Side-dependent effect in the response of valve endothelial cells to bidirectional shear stress

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Abstract

Endothelial cells covering the aortic and ventricular sides of the aortic valve leaflets are exposed to different stresses, in particular wall shear stress (WSS). Biomechanical stimuli actively regulate valve tissue structure and induce remodeling events leading to valve dysfunction. Endothelial to mesenchymal transformation (EndMT), for example, has been associated with aortic valve disease. The biomechanical response of cells at different sides of the leaflets has not been clearly characterized. To analyze the mechanical response of valve endothelial cells (VECs) we developed a unique fluid activation device that applies physiologically relevant pulsatile WSS. We characterized the morphology and function of adult porcine aortic VECs derived from the opposite sides of aortic valve leaflets following exposure to different pulsatile WSS. We found that elongation and orientation of cells in response to pulsatile WSS depends on their side of origin. Quantification of gene expression confirms phenotypic differences between aortic and ventricular VECs. Aortic VECs exposed to pulsatile WSS similar to that in vivo at the tip of aortic side of the valve leaflet upregulated pro-EndMT (*ACTA2*, *Snail*, *TGF β 1*) and inflammation (*ICAM-1*, *VCAM-1*) genes, whereas expression of endothelial markers like *PECAM-1* was decreased. Conversely, ventricular-VECs showed strong increase of *PECAM-1* expression and no activation of pro-EndMT marker. Finally, we found that stress-induced genes are upregulated in both cell types, at higher levels in ventricular compared to aortic VECs. Application of physiological shear stress levels using a fluid activation device therefore reveals functional differences in VECs derived from opposite sides of the aortic valve leaflets.

Introduction

The aortic valve maintains unidirectional blood flow between the left ventricle and the ascending aorta. Aortic valve leaflets are composed of three highly organized layers of extracellular matrix (ECM) populated by valve interstitial cells and covered by a monolayer of valve endothelial cells (VECs). The distinct layers of ECM within the leaflet are mainly composed of collagen fibers at the aortic side (*fibrosa*), elastin at the ventricular side (*ventricularis*) and proteoglycan and glycosaminoglycan (*spongiosa*) in between [1]. The composition of ECM components contributes to the structural and mechanical properties of the valve. Collagen provides tensile strength to the valve leaflet during opening and transfer the load to the aortic wall when the valve is closed [2]. Elastin facilitates the fast opening of the valve leaflet. The spongiosa absorbs shocks during cardiac cycle and also facilitates the relative internal rearrangements to protect the valve leaflet from damage [3]. VECs surrounding the valve leaflets are critical for circulatory function and blood-tissue interaction and can respond to changing stimuli by altering their normal functions [4]. Experimental studies have reported that aortic valve leaflets experience a wide range of wall shear stress (WSS) depending on the side (aortic or ventricular) and region (the base, belly and tip regions) at which they are located [5]. The three sub-regions of the aortic valve leaflet have been anatomically characterized by Cao and Sucosky (2017) as a region aligned along the circumferential direction, and spanning on third of the total length of the leaflet (Figure S1). While the ventricular side experiences a strong pulsatile unidirectional WSS, the aortic side experience a much lower recirculating WSS. Therefore, a WSS is a mechanical stimulus that greatly differs on either sides of the valve leaflets, and plays an important role in the development of side-dependent valve disease [6, 7]. Indeed, abnormal blood velocity and pressure can apply environmental changes, which will have consequences at the macro- to microscales promoting maladaptive tissue remodeling. Such mechanical stimuli can activate VECs, which transduce this mechanical signal to a

biochemical signal that leads to changes in gene expression and/or protein secretion, particularly of ECM components in the valve [8, 9].

At the macroscale, hemodynamic forces induce WSS on the side of the valve leaflets as well as deformation of the valve tissue under cyclic stretch. WSS modulates the function of valve cells and impacts the physiology and pathologies of these cells [10]. Recent 3D fluid structure interaction valve numerical modeling has investigated the macroscopic scale temporal and regional WSS characteristics on aortic valve leaflets under physiological flow [11, 12]. These studies confirmed side-specific differences in WSS pulsatility and magnitude between the base, belly and tip regions of the leaflets. High WSS, essentially unidirectional and pulsatile, with values comprised between 46 and 70 dyne/cm² near the base and the belly and reaching 93 dyne/cm² at the tip of the leaflet were found on the ventricular side, but were one order of magnitude lower and bidirectional on the aortic side.

Identifying how VECs respond to mechanical forces is critical to better understand aortic valve disease. The side-specificity of valve disease such as calcification might be attributed to the inherent differences in the VECs lining the aortic and ventricular sides of valve leaflets [13]. Interestingly, ~10% of VECs in human valve have been shown to undergo endothelial to mesenchymal transformation (EndMT) during development decreasing to ~1% in adult valves, suggesting heterogeneity in the phenotype of these cells [14]. To further investigate side-dependent differences in VECs we designed and used an original fluid activation device to apply controlled *in vivo* like WSS on the surface of the cells. The flow profile within the bioreactor was characterized both computationally and experimentally. Exposure of different pulsatile shear stress magnitudes to VECs confirmed a side-dependent effect. Porcine aortic VECs isolated from the two sides of the leaflets elongated and aligned differently according to the pulsatile WSS applied. Moreover, we observed a side-dependent gene expression pattern that reflected a differential response of these cells to biomechanical forces. Our results showed

97 that aortic-VECs undergo EndMT associated with a decrease of endothelial gene expression
98 and an increase of inflammation genes, whereas expression of *PECAM-1* was highly increased
99 in ventricular-VECs. Therefore, by using an original fluid activation device we highlight the
100 side-specific phenotype of VECs lining on the two sides of the aortic valve leaflets. These data
101 help to better understand how heterogeneity of VECs contributes to their function on different
102 sides of the aortic valve leaflets under physiological and pathophysiological conditions.

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Materials and Methods

Bioreactor design, development and functioning

A schema of the bioreactor composed of an original homemade fluid activation device linked to a specific 2D flow chamber is shown in Figure 1. An upstream glass reservoir bottle with screw cap pressure compensation (0.2 and 0.45 μ m filters) contains the culture medium (Figure 1A). Its volume depends on the duration to which the valve cells are submitted to WSS. Two stainless steel syringes (kdScientific) of 4.85mm inside diameter and 168.7mm overall barrel length were used (Figure 1B). One of the two syringes, filled with medium before the beginning of the experiment, injects medium in the 2D flow chamber in response to a computer generated signal. This syringe is emptied, whereas the other one, mechanically enslaved to it in an opposite manner, fills up. When the medium volume contained in one syringe tends to zero, a 3- way solenoid operated pinch valve (Bio-Chem, 100PD3MP12-02S) is automatically actuated. Meanwhile the second syringe, which is full, injects the medium into the flow chamber. C-flex tubing (3.2mm OD, 1.6mm ID, St Gobain) was used for the pinch valve to avoid potential contaminations. A downstream glass reservoir bottle was connected to the flow chamber outlet to collect the medium. Special attention was paid to avoid air bubbles within the syringes, tubing, and flow chamber.

A computer-controlled trio motion coordinator was used to drive a stepper motor (HS200-2231, Danaher motion) linked to a rolled ball screw (W1001MA-3PY-C3Z2, NSK). A gear ratio system was also added to improve displacement accuracy. This set up, using preloaded rolled ball screws with 0mm of axial play and a stepper motor with a small rotor inertia ($J=340\text{ g cm}^2$) enabled fast accelerations and decelerations with high accuracy.

The choice of the technical specificities used to move both syringes as well as the linking system between the engine and the syringes are the major key points that allow this original fluid

activation set-up to reproduce the WSS to which the valve cells are exposed *in vivo* in a perfectly controlled manner (see below for the validation of the activation device).

This fluid activation device is actually more adapted than commercial systems to mimic the rapid changes in flow direction. It is able to master pulsatility, accelerations, decelerations and fast variations of flowrate generating pathophysiological flow waveforms. This demonstrated its superiority in comparison to previous approaches [15-17] that used 2D flow chamber, plastic syringes and a peristaltic pump that generated steady or oscillatory flows, which do not achieve hemodynamic conditions on valvular leaflets. Finally, although Mohammed et al. [18] have used an interesting piezoelectric pumping system for studying aortic endothelial cells under pulsatile flow, their system cannot drive the values of flow rate we need in our system.

In order to impose controlled WSS on a surface we needed to master the flow behavior in a flow chamber. It is now well known that a 2D parallel plate flow chamber, with specific geometrical dimensions, permits such a control.

Assuming fully developed, laminar and incompressible flow, there exists an analytical relation between the imposed flow rate Q within the chamber and the WSS induced on its walls: $Q = \frac{WSSlh^2}{6\mu}$ where l , h and μ are the width, the thickness of the flow chamber and the fluid dynamic viscosity respectively. This relation remains true when assuming unsteady flow given the value of the frequency parameter. Consequently, $Q(t) = \frac{WSS(t)lh^2}{6\mu}$ (equation 1) and the wall shear stress on endothelial cells will be $WSS(t) = \frac{6\mu}{lh^2} Q(t)$.

The designed flow chamber was composed of three parts (Figure 1A). The upper and lower covers, 10mm thick, were machined using plexiglass. In between, the flow chamber itself, $l=21\text{mm}$ and $L=90\text{mm}$, was machined using a $250\mu\text{m}$ thickness Lexan 8010 film sheet; inlet and outlet diameters were of 4mm. The fourteen stainless steel screws used for assembling the three parts ensured that the flow chamber was leak-tight. Tygon™ tubing (3.2mm OD, 1.6mm

ID, St Gobain) and connectors in polypropylene (Ark Plas Products™) were used to associate the flow chamber to the activation device components.

As this flow chamber is 2D, it was important to include a 3D scaffold to culture the VECs, which did not change the shear stress at the flow chamber walls but allowed for a 3D cell culture environment. To do so, elliptical holes, with major and minor axis dimensions of 26mm X 9.8mm respectively and 2mm of depth, were drilled within the lower cover of the flow chamber (Figure 1A). They were then filled with a 3D collagen gel. 3D collagen gels at a concentration of 1.5 mg/mL collagen type I were made by addition of Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fischer Scientific), 10% fetal bovine serum (FBS, Thermo Fischer Scientific), 0.1M NaOH and rat tail collagen (10 mg/mL concentration, BD Biosciences). An aliquot of the collagen solution was pipetted into each well and allowed to gel for at least 1hr at 37°C in 5%CO₂. VECs were seeded on the surface of the 3D collagen gels. It is worth noting that the 3D collagen gel is not a model of the leaflet tissue, but provides a 3D cell culture environment.

The dynamic viscosity of the working medium fluid was measured using a rheometer HaakeMars III, fluid $\mu=0.945\text{e}^{-3}$ Pa.s. The entire set up was placed in an incubator and kept at 37°C and 5% CO₂ during all the experiments.

Porcine Aortic Valve Endothelial Cells Isolation and Culture

Porcine aortic valve endothelial cells (VECs) were isolated from the aortic or the ventricular side of the aortic valve leaflets as previously described [16]. Porcine hearts were kindly donated by the "abattoir Saint-Saturnin-lès-Apt". Briefly, aortic valve leaflets were isolated and incubated in collagenase type II/DMEM (Life Technologies) for 3x7 min. After incubation cells were gently scraped to isolated VECs from the two sides of the leaflets. After isolation aortic or ventricular VECs were seeded and cultured on flask coated with 50ul/mL rat collagen type I

(10 mg/mL concentration, BD Biosciences). VECs were cultured at 37°C and 5% CO₂ in Endothelial Cell Growth Medium (Promocell) supplemented with manufacturer's adjuvants and 5% FBS. Endothelial cell phenotype was confirmed by qPCR. Thus, the low expression levels of *ACTA2* validated the lack of contamination with other cell types such as interstitial cells in the culture. The cobblestone-like morphology of valvular endothelial cells was confirmed at passage 3, and cells were used at passages 3 to 6 [16].

Immunohistochemistry

Cells on collagen gels were fixed in 4% buffered paraformaldehyde during 10 min at room temperature. Fixed cells were washed for 15 min three times with PBS and permeabilized with 0.2% Triton-X 100 (Sigma) in PBS for 10 min. Cells were washed another three times with PBS before a saturation with 3% BSA and 10% Fetal Bovine Serum (Life Technologies). Cells were incubated overnight with Phalloidin-iFluor 488 Reagent (F-actin; abcam) and DAPI (4',6-diamidino-2-phenylindole) was used at 300 nM as a counterstain to distinguish cell nuclei. Cells were photographed with a confocal LSM800 (Zeiss).

Morphology Analysis

10 representative confocal images were taken on static and exposed cells on 3D collagen I gels that were fixed and stained with Phalloidin-iFluor 488 Reagent. The images were analyzed using Image J2 software [19]. Images from gels exposed to WSS were aligned so that the image horizontal corresponded to the direction of flow. Using Morpholib plugins on Image J2 software, cell area and cell perimeter were analyzed. Orientation angle between the horizontal (flow direction) and the majority of F-actin filaments was also determined. Based on cell area (A) and cell perimeter (P), Elongation index (Elong I) and Circularity index (shape index - SI) of cells (static or exposed to the flow) were determined ($SI = 4 \pi A / P^2$ and $Elong I = P^2 / 4$

π A). Cell alignment was assessed using angle orientation data. Frequency of cells upon angle orientation between 0-90°C were analyzed using Matlab software. Angle 0° were used as reference to cell parallel with the flow and 90° as reference to cell perpendicular with the flow. Angle orientation data from static conditions or cells exposed to fibrosa WSS (base, belly and tip) were represented in polar histogram obtained using Matlab software.

Real time quantitative PCR (qPCR)

Cells were first removed from the collagen gels. Cells were lysed on Trizol (Life Technologies) and RNAs were extracted using RNeasy mini Kit (Qiagen). Reverse transcriptions were performed by using first strand cDNA synthesis kit (Agilent) per manufacturer's instructions. LightCycler 480 SYBR Green I Master mix (Roche) was used for quantitative real-time qRT-PCR analysis with a LightCycler 480 (Roche) following the manufacturer's instructions. Each experiment was performed with $n \geq 3$. Samples were normalized to *TBP* as an endogenous housekeeping gene. mRNA expression levels for each gene were calculated using the comparative cycle threshold ($\Delta\Delta CT$) method. Expression levels in the static condition were set to 1. Primers used are listed in Table 1.

Statistical Analysis

All experiments were performed with $n \geq 3$. Data is expressed as mean $\pm$ SEM. Statistical significance was determined using Student's *t* test to compare variances. For non-parametric data, the Mann-Whitney test was used to calculate significance between the medians. Interactions between conditions were initially tested with ANOVA statistics (<http://biostatgv.sentiweb.fr/>). This test was used when it was necessary to decide whether several independent groups defined by the *k* modalities of the study factor were from the same population. *p* value < 0.05 was considered significant for this study.

Results

During the cardiac cycle, wall shear stress (WSS) is considered as one of the most important hemodynamic parameters for valve endothelial cells (VECs). Previous transcriptional analysis of VECs isolated from aortic valve leaflets have identified side-specific differences in VECs expression suggesting heterogeneity in the VEC phenotype [13, 20]. In order to assess the side-specificity of VECs we designed and developed an original fluid activation device able to apply a physiological controlled pulsatile WSS on the surface of VECs isolated from alternate sides of the leaflets. The temporal evolution of the WSS radial component obtained by Cao et al, 2016 (Figure 2A-C) at the base, belly and tip regions of the aortic side of the leaflets have been modeled using this original fluid activation device (see Figure S1) [11].

To analyze WSS amplitudes and waveforms, it was important to determine their amplitude WSS variation (ΔWSS) as well as their maximum, minimum and mean ($\overline{WSS}$) values at base, belly and tip regions (Table 2).

Table 2 shows large amplitude WSS variations, more particularly at belly and tip regions. Both positive and negative WSS values are high compared to the mean values and the radial WSS component temporal evolutions (Figure 2A-C) highlight a significant alternation between these positive and negative values. To more accurately capture this bidirectional oscillatory

waveform, oscillatory shear index, $OSI = \frac{1}{2} \left(1 - \frac{|\int_0^T WSS dt|}{\int_0^T |WSS| dt} \right)$, [21], was also calculated. $|WSS|$

is the norm of WSS and T, the period of cardiac cycle. This index, which is a hemodynamic factor related to oscillation flow, can vary from 0 to 0.5. When considering a WSS component, OSI will be equal to 0 if WSS is consistent and 0.5 if highly oscillatory. In the present study, we found $OSI_{base}=0.38$, $OSI_{belly}=0.4$ and $OSI_{tip}=0.45$. With such a high temporal oscillation of WSS during the cardiac cycle, it is obvious that VECs are exposed to strongly oscillating WSS signals far from static and steady flow conditions. In this latter case, we can also note that it

would not be appropriate to subject VECs to WSS mean values that are low compared to maxima or minima values encountered.

Validation of the flow device

The flow rates were generated by controlling the displacements of the syringes, (D_s), using a computer. A direct relation exists between D_s and the fluid volume, ϑ_f , injected, by the syringe:

$\vartheta_f = S_{sy} D_s$. with S_{sy} being the syringe section. Considering t_i the time of fluid injection $Q(t)$ is

derived by $Q(t) = \frac{\vartheta_f}{t_i} = \frac{S_{sy} D_s}{t_i}$ (equation 2).

To validate our original fluid activation device, it was therefore important to record the syringe-imposed displacements to assess their spatio-temporal accuracy. Three different WSS signals computed at base, belly and tip of the aortic side were extracted from results reported in Cao et al., 2016 (Figure 2A-C). Flow rates corresponding to these signals were then determined using equation (1) and associated displacements of the syringe calculated using equation (2). Figure 2 shows the comparison between syringe-imposed displacements to generate radial WSS signals related to the belly, base and tip of the aortic side and the measured ones using Solartron Metrology displacement sensors (Figure 2G-I). The results highlight that the fluid activation device is able to precisely reproduce the complex and fast displacement changes, which, in turn, induce the WSS changes occurring during the cardiac cycle.

Response of endothelial cells to mechanical environment

We investigated the orientation and shape of aortic and ventricular VECs (aortic-VECs and vent-VECs) following static culture or 1hr exposure to different pulsatile WSS as predicted in the belly, base and tip regions of the aortic side [11]. Under static conditions, we found no difference between aortic- and vent-VECs since the two cell types were randomly oriented (Figure 3). Following exposure to pulsatile WSS predicted in the tip region of aortic side of the leaflets, aortic-VECs did not display clear orientation. Cells were randomly oriented, as more

than 60% had the angle of orientation ranged from 30° and 60° (Figure 3A,B). Elongation and shape index showed that aortic-VECs were mainly rounded (Figure S2). However, ventricular-VECs exposed to a similar pulsatile WSS adopted an azimuthal orientation (Figure 3C, D). We found that 60% of vent-VECs were orientated with the angle of orientation ranged from 45° and 90°. Cell shape index analysis revealed that at equal magnitude of pulsatile WSS, vent-VECs have a more elongated shape than aortic-VECs (Figure S2). Together these results show that VECs isolated from the opposite sides of aortic valve leaflets have different phenotypes.

Side-dependent differential gene expression

To further investigate the phenotype of the two cell types we analyzed the expression of different markers after an exposure of 1hr to different pulsatile WSS (related to the belly, base and tip regions of the aortic side). For the aortic-VECs we quantified changes in pro-EndMT (*ACTA2*, *Snail*, *TGFβ1*) genes following exposure to pulsatile WSS (Figure 4A). Compared to static control, exposure to pulsatile WSS predicted in the tip region at the aortic side significantly upregulated pro-EndMT gene expression (*ACTA2* 4.3±0.3 fold increase over static control, $p<0.05$). Interestingly, the upregulation of *ACTA2* expression was proportional to the magnitude of the pulsatile WSS applied (Figure 4B). However, ventricular-VECs exposed to similar pulsatile WSS did not show comparable activation of *ACTA2* compared to aortic-VECs (Figure 4B). Similarly, we did not observe significant fold change for other pro-EndMT markers (Figure S3). We further analyzed the expression of the endothelial gene *PECAM-1* in the two cell types. Expression of *PECAM-1* was increased in aortic-VECs exposed to pulsatile WSS predicted in the base region of the aortic side of the leaflets (3.2±0.7 fold increase over static control, $p<0.05$), whereas it was downregulated in cells exposed to pulsatile WSS predicted in the belly and tip regions (Figure 5A). Conversely, *PECAM-1* expression was strongly upregulated in ventricular-VECs after exposure to the three pulsatile WSS applied (Figure 5B). For instance, expression of *PECAM-1* was highly increased in vent-VECs exposed

301 to pulsatile WSS predicted in the tip region (60 ± 10 fold increase over static control, $p < 0.05$).

302 As activation of inflammatory mediators is induced by abnormal shear stress [22], we analyzed

303 the expression of 2 inflammatory markers, *VCAM-1* and *ICAM-1*. Expression of these genes

304 was upregulated in both cell types with some differences (Figure 6). Expression of *VCAM-1*

305 and *ICAM-1* was increased in aortic-VECs, with the highest activation observed in cells

306 exposed to pulsatile WSS with the greatest differential (Figure 6A; 49.5 ± 3.7 fold increase after

307 exposure as predicted in tip region over static control, $p < 0.05$). ~~Upregulation of *NF- κ B*~~

308 ~~expression in aortic VECs confirmed the activation of inflammation in this cell type with~~

309 ~~(Figure S4).~~ Although expression of *VCAM-1* and *ICAM-1* were both increased in vent-VECs

310 (Figure 6), activation of *VCAM-1* was significantly greater in this cell type compared to its

311 upregulation in aortic-VECs (Figure 6B; 265 ± 17 fold increase after exposure as predicted in tip

312 region over static control, $p < 0.05$). Thus, these results suggest that the pulsatile fibrosa WSS

313 activates expression of inflammatory markers in the vent-VECs. To analyze the activation of

314 stress-related genes in both cell types we quantified expression of *NRF-2* and *EGR-1*, which

315 are known to respond to shear stress in endothelial cells [23, 24]. Expression of *NRF-2* and

316 *EGR-1* was upregulated in the two cell types, but was greater in vent-VECs compared to aortic-

317 VECs (Figure 7A,B). Together these findings indicate that aortic- and vent-VECs behave

318 differently to pulsatile WSS predicted in the different regions of the aortic side of aortic valve

319 leaflets.

Discussion

Although the aortic valve is covered by a monolayer of endothelial cells on its two sides (aortic and ventricular) the response of valve endothelial cells (VECs) to shear stress remains poorly understood. Here we used a fluid activation device to show that VECs respond differently to similar WSS depending on their side of origin. Shear stress is known to play a leading role in endothelial cell migration and vessel remodeling. Shear stress has also been shown to result in directed migration of endothelial cells against the direction of flow [25]. Our findings showed differences in the alignment of aortic- vs vent-VECs to the direction of the flow. We found that the orientation angle of 60% of vent-VECs was ranged between 45° to 90° when the magnitude of the pulsatile WSS was highest (corresponding to WSS at the tip of the leaflet). Conversely, at a similar pulsatile WSS magnitude less aortic-VECs were oriented with this angle suggesting these two cell types align differently in response to flow. The alignment behaviors that we observed in these two cell types may thus reflect a specific response to high WSS. The exact mechanism by which VECs sense shear stress remains unclear. Several endothelial cell flow sensors have been proposed for endothelial cells including integrin complexes, ion channels, caveolae and complex including PECAM-1, VE-cadherin and VEGFR2 [26]. Our observations suggest that the expression of *PECAM-1* was highly upregulated in vent-VECs compared to aortic-VECs confirming that cell-cell contacts may be implicated in flow sensing [27]. Our data are consistent with previous study that suggests distinct phenotype between VECs and vascular endothelial cells in their response to blood flow [16]. Analysis at the transcriptional and protein level has confirmed a transcriptional difference between VECs and aortic endothelial cells [10]. These data suggest that valvular endothelium acts as a distinct organ system, which may explain the difference find between valvular and vascular pathology.

Several studies have also shown EndMT on the aortic side of adult valves, which is exposed to bidirectional shear stress and is often the site of inflammatory and calcific degeneration [28,

29]. On the contrary, EndMT is absent at the ventricular side of the valve [30]. At this side, the VECs are exposed to high unidirectional shear stress. Consistent with these observations, our data show that bidirectional WSS signal induces EndMT in aortic-VECs but not ventricular-VECs.

We showed that inflammatory markers (*VCAM-1* and *ICAM-1*) are upregulated in both populations of VECs in response to physiological bidirectional WSS. The difference of *VCAM-1* upregulation between the aortic- versus vent-VECs is consistent with a previous study showing differential expression of VCAM-1 on opposite sides of normal aortic valves [13]. The upregulation of *VCAM-1* and *ICAM-1* has already been demonstrated in response to $\text{TNF-}\alpha$, suggested a role for the hemodynamic environment in valve inflammatory process [31]. However, our results differ from a previous study, which demonstrated that exposure of the aortic, but not ventricular, surface of the aortic valve leaflet to bidirectional shear stress increases expression of inflammatory markers [22]. The WSS signal used in this previous study, although bidirectional, was not physiological. More particularly the accelerating and decelerating slopes as well as the frequency of flow direction changes were lower than those physiologically observed. Another difference is that we used isolated VECs and not a leaflet. Therefore, cells within the endothelium in intact leaflets, may be less sensitive to shear stress than isolated cells. Indeed, it is known that the trilaminar structure of the leaflet protects the valve, and so the VECs, from its environment (hemodynamic and biomechanics). Although VECs were cultured directly on the collagen gel we observed that exposure to particular conditions induced EndMT and probably invasion in the matrix, even though the collagen gel has different stiffness than the ECM forming the leaflet tissue. Assessment of the effect of the difference in stiffness has demonstrated that the phenotype of VECs responds to changes of the 3 D culture environments [32, 33]. Thus, it would be important to work on a 3D cell culture environment that reproduces the physiological condition.

Our results confirm a previous study showing that VECs on aortic and ventricular surfaces have differential gene expression profiles and, therefore, distinct phenotypes [13]. The observation of morphological and functional differences between VECs isolated from the two sides of aortic valve leaflets exposed to identical conditions suggests that the side-specific phenotypic differences are in part intrinsic. Recent transcriptomic analysis of postnatal heart valves at single cell resolution demonstrated that the endothelial cell population is separated into three distinct subpopulations that display specific spatial locations in the valve leaflets [20]. The VECs subset, which is characterized by classic endothelial markers, can be found on both sides of porcine aortic valve leaflets. However, a particular subset of VECs is present in regions of high mechanical stress, where valve leaflets are exposed to high shear [20]. Interestingly, a recent study demonstrated restricted expression of Wnt9b in endothelial layer of zebrafish heart valves in response to fluid forces [34]. Altogether these findings suggest a contribution of biomechanical forces to maintain VEC diversity in different region of the valves.

Conclusions

In the current study, we designed and developed a unique fluid activation device that applies physiologically relevant pulsatile WSS (related to the belly, base and tip regions of the leaflet). We identified a clear phenotypic difference between VECs isolated from the opposite sides of aortic valve leaflets. Our model replicates the physiological radial component of WSS predicted in different regions of the aortic side of the aortic valve leaflet. We observed that aortic-VECs undergo EndMT when bidirectional WSS, characterized by high OSI value, is applied, whereas vent-VECs are less sensitive to this WSS feature. We found that VECs isolated from the two sides of aortic valve leaflets induced inflammatory process, which probably respond to the activation of a stress program. Our findings provide an improved understanding of phenotypic

394 differences between VEC populations in their environment and suggest that vent-VECs are less
395 subject to valve disease.
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Figure legends

Figure 1: Schematic illustration of the fluid activation device. (A) Scheme illustration of the flow chamber. (B) Scheme of the bioreactor set-up for physiological wall shear stress experiments.

Figure 2: A unique fluid device system producing a pulsatile WSS. Comparison between imposed piston displacements to generate bidirectional WSS signals related to the belly of the *fibrosa* leaflet side and the measured ones using Solartron Metrology displacement sensors. Note that the fluid activation device is able to reproduce the complex and fast displacement changes inducing the WSS changes occurring during the cardiac cycle. (A,D,G) Radial WSS signals at belly, base and tip regions of the aortic side of the aortic valve leaflets from Cao et al, 2016. (B,E,H) Associated flow rates. (C,F,I) Comparison between the displacements imposed to generate the flow rates associated with WSS (blue symbol) and the measured values (black symbol).

Figure 3: Valve endothelial cell alignment following exposure of wall shear stress. (A-D) Valve endothelial cell (VECs) derived from opposite sides (aortic and ventricular) of aortic valve leaflets were cultured under static conditions or exposed to different pulsatile wall shear stress (WSS) as predicted in the base, belly and tip regions of the aortic side of the leaflets. (A,C) F-actin is shown in green; nuclei are labeled with DAPI (blue). Orientation angle of VECs was determined in static condition or following exposure of different pulsatile WSS and displayed as a rose plot to represent the frequency and directionality of the cells. Quantification of the angle between the nucleus and flow showed that 60% of vent-VECs aligned between 45° and 90° to the direction of flow. **White arrow indicates the direction of the flow.**

Figure 4: Valve endothelial cell phenotype following exposure to wall shear stress. (A) EndMT-related gene expression in valve endothelial cells (VECs) derived from the aortic side following exposure of pulsatile wall shear stress (WSS) related to tip region of the aortic side of aortic valve leaflets. (B) *ACTA-2* expression in VECs derived from opposite sides (aortic and ventricular) of aortic valve following exposure of different pulsatile WSS (as predicted in the base, belly and tip regions of the aortic side). qRT-PCR experiments were performed in triplicate (n= 3 for each conditions) and expressed as mean $\pm$ SEM (* p <0.05 using Mann-Whitney test). Expression levels in the static condition were set to 1 and all conditions were compared to this reference.

Figure 5: *PECAM-1* expression following exposure of wall shear stress. (A,B) *PECAM-1* expression in aortic-VECs (A) and ventricular-VECs (B) following exposure of different pulsatile WSS (as predicted in the base, belly and tip regions of the aortic side). All experiments were performed in triplicate (n= 3 for each conditions) and expressed as mean $\pm$ SEM (* p <0.05 versus control (static condition) according to ANOVA followed by Mann-Whitney test). Expression levels in the static condition were set to 1 and all conditions were compared to this reference.

Figure 6: Expression of inflammatory markers after exposing valve endothelial cells to wall shear stress. *IVAM-1* and *VCAM-1* expression in VECs derived from opposite sides (aortic and ventricular) of aortic valve leaflets following exposure of different pulsatile WSS (as predicted in the base, belly and tip regions of the aortic side of aortic valve leaflets). All experiments were performed in triplicate (n= 3 for each conditions) and expressed as mean $\pm$ SEM (* p <0.05 versus control (static condition) according to ANOVA followed by Mann-

Whitney test). Expression levels in the static condition were set to 1 and all conditions were compared to this reference.

Figure 7: Expression of stress-related genes after exposing valve endothelial cells to wall shear stress. (A,B) *NRF-2* (A) and *EGR-1* (B) expression in VECs derived from opposite sides (aortic and ventricular) of aortic valve leaflets following exposure of different pulsatile WSS (as predicted in the base, belly and tip regions of the aortic side of aortic valve leaflets). In pulsatile WSS as predicted in the tip region the expression of *NRF-2* was 2.93 ± 1 fold increased over static control ($p < 0.05$) in aortic-VECs and 6.21 ± 0.1 fold increased over static control ($p < 0.05$) in vent-VECs. Similarly, in pulsatile WSS as predicted in the tip region the expression of *EGR-1* was 2.86 ± 0.37 fold increased over static control ($p < 0.05$) in aortic-VECs and 71.5 ± 3 fold increased over static control ($p < 0.05$) in vent-VECs. All experiments were performed in triplicate ($n = 3$ for each conditions) and expressed as mean $\pm$ SEM ($*p < 0.05$ versus control (static condition) according to ANOVA followed by Mann-Whitney test). Expression levels in the static condition were set to 1 and all conditions were compared to this reference.

Supplementary figure legends

Figure S1: Schematic representation of the aortic valve. (A) Upper view of the aortic valve.

(B) Lateral view of one the aortic valve leaflets. Inset shows the base, belly and tip regions on the aortic valve leaflet. This drawing was inspired by the study by Cao and Sucosky (2017).

Figure S2: Modulation of endothelial cell morphology following exposure of wall shear stress. Circulatory index shows that aortic and ventricular valve endothelial cells (VECs) have different morphology following exposure to pulsatile wall shear stress (WSS) as predicted in the base, belly and tip regions of the aortic side of the leaflets.

Figure S3: Valve endothelial cell phenotype following exposure to wall shear stress. (A) EndMT-related gene expression in ventricular valve endothelial cells (VECs) following exposure of pulsatile wall shear stress (WSS) related to tip region of the aortic side of aortic valve leaflets. qRT-PCR experiments were performed in triplicate (n= 3 for each conditions) and expressed as mean $\pm$ SEM. Expression levels in the static condition were set to 1 and all conditions were compared to this reference.

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A **B**

Figure 3

A **B**

Figure 4

A **B**

Figure 5

Figure 6

A **B** **Figure 7**