QATAR UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

# CADMIUM, AN ENVIRONMENTAL CONTAMINANT WITH A POTENTIAL ROLE IN MODULATING CARDIAC MATRIX METALLOPROTEINASES

BY

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ABSTRACT

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Title: Cadmium, an Environmental Contaminant with a Potential Role in Modulating Cardiac Matrix Metalloproteinases

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Cadmium (Cd) is a toxic heavy metal that is found in the environment from both natural and anthropogenic sources. It is a xenobiotic environmental pollutant with no known essential biological functions. Exposure to cadmium has been implicated as a risk factor for cardiovascular disease by inducing inflammation and promoting fibrosis of cardiac tissue. The aim of this study was to evaluate the role of low dose chronic cadmium exposure in modulating cardiac matrix metalloproteinases (MMPs) in the heart of rats. Adult male Sprague-Dawley rats were exposed to 15 ppm $CdCl_2$ in drinking water for 10 weeks followed by withdrawal of cadmium treatment for 4 weeks and their heart tissue were obtained. Inflammatory status in the cardiac tissue was evaluated by real-time PCR while protein expression and enzyme activity of MMP-2, MMP-9 and their endogenous inhibitors (TIMP-1 and TIMP-2) was evaluated by western blotting and gelatin zymography, respectively. Results show that the administered cadmium dose incites an inflammatory response until week 10 that is slightly diminished after 4 weeks. At the protein level, cadmium incites a differential effect on the expression and activity of gelatinases and their endogenous inhibitors in an exposure-dependent manner. In conclusion, the present study provides substantial evidence of cadmium-induced imbalance in the MMP-TIMP system in the cardiac tissue. This imbalance

may be mediated by cadmium-induced inflammation that could contribute to various cardiovascular pathologies.

# DEDICATION

*“If I have seen further, it is by standing on the shoulders of giants”*

*-Sir Isaac Newton*

*This thesis is dedicated to the team where I have seen further.*

*To Laboratory Animal Research Center*

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# TABLE OF CONTENTS

DEDICATION ..... v

ACKNOWLEDGEMENTS ..... vi

LIST OF TABLES ..... x

LIST OF FIGURES ..... xi

Chapter 1: Introduction ..... 1

Cadmium and cardiovascular disease ..... 4

Chapter 2: Literature review ..... 6

Matrix metalloproteinases ..... 6

Tissue inhibitors of metalloproteinases ..... 11

Role of cadmium in modulation of matrix metalloproteinases ..... 14

Research objectives ..... 16

Chapter 3: Methodology ..... 17

Treatment of animals and tissue collection ..... 17

Preparation of tissue homogenates ..... 18

RNA extraction and quantification ..... 18

Gene expression by real time-PCR ..... 19

Protein extraction and quantification ..... 20

Protein expression by Western Blotting ..... 20

Zymography ..... 21

Statistical analysis ..... 22

Chapter 4: Results ..... 23

Quantity and quality of extracted total RNA ..... 23

Level of gene expression of inflammatory mediators in heart tissue ..... 24

Quantification of total crude protein ..... 25

Level of protein expression of MMPs in heart tissue ..... 27

Level of protein expression of TIMPs in heart tissue ..... 29

Gelatinolytic activity of MMPs in heart tissue ..... 32

Chapter 5: Discussion ..... 37

Chapter 6: Conclusion ..... 44

References ..... 45

LIST OF TABLES

Table 1: Classification of matrix metalloproteinases.....8

Table 2: Source of TIMPs in the heart and their biological roles.....13

Table 3: Details corresponding to reagents used in western blotting .....21

Table 4: Quantity and quality of total RNA extracted from heart tissue of male Sprague-Dawley rats.....23

Table 5: Quantification of crude total protein by Bradford assay from heart tissue of male Sprague-Dawley rats. .....26

LIST OF FIGURES

Figure 1: Components of matrix metalloproteinases (MMPs). ..... 7

Figure 2: Illustration indicating the mechanism activation and inactivation of MMP.. ..... 9

Figure 3: Basic structure of tissue inhibitor metalloproteinase. ..... 11

Figure 4: Scheme illustrating the timeline of the study design..... 18

Figure 5: Gene expression of inflammatory mediators in heart tissue of Cd-treated male Sprague-Dawley rats using real time-PCR.. ..... 25

Figure 6: Graph of standard calibration curve for Bradford protein assay. ..... 26

Figure 7: Representative image of Commassie Brilliant Blue stained 9 % SDS-PAGE indicating band pattern of 40 µg crude protein. ..... 27

Figure 8: Representative immunoblots for active MMP-2 (64 kDa) and GAPDH (40 kDa) at week 5 (A), week 10 (B) and week 14 (C). ..... 28

Figure 9: Effect of Cd-treatment on expression of MMP-2 in heart tissue of male Sprague-Dawley rats. ..... 29

Figure 10: Representative image of Commassie Brilliant Blue stained 12 % SDS-PAGE indicating the band pattern of 50 µg crude protein. ..... 29

Figure 11: Representative immunoblots for TIMP-1 (23 kDa), TIMP-2 (24 kDa) and GAPDH (40 kDa) at week 5 (A), week 10 (B) and week 14 (C). ..... 30

Figure 12: Effect of Cd-treatment on expression of TIMP-1 in heart tissue of male Sprague-Dawley rats. ..... 31

Figure 13: Effect of Cd-treatment on expression of TIMP-2 in heart tissue of male Sprague-Dawley rats. ..... 32

Figure 14: Representative image showing gelatinolytic activity of MMP after 24

hours incubation. ..... 32

Figure 15: Gelatinolytic activity of pro-MMP-2 after 24hr incubation. ..... 33

Figure 16: Representative image showing gelatinolytic activity of MMP after 60+ hours incubation. ..... 34

Figure 17: Gelatinolytic activity of pro-MMP-9 after 60+ hr incubation. ..... 34

Figure 18: Gelatinolytic activity of pro-MMP-2 after 60+ hr incubation. ..... 35

Figure 19: Gelatinolytic activity of active MMP-2 after 60+ hr incubation. ..... 36

# Chapter 1: Introduction

Continuous industrialization and urbanization globally is widely evident. Industrial innovation and progression has led to increased metal contamination globally, increased production as well as consumption of heavy metals including cadmium (Ali, Khan, & Ilahi, 2019). Cadmium is the 48$^{th}$ element and a member of group 12 elements in the periodic table. In its pure form, cadmium is a silver-white malleable metal with a crystalline hexagonal form and molecular weight of 112.4 (Hooser, 2018). It is a naturally occurring heavy metal usually found with zinc, lead and copper ores in the Earth's crust with concentrations of 0.1-0.5 ppm. It is a heavy metal of important industrial utility with approximately 24,000 metric tons being produced globally per year (Tolcin, 2016). Its production is mainly employed in the manufacturing of nickel-cadmium batteries, some pigments, thermoplastic stabilizers, metallic coatings and alloys. As of 2012, the industrial use of cadmium globally was reported to be 79 % in nickel-cadmium batteries, 11 % for pigments, 7 % for metallic coatings and 3 % for miscellaneous applications (Nordberg, Nogawa, & Nordberg, 2015). In the environment, cadmium has natural as well as anthropological origins, that circulates often between the three matrices of the environment namely, air, water and soil. An aspect that contributes to its movement in these matrices is its high persistence due to non-biodegradability. Furthermore, this heavy metal is reported to have a high rate of transfer from soil-to-plant, permitting its entry into the food chain (Lamb et al., 2016).

Despite its importance in industrial applications, recently, it has been identified as one among 126 priority pollutants (EPA, 2014), a Class I carcinogen (IARC, 2012) and is considered a pollutant of the environment and toxicant for health (ATSDR, 2012; WHO, 2003). A cause for concern to cadmium exposure is due to its long biological

half-life of about 10-30 years (Nordberg et al., 2015). In the early 1950s, a large volume of data was reported concerning the toxicities associated with cadmium exposure in humans and in laboratory animals. Cadmium exposure can occur by inhalation, ingestion and dermal absorption. Additionally, smoking can be a source of exposure. The efficiency of cadmium adsorption via inhalation is about 25-50 % which is relatively higher than that through ingestion ranging from 1-10 % (Asagba, 2013). The absorption of cadmium from food in the gastrointestinal tract is about 3-5 % however this percentage may increase in individuals with nutritional deficiencies (Rani, Kumar, Lal, & Pant, 2014). Dermal absorption of cadmium is usually low below 1 % ranging between 0.2-0.8 % (Rani et al., 2014).

For the non-smoking population, the major source of exposure is by ingestion of cadmium contaminated food and water (ATSDR, 2012). Following oral exposure, cadmium is preferentially absorbed in the duodenum and proximal jejunum. Immediately after this uptake, cadmium primarily binds to albumin and other large proteins as well as metallothionein (MT) in the blood plasma. As a subsequence of cadmium uptake into the organism, cadmium is widely transported and distributed to various tissues via the blood circulation. Particularly in the liver, the presence of cadmium induces the synthesis of MT, a ubiquitous low molecular mass, high cysteine content metal-binding protein. Also here, cadmium forms complexes with MT and other proteins like glutathione (GSH). The cadmium as $Cd^{2+}$ ions is then secreted either into the bile or released as complexes of Cd-GSH or Cd-MT into circulation and taken up by target tissues where its toxic effects ensues (Thévenod, 2009). In fact, more than half of the cadmium burden is accumulated in the liver and kidney due to their capacity to produce MT and sequester cadmium (Vardhan, Kumar, & Panda, 2019). In the kidney, the tubular cells absorb the Cd-MT complex which are

transported into the lysosomes and catabolized. Lastly, cadmium may be removed from the body via urine for a meagre amount of 0.005-0.01 % of the total cadmium load (Vardhan et al., 2019). Hence, the ensuing toxic impact of cadmium causes adverse impacts on the various organs of the body.

Reports on the toxicological properties of cadmium varies based on the route, dose and duration of exposure. A well-documented indication of the toxic effects of chronic cadmium exposure is characterized by excretion of low molecular weight proteins, calcium, metallothionein and intracellular enzymes, ultimately leading to renal dysfunction (Rana, Tangpong, & Rahman, 2018). During acute exposure, the critically affected organ is the liver characterized by hepatocyte injury and release of inflammatory cytokines and chemokines (Matović, Buha, Đukić-Ćosić, & Bulat, 2015). However, the reports of nephrotoxicity and hepatotoxicity are endpoints indicating the strain of cadmium on the body. Over the past few years, cadmium has been reported to impart its toxic effects  even at low doses on other organ systems including the nervous system (Méndez-Armenta & Ríos, 2007), immune system (Marth, Barth, & Jelovcan, 2000), reproductive system (Thompson & Bannigan, 2008) and the cardiovascular system (Tinkov et al., 2018). With regards to the cardiovascular system, there is an increasing amount of epidemiological evidence showing that cadmium has an impact on cardiovascular health (Deering et al., 2018; Fagerberg et al., 2015; Gao et al., 2018; M.-S. Lee, Park, Hu, & Lee, 2011; Noor, Zong, Seely, Weisskopf, & James-Todd, 2018). However, the mechanism by which it renders its effects is not clearly understood. One of the widely cited mechanisms to initiate the toxicity of cadmium centers on oxidative stress that weakens the antioxidant defenses, enhances oxidative damage (Solenkova et al., 2014) and activates defense mechanisms (Ghosh, N, & Indra, 2018). These mechanisms are not

mutually exclusive but may work in a cohesive pattern to overcome the cadmium-induced damage. Among published literature, an important node in the signaling mechanism is the activation of nuclear factor-κB that mediates the downstream cascade via cytokine secretion.

**Cadmium and cardiovascular disease**

Epidemiological studies have reported the association of cadmium exposure with various cardiovascular diseases including hypertension (Garner & Levallois, 2017; B.-K. Lee et al., 2016; M.-S. Lee et al., 2011; Tellez-Plaza, Navas-Acien, Crainiceanu, & Guallar, 2008), coronary heart disease (Tellez-Plaza, Jones, Dominguez-Lucas, Guallar, & Navas-Acien, 2013), peripheral artery disease and atherosclerosis (Navas-Acien et al., 2004), stroke and heart failure (Agarwal, Zaman, Murat Tuzcu, & Kapadia, 2011). Studies have also reported the deposition and accumulation of cadmium in the heart and arterial tissue in both humans (Egger et al., 2019) as well as animal models (Erdem, Yazihan, Kocak, Sayal, & Akcil, 2016; Young et al., 2019). Histopathological studies of cardiovascular tissues after cadmium exposure have reported alteration of tissue structure and integrity, fibrosis and depletion of collagen fiber (Saleh & Awadin, 2017; Sangartit et al., 2014). A closer view of the cardiac tissue showed profound degeneration in parallel with capillary dilation, congestion of the vasculature and necrosis of the myocardial fibers (Bhattacharjee et al., 2019). Another study also reported that the hearts of rats treated with 10 mg/L $CdCl_2$ drinking water for 90 days showed pronounced change in morphology in the cardiomyocytes and network of myocardial interstitial fibrillar collagen (Veličkov et al., 2013). Erdem et al. (2016) reported that the heart tissue accumulated the lowest amount of cadmium after liver and kidney. However, from the previously cited studies, it can be inferred that even at low concentrations of

cadmium, there are biochemical and molecular alterations in the heart. It is not well understood yet how cadmium imposes varying degrees of effects on the heart tissue that leads to cardiovascular injury. From a mechanistic perspective, the detrimental remodeling reported in cardiac tissue after cadmium exposure may be attributed to the dysfunction of the expression or activity of matrix metalloproteinases and their corresponding inhibitors.

# Chapter 2: Literature review

## Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of extracellular zinc-dependent proteases that process components of the extracellular matrix (ECM). These proteases comprises of both soluble and membrane-bound endopeptidases that are expressed across majority of tissues with some members being specific to cell types (Jobin, Butler, & Overall, 2017). In 1962, MMPs were first discovered in the study of tadpole tail metamorphosis and came to be known as matrix proteins that collectively degraded the ECM proteins (Iyer, Patterson, Fields, & Lindsey, 2012). Initially, these proteins were classified based on substrates and were considered as broad, non-specific ECM-degrading enzymes with vital roles in turning over ECM components (Hannocks et al., 2019). Between the late 1980s and 1990s, additional members of the MMP family were discovered and studied to gain a clearer understanding of their role in physiology (Iyer et al., 2012). However, this concept of the role of MMPs was revised as these enzymes were found to have a much more broader range of substrates and it was suggested that they play more of a processing rather than a degrading role (Hannocks et al., 2019). Over time, it came to be understood that MMPs play an active role in the repair and remodeling of tissues including cardiovascular remodeling. MMPs cleave the ECM that enables the cell migration to make the repair possible. The role of MMPs are not restricted to changes of the structure of tissues. MMPs process both extracellular as well as cell-surface non-ECM proteins including receptors, peptide hormones and cytokines, therefore playing an important role in signaling in homeostasis and pathology (Jobin et al., 2017). They also function as activators of pro-MMPs. Given their role at the level of the tissue, MMPs are regulated under tightly controlled mechanisms.

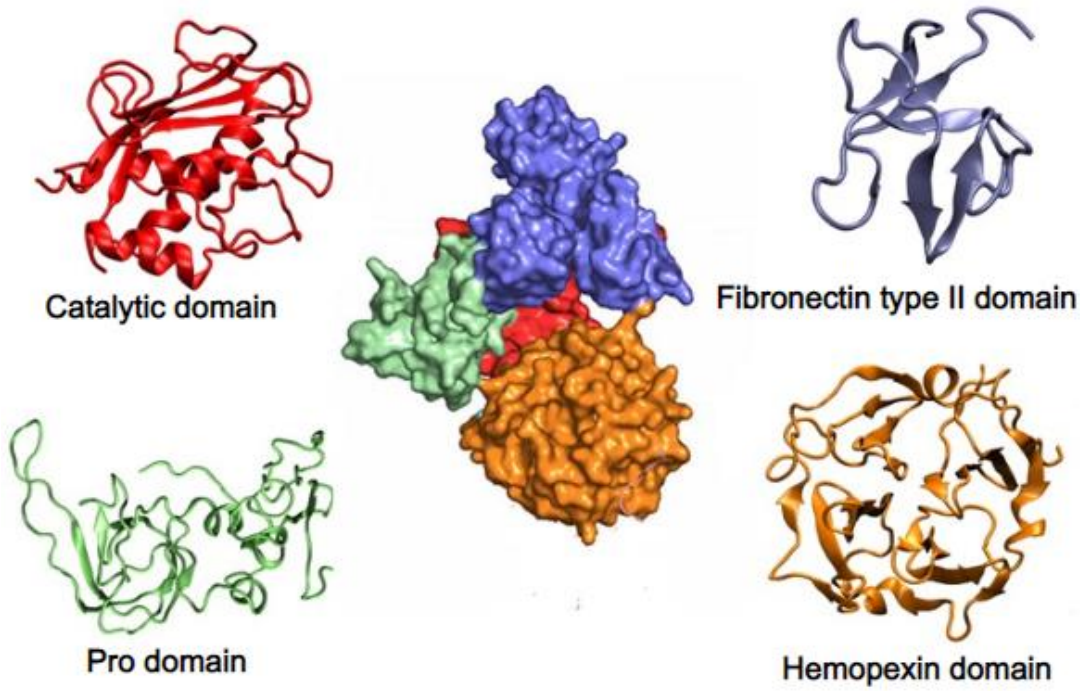


*Figure 1*: Components of matrix metalloproteinases (MMPs). Catalytic domain indicated in red, pro-domain indicated in green, fibronectin type II domain indicated in blue and hemopexin domain indicated in orange. PDB ID: 1GXD (Murphy & Nagase, 2008)

Nomenclature of MMPs are based on structural organization and substrate precedency. To date, 28 MMPs have been described in mammals, specifically in humans, 24 members with 6 groups of the MMP family are known (Myasoedova, Chistiakov, Grechko, & Orekhov, 2018; Nguyen, Mobashery, & Chang, 2016). Members of the MMP family share 4 components in their basic structure - 1) an N-terminal pro-peptide domain, 2) cysteine-containing switch zinc motif that is the site of catalysis and activator regulation, 3) C-terminal proline rich hinge and 4) a repeated hemopexin domain that is recognized by inhibitors (*Figure 1*). The MMPs are classified and named as per the table below (Table 1).

Table 1: Classification of matrix metalloproteinases

| Group | Members |
|---|---|
| Collagenases | MMP-1, MMP-8, MMP-13 |
| Gelatinases | MMP-2, MMP-9 |
| Stromelysins | MMP-3, MMP-10, MMP-11 |
| Matrilysins | MMP-7, MMP-26 |
| Membrane-type | MMP-14, MMP-15, MMP-16, MMP-24 |
| Membrane anchored | MMP-17, MMP-25 |
| Non-classified | MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-28 |

MMPs play a vital role in physiological and pathological tissue remodeling and hence control of their activity and function are vital to homeostasis. These controls occur at three levels. Firstly, at the level of the expression of their genes, then at the level of activation of the pro-enzymes and lastly by the inhibition of the catalytic activity. MMP expression can be modulated at the level of transcription by various physiological signals including growth factors, cytokines and matrikines (Li, McTiernan, & Feldman, 2000). Each MMP gene has a unique promoter that contains binding sites for various transcription factors. MMPs are synthesized as inactive zymogens wherein their activation occurs by the proteolytic cleavage of the N-terminal propeptide. This forms the first step in their regulation. This activation process is extracellularly mediated by serine protease plasmin as well as other proteinases including some other MMPs as well as autolytic activation. Depending on the tissue type and physiological process involved, the regulatory process of MMPs differs. Cytokines are significant regulators of MMP gene expression. It has been shown that both tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) may indirectly activate MMP genes due to prolonged activation of the signaling cascade (Li et al., 2000). Some MMP genes expression can also be modified by the substrates of MMPs, cell-cell and cell-ECM adhesion molecules and agents of cell shape alteration (Li et al., 2000).

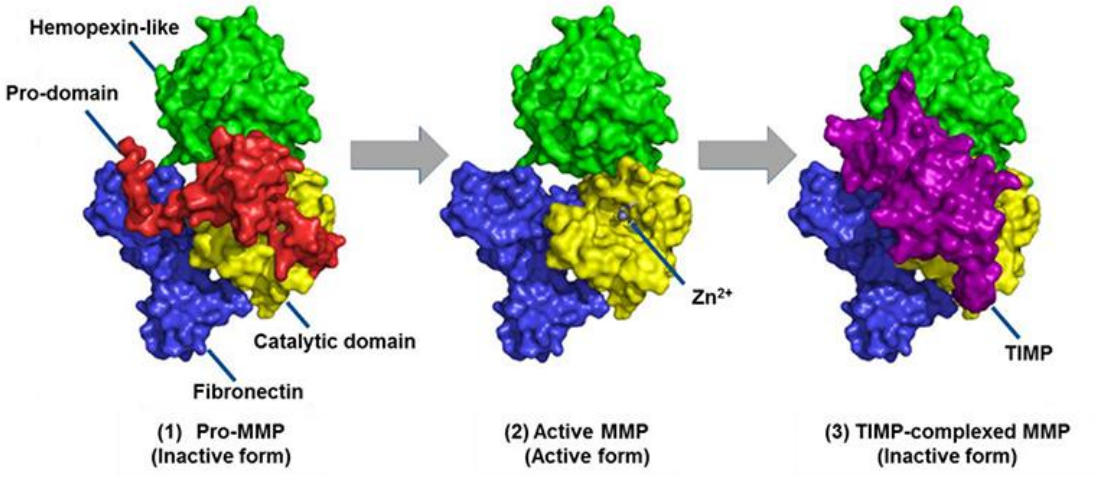


*Figure 2*: Illustration indicating the mechanism activation and inactivation of MMP. By this mechanism, the inactive proenzyme form is activated and inactivated by tissue inhibitor of metalloproteinases (Nguyen et al., 2016).

MMPs are secreted as inactive proenzymes that are activated by the cleavage of the pro-domain which is the removal of an amino-terminal propeptide to expose the active site for catalysis (*Figure 2*). It is believed that the latency of MMPs are rendered by the interaction of the cysteine-rich residue of the pro-domain with the zinc moiