increases in activation on stiff hydrogel compared to the soft hydrogel while grown in FBS, with no change in activation of the male cells on stiff hydrogels compared to soft hydrogels [11]. The differences in activation patterns found in our study and the published study may be due to inherent differences in responsiveness of these two cell types that are derived from different tissues.

The highest incidence of PAH is in females between the ages of 30-60 years old (average age = 36 ± 15 years old) and studies have shown that higher levels of estradiol are associated with PAH development in both females and males [1-4]. Therefore, to study the influence of circulating sex hormones on hPAAF activation, we used human sera separated by sex and age. When assessing the effect of sex-matched, age-separated human sera on hPAAF activation, female hPAAFs were responsive to increases in microenvironmental stiffness when grown in pooled sera from either female or male donors age < 50. We hypothesized that female hPAAFs would demonstrate increased activation when cultured with age < 50 female sera, as this serum had the highest pooled concentration of estradiol (115.7 pg/mL), and high levels of estradiol are associated with increased PAH development. However, when cultured with sera from younger male donors where estradiol was low, female hPAAF were also responsive to microenvironmental stiffening but showed higher levels of

activation on soft substrate. Interestingly, when grown with sera from donors ≥ 50 of either sex, hPAAF activation was higher on soft substrates, similar to the younger male sera, and there were no changes in activation with increases in stiffness, recapitulating what was seen when female hPAAF were grown in FBS. This was interesting as estradiol concentration in the FBS was low, similar to the levels in the ≥ 50 female and male sera (Table 2), suggesting that estradiol may be a key regulator of activation levels in female hPAAF, especially on soft substrates.

Male hPAAFs cultured with age < 50 sera from either sex showed an increase in activation in response to stiffening, similar to levels measured when grown with FBS. When grown with age ≥ 50 sera, male hPAAFs demonstrated an increased activation on soft substrates and only showed responsiveness to stiffening when cultured with male ≥ 50 sera. The significant increases in activation of the male hPAAFs grown in the older male sera on the stiffened hydrogels could correlate to why males tend to have worse outcomes, as males tend to develop PAH at older ages than females do [2, 3, 22, 23].

We next used sex-mismatched sera to further understand the role of sex hormones versus innate sex differences in hPAAF activation. This experiment enabled investigation of how sex-mismatch sera compositions and sex hormone concentrations impacted cellular activation in response to microenvironmental stiffening. Female hPAAFs showed increased levels of activation in sera from males of all ages and did not respond to microenvironmental stiffening. These findings seem to be contradictory to previous studies looking at the protective effects of testosterone within the pulmonary vasculature[3], as the male serum has much higher concentrations of testosterone

compared to the female serum. These differences may be related to other differences in the serum composition. A study by Ramsey et al. found there are 77 differentially expressed analytes between female and male sera [16] and another study examining the impact of serum composition on fibroblast activation found that increased inflammatory cytokines in sera from patients undergoing aortic valve replacement increased aortic leaflet fibroblast activation [15].

Male hPAAFs showed similar levels of activation on soft and stiff microenvironments when cultured with sera from young donors of either sex and with FBS. This suggests that the high levels of predominately female sex hormones (estradiol or progesterone) may be allowing the cells to behave similarly under these different conditions. However, when male cells were grown in mismatched (female) age ≥ 50 sera, very high levels of activation on both soft and stiffened conditions were measured, higher than when the male cells were grown in any other serum composition. While studies have found that pulmonary vessels significantly stiffen with age [24], few studies have investigated how serum composition changes with age between the sexes outside of sex hormone concentrations. One study on serum biomarker levels between younger and older males found differences in nine types of cytokines levels between the age groups [25]. The results of our cytokine analysis supported the hypothesis that increased inflammatory signaling in older male serum could have initiated higher levels of activation; however, the same trend was not clear for female serum (Figure S3). Together, these data underscore the need for further identification of components driving fibroblast activation in response to microenvironmental stiffening within human serum.

Lastly, our analysis using a design of experiments approach demonstrated microenvironmental stiffness was the only individual variable to significantly impact fibroblast activation. Next, interaction effects were examined between the five factors studied here: substrate stiffness, cell sex, estradiol concentration, progesterone concentration, and testosterone concentration. The interaction between sex hormone concentrations and stiffness also significantly influenced fibroblast activation. As sex hormone concentrations increased, the fold change in activation between soft and stiffened conditions in both female and male cells increased as well. This trend was consistent among all three sex hormones measured: estradiol, progesterone, and testosterone. Interaction analysis showed that at low concentrations of estradiol and progesterone (Figure 5D and E), female hPAAFs were not responsive to stiffening, while higher sex hormone concentrations increased female hPAAF sensitivity to microenvironmental stiffness. The analysis also confirmed our findings that male hPAAFs were always sensitive to increases to microenvironmental stiffness, and that sensitivity increased with higher sex hormone concentrations.

Microenvironmental stiffness was the factor that affected both female and male hPAAFs the most, however male hPAAF activation was influenced twice as responsive to changes in compared to female hPAAFs (Figure 5G and H). Female hPAAFs were secondly impacted by the compound effect of estradiol and stiffness. Estrogens can increase prevalence of PAH but are also cardioprotective and protect against severe disease progression [3-5]. Interestingly, in this work, high levels of estradiol were correlated with lower levels of activation on soft surfaces while demonstrating increased activation on stiffened surfaces. These findings suggest that increased estradiol

concentrations increase female hPAAF sensitivity to microenvironmental stiffness changes and therefore it may be possible to model the estrogen paradox observed in the PAH patient population *in vitro.*

Male hPAAFs were secondly impacted by the compound effects of progesterone and stiffness. These results were unexpected because progesterone is not typically considered a primary sex hormone in males, further highlighting the need for considering the concentration of progesterone when culturing male hPAAFs. The FBS evaluated here had the highest concentration of progesterone. Progesterone is the most dominate hormone present during pregnancy; therefore, it is unsurprising that FBS, which is isolated by blood collection from a fetal calf heart, has high concentrations of progesterone [3, 14]. However, the combination of male cells with FBS-supplemented medium is one of the most common modalities for investigating fibroblast activation *in vitro* [11, 12, 26-28]. Therefore, it is critical for researchers to report cell sex and serum hormone concentrations when performing activation assays *in vitro.*

Together, the mixed results evident in our appropriate and inappropriate sex-hormone data support the estrogen puzzle identified by other researchers in PAH. However, our study shows that high levels of estradiol exacerbated female hPAAF activation in response to increased microenvironmental stiffness, suggesting that estradiol increases female hPAAF sensitivity to changes in stiffness. Thus far, the work in this study supports developments in the field of PAH looking at the biological differences of sex-specific pulmonary artery cell types and sex differences within circulating molecules. Future work will challenge hypotheses related to cellular activation in PAH in 3D to achieve a more physiologically relevant geometry of the

pulmonary artery [29], which could include all three pulmonary artery cell types. A limitation of this work was cell lines used where derived from prepubescent donors, which could impact how biochemical cues from sera donors older than cell donors influence cellular activation. Additional investigations could include incorporation of cells and/or sera derived from PAH patients to better replicate PAH pathogenesis. A comprehensive analysis of other differential circulating factors in control versus PAH patient-derived sera would also further this line of investigation.

## 5. CONCLUSION

In this study, we measured differences in sex-specific hPAAF activation in response to increases in microenvironmental stiffness and sex-matched or mis-matched, age-separated human donor sera. Overall female hPAAFs tended to have increased levels of activation on soft and stiffened conditions, unless cultured in younger female sera. The results from this study suggest that higher sex-hormone concentrations increased female hPAAF sensitivity to increased microenvironmental stiffness. Male hPAAFs were more activated on stiffened microenvironments than on soft microenvironments regardless of the concentration of the sex hormones. This study found that male hPAAFs were more sensitive to increases to microenvironmental stiffness, and that sensitivity increased with higher sex hormone concentrations, especially in response to progesterone. Collectively, these results suggest that it is critical to intentionally design experiments considering cell sex and serum source as biological variables when conducting *in vitro* experimentation. Carefully controlling these aspects of cell culture will lead to improved models of the estrogen puzzle observed in PAH patients *in vitro*.

### Author Disclosures

Chelsea Magin reports a relationship with the Colorado Bioscience Institute that includes board membership. Chelsea Magin reports a relationship with Boulder IQ that includes consulting or advisory. Chelsea Magin reports a relationship with the University of Iowa, Colorado State University, and the Vanderbilt University that includes speaking and lecture fees. Chelsea Magin has patent #PCT/US2019/012722 pending to

University of Colorado. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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**Data Availability**

The data that support the findings of this study are openly available at the following URL/DOI: https://doi.org/10.17632/gs44hgz895.1.

## REFERENCES

[1] D.J. Levine, Pulmonary arterial hypertension: Updates in epidemiology and evaluation of patients, 2021.
[2] K.W. Prins, T. Thenappan, World Health Organization Group I Pulmonary Hypertension: Epidemiology and Pathophysiology, Cardiol Clin 34(3) (2016) 363-74.
[3] J. Hester, C. Ventetuolo, T. Lahm, Sex, Gender, and Sex Hormones in Pulmonary Hypertension and Right Ventricular Failure, Compr Physiol 10(1) (2019) 125-170.
[4] T. Hye, P. Dwivedi, W. Li, T. Lahm, E. Nozik-Grayck, K.R. Stenmark, F. Ahsan, Newer insights into the pathobiological and pharmacological basis of the sex disparity in patients with pulmonary arterial hypertension, Am J Physiol Lung Cell Mol Physiol 320(6) (2021) L1025-L1037.
[5] Y.N. Martin, C.M. Pabelick, Sex differences in the pulmonary circulation: implications for pulmonary hypertension, Am J Physiol Heart Circ Physiol 306(9) (2014) H1253-64.
[6] C.E. Ventetuolo, A. Praestgaard, H.I. Palevsky, J.R. Klinger, S.D. Halpern, S.M. Kawut, Sex and haemodynamics in pulmonary arterial hypertension, Eur Respir J 43(2) (2014) 523-30.
[7] F. Liu, C.M. Haeger, P.B. Dieffenbach, D. Sicard, I. Chrobak, A.M. Coronata, M.M. Suarez Velandia, S. Vitali, R.A. Colas, P.C. Norris, A. Marinkovic, X. Liu, J. Ma, C.D. Rose, S.J. Lee, S.A. Comhair, S.C. Erzurum, J.D. McDonald, C.N. Serhan, S.R. Walsh, D.J. Tschumperlin, L.E. Fredenburgh, Distal vessel stiffening is an early and pivotal mechanobiological regulator of vascular remodeling and pulmonary hypertension, JCI Insight 1(8) (2016).
[8] J. Baum, H.S. Duffy, Fibroblasts and myofibroblasts: what are we talking about?, J Cardiovasc Pharmacol 57(4) (2011) 376-9.
[9] W. Sun, S.Y. Chan, Pulmonary Arterial Stiffness: An Early and Pervasive Driver of Pulmonary Arterial Hypertension, Front Med (Lausanne) 5 (2018) 204.
[10] E. Aikawa, Immunohistochemistry, Comprehensive Biomaterials2011.
[11] B.A. Aguado, C.J. Walker, J.C. Grim, M.E. Schroeder, D. Batan, B.J. Vogt, A.G. Rodriguez, J.A. Schwisow, K.S. Moulton, R.M. Weiss, D.D. Heistad, L.A. Leinwand, K.S. Anseth, Genes That Escape X Chromosome Inactivation Modulate Sex Differences in Valve Myofibroblasts, Circulation 145(7) (2022) 513-530.
[12] T. Bertero, K.A. Cottrill, Y. Lu, C.M. Haeger, P. Dieffenbach, S. Annis, A. Hale, B. Bhat, V. Kaimal, Y.Y. Zhang, B.B. Graham, R. Kumar, R. Saggar, R. Saggar, W.D. Wallace, D.J. Ross, S.M. Black, S. Fratz, J.R. Fineman, S.O. Vargas, K.J. Haley, A.B. Waxman, B.N. Chau, L.E. Fredenburgh, S.Y. Chan, Matrix Remodeling Promotes Pulmonary Hypertension through Feedback Mechanoactivation of the YAP/TAZ-miR-130/301 Circuit, Cell Rep 13(5) (2015) 1016-32.
[13] A.N. de Faria, D.C. Zancanela, A.P. Ramos, M.R. Torqueti, P. Ciancaglini, Estrogen and phenol red free medium for osteoblast culture: study of the mineralization ability, Cytotechnology 68(4) (2016) 1623-32.
[14] C. Tekkatte, G.P. Gunasingh, K.M. Cherian, K. Sankaranarayanan, "Humanized" stem cell culture techniques: the animal serum controversy, Stem Cells Int 2011 (2011) 504723.
[15] B.A. Aguado, K.B. Schuetze, J.C. Grim, C.J. Walker, A.C. Cox, T.L. Ceccato, A.C. Tan, C.C. Sucharov, L.A. Leinwand, M.R.G. Taylor, T.A. McKinsey, K.S. Anseth,

Transcatheter aortic valve replacements alter circulating serum factors to mediate myofibroblast deactivation, Sci Transl Med 11(509) (2019).
[16] J.M. Ramsey, E. Schwarz, P.C. Guest, N.J. van Beveren, F.M. Leweke, M. Rothermundt, B. Bogerts, J. Steiner, L. Ruta, S. Baron-Cohen, S. Bahn, Molecular sex differences in human serum, PLoS One 7(12) (2012) e51504.
[17] X. Tong, J. Lai, B.-H. Guo, Y. Huang, A new end group structure of poly(ethylene glycol) for hydrolysis-resistant biomaterials, Journal of Polymer Science Part A: Polymer Chemistry 49(6) (2011) 1513-1516.
[18] E.R. Ruskowitz, C.A. DeForest, Proteome-wide Analysis of Cellular Response to Ultraviolet Light for Biomaterial Synthesis and Modification, ACS Biomater Sci Eng 5(5) (2019) 2111-2116.
[19] C.L. Petrou, T.J. D'Ovidio, D.A. Bolukbas, S. Tas, R.D. Brown, A. Allawzi, S. Lindstedt, E. Nozik-Grayck, K.R. Stenmark, D.E. Wagner, C.M. Magin, Clickable decellularized extracellular matrix as a new tool for building hybrid-hydrogels to model chronic fibrotic diseases in vitro, J Mater Chem B 8(31) (2020) 6814-6826.
[20] T.K.L. Meyvis, D.S.S. C., D. J., H.W. E., Rheological monitoring of long-term degrading polymer hydrogels, Journal of Rheology 43(4) (1999) 933-950.
[21] P.B. Dieffenbach, M. Maracle, D.J. Tschumperlin, L.E. Fredenburgh, Mechanobiological Feedback in Pulmonary Vascular Disease, Front Physiol 9 (2018) 951.
[22] D.B. Taichman, J. Mandel, Epidemiology of pulmonary arterial hypertension, Clin Chest Med 34(4) (2013) 619-37.
[23] K. Kozu, K. Sugimura, T. Aoki, S. Tatebe, S. Yamamoto, N. Yaoita, T. Shimizu, K. Nochioka, H. Sato, R. Konno, K. Satoh, S. Miyata, H. Shimokawa, Sex differences in hemodynamic responses and long-term survival to optimal medical therapy in patients with pulmonary arterial hypertension, Heart Vessels 33(8) (2018) 939-947.
[24] D. Sicard, A.J. Haak, K.M. Choi, A.R. Craig, L.E. Fredenburgh, D.J. Tschumperlin, Aging and anatomical variations in lung tissue stiffness, Am J Physiol Lung Cell Mol Physiol 314(6) (2018) L946-L955.
[25] C. Banerjee, J. Ulloor, E.L. Dillon, Q. Dahodwala, B. Franklin, T. Storer, P. Sebastiani, M. Sheffield-Moore, R.J. Urban, S. Bhasin, M. Montano, Identification of serum biomarkers for aging and anabolic response, Immun Ageing 8(1) (2011) 5.
[26] M. Das, E.C. Dempsey, J.T. Reeves, K.R. Stenmark, Selective expansion of fibroblast subpopulations from pulmonary artery adventitia in response to hypoxia, Am J Physiol Lung Cell Mol Physiol 282(5) (2002) L976-86.
[27] M.G. Frid, M. Li, M. Gnanasekharan, D.L. Burke, M. Fragoso, D. Strassheim, J.L. Sylman, K.R. Stenmark, Sustained hypoxia leads to the emergence of cells with enhanced growth, migratory, and promitogenic potentials within the distal pulmonary artery wall, Am J Physiol Lung Cell Mol Physiol 297(6) (2009) L1059-72.
[28] L.A. Cussac, G. Cardouat, N. Tiruchellvam Pillai, M. Campagnac, P. Robillard, A. Montillaud, C. Guibert, P. Gailly, R. Marthan, J.F. Quignard, J.P. Savineau, T. Ducret, TRPV4 channel mediates adventitial fibroblast activation and adventitial remodeling in pulmonary hypertension, Am J Physiol Lung Cell Mol Physiol 318(1) (2020) L135-L146.
[29] D. Davis-Hall, E. Thomas, B. Pena, C.M. Magin, 3D-bioprinted, phototunable hydrogel models for studying adventitial fibroblast activation in pulmonary arterial hypertension, Biofabrication 15(1) (2022).