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Piezo1: Proteins for mechanotransduction and integration of endothelial shear stress & intravascular pressure

Juan Garcia Robledo M.D., *The University of Western Ontario*

Supervisor: Dr. Dwayne Jackson, *The University of Western Ontario*

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Abstract

Piezo proteins are transmembrane ion channels, specialized in detecting mechanosensitive stimuli and transduce mechanical forces into biochemical signals. Piezo proteins research has helped understand physiological mechanisms, but the integrative role that Piezo1 plays in the regulation of the microvasculature has remained unstudied. Our main objective was to characterize ex vivo microvascular responses to the blockade of Piezo1 mechanotransduction in male (n=29) and female (n=24) Sprague-Dawley (SD) rats. Gracilis arterioles (GA) and middle cerebral arterioles (MCA) were harvested for ex-vivo vessel preparations. After vessel viability confirmation, every vessel was submitted to myogenic and flow challenges under control conditions and after Grammostola Mechanotoxin 4 (GsMTx4) incubation to blocking Piezo1 channels, to quantify the homeostatic response of arterioles before and after Piezo1 antagonism. We are able to report Piezo1 as indispensable component in vascular smooth muscle cells (VSMC) and Endothelial cells (EC) to sense and change vessel diameter based on intravascular pressure and shear stress, correspondingly. Also, we report for the first time a heterogeneous response in males and females after Piezo1 antagonism in representative resistance arterioles from the skeletal muscle and cerebral circulation.

Keywords

Piezo 1, Ion channels, Mechanotransduction, Mechanosensitive proteins, Microcirculation, Homeostasis, Myogenic response, Shear-induced dilation, Gender dimorphisms.

Summary (Lay Audience)

Piezo proteins are found in cell's membranes and specialize in detecting mechanical forces (e.g. friction and pressure) and translating this information into signals that promote proper cellular function. To date, the study of Piezo proteins has helped understand diverse cellular mechanisms and their importance in the context of heart and blood vessel diseases. Nevertheless, Piezo1's role in the regulation of the smallest vessels in the body and how Piezo 1 function may vary between males and females, has remained unstudied. We focused on blood vessels from the skeletal muscle and cerebral circulations, due to their natural differences in regulating blood flow. The main objective of our project was to understand Piezo 1's role in their unique regulation properties and if the negative effects of Piezo 1 dysfunction impact differently on males and females. By approaching our project contemplating sex and anatomical differences, we have opened up the field to using Piezo1 as a potential target for therapies to provide more effective treatments for cardiovascular diseases in males and females.

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“La educación es fundamental para la felicidad social; es el principio en el que descansan la libertad y el engrandecimiento de los pueblos.” -Benito Pablo Juárez García-

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“If I mentioned all of my skeletons, would you jump in the seat? Would you say my intelligence now is great relief? And it's safe to say that our next generation maybe can sleep with dreams of being a lawyer or doctor Instead of a boy with a chopper”. – Kendrick Lamar Duckworth-

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Chapter 1

Introduction to Microcirculation and Piezo Proteins

1.1 Introduction & Background

The principal objective of this chapter is to give the reader an overall perspective on the importance, function, and regulation of the microcirculation. We will also describe the anatomic components of the microvasculature and review the concepts of shear stress-mediated dilation and myogenic constriction, in order to underscore the important role these physiological mechanisms play in maintaining homeostasis in the microvasculature. Additionally, our aim will be to update the reader on gender-based dimorphisms in vascular regulation. Finally, we will describe the physiological properties associated with mechanosensitive proteins, elucidate how Piezo1 enables endothelial cells (EC) and vascular smooth muscle cells (VSMC) to sense forces that eventually will be transduced into electrochemical signals and initiate shear stress- and myogenic-dependent vessel control.

1.1.1 Overview of the microcirculation & homeostasis (control & regulation)

The cardiovascular system is composed of the heart and an extensive system of blood vessels serving as an interconnected organic unit to efficiently transport blood to all body tissues. In the context of Walter Cannon's concept of homeostasis, described it as the ability of the organism to maintain a stable internal environment, the cardiovascular system supplies a continuous blood volume to tissues/organs during physiological conditions, physiological stress (e.g. exercise, emotional stress) and pathological conditions. In order to maintain such a stable internal environment in tissues, the vasculature is under continuous modulation of pressure and flow through changes in lumen dimensions and by redistribution of circulation through vascular beds in accord with the changing metabolic needs of specific tissues and body activity. It is the result of this balancing mechanism, along with a plethora of mediator pathways, that the functional cellular elements in tissues can satisfy their metabolic needs in a specialized manner (12–14).

Each of the components comprising the cardiovascular system contributes essentially to the maintenance of homeostasis (14,15). In order to achieve a balanced tissue

environment, blood has to be made available to the various organs of the body through the pumping action of the heart and distributed by way of large vessels and a succession of arterial branches of diminishing caliber. Nevertheless, the basic element of vascular homeostasis occurs within the microcirculation (arterioles, capillaries, and venules) by adjusting the surface area available for metabolic exchange and maintaining exchange between blood and the parenchyma at a prescribed level (13,16). Although definitions and borderline diameters for macro- and micro-circulation can vary, it is widely accepted that diameters from smaller microvascular components remain consistent regardless of mammalian species (12,14,17,18). Moreover, recognition of microvascular architectural and functional similarities between species has blossomed into a rich area of investigation that has shaped our understanding of the biochemistry and cell signaling pathways influenced by pressure and flow sensors (16,19–21).

With progressive diminishing diameter in the successive vascular branches of every network, maintenance of structural integrity and efficient exchange comes with physiological challenges to maintain adequate arteriolar tone and normalize the effects of acute and chronic pressure and flow disturbances. Some of the controls that acutely modulate lumen dimensions and flow redistribution among the vascular bed include the sympathetic nervous system, baroreflex mechanisms, metabolic feedback, shear stress-induced vasodilation and myogenic constriction (18,20). However, to obtain vascular homeostasis under conditions where persistent perturbations in pressure-flow relationships develop, arteries present dramatic changes in composition, structure, and function; a fundamental and often ignored characteristic of arteries to maintain their composition, geometry, and function over long periods despite the variable changes in mechanical loading (18,22).

Though, is clear that a myriad of mediator pathways are implicated in vascular adjustments needed to achieve vascular homeostasis, our efforts will be focused on the effects that myogenic control and shear stress dilation have over the modulation and control of perfusion within the microcirculation. The microcirculation's role in vascular homeostasis essentially depends on multiple specialized molecules in cells transducing superficial stresses near the vessel walls that result from the hemodynamic conditions

inside blood vessels (20,23–25). Pressure-induced myogenic contraction, intrinsic to vascular smooth muscle cells, and the vasorelaxing influence of endothelial cells via the release of vasodilator autacoids in response to shear stress, have been widely described since the beginning of the twentieth century, but complete understanding of the effector biochemical signals involved in acute and chronic regulation of VSMC & EC cellular function and morphology are yet to be defined (26–29).

The definition of myogenic contraction and endothelial shear-induced dilation, along with previous work done to accurately describe these mechanisms, will be described in more detail on the next sections.

1.1.2 Microvascular techniques and advancements

In 1628 “An Anatomical Exercise on the Motion of the Heart and Blood in Living Beings" by William Harvey first established the circulation of the blood and postulate the existence of “invisible pores to the flesh” to explain a path for arterial blood flow in tissues that connects to the venous counterpart and return to the heart (1–3). Since then, subsequent contributions focused on the specialized structure of the microcirculation (2). It was not until research by Poiseuille in the 1830s that attention in the field was redirected to understanding the hemodynamical properties that rule the microcirculation (1).

Development of ex vivo methods like wire-mounted vessels, and perfused and nonperfused isolated microvessel preparations have provided the opportunity to control and measure the numerous variables modulating the physiological behavior of resistance arterioles. While the physiological behavior is better observed under intact conditions, vascular adjustments under these conditions are not easily attributed to specific effector mechanisms (4). The importance of cannulated microvessel techniques relies upon their flexibility for study, and these have been found to be representative of in vivo physiological performance (4). Initially described by Uchida, et al. in 1967 and further enhanced by Duling, et al. in 1981, studies in isolated pressurized microvessels (12-112 μm) have provided much of our current understanding of the intrinsic microvascular vasomotor response and its underlying mechanisms where the effects of pressure could

be clearly distinguished from flow, metabolic, neural, and endothelial influences (5–8). Nevertheless, more than a century of research efforts have failed to create consensus with regard to the microvascular intracellular signaling pathways that pair supply/demand to multi-fold metabolic rate changes and the conversion of mechanical forces into electrical responses (mechanosensory transduction) remains a major biological question (4,9,10). One of the hypotheses proposed to explain the coupling mechanism is the observation of stretch-activated ion channels (SAC) serving as “sensors” of mechanical forces and allowing the influx of extracellular calcium, resulting in a vascular tone increase. The conundrum that this hypothesis presents comes with the conceptual framework that defines SAC's properties. Activation of mechanosensitive channels would elicit calcium influx refractory to calcium channel blockers, and the resulting potassium conductance increase would hyperpolarize the cell. However as Harder et al. reported, their activation comes associated with membrane depolarization, can be blocked by calcium (Ca++) blockers and can be up- or down-regulated by potassium channel modulators (agonists and antagonists) (11).

Even though ex-vivo isolated vessel preparations have been groundbreaking, there are inherent strengths and limitations to these techniques. When carried out effectively, isolated preparations allows precise control of experimental conditions (luminal pressure, shear stress, superfusate & perfusate solutions, etc.) while eliminating confounding variables such as autonomic innervation, hormonal and metabolic effects (12–14). Ironically, the main disadvantages to ex-vivo preparations derive from the loss of the neuro-humoral, metabolic and intercellular conduction input (14,15). Additionally, the limitation of this preparation to feed arteries and trauma due to the dissection of the vessels can also be problematic (14).

Technological advancements and increased sophistication of in vivo microcirculatory techniques including skeletal muscle, cheek pouch and brain preparation, to name a few, have made possible a more convenient approach to extrapolate results carried out in studies on small mammals (16). It has been mentioned that the main disadvantages to ex-vivo preparations derive from the loss of the neuro-humoral, metabolic and intercellular conduction input (14,15). On the other hand, knowing and quantifying the effect of in

vivo physiological disturbances on intact microvascular beds convey definite advantages to the interpretation of the true effect of any given agent (e.g. pharmacological or experimental agent) (2,16,17). Classical vascular beds, as the skeletal muscle and brain, have been employed and defined as representatives of the microcirculation due to their interpretative strengths. Skeletal muscle comprises nearly one-half of the body mass and has been shown to contain practically every vascular receptor known and studied (16,17). However, major limitations from these studies of skeletal muscle arteriolar network structure stem from the approach used to collect the geometric and topological data, as well as the inherent anatomy of the skeletal muscle preparations and frequently used networks(terminal/distal networks) (16). The cranial window in vivo preparation provides a considerable amount of data on a relatively large surface area of brain tissue in the rat, by means of measurements of the microvasculature (e. g. pial, distal MCA), and high correlation with pathological states as cardiovascular disease, as well as aging (16,18,19). Nonetheless, its major limitation comes from the technical difficulties associated with the technique, although these can usually be prevented by being extremely meticulous during the surgical procedures (19).

1.1.3 Microcirculation: Male & Female differences

Significant differences in metabolic demand, autonomic innervation, overall higher blood pressure in males and higher prevalence and earlier settings of cardiovascular diseases in males, have prompted decades of studying the effects in the cardiovascular system of the gender-specific sex hormones to elucidate the cardiovascular protective effect found in female subjects (20–24).

Historically, gender-based dimorphisms and the role they play in microvascular regulation has been understudied. Most of the previous studies in the field have been performed on animals of one sex or the other; whether these decisions have been taken to prevent hormonal influences as confounding variables or because intrinsic behavioral differences between genders facilitate certain studies, inevitably these single-sex studies have impaired our understanding on microvascular regulation and the role gender plays in pathological states. (20,25).

Byrom's postulates on the effect of estrogens and progestogens over vasopressin and other vasoactive autacoids in 1938, and later work in the field by Lloyd in 1959 to explain the heterogeneous response to oxytocin in mesenteric vessels were remarkable milestones in vascular research (26,27). Nevertheless, critical new insight into the responsiveness to catecholamines on microcirculatory blood vessels came from Dr. Burton Altura, who presented firm support on the effect female hormones play in terminal arterioles. Until his research, it was generally accepted that microcirculatory vessels responsiveness to vasoactive peptides was not influenced by sex (26,27). Confirmation of estrogenic receptors in endothelial cells was given by Colburn & Buonassisi, who provided understanding of the estrogenic contribution in regulating blood supply during cyclical functional variations in the female reproductive system and, thus, improve fertilization likelihood (28). Continuous research efforts for over forty years have led researchers to suggest estrogenic and other sex hormones exert nongenomic rapid effects over vascular reactivity through a variety of mechanisms that have not been completely understood.

One of the more prominent ideas shaping this field has been the role that sex hormones play on the cardioprotective effect. Studies have defined the 17α-estradiol or 17β-estradiol effect the most; where it has been reported to produce an acute and clinically relevant relaxation from females through endothelial nitric oxide synthase (eNOS) activation and an increase of nitric oxide (NO) release (29). Nevertheless, since the 1940's studies on males and the effect of testosterone have also been associated with an improvement on certain cardiovascular pathologies (30). Positive effects over angina pectoris symptoms and on exercise-induced myocardial ischemia have been reported by Hamm & Walker in '42, Sigler in '43 and Lesser in '46 but have failed to effectively conclude on testosterone's benefit in myocardial ischemia and coronary artery disease (30). Possible mechanisms discussed for this beneficial effect included an improvement of oxygen-carrying capacity of red cells, an increase in blood hemoglobin levels and dilatation of the coronary arteries or their collaterals. However, the negative effects of testosterone remain controversial (e.g., higher coronary artery disease and hypertension prevalence in males) (20,30). Multiple meta-analyses and review studies have not been

able to associate testosterone with exacerbating the development of cardiovascular diseases; in contrast, low testosterone levels are more a marker of poor health. An association has been reported between low testosterone levels and an increased cardiovascular disease (CVD) mortality, but still studies have lacked the power to demonstrate a relationship with CVD morbidity (20,31,32). Therefore, we will focus our attention on more studied gender dimorphism postulates, in particular, estrogenic and autonomic innervation.

1.1.3.1 Estrogenic effect over microvascular regulation

It has been postulated that the activation of eNOS in endothelial cells by estrogen receptors α (ERα) is dependent on the PI3-kinase-Akt pathway or mitogen-activated protein (MAP) (20). These two intracellular signal transduction pathways involving the nongenomic activation of eNOS have been well documented. However, the specific mechanism to which endothelial estrogenic dependent vasodilation is specifically achieved remains controversial (20,21). Moreover, it remains uncertain whether the nongenomic activation of eNOS by estrogen is dependent or independent of increases in intracellular Ca2+ and endothelial intervention. Collins et al. in ’93 suggested that 17α-estradiol might behave as a Ca2+ channel antagonist, but this postulate was mostly based on previous evidence on estrogenic calcium antagonist properties and mainly a hypothetical conclusion (33). A year later Salas et al. determined that the relaxation evoked by 17α-estradiol, with a shared effect by 17β-estradiol, of coronary arteries may be independent of endothelial modulation. They observed vasorelaxation was present under conditions where the production of NO was inhibited or in coronary arteries whose endothelium had been removed but still, e-NOS showed calcium-dependent activation (20,33,34). Nevertheless, in 1997 Caulin-Glaser’s in-vitro studies concluded that 17β-estradiol’s main effect appeared to be mediated through estrogen receptors activating eNOS function and did not elicit any intracellular calcium influx (35). Calcium dependence studies showed that calcium-mediated vasorelaxation depended on the NOS isoform involved. Both eNOS and iNOS (inducible nitric oxide synthase) have been detected in EC under certain conditions, but it is the eNOS isoform that is calcium-dependent, whereas iNOS is not (36).

Although, estrogenic direct calcium antagonistic effects are mainly attributed to elicit their effects on VSMC, wherein estrogenic response seems to be independent of estrogenic receptors and directly block Ca2+ channels of smooth muscle, decrease Ca2+ VSMC sensitivity or even increase calcium efflux (20,37). The endothelial estrogenic effect has been associated with ERα as responsible for the nongenomic activation of eNOS; however, a recent study demonstrated that ERβ is also able to stimulate the nongenomic activation of eNOS in endothelium caveolae (20). It is worth mentioning that both Ca2+ antagonist mechanisms are elicited by activation of membrane adenyl cyclase and atrial natriuretic factor-stimulated guanylate cyclase activity (20,29).

Studies in small mammals have helped researchers to affirm this mechanism may contribute significantly to the protective effect of estrogen on the development and progression of atherosclerotic disease, in the coronary and other arteries of women. In addition, estrogen-mediated activation of eNOS may be of major importance to the regulation of uterine and placental blood flow during normal physiological pregnancy conditions (38). Further studies on acute eNOS activation by estrogen will continue to enhance our understanding of the role of this hormone in vascular biology.

Nevertheless, the estrogenic and progestin effect over blood flow regulation can explain only 50% of microvascular changes. Hormonal interaction with other molecules and secondary by-products leaves open the question, whether hormonal heterogenous effects over the microcirculation, "single-handedly" can explain premenopausal cardioprotection in a female subject.

1.1.3.2 Autonomic nervous system gender-based dimorphisms

The autonomic nervous system and its divisions' primary function is to assist tissues/organs in maintaining homeostasis. Specifically, the sympathetic division of the autonomic system act as an essential modulator of the peripheral circulation and blood pressure (39,40). Sure enough, the association between increased sympathetic activity and cardiovascular diseases has been reported multiple times in human and animal models.

In an augmented sympathetic nerve activity state, the cardiovascular system undergoes several pathophysiological changes. Cardiac output decreases due to a diminished stroke volume and β−adrenergic responsiveness, as well as a vascular resistance augmentation secondary to vascular hypertrophy. Alexander V. Ng, et al. confirmed gender as an important determinant effect, for the first time, of augmented efferent sympathetic nervous system activity and catecholamines increases in plasma through directly recorded sympathetic nerve activity to skeletal muscle (MSNA) (41). Although previous reports have effectively associated augmented MSNA to age (42,43), Alexander V. Ng postulate, underscored the importance of understanding gender dimorphisms in high sympathetic states where these pathophysiological changes contribute to hypertension, ventricular and vessel's media layer hypertrophy among the physiological changes mentioned above and to which association to elevated incidences for the plethora of pathologies that comprise the CVDs represent the best "target" to which medical treatment is directed in everyday practice until present times (41,44,45). Nonetheless, regardless of multiple studies correlating increased MSNA to older male and female subjects and males < 50 years, human and animal studies have failed to confirm the mechanism to which the individual "weight" of gender and age play as significant determinants to explain the augmented sympathetic activity in older (≥ 50 years) males and female subjects (42,43,46,47).

The apparent discrepancies on reports showing inconsistent results over the significant effect sex hormones play to determine cardiovascular dimorphisms accentuates the need of attention into techniques that allow direct observation at all levels of the arteriolar hierarchy in the overall microvascular network, as well as to other cellular mechanisms. As reported previously, heterogeneous regulation exists throughout the microcirculation (2,3,42). Moreover, this focus has helped clarify regional variations in vascular regulation and has provided a rich area of research into other related sympathetic mechanisms and receptors involved in gender dimorphisms. For example, concentrations of core vesicles containing neuropeptide Y (NPY) are found in greater amounts with decreasing vessel size & and exerts a cooperative vasoconstrictive effect with noradrenaline (NA) through the respective activation of neuropeptide Y type 1 (Y1R) and adrenergic α1 (α1R) receptors. Within this scenario, it is logical to expect its contribution to basal blood

pressure regulation but its contribution is still debated (48). However, series of studies by De Potter et al. and Jackson et al. presented evidence to support the postulate of NPY as a critical regulator of baseline blood pressure (BP), especially in vessels of higher order on the overall vascular architecture, where they are chronically mediated by NPY (48–51).

Series of studies by Jackson et al. focused on gender and estrogen as important contributors to NPY modulation in sympathetic activity regulation, in accord with previous findings, female reported lower levels of baseline sympathetic activity. Thus, it was hypothesized that female subjects would have decreased NPY vascular control. Since NPY & NA balanced synergetic input is required for maintenance of vascular tone in skeletal muscle (24,41,52,53). Moreover, a novel finding in these studies was that, in contrast to males, female rats did not exhibit basal endogenous Y1R control of hindlimb vascular conductance (VC) despite the fact of Y1R a bioavailable neuropeptide Y (24). The observed differences and the lack of endogenous NPY receptor activation suggested that limited bioavailability of NPY. It was later hypothesized by the same group, that female rats have augmented proteolytic processing of NPY, augmented autoinhibitory Y2R NPY receptor expression, and activation. Nevertheless, the mechanisms to which these three elements would limit NPY bioavailability and estrogenic influence over neurogenic vasomotor control was not confirmed and the NPY bioavailability dimorphism between genders was suspected to be due to estrogen’s impact on NPY release and Y1R expression, but not Y2R expression or peptidase activity (52,54).

Whether small sample size, the inclusion of subjects taking hormone replacement therapy or intrinsic socio-cultural differences in human populations have skewed results in studies previously referred. It has not been overlooked the need for larger and longitudinal studies in order to accurately establish the effects of gender on sympathetic nerve activity and its relationship with CVDs. In regard of this matter, is worth to mention that the significance of understanding gender dimorphisms are underscored in the conflicting results of two vastly referenced human studies on gender dimorphisms and the role sex hormones play in a clinical setting, what has become known as gender-related cardioprotection, the Heart Estrogen/Progestin Replacement Study (HERS) and the Nurses' Health Study. While the HERS demonstrated that hormone therapy did not

reduce the risk for cardiac events and even remarks the increase for Coronary Heart Disease (CHD) in the first use of the Hormone replacement therapy and a later reduction in the fourth and fifth year. Yet, the Nurses' reported an overall reduction in cardiovascular events of about 40-60%(20,55,56). The contradictory results reported in these two studies exemplify the need to elucidate other factors contributing to sex differences in microvascular autoregulating mechanisms since they can potentially bring specific target therapies for males and females with CVD's. Whether by lack of sex-specific clinical trials or underrepresentation on the ones developing, these and many other obstacles contribute to maintaining CVD's as the leading cause of death for women in both industrialized and developing nations (57,58).

1.1.4 Microcirculation: Differential blood flow control in skeletal muscle and cerebral

A number of mechanisms have been broadly described in multiple physiological and medical literature as imperative mechanisms to control local blood flow, some of them include autonomic nervous stimuli, myogenic responses, shear-dependent responses, metabolic responses, conducted responses propagated along vessels, and communication between paired vessels (39,59–61). Manifold studies have described the previously mentioned mechanisms and the biological response that effector cells wield in the overall circulation. However, special consideration to myogenic control and shear stress-induced dilation will be taken, since they will be the focus of the next subsections.

Both mechanisms exert local regulation of blood flow to organs/tissues and supply correspondingly to the metabolic demands. For example; the rate of blood flow in skeletal muscle varies directly with the contractile activity and the specific muscle being studied. In resting muscle, most of the capillary bed sub-perfused and total blood flow is between 1.4 to 4.5 mL/minute/100 g. Nonetheless, during aerobic exercise arterioles and small arteries relax and muscle blood flow may increase up to nearly 100-fold (39,61,62). Whereas in the brain, blood volume and extravascular fluid must remain relatively constant; with any given change in one of these fluid volumes, it must be accompanied by a reciprocal change in the other. In human studies, it has been reported that the rate of cerebral blood flow is maintained within a narrow range and averages a rate of 55

mL/minute/100 g (39).

With this heterogeneous control of blood flow, an optimized distribution of oxygenated blood can be achieved by distributing just at a slightly higher rate to the tissue that needs oxygen the most, in order to maintain the metabolic needs of the organ/tissue and, under baseline conditions, never experience hypoxia; all of this while maintaining minimum workload to the heart.

Table 1 : Blood to Different Organs and Tissues under basal conditions – Adapted from "Table 17-1: Blood Flow to Different Organs and Tissues Under Basal Conditions"; Unit IV, Chapter 17, page 192. from Guyton & Hall Textbook of Medical Physiology 12th Ed.

	Percent of Cardiac Output	ml/min	ml/min/100g of Tissue Weight
Brain	14	700	50
Heart	4	200	70
Bronchi	2	100	25
Kidneys	22	1100	360
Liver	27	1350	95
Portal	(21)	1050	
Arterial	(6)	300	
Muscle (inactive state)	15	750	4
Bone	5	250	3
Skin (cool weather)	6	300	3
Thyroid gland	1	50	160
Adrenal glands	0.5	25	300
Other tissues	3.5	175	1.3
Total	100.0	5000	

It is important to mention the ratio to which both mechanisms produce their effect are interchangeable in some tissues. Although, regulation of cerebral blood flow (CBF) involves interplay among myogenic, shear stress, metabolic, and neural mechanisms (regional neural activity) intracranial arterioles respond immediately to changes in cerebral perfusion pressure (39,60,63,64).

In general, cerebral blood flow is fixed to stringent ranges of intraluminal arterial

pressure (60 – 160 mmHg) to prevent pathophysiological states (e.g. ictus, cerebral edema). However, reliability on myogenic responsiveness for the autoregulation of CBF not only assists on said preventive measurements; but also, the considerably larger myogenic tone provides cerebral vessels with an apparently larger capacity to increase blood flow depending on neural activity demand (64). This is in accordance to Carlson et al. theoretical model, by combining predicted values of the myogenic and metabolic responses they were able to produce the strongest autoregulation as observed by experimental results; supporting the concept that information about metabolic tissue status is communicated to upstream vessels by conducted responses (39,59,61).

It is important to highlight that while the contribution to local blood flow regulation from shear stress and myogenic constriction play an important role as components of the circumferential tension exerted inside the vessel walls, they do not represent all known mechanisms but do represent individual fractions of the dominant stress to which the vasculature is subjected to. Regardless, metabolic variability in demands within different tissues/organs may modulate the contributions of response mechanisms to autoregulation (59). Therefore, the importance of running comparative studies considering skeletal muscle and cerebral vasculature has not passed unattended by our group, expecting differential vascular effects, our current project focuses on describing phenotypical differences on vasculature derived from both tissues.

1.2 Myogenic Control

1.2.1 Concept

The myogenic effect was first described as the ability of a vessel to vasoconstrict or vasodilate when intravascular pressure correspondingly rises or decreases (6,65). It pertains to the intrinsic ability of smooth muscle cells in the vessels to modulate their tone after an asymmetrical force leads to the deformation of the vascular wall. Traditionally, it was assumed that the myogenic response was limited to VSMC transduction and independent of any neural or humoral intervention. Nevertheless, in the '80s and '90s a series of studies reported the ability of the endothelium to maintain a certain degree of control over VSMC tone in response to chemical and physical stimuli

(6,20,66).

Despite a multitude of studies that have brought knowledge to the cellular mechanisms involved in the myogenic response, it has been difficult to establish a definitive cause-effect relationship. But a direct Ca++-dependent initiation of contraction or intracellular release of Ca++ stores secondary to unspecific cation influx into the cell seems to be the most plausible sequence of events proposed by a series of studies (39,67).

1.2.2 Previous work

Historically, characterization and description of the vascular mechanobiological properties can be traced to the early twentieth century. In 1902, Bayliss established the idea of the myogenic response (6). He considered this response to be independent of central nervous system excitation and of myogenic nature, but too rapid to be metabolite mediated. Though Bayliss' observations were groundbreaking, it was not until almost fifty years later that Folkow's denervated preparations confirmed VSMCs' pressure-dependent capacity to generate active tone (30,31).

The myogenic tone plays a significant role in various physiological mechanisms, but two functions stand out: establishment of basal vascular tone and autoregulation of local blood flow and capillary pressure (6). However, the signaling mechanism required for VSMC to elicit vasoconstriction after distention of the wall is still not completely understood. Yet, it has been proposed and widely accepted that an activation of membrane calcium channels in VSMC is secondary to length-dependent activation (67,69).

It is well-known that vascular initial length serves as a modulator for sympathetic agonists. Since sympathetic agonists commonly potentiate the myogenic response, it is likely that a functional overlap exists for the two pathways (6). Though possible, most evidence to support the postulate is indirect and limited. Davies et al. have provided experimental evidence supporting this mechanism in isolated vessel experiments, but the sites sensitive to distortion or the presence of mechanosensitive ion channels provide another alternative for the translation of the distorting force into a biochemical event (67).

It was in 1954 when Bulbring's study in guinea-pig taenia coli first proposed "a close correlation between membrane potential, the rate of discharge and tension"; nonetheless, it was not until 1969 that Uchida & Bohr presented for the first time the postulate that the myogenic response might reflect an improved excitation-contraction coupling resulting from membrane depolarization and increased Ca++ permeability (6,70). Even though there is a myriad of evidence supporting this postulate, the electromechanical coupling cannot fully account for myogenic behavior and other mechanisms must be involved to modulate cell depolarization; e.g., changes in Ca++ sensitivity (6).

Other intracellular mechanisms have been involved in the modulation of VSMC depolarization and are discussed in detail in review papers such as Davis & Hill, heavily referenced in this subsection (6). Nevertheless, it is important to consider the modulating role that estrogen plays in females' myogenic response. Kaley & Huang, besides their detailed review of the estrogenic role in the cardiovascular function, reported results obtained in this matter (20). Kaley & Huang concluded estrogen contributes to the regulation of peripheral resistance, as a consequence of a complex regulating mechanism. Overall, their evidence suggested the estrogenic influence on potentiating the basal release of endothelial NO, influencing estrogen receptor activation of the NO/cGMP cascade, which attenuates myogenic response, or normalizing altered prostaglandin synthesis preventing the production of prostaglandin H2 (PGH2) (20), with similar results on female OVX (ovariectomized) and OVX+OVE (estrogen replacement) rats. Additionally, the genomic disruption of ERα obliterates the modulation of the myogenic response mediated by estrogen. Their series of studies concluded that estrogen regulates arteriolar responses to intravascular pressure in both, physiological and pathological conditions & intervenes in the modulation of a lower basal tone on female microvessels (20).

1.3 Shear stress/flow-induced dilation

1.3.1 Concept

Shear stress-induced dilation is defined as a vasodilation response due to increases in wall shear stress (WSS). As blood (a viscous fluid) flows through a vessel, it exerts a

force parallel to the vessel wall as a result of the friction between the stationary cell-free layer and the layer of blood, immediately below, moving faster in response to the pressure gradient. This drag is known as wall shear and, the units of force elicited measured in dynes/cm^2 (71).

Equation 1: Shear stress

$$\tau = 4\ \eta Q/\pi r^3$$

In agreement with the shear stress (τ) formula, increases in flow rate and viscosity are directly proportional to increases in wall shear and depends heavily on the integrity from the vascular endothelium (20,72,73). In conjunction with exposure of mechanical forces, endothelial cells in-vivo, maintain a balance along to chemical stimuli to promote transmitting and transducing information from the blood to the rest of the vessel wall. These signal transduction mechanisms range from extremely rapid responses like electric activation currents through ion channels to relative slow structural changes elicited by gene expression regulation. Moreover, endothelial shear sensing is indispensable activating many of these responses. Thus, most of them are not fully understood. (74,75)

A plethora of studies has focused on synthesis and release of vaso-relaxant factors by ECs, in order to elucidate some of the mechanisms involved in the shear stress response. Prostaglandin I2 (PGI2), Nitric oxide (NO) & Endothelium-dependent hyperpolarizing factor (EDHF) have been well described and understood as endothelium-dependent mediators, by-products of the shear stress-dependent response (20,39,59,72,76). Although the intracellular signaling processes have been extensively studied, the cellular mechanism responsible for mechanotransduction of shear stress remains elusive.

1.3.2 Previous work

First reports of vasodilation secondary to increases in blood flow are attributed to Schretzenmayr; nonetheless, the ability of the vascular endothelium to sense and respond to the flow of blood was observed more than a century ago by Rudolf Virchow (77). In addition, Shchetzenmayr was able to make a key observation and suggested for the first time the relevance of preservation of this mechanism to prevent pathophysiological states

(78). The cellular mechanisms by which vessels could exert vasodilation remained obscure for 50 years, due to little interest in the matter (79). It was not until the development of ex-vivo techniques and the innovative work by Furchgott et al. that reports on the role of endothelial cells' functional contribution to active tone by "releasing a substance (or substances) which in turn acts on the smooth muscle cells in the media to activate relaxation" was first postulated and posteriorly confirmed by other groups (80,81).

Arguably, short-term responses to shear stress, like intracellular signaling processes and the excretion of flow-mediated humoral agents, have been predominantly the focus of studies (68). NO synthesis and its flow-mediated release were suggested by Rubanyi et al. in the mid-'80s, although failed to properly characterize the flow-mediated humoral factor in question, which they referred to as an endothelium-derived relaxing factor (68,76). Through bioassay experiments, they were able to demonstrate, though with limitations, that the endothelial-based dilation observed in their ex-vivo preparations was due to a non-prostacyclin relaxing mediator that increased its bioavailability after superoxide dismutase infusion (76). Until the early 90's most of the research efforts were performed on larger arteries and conduit vessels. Thus, conflicting observations were still reported in large vessel preparations, and whether shear stress-mediated dilation was observed to be endothelium-dependent or independent depended on the particular experimental circumstances (68). Bevan's suggestion on the variability seen, and the motive for these difficult to resolve and seemingly conflicting observations, was that these issues were due to heterogeneous endothelium effects between large and small blood vessels (68). This later was confirmed by multiple studies on modeling techniques in skeletal muscle, cardiac muscle and cerebral tissue (17,59,63,75,82–85).

Nevertheless, research in coronary arteries and coronary beds by Kuo et al. (1990 & 1992) and Pohl & Busse (1990) reported the preservation of endothelial flow-mediated control in resistance arterioles (72,73,81,86–89). Further improvements on isolated vessel and in-vivo techniques allowed clarity to elucidate the biochemical cascade involved in the flow-dependent response, as shown in Figure 1 (73). The mechanism studied the most has been the modulation of vasodilation through NO (72,76,86,87,89). Nitric oxide

induces an increase in cellular cGMP (cyclic guanosine monophosphate). The increase of cGMP results in the inhibition of agonist-induced phosphatidylinositol hydrolysis, stimulation of the plasmalemmal Ca++ pump, as well as augmentation of Ca++ uptake into the sarcoplasmic reticulum. Overall, the increase in intracellular calcium efflux and activation of Ca++ dependent K+ channels contributes to a hyperpolarizing state of the EC. In addition to the reduction of intracellular free Ca++, cGMP may also be involved in a reduction of the Ca++ sensitivity of the contractile apparatus by dephosphorylation of myosin light chains (92).

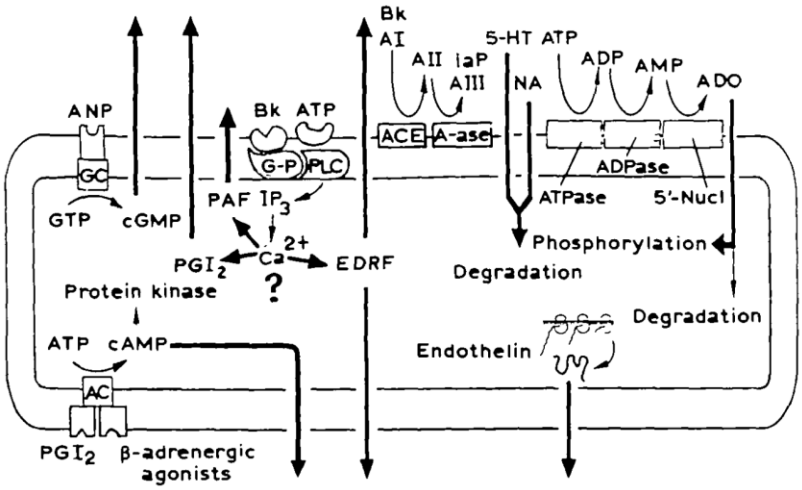

Figure 1: Diagram of endothelial functions which directly or indirectly affect vascular tone.

Although a plethora of hypotheses contemplating transduction of the mechanical environment to which ECs are exposed has been growing in acceptance, work still has to be done to fully understand the mechanobiological mechanisms that influence flow-induced dilation and local regulation of blood flow. One of the most promising mechanisms is the discovery of stretch-activated ion channels (90). Although we will discuss this hypothesis further in this chapter, it is significant to mention that ion-specific stretch-activated channels are present on endothelial cells, and blocking these channels has been shown to inhibit the induction of NO and expression of eNOS and TGFb by shear stress (77,90).

As mentioned in subsection 1.1.3.1, it is important to consider the modulating role that estrogen plays in females' shear stress response. Kaley & Huang's review took into consideration the role estrogen plays in the regulation of vascular flow-dependent responses (20). They were able to demonstrate an upregulating mechanism of endothelial autacoid synthesis (NO, PGs & EDHF) elicited by estrogen that enhanced vascular responses to flow, and the magnitude of the dilation to shear stress was significantly greater in arterioles of females, but not in males (20). Additionally, they were able to confirm this postulate, since OVX female rates had abolished the enhanced NO-mediated dilation and were able to revert the obliterated dilator response once estrogen replacement was installed, even in OVX+OVE hypertensive rats (20). They concluded that the estrogen-dependent vasodilator response is caused by transcriptional upregulation of eNOS by estrogen (confirmed in cell cultures of pulmonary artery endothelial cells), modulated by Ca++ dependent modulation of eNOS. Additionally, Huang & Kaley were able to reveal a novel mechanism, by which EDHF contributes to the maintenance of shear stress-sensitive regulation, particularly in female rats in deficient NO synthesis states, suggesting that EDHF synthesis is a back-up mechanism to maintain flow-induced dilation in pathological conditions (20).

We have commented previously that the understanding of shear stress-dependent vasodilation response has focused its attention on short-term responses elicited by neurohumoral mediators or force mechanotransduction interacting with ECs, glycocalyx & endothelial surface layer (75,90). Nevertheless, a couple of reviews have stressed the importance of blood flow in modulating not only local vasodilation but also to long-term effects that regulate local adaptative structural changes, influencing vessel size, diameter and length (68,77,91). These structural changes include damage and repair near branch points and bifurcations, and chronic vessel wall changes that appear to be evoked by transcriptional changes mediated via shear stress responsive promoter elements (SSREs) that bind transcription factors, which are also activated by shear stress (73,77,90).

Activation of endothelial stimulus-response coupling to shear-stress comes at the expense of a fine-tuned balance between intravascular pressure and shear stress sensing by the endothelium, to which Secomb & Pries defines as a pressure-shear hypothesis (92).

Overall the pressure-shear hypothesis estates that vascular equilibrium is given by a characteristic relation between wall shear stress and pressure that would trigger short- or long-term structural adaptations to the vessel (92).

Due to intrinsic variations in the design of vascular networks between different tissues and natural diameter decreases in the vascular components of the microcirculation, shear stress values cannot be assumed to elicit the same responses to same shear-stress stimulus, as Chilian et al. reported in vessels from 25 – 130 μm from the coronary microcirculation (93). Nevertheless, as reported by Kaley G. et al. in cremasteric arterioles & further explored by Secomb et al. using the same experimental shear stress and a representative segment model study on vessels ranging from 40 to 300 μm, maximal sensitivity to wall shear stress occurs in the physiologically relevant range of 10 to 50 dyne/cm2 (82,94) and in the presence of intact endothelium wall shear stress remain relatively constant and supports the postulate that wall shear stress is a controlled parameter in vascular networks. For example, as reported by Kaley & Koller in cremasteric arterioles the presence of endothelium mean control wall shear stress was 26.58 dyne/cm2, within physiological values as reported by other groups (95,96). In Long-Evans male rats harvested middle cerebral & penetrating arterioles reported wall shear stress values between 11 to 60 dyne/cm2 during physiological conditions (97). In rabbit mesenteric arterioles (17-32 μm) Tangelder et al. reported that the range of wall shear stress was 4.72 to 41.72 dyne/cm^2 (98) and in rat cremaster muscle preparation, Mayrovitz & Roy observed a reasonably constant wall shear rate (mean 42.53 dyne/cm^2) in vessels ranging from 6 to 108 μm in diameter (96). Similarly, in pial arterioles (35.4 - 177.8 μm), a constant wall shear stress (range 26.95-29.15 dyn/cm^2) was found (99).

However, during stenotic conditions shear stresses can reach levels above several hundred dynes per square centimeter (30.4-380 dyne/cm2) promoting platelet aggregation, similar to the effect present in a region of endothelial cell desquamation after atherosclerotic plaque rupture (100). Higher shear-stress values have been reported in Gracilis arterioles of Spontaneously hypertensive rats cannot maintain constant shear stress during increases in flow rate; hence, shear stress reaches a much higher value (90 dyne/cm2) (101). In the late 90’s clinical data confirmed that the initial phase of severe

sepsis or septic shock is characterized by low pressure and low systemic flow, therefore reduced shear stress (102). As reported by Nohé et al., flow conditions in systemic hyper-inflammation states, impaired chemotaxis, and decreased leukocyte recruitment persist despite persistent upregulation of leukocyte integrins (102). Contrary to what was hypothesized before, cell adhesion to surfaces or other cells varies inversely with the magnitude of applied shear forces. Cell detachment or failure to adhere may be due to either mechanical disruption of existing molecular bonds or the limited time allowed for bonds to form between membranes and surfaces (103). Hence, low shear stress promotes a deregulated and paradoxical maldistribution of activated leukocytes during sepsis.

1.4 Piezo proteins: Discovery, properties, pathophysiology

Briefly discussed in the previous section, mechanobiology concepts and mechanotransduction modulated cell activity has been growing into acceptance as emerging theories of how mechanical forces affect the cells. Mechanical forces are detected by specialized molecules in cells and transduced into electrochemical signals that modify acute and chronic regulation of cellular function and morphology. For example, it has been well documented that force-induced strains on the cellular plasma membrane, on nearly all cell types, are able to alter gene expression by activation of secondary chemical pathways or through the cytoskeleton transcriptional activators and repressors (77,90,104). One of the most promising theories is the discovery of stretch-activated ion channels, eliciting sensor-like effects (90,104). Recently, we have been seeking to understand how the various “sensors” in the microcirculation integrate to optimize energy transfer and blood flow efficiency across intact microvascular networks.

1.4.1 Discovery & distribution

Mechanically activated ion channels form pores in the plasma membrane with increasing mechanical stimuli. When open, a membrane current is generated by the passive diffusion of charged ions down their electrochemical gradient (105). Although most channels exhibit selectivity for specific ions, there are also non-selective cation channels that pass a combination of cations (Na+, Ca++, K+), modulated by the inner pore lining of the ion channels which determines the ion selectivity and flux of the permeating cations (104).

The existence of ion channels that are activated by mechanical inputs was first proposed by Bernard Katz in 1950 and several years later reiterated by Georg von Békésy (105). Nonetheless, one of the most exciting developments in this area has been the identification of Piezo (FAM38a) mechanosensitive proteins (9,15,105). The identification of Piezo proteins has been attributed to Coste et al. the identification of Piezo proteins (9,105–108). Having identified a cell line that produced robust mechanically-induced currents (Neuro2A), they generated multiple silencing RNAs for each gene, introduced them to each cell and tested for electrical current attenuation. As a result, the Fam38a gene sequence was identified and later renamed as Piezo1. However, Coste et al. isolated a second Piezo channel after observing that Piezo 1 was scarcely expressed in dorsal root ganglion neurons (DRG). Indeed, Fam38B, posteriorly named Piezo2, are closely related to Fam38A and were initially identified and cloned from DRG neurons & lung (9,107,108).

Piezo ion channels have been classified into two isoforms: Piezo1 and Piezo2. Both are considered inherently mechanosensitive (105). Piezo proteins have been heterogeneously found in multiple tissues across the body (9,108,109). Nevertheless, there is a perceivable relationship between the prevalence in the expression of a certain subtype of Piezo receptors and the tissue/cell type (109). This could be interpreted as a “tailored” presence of Piezo proteins that modulate mechanical transduction depending on the specialization on any given tissue/cell where they are found. For example, Piezo1 proteins are primarily expressed in non-sensory tissues exposed to fluid pressure and flow (e.g., kidneys, red blood cells smooth muscle cells & endothelial cells) (109–113), whereas Piezo2 proteins are predominantly found in sensory tissues (e. g., DRG, and Merkel cells) (9,109).

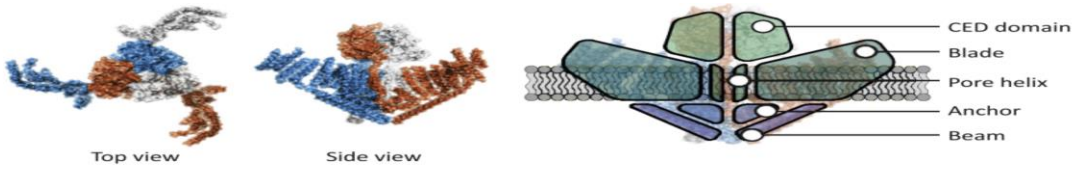

Figure 2: Piezo 1 structure obtained by cryo-electron microscopy (cryo-EM).

Furthermore, as reported in recent studies by Liedtke et al., a synergistic role between both Piezo channels in tissues (chondrocytes) exposed to high-strain mechanical forces was suggested in order to modulate mechanical loads. Although the Piezo1/2 distinct distribution pattern is apparently conserved in other species, further studies are needed, and extrapolation of Liedtke et al. results cannot be assumed in other tissues since the expression of isolated isoforms has also been reported by Patapoutian et al. (114,115).

1.4.2 Mechanosensation & Transduction (Activation)

Mechanotransduction is a sensing mechanism conserved through evolution involving mechanosensitive ion channels, highly sensitive molecules coupled as clusters in the cell's membrane and specialized in conferring force sensitivity (9,108,116,117). In order for Piezo channels, like other mechanosensitive ion channels (MS), to transduce the transmitted force into electrochemical signals, two mechanisms have been hypothesized: 1) ‘Force-from-lipids' model, where the stimuli are transmitted directly to MS channels via deformation of the bi-lipid membrane, and 2) ‘Force-from-filaments’ model, where the interaction between the MS channel and the extracellular matrix (ECM) or cytoskeletal proteins are elicited by tethering and resulting in gating of the channel (114,118).

Recent evidence that Piezo1 is gated, at least in part, by direct membrane tension (‘force-from- lipids’ principle) strongly negates the reliance of gating on structural scaffold proteins and confirms the evolutionary conservation of this gating mechanism (105). Mechanical perturbation of reconstituted mouse Piezo1 protein in droplet interface bilayers (containing no other cellular components) opens Piezo1 with an estimated force of 3.4 mN/m, and even lateral membrane tension as low as 1.4 mN/m activates the channel in cellular membranes (119). Nevertheless, Patapoutian et al., Grandl et al., and Pathak et al. provided confirmation that mechanical indentation of the apical cell surface with a blunt glass probe or the application of suction (negative pressure) to a membrane patch results in stretching of the membrane, and both manipulations activate Piezo proteins (104,107,114,120). Such global or localized stretching of the cell membrane argues in favor of the ‘force-from-filaments’ principle and similar mechanisms are associated with somatosensory nerve endings in the skin, as well as blood flow-mediated

forces by endothelial cells within arteries and veins (111,121).

Despite conflicting results in support of either principle, a unifying mechanism has been proposed by Martinac et al. (112), where the lipid bilayer is sufficient to mechanically gate Piezo channels but its fine-tune properties depend on scaffold proteins. Though unequivocal evidence on the gating mechanism still awaits, research efforts will continue to be made in order to elucidate this and other mechanobiology questions.

1.4.3 Phenotypes

Studies in humans and small mammals have identified Piezo 1/2 loss-of- and gain-of-function phenotypes associated with multiple congenital diseases. In human phenotypes, Piezo 1/2 loss-of-function is associated with less severe conditions, compared to those in animal models. In knockout mice, biallelic deletions of Piezo 1/2 have resulted in embryonic (111) or perinatal lethal conditions (122).

Loss-of-function Piezo1 phenotypes in humans have been associated to congenital lymphatic dysplasia, a pathological state where lymphatic vessels fail to constrict and drain from the extracellular space leading to lymphedema, while gain-of-function Piezo 1 phenotypes are related to autosomal hereditary xerocytosis, a hereditary hemolytic anemia characterized by a gradient shift where intracellular K+ content is decreased and intracellular Na+ increased, resulting in cation leak across the membrane and erythrocyte dehydration (123). A more detailed review of the human loss-of- and gain-of-function phenotypes mentioned can be found on Alpert et al. review on diseases of Piezo channel mutations, and we refer the reader to this work for better understanding of the gene families associated with these pathological states.

Although, the reported effects of loss-of-function Piezo 1 phenotypes from animal models shall be further studied and extrapolation on human phenotype findings must be measuredly considered, results reported by Ranade et al. (human HUVEC cells) and Li et al. (mouse endothelial cells) are in line with the known contribution by ECs in atherogenesis and vascular architecture orientation (111,117,122,123). As a result of “sensing” shear stress, Piezo1 contributes to normal cellular and stress fiber alignment

along the axis of the shear vector (123). Also, reports from studies carried out to elucidate Piezo1 phenotype in erythrocytes were consistent with the human loss-of-function phenotype fount in red blood cells of most patients with generalized lymphoid dysplasia (123).

As mentioned, the extrapolation of the results and associations to Piezo 1 phenotypes in human pathology must be done carefully. Nonetheless continuous research efforts to understand Piezo 1 phenotypical variations in healthy and pathological states are continuously being made and should bring into fruition understanding on Piezo 1 proteins mutations as a risk variant on diploid and haploid associated disorders. An example of this is The Exome Aggregation Consortium (ExAC) database, a database of large-scale sequencing projects, as part of various disease-specific and population genetic studies.

1.4.4 Role in cardiovascular pathological conditions

A series of studies on Piezo1 have been capable to associate Fam38a with pathophysiological conditions such as hypertension (9,111), promoting inward eutrophic remodeling due to altered fiber organization, or an increase in intracellular calcium by malfunction of the actin cytoskeleton. We have already discussed in previous sections the importance of Piezo1 as a determinant of vascular structure in both development and architectural remodeling (111,117,122,123). As Retailleau et al. reported, Piezo1 in smooth muscle cells is associated with structural changes in the myocytes by eliciting a trophic effect during hypertension. Also, without the protective effect of the cytoskeletal protein filamin A, a protein that cross-links actin into either networks or stress fibers, and increase of Piezo1 gating, arteriolar smooth muscle cell remodeling occurs influencing arterial wall thickness without the need of a hypertensive state (107,117–119). Moreover, the role of Filamin A mutations extends into other pathophysiological processes, as they have been associated with a propensity for aortic dilatation, aneurysms, abnormalities of the microcirculation, and premature stroke as complications from perinodular heterotopia (111,124–126).

In a series of studies performed by Dr. Kaufman et al. on a femoral ligated rat model, they reported that after Piezo1 blocking an immediate reduction to exercise pressor reflex

was elicited (112,113). The mechanically sensitive component of the reflex plays a role in the increase of sympathetic nervous activity (SNA), blood pressure (BP), heart rate (HR), myocardial contractility, and peripheral vasoconstriction, an elemental function for adaptation whilst performing intense physical activity (127). Also, exercise pressor reflex provides an indirect perspective on peak workload placed on the heart, as well as workload placed on the heart throughout the contraction period (113). Secondary to an exaggerated sensitization of the mechanical component of the exercise pressor reflex (128,129). Persistence of the exaggerated sensitization state of the exercise pressor reflex has been previously associated with abnormal hemodynamic responses that contribute to the development of Heart Failure & Peripheral Vascular Disease (113,129,130). Elucidating the contribution of Piezo 1 modulating the mechanosensitive component of these mechanisms may serve as a novel target in the treatment for adverse cardiovascular events such as myocardial ischemia or infarction, cardiac arrest, and stroke (131–133).

1.4.5 Integration of forces

Piezo1 co-expression in mammalian vascular smooth muscle cells (VSMC) and endothelial cells (EC) (127,134) supports the postulate that pressure and shear sensing, in arterioles, occurs concurrently and by the same sensor. In the endothelium, Li et al. reported dependence on Piezo1 activity of shear stress sensing in the endothelium, resulting in a suppressed shear stress-induced Ca++ influx and altered sensitivity due to altered fiber and cell organization after Piezo1 depletion or specific inhibition with Grammostola Mechanotoxin #4 (GSM) (111,117). Furthermore, they reported a total reduction in eNOS and eNOS (serine 1177), results which were later confirmed by Wang et al. and Baptiste et al. (111,117,127,134). Wang suggested the reduction of NO synthesis was due to an ATP reduction dependent on Piezo1 in endothelial cells. However, the mechanism underlying the release of ATP from endothelial cells is still poorly understood (134).

Moreover, Retailleau et al. work reported the influence of Piezo1 on smooth muscle cells and how Piezo1 elicits arterial remodeling. However, he failed to report any effect on myogenic response in spite of demonstrating that stretch-activated channel (SAC) activity in caudal arteries was critically dependent on Piezo1 function and that SACs have been

referenced in multiple studies as required for triggering a myogenic response (6,111,135). In a smooth muscle cell Piezo1 conditional knockout mouse model, Retailleau et al. were able to demonstrate increased Piezo1-mediated structural remodeling in SMC, either by mechanical stress or removing Filamin A (FlnA), and it was associated with structural defects and remodeling of caudal artery even in the absence of induced hypertension. Furthermore, the Piezo1-mediated rise in Ca++ is correlated with greater activity of transglutaminases, a cross-linking enzyme in small artery remodeling. Interestingly, the removal of a single Piezo1 allele was sufficient to prevent remodeling (9,111).

Regardless of research efforts in the last 9 years, the mechanisms of the regulation (myogenic control and flow-induced dilation) and the chemical cascade upregulated by Piezo activation is still understudied. Additionally, gender dimorphisms on Piezo1-mediated shear and pressure sensing are still unknown. Thus, the focus of our current project is to determine gender dimorphisms in shear stress and myogenic control and the role Piezo1 plays on local blood flow regulating mechanisms.

1.5 Purpose

As we have discussed in this first chapter, understanding the setup, biomechanical properties and local regulating mechanisms of the microvasculature are of great relevance to elucidate the implications of cardiovascular diseases, still considered, the biggest killer worldwide. Cardiovascular diseases comprise 31% of all deaths each year worldwide and just in Canada are the cause of 25% of the premature deaths in males and 15% in females aged 30-60 years (136). Treatment for all the individual ailments that constitute CVD's, by most part change the natural history of the pathology and their goals are focused on improving survival rates and reducing morbidity. Nevertheless, CVD treatment that statistically reduces mortality still remains challenging, due to the heterogeneous pathophysiological mechanisms.

By far, intracellular signaling processes have been the most extensively studied and have been the focus of the current pharmacopeia and treatment therapy options available in the present day. Nevertheless, the cellular mechanism responsible for mechanotransduction of the myogenic and shear stress responses remains elusive. Thus, our project bears

importance on understanding mechanotransduction signals that Piezo1 proteins elicit, in an attempt to provide new therapeutic targets or diagnostic efforts to improve CVD mortality and morbidity statistics in males and females.

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Chapter 2

Piezo 1: A fundamental piece to transduce shear and circumferential stress in the microcirculation

2.1 Introduction

Bayliss's description attributing the phenomena of myogenic response to intrinsic regulation properties of the blood vessels without any input of the central nervous system was the first description of mechanoreception properties attributed to blood vessels (1). Mechanoreception is the most widely distributed sensory modality providing the ability to sense and adapt to mechanical stress in the environment. Its contribution has been associated with evolutionary needs from our prokaryotic ancestors reducing osmotic stress and to proprioception, touch, hearing, etc. on more complex organisms comprised by a homeostatic relationship of eukaryotic cell complexes (2–4).

Transmembrane proteins, referred to as mechanical sensitive ion channels (MSCs), are responsible to convert said mechanical stresses into electro-chemical signals according to the cellular needs. In eukaryotic cells research efforts on MCSs has focused on the TRP family; although data suggests involvement in said sensory modality, their role in mechanotransduction has never been established and doesn't fulfill all the criteria for transferable mechanical properties (3,4). Therefore, other candidates have been described as better representatives of cells mechanosensitive properties; Potassium selective channels (K2p) family as TREK-1, TRAAK or NOMPC from the TRP family, to name some, have been confirmed to be activated by a number of different mechanical stimuli and fulfill the criteria described by Árnadottir et al. and Christensen et al. (4,5)

One of the major advancements in this area of research has been the discovery and description of Piezo proteins by Coste et al. (3,6,7). Piezo proteins are bona fide mechanical channels named after the Greek word πίεση (píesi) meaning pressure, from which two isoforms have been identified (Piezo 1 & Piezo 2) and deemed essential components of mechanically activated channels since they fulfill many requirements of the criteria for true mechanically activated ion channels, as they are pore-forming subunits, confer mechanically activated currents in a heterologous system and are necessary components for cell's mechanical responses in many cellular lines. (7–10). A particularity of Piezo proteins that confer great importance in the overall transduction of mechanical sensitization of cells is that they represent two of the human proteins that are

predicted to have the highest number of transmembrane segments (8). Thus, probably contributing more to the detection of mechanical forces compared to other known ion channels through its unique structure and associated molecules in the overall cell's architecture (8,11).

As previously mentioned, Piezo1 proteins are essential components in the cellular membrane contributing to the mechanical sensitization transduction in ECs & VSMCs. Li et al. reported endothelial cells' dependence on Piezo1 to sense shear stress and flow sensitivity, a result of altered fiber and cell organization after Piezo 1 inhibition with GSM (12). Moreover, Retailleau et al. reported the secondary effect of Piezo1 inhibition on smooth muscle cells altering arterial remodeling (7). Although Piezo 1 role played in flow-induced dilation and the myogenic response has not been properly clarified, its co-expression in mammalian vascular smooth muscle cells (VSMC) and endothelial cells (EC) (13,14), supports the postulate that pressure and shear sensing, in arterioles, occurs concurrently and by the same sensor.

Over the last year, the purpose of our work was to ultimately understand how Piezo1 proteins serve in the integration of mechanical forces that elicit opposite responses, myogenic constriction in response to increases of intraluminal pressure and flow-induced dilation in response to increased shear stress, as well as their role in optimization of energy transfer and blood flow efficiency. Besides, to understand Piezo 1 role in the regulating mechanisms, the main objective in our current project focused on determining gender dimorphisms in shear stress and myogenic control in two different vascular locations, cerebral and musculoskeletal vascular networks. As referenced previously, metabolic variability in demands within different tissues/organs may modulate the contributions of response mechanisms to autoregulation (15). Thus, it is important to run comparative studies between skeletal muscle and cerebral vasculature since it is logical to expect differential vascular effects. Our overall hypothesis is that Piezo1 is a fundamental component in EC's and VSMC's from resistance arterioles to optimize rheological efficiency and blood flow distribution via shear and circumferential-stress sensing.

2.2 Methodology

2.2.1 Experimental Data Acquisition

Sprague Dawley rats Males n= 29 & Females n= 25 (~10 weeks of age; M= 414 mg, F= 264 mg) all protocols received prior approval from the Council on Animal Care at the Western University. All rats received an anesthetic compound solution of α-chloralose (160 mg/kg) and urethane (1000 mg/kg) via IP injection.

Table 2: Gracilis & middle cerebral arterioles. Data presented as mean ±SEM

	Males			Females		
	Mean	SD	SEM	Mean	SD	SEM
Gracilis arteriole (GA)	113.05	18.88	4.22	116.00	19.23	5.55
Middle cerebral arteriole (MCA)	134.70	22.50	5.03	140.70	13.42	4.25

After adequate induction of anesthesia Gracilis (GA) and middle cerebral arterioles (MCA), were harvested and hung on both sides to glass pipettes in ex vivo single vessel heated chamber bathed in physiological HEPES buffer solution. Posteriorly, single vessel chambers were connected to a set-up of peristaltic pumps to regulate flow rates and intraluminal pressure changes (Living Systems Instrumentation LLC., Fairfax, Vermont, USA) and equilibrated for 30 minutes with an open flow. Following, vessels were pressurized at 80% of MAP for another 30 minutes until they spontaneously developed active tone. Confirmation of vessel viability was carried after spontaneous vascular tone development through increasing concentrations of vasoconstrictors and dilators peptides; serotonin (5-HT) and acetylcholine (Ach) for middle cerebral arterioles, and

Phenylephrine (Pe) and Acetylcholine (Ach) for Gracilis arterioles (Fig1). After corresponding drug response curves and viability test confirmed proper function of VSMCs and ECs, vessels were exposed to randomized pressure challenges going from 5 to 160 mmHg, at 20 mmHg incremental intervals; and Flow challenges selecting 5 μl/min (4.9 dyne/cm^2 in GA's & 2.9 dyne/cm^2 in MCA's), 10 μl/min (9.8 dyne/cm^2 in GA's & 5.9 dyne/cm^2 in MCA's), 30 μl/min (29.3 dyne/cm^2 in GA's & 17.6 dyne/cm^2 in MCA's) & 60 μl/min (58.6 dyne/cm^2 in GA's & 35.13 dyne/cm^2 in MCA's). Shear stress was calculated employing of Equation 1 (Ch 1, Sec 1.3.1), where Q was the perfusate flow rate and viscosity for HEPES buffer η=.83 (20) and was decided to be the only buffer utilized for this protocol. Furthermore, it is important to differentiate flow rate vs. shear stress since same flow rates can result in heterogeneous shear stress values depending on the design of vascular networks between different tissues, hierarchical diameter decreases in the network and viscosity, as described previously in Chapter 1. Both flow rates and equivalent shear stress are representative of physiological conditions in skeletal muscle and cerebral circulation, since only one buffer was used (same viscosity), flow rate was preferred to present the data. Arterioles diameter was recorded with LabScribe myogram software (version 4, iWorx Systems Inc., Dover, NH, USA), synchronized to a monitor & video dimension analyzer to track diameter changes.

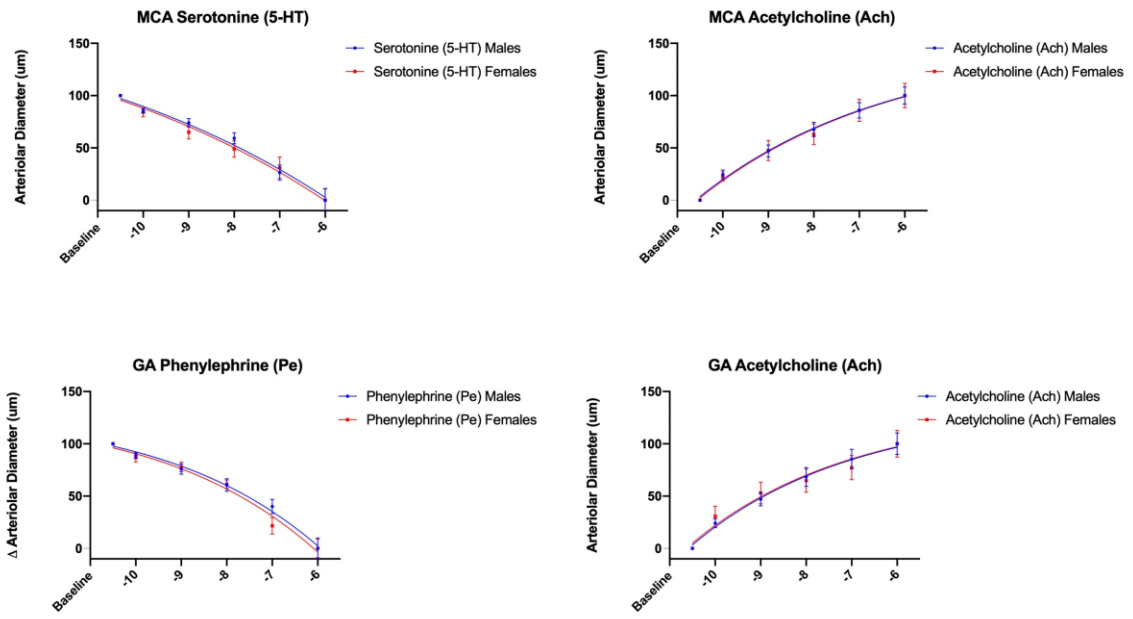

Figure 3: EC50 Drug response curves - Arteriolar diameter response to higher drug concentrations to determine endothelial and vascular smooth muscle viability. Serotonin (5-HT) and Acetylcholine (Ach) for middle cerebral arterioles. Phenylephrine (Pe) and Acetylcholine (Ach) for Gracilis arterioles.

2.2.2 Statistical Analysis

Statistical analysis was performed in Prism (version 8.2.0, GraphPad Software Inc., La Jolla, CA, USA). Two-way ANOVA with Tukey's multiple comparison test to determine significant differences between control and GSM treated groups at any given flow rate. Moreover, student t-test was employed to determine significant differences between gender groups. To asses Piezo 1 role in the regulation of myogenic effect, Two-way ANOVA with Tukey's multiple comparisons was applied to evaluate significance between component groups in myogenic challenges. All values analyzed for flow and myogenic challenges were presented as mean ± SEM.

2.3 Results

2.3.1 Grammostola Mechanotoxin 4 EC50 determination

Initially, we determined to use Grammostola Mechanotoxin 4 (GSM/GsMTx4) as Piezo 1 blocking agent due to previous studies reports that have shown more than 80% of Piezo 1 mediated activating currents. To establish GSM effective concentration, we ran a small set of experiments to determine the concentration where the compound starts to induce its deleterious effect over the autoregulating mechanism. Increasing concentrations from GSM (10^{-10}-10^{-6}) were incubated in the superfusate or through the intraluminal PSS perfusion and run pressure and flow challenges to determine the EC50. As result, we obtained a GSM EC50 between 10^{-9}-10^{-7}. Previous pharmacological studies on this compound, done in our lab, on in-vivo gluteus maximus preparations reporter an EC50 OF 10^{-7}; thus, we deemed essential to use this concentration to assure proper Piezo 1 proteins blockade.

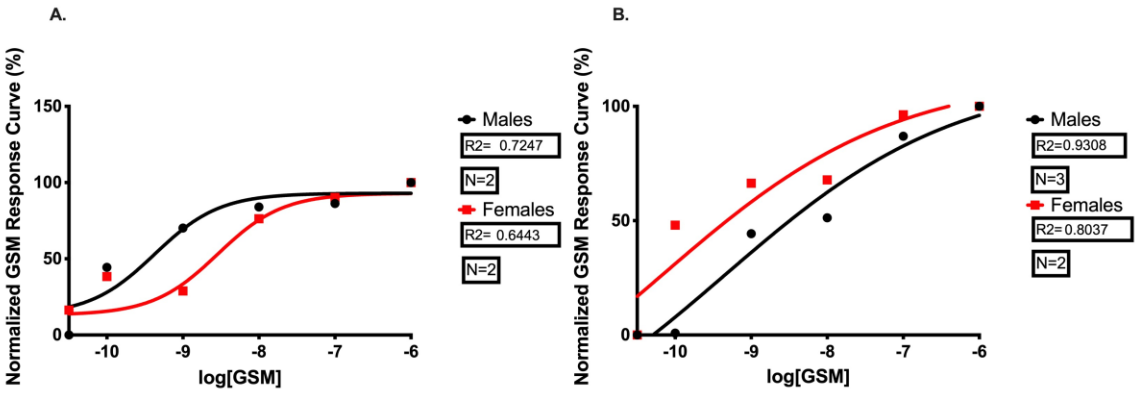

Figure 4: GSM EC50 normalized response curve. Arteriolar diameter changes to incremental GSM incubations done in gracilis arterioles. A. GSM superfusate incubation B. GSM intraluminal incubation.

2.3.2 Piezo 1 effect over endothelial Flow-Induced Dilation

Arteriolar diameters were recorded continuously at a rate of 100 samples/sec for ten minutes with LabScribe software. After all, values being exported, the individual 60,000

points in time were averaged on all conditions and normalized to weight to tease out the influence of the 150 mg difference between genders weight (Males: 414 gr., Females: 264 gr.).

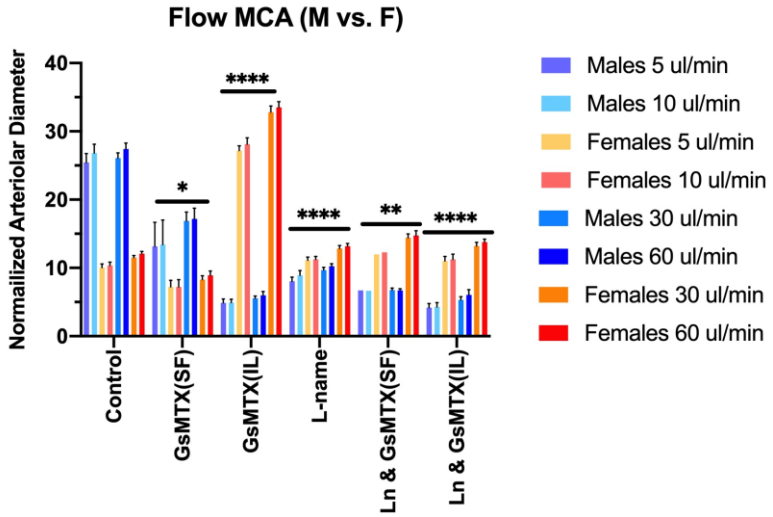

Figure 5: Relationship between changes in arteriolar diameter and treatment conditions at different flow rate challenges for Middle cerebral arterioles (MCA). Data presented as percent change ± SEM (Males n= 29; Females n=22) (*,**,**** Significantly different from control condition at $p < 0.0005$).

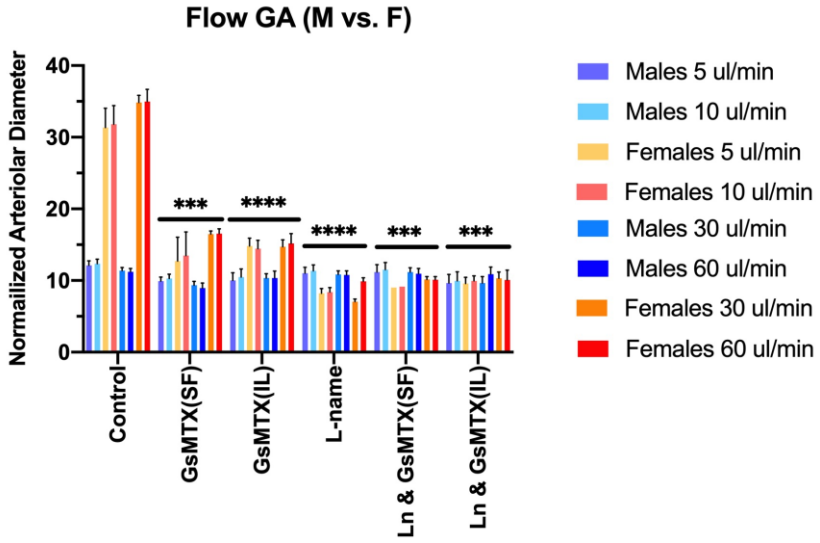

Figure 6: Relationship between changes in arteriolar diameter and treatment conditions at different flow rate challenges for Gracilis arterioles. Data presented as

percent change ± SEM (Males n= 29; Females n=22) (***,****Significantly different from control condition at $p < 0.005$).

Arterioles exposed to Piezo1 antagonism were significantly different from control groups, and we can reject the null hypothesis since the significant overall main effect was observed on all harvested MCA vessels incubated with GSM(SF) & GSM(IL) in a completely different set of both harvested vessels. Since the overall response to GSM was a decrease in flow-induced dilation, with exception of Piezo1 antagonism in middle cerebral arterioles from female subjects, we were determined to validate if the apparent GSM interaction measured did not result of cross-reactivity to nitric oxide synthase. Thus, we used N-Nitroarginine methyl ester (L-name) from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), a known Nitric oxide synthase (NOS) inhibitor, by itself, plus in combination with GSM, and repeat flow challenges to tease out the effect of NOS blockade as the reason of the impaired vasodilation. Also, after the respective flow challenges with GSM, we added once again ACH to determine if the detriment vasodilating response, was due to a decrease in NO bioavailability.

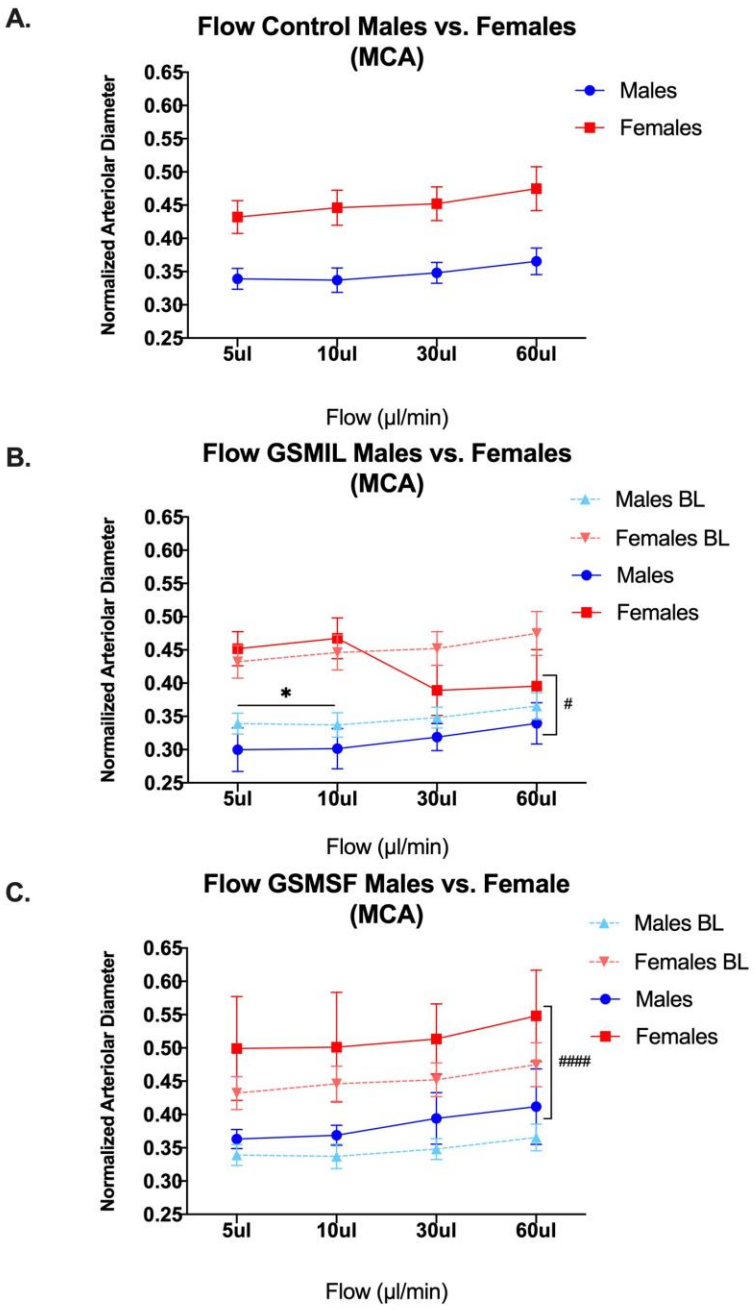

Figure 7: Main effect of flow rate increases and its relationship to vasodilation at control conditions and after GsMTx4 (GSM) antagonism for Middle cerebral arterioles (MCA). A. Control conditions for Male (n=29) and Females (n=22). B. Main effect on arteriolar diameters after GSM intraluminal incubation, control condition is represented as baseline (BL) in dashed lines (Males n= 12; Females n=9). C. Main effect on arteriolar diameters after GSM superfusate incubation, control condition is represented

as baseline (BL) in dashed lines (Males n= 10; Females n=9). All data is presented as percent change ± SEM ([*] Significantly different from control condition at $p < 0.05$, [#,###] GSM response significantly different between genders at $p < 0.005$).

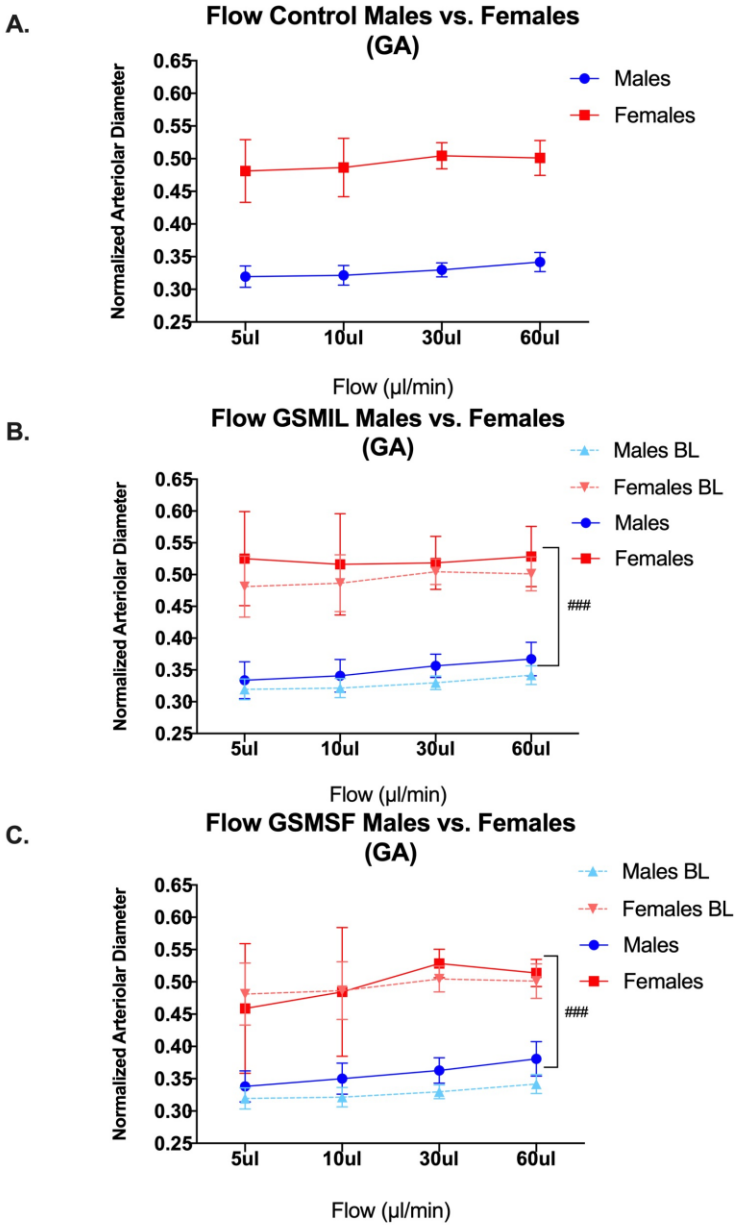

Figure 8: Main effect of flow rate increases and its relationship to vasodilation at control conditions and after GsMTx4 (GSM) antagonism for Gracilis arterioles (GA). A. Control conditions for Male (n= 29) and Females (n=19). B. Main effect on arteriolar diameters after GSM intraluminal incubation, control condition is represented

as baseline (BL) in dashed lines (Males n= 14; Females n=10). C. Main effect on arteriolar diameters after GSM superfusate incubation, control condition is represented as baseline (BL) in dashed lines (Males n= 12; Females n=8). All data is presented as percent change ± SEM ($^{###}$ GSM response significantly different between genders at $p < 0.05$).

After confirming that GSM antagonistic effect plays a significant negative role on shear dependent dilation regulation by Piezo 1, we replotted the same data as a relationship between flow rates and arteriolar diameter changes (Fig4 & Fig5) in order to understand if location of where the incubation takes place weights as a significant factor in the main effect interaction, as well as to determine sensitivity changes across the increasing flow rates.

2.3.3 Piezo 1 effect over Myogenic control

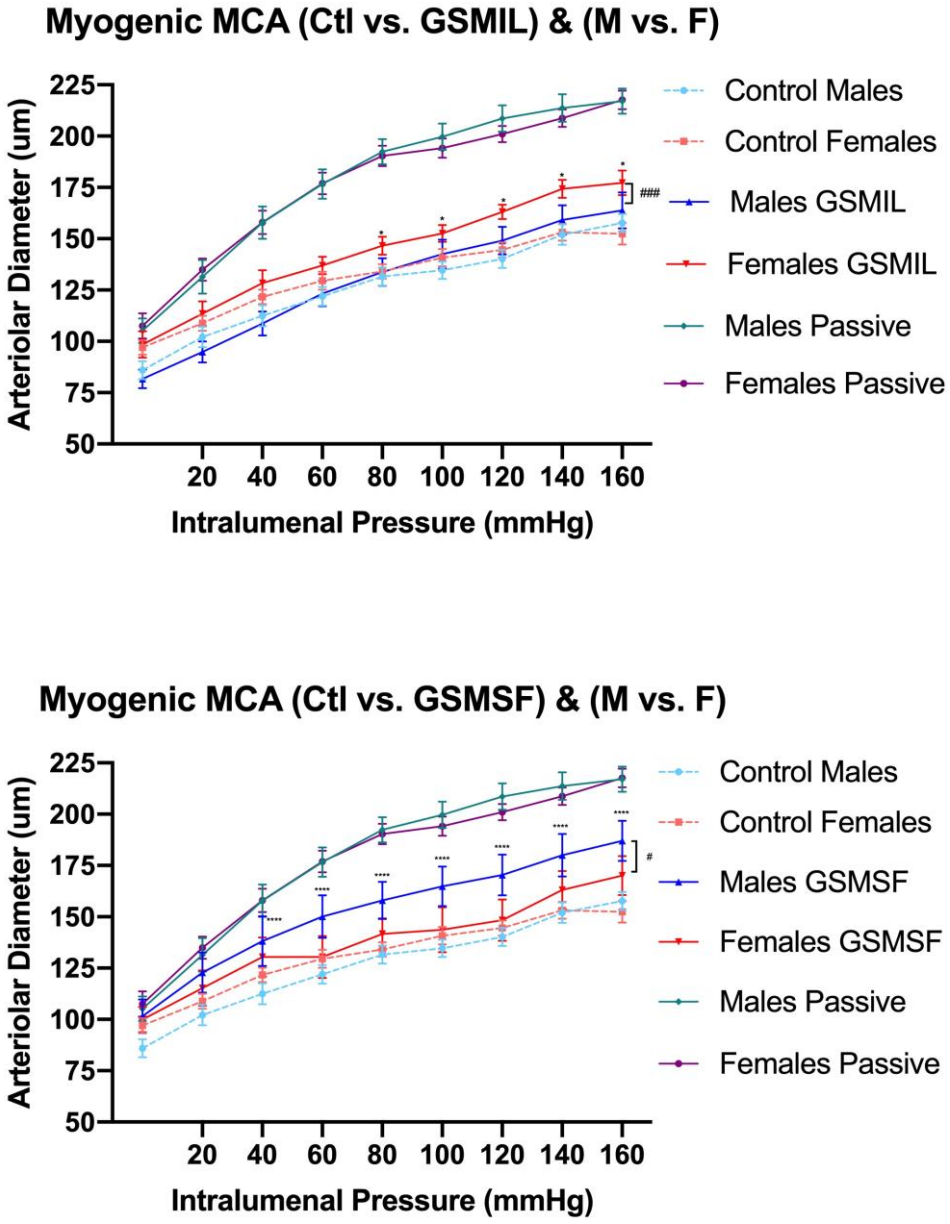

Figure 9: Relationship between changes in arteriolar diameter and treatment conditions at increasing intralumenal pressure for Middle cerebral arterioles (MCA). Data presented as raw arteriolar diameter change ± SEM; Males n= 27, Females n= 24 (*,**** Significantly different from control condition, GSMIL Females = $p < 0.013$, GSMSF Males = $p < 0.0001$; $^{\#,\#\#}$ GSM response significantly different between genders, GSMIL at $p < 0.03$ & GSMSF at $p < 0.0001$).

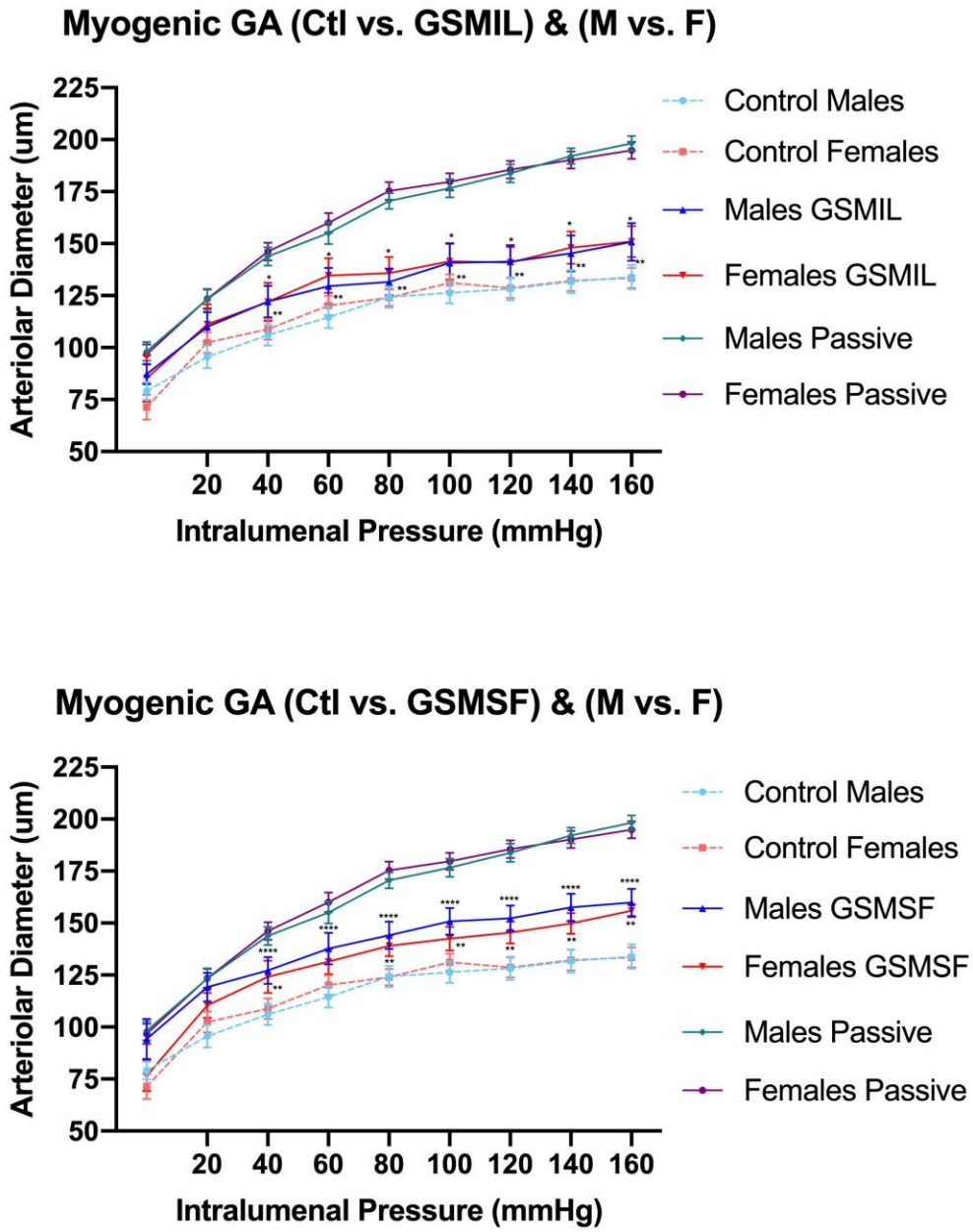

Figure 10: Relationship between changes in arteriolar diameter and treatment conditions at increasing intraluminal pressure for Gracilis arterioles. Data presented as raw arteriolar diameter change ± SEM; Males n= 28, Females n= 22 (*,**,**** Significantly different from control condition, GSMIL Males = $p < 0.0030$, Females = $p < 0.015$; GSMSF Males = $p < 0.0001$, Females = $p < 0.0029$).

As we stated in the first chapter, myogenic constriction as a response to increasing intraluminal pressure (Fig6 and Fig7) play an indispensable role in homeostatic regulation. To test whether Piezo 1 activity significantly contributes to the mechanical sensitization of circumferential stress, thus triggering myogenic constriction, we tested the hypothesis that myogenic response in females will be markedly altered in skeletal muscle arterioles result of loss of active tone and loss in sensitivity to greater shifts of pressure and plotted as a relationship between intraluminal pressure changes and arteriolar diameter. We found that after Piezo 1 antagonism there is a significant difference in myogenic control, were males and females present a significant loss of active tone after GSM incubation on both representatives of the peripheral circulation studied, with a particular significant contrast in the cerebral circulation.

2.4 Discussion

The purpose of this work was to determine for the first, to our knowledge, Piezo 1 impact in the transduction of forces and the role it plays in the regulation of myogenic response and shear-induced dilation. As well as, gender-based dimorphisms result of Piezo 1 mechanosensitive effect. As we observe in Fig2 and Fig3., GSM antagonism on Piezo 1 compromises dilation on both types of harvested vessels and its effect was independent of eNOS activity. Particularly and contrary to was expected, male data presented in Fig.2, show a greater detriment on flow-induced dilation. Nevertheless, we did observe the expected adverse effect after intraluminal incubation males across all the flow rates, presenting a decrease of 78.8% compared to superfusate incubation were the results of 40.4% in a decrease of vasodilation response. Moreover, females presented a 28.5% decrease after superfusate incubation, but we were surprised to see that after intraluminal incubation instead of observing a decrease, results showed an increase of almost 2 times the dilation vascular response observed under baseline conditions. In contrast, GA data reported in fig.3 reports the expected effect after Piezo 1 antagonism, where both genders seemed to be significantly affected by GSM antagonism to Piezo1 channels but seemingly, females were affected in a higher magnitude, regardless of where incubation of GSM had taken place. After intraluminal incubation females, across all the flow rates, presented a dilation decrease of 55.6% compared to superfusate incubation were the

results were 55.5% decrease in vasodilation. As mentioned, males seemed to present a negative effect on shear-dependent dilation of lower magnitude with an 18.2% decrease after superfusate incubation and a 13.9% decrease in vasodilation after intraluminal incubation.

To consider Piezo1 effect over sensitivity in MCAs & GAs Fig4 & Fig5 report that under control conditions females have significantly greater dilation response & greater sensitivity to flow rate changes compared to males. As shown in Fig. 4, after GsM incubation IL, we observed a proportional downward shift and decrease in sensitivity on males and females compared to control conditions(BL), where males presented a significant downward shift on the lower flow rates (5 ul/min & 10ul/min) and although females reflected greater compromise in sensitization on the higher flow rates did not shown significant differences mainly to greater variability on the data obtained at higher rates (30 ul/min & 60 ul/min). Contrarily, after GSM superfusate incubation both genders seemed to preserve flow shear sensing properties and present even present an upward shift in dilation magnitude, being females with a significantly greater magnitude compared to males.

Figure5 shows Flow in GAs, where the persistence of female significantly greater response and greater sensitivity to flow rate changes compared to males were still observed in skeletal muscle arterioles. Regardless of GsMTx4 incubation, we reported the preservation in sensitivity among both genders, as well as the significant greater magnitude in females flow-dependent dilation response as seen under control conditions.

At physiological intraluminal pressures myogenic response in MCAs (Fig6) we were not able to report any differential response between genders at baseline conditions (BL). Nonetheless, when Piezo1 antagonist GSM was incubated IL, females reported a significant loss of 40% of active tone compared to baseline, but not in males. Although males had a similar shift of 33.3% in arteriolar diameter compared to baseline conditions it was not significantly different to control conditions (BL). After superstate incubation of GSM males, compared to females, had a compromised myogenic response by active tone loss of 39.8%, significantly different from its control state. Although females had a

similar shift of 22.8% in response to Piezo1 antagonism it was not significant. Furthermore, regardless of the incubation type, the differential effects after Piezo 1 antagonism were significantly different between genders.

In contrast to cerebral circulation, impairment of the myogenic response in GAs after Piezo1 antagonism did not show gender dimorphism in spite of the differential GSM applications. Though, we were able to report a significant loss in myogenic response with an overall effect versus control conditions result from a loss of active tone of 41.5% and 40.8% in males and females respectively.

2.5 Limitations

Although, we choose to employ isolated preparations in our experimental protocol to precisely control most of the experimental conditions (luminal pressure, shear stress, superfusate & perfusate solutions, etc.) and elucidate phenotypical variances, as well as gender-based dimorphisms in this project, as we deemed to be a better fitting model. One of the main disadvantages derived from ex-vivo preparations come from the loss of other regulating mechanisms constantly counterbalancing (neuro-humoral, metabolic and intercellular conduction input) (16,17) and one of the main problems in this matter we encountered in our flow challenges, as previously discussed.

Moreover, flow rates employed in our experimental protocol were selected as a result of initial flow response curves to determine the capacity of the experimental operating systems to autoregulate intraluminal pressure as a response to increases in flow rates. Although, the chosen flow rates concur within the physiological intraluminal pressures observed on in-vivo gluteus maximus preparations and modeling techniques from previous studies from our lab (18), there is a concern that 30 ul/min & 60 ul/min rates are three to six orders of magnitude above the physiological ranges expected in vessels of the same order (18). Nonetheless, the literature has reported different ranges correspondingly to the area where the vessels for the ex-vivo preparations were harvested and established a three times value deviation between experimental and in vivo values, due to viscosity differences between blood and saline solution (4).Thus, the effects of the higher flow rates shall be carefully considered as representatives of a hemodynamic state where the

flow rate is at maximum functional capacity and serve as a threshold limit for posterior projects.

2.6 Conclusion

As stated in the first chapter, gender-based dimorphisms and differential effects in local regulation mechanisms were expected in cerebral and musculoskeletal circulations. When compared versus males, female middle cerebral vessels tend to preserve better shear-induced dilation after Piezo1 mechanosensitive properties were blocked with GSM, except for direct Piezo1 antagonism in the endothelium, where higher flow rates were greatly compromised and sensitivity to higher flow rates approach male control values. Although this effect was not significant, linear regression analysis resulted in a negative trend ($Y = -0.001158*X + 0.4506$) and most likely by getting closer to a balanced sample size of n=15, significance on the interaction will be present. As expected, male and female musculoskeletal arterioles seemingly preserve flow better regardless of Piezo1 antagonism directly in the endothelium or in smooth muscle cells, but still, we were able to elucidate Piezo1 in ECs as a determinant for shear load sensitivity.

Piezo1 properties to bellwether pressure loads in SMC, were more visible in males compared to females especially on cerebral arterioles, knowingly more dependent on sensing pressure variations to protect encephalic tissue. GAs was overall affected by GSM antagonism over Piezo 1 channels but regardless of GsMTx4 incubation (SF or IL) loss of pressure sensing related to Piezo1 was still observed on musculoskeletal vasculature.

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Chapter 3

General Discussion

3.1. Contributions

The main purpose of this graduate thesis serves as a first approach to understand Piezo 1 as a fundamental piece of "the puzzle" that is understanding homeostatic regulation in the microvascular networks. Furthermore, for the first time, we approach Piezo 1 mechanosensitive properties from a more comprehensive approach to its differential effects in both cell lines that encompass the vasculature and the way the impact local regulating mechanisms, in particular, myogenic constriction & shear-induced dilation.

In addition, we explored for the first time Piezo 1 interactions and differential effects on resistance arterioles from skeletal muscle and cerebral circulation, which we have addressed previously in the first chapter of this dissertation, have been prominently reported to address their local regulation in specific ways that correlate to their physiological needs (39,61,62,127). Although functional approaches to determine Piezo1 impact in myogenic control and flow-induced vasodilation have been reported previously (111,127,134), these research groups have appeal to understand the role of Piezo 1 just in the context of the functional detriment to the regulating mechanisms in vessels from Piezo knockout subjects and didn't considered gender dimorphisms in their studies.

Having a preference for ex-vivo isolated vessel techniques besides from their practical benefit to control, detailed out in the first chapter (sec.1b), we were able to select and test under specific physiological scenarios in a heterogeneous group model with diverse gender, arterioles, and mechanisms of incubations. With this model, we were able to report about the indispensable role Piezo1 mechanotransduction properties plays into regulation of myogenic control and shear-induced dilation, as well as report for the first time male and female differences dependence to this mechanosenstive ion channel in different circulatory systems.

Nonetheless, as a secondary and more global purpose, this thesis serves as an attempt at our lab to keep working in understanding and develop tools that help bring clarity in an understudied area of research that is cardiovascular research and moreover, gender differences. By approaching Piezo1 mechanotransduction properties contemplating gender dimorphisms and anatomical differences, opens a field of Piezo 1 proteins as

potential biomarkers or target therapies providing a more effective treatments for cardiovascular diseases in males and females.

Piezo 1 potential as prognostic & predictive biomarker is exemplified by its recently established relationship to cell migration, a defining characteristic of highly metastatic cancer cells. MCF-7, a highly malignant breast cancer cell line identified with higher Piezo 1 concentrations or PC-3, associated to a highly aggressive form of prostate cancer, mechanical activated currents can be blocked by GsMTx4 (8,9). Furthermore, biomarker and treatment possibilities can be exemplified by recent reports on MicroRNA-103a, which directly targets and regulates Piezo1 gene expression. MicroRNA-103a, has been associated with biomarker qualities for CVD's as Hypertension or Acute myocardial infarction and as etiological factor for Hypertension. Nonetheless, further studies are needed to clarify the role of miR-103a and Piezo1. As other MicroRNA's, miR-103a can target multiple genes, but it is still unknown how many of these genes are involved in regulatory effects on the biological functions of endothelial cells (7).

3.2. Future work

Although, we tried to quantify Piezo 1 concentrations in different homogenates from the same vascular tissues employed in our pressure and flow challenges, as well as in other conduit vessels (renal artery, Iliac artery, abdominal & thoracic aorta), lung tissue and gluteus maximus tissue, excised from the same males and females. We were not able to collect enough tissue to run consistent Piezo 1 protein quantifications in MCA & GA, due to the need for greater dilution ratios or small volumes of microvessel homogenates. Nonetheless, we are aware that the effects found in our experiments can be the result of quantifiable differences in the gender and microvessels selected for this project.

Thus, as next step we are aware that smaller volumes, though more concentrated homogenates, would require the use of Piezo 1 Enzyme-Linked Immunosorbent Assay (ELISA) kits to finally confirm if the deleterious effects reported in our result section are result of a heterogenous presentation of Piezo 1 proteins in the vasculature of cerebral and skeletal muscle circulation. Also we will repeat the ELISA with enzyme digested cell cultures of VSMCs and ECs, to quantify Piezo1 differences in the membranes of the

individual cellular components of the vasculature, which will enrich our understanding of the role Piezo 1 plays as a determinant factor not only in regulating mechanisms of the microcirculation but as well as in other conduit vessels and vascular systems.

Furthermore, we will thrive to analyze and increase the sample size for our IVVM experiments to have a better perspective on the in-vivo factors that impact the overall regulation of the microvasculature and functional relevance to Piezo1 blockade. As previously mentioned in chapter 2 of this dissertation, shear stress variations found between ex-vivo and in-vivo models influence the way we may interpret endothelial responses to shear stress, considering viscosity disparity due to the perfusate solution in ex vivo preparations and contemplate the interaction with other regulating mechanism in order to understand better the negative effects of Piezo1 antagonism. It is important to note that it will take three times the volume flow with saline to elicit the same endothelial shear stress responses with ex-vivo preparation, compared to in-vivo experimental models as IVVM, due to viscosity differences between blood and saline perfusate. Additionally, loss of regulating mechanisms in ex-vivo models, but found in-vivo, like neuro-humoral, metabolic or intercellular conduction, make IVVM the next best step to further explore Piezo 1 phenotypical variances under physiological and pathological conditions.

Finally, we will develop model studies with endothelial-specific and SMC specific knockout mice. With our current approach was impossible to determine phenotypical differences. Piezo1 gain of function or loss of function variations have not been correlated to differences between gender and harvested vessels, nor if the phenotypical variations play a role in the incidence of pathologies mentioned thought this dissertation. Although the specific endothelial knockout is lethal, we will work around this condition with tamoxifen conditional knockouts for the endothelial variable.

Our project is part of continuous effort in our lab for over a decade to understand and develop tools & techniques that help bring clarity in an underfunded area, that is cardiovascular research and the role sex differences play in autoregulation of the microvasculature. Therefore, we will work to add Piezo 1 mechanosensitive properties in

our computational models to properly predict their outcome in control conditions and conditions such as hypertensive, X syndrome and diabetic, where Piezo 1 reports have shown significant effects as marker or treatment targets.

3.3. References

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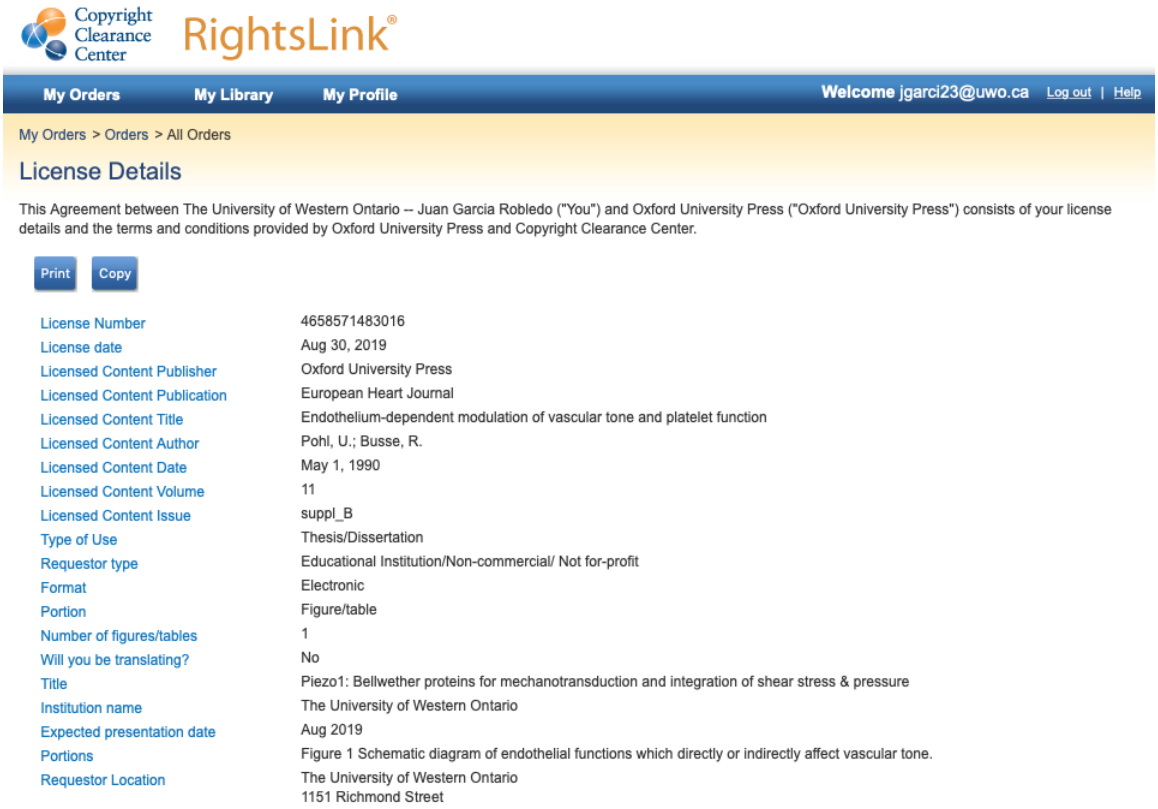

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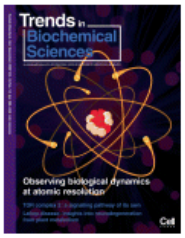

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Official Notification of ACC Approval: A MODIFICATION to Animal Use Protocol **2017-029** has been approved.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15 http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
   c) http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm
2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training ([training@uwo.ca]training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance
   the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets, http://www.uwo.ca/hr/learning/required/index.html

List of abbreviations and Symbols

SD: Sprague Dawley

MCA: Middle Cerebral Arteriole

GA: Gracilis Arteriole

GsMTx4 or GSM: Grammostola Mechanotoxin 4

EC: Endothelial Cells

VSMC: Vascular Smooth Muscle Cells

SAC: Stretch-activated ion Channels

Ca++: Calcium

eNOS: Endothelial Nitric Oxide Synthase

iNOS: Inducible Nitric Oxide Synthase

NO: Nitric Oxide

CVD: Cardiovascular Disease

ERα: Estrogen Receptors α

ERβ: Estrogen Receptors β

MAP: Mitogen Activated Protein

MSNA: Sympathetic Nerve Activity to Skeletal Muscle

NPY: Neuropeptide Y

NA: Noradrenaline

Y1R: Neuropeptide Y Type 1 Receptor

α1R: Adrenergic α1 Receptor

BP: Blood Pressure

VC: Vascular Conductance

HERS: Heart Estrogen/Progestin Replacement Study

CHD: Coronary Heart Disease

CBF: Cerebral Blood Flow

cGMP: Cyclic Guanosine Monophosphate

PGH2: Prostaglandin H2

OVX: Ovariectomized

OVE: Estrogen Replacement

WSS: wall shear stress

τ: Shear stress

η: Viscosity of fluid

Q: Flow rate

π: Pi

r: Radius of tube

PGI2: Prostaglandin I2

EDHF: Endothelium-dependent Hyperpolarizing Factor

K+: Potassium

TGFb: Transforming growth factor beta

Na+: Sodium

FAM38a: Piezo 1

DRG: Dorsal Root Ganglion Neurons

MS or MSC: Mechanosensitive Ion Channels

ECM: Extracellular Matrix

HUVEC: Human Umbilical Vein Endothelial Cells

ExAC: Exome Aggregation Consortium

SNA: Sympathetic Nervous Activity

HR: Heart Rate

FlnA: Filamin A

TRP: Transient receptor potential channel

K2p: Two pore domain potassium channels

TREK-1: Potassium channel subfamily K member 2

TRAAK: Potassium channel subfamily K member 4

NOMPC: No Machanoreceptor Potential C

IP: Intraperitoneal injection

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid Buffer

5-HT: Serotonin

Ach: Acetylcholine

Pe: Phenylephrine

GSM(SF): Grammostola Mechanotoxin 4 incubated in the superfusate

GSM(IL): Grammostola Mechanotoxin 4 incubated intraluminal

BL: Baseline

SEM: Standard Error of the Mean

ELISA: Enzyme-Linked Immunosorbent Assay

IVVM: Intravital Video-Microscopy

Curriculum Vitae

Name:	Juan Garcia Robledo
Post-secondary Education and Degrees:	Universidad de Monterrey (UDEM) Monterrey, Nuevo Leon, Mexico 2005-2012 M.D. The University of Western Ontario London, Ontario, Canada 2017-2019 MSc.
Honours and Awards:	Consejo Nacional de Ciencia y Teconologia (CONACYT) Scholarship 2017-2019
Related Work Experience	Research Assistant The University of Western Ontario - A.C. Burton Laboratory for Vascular Research 2017-2019
Publications:	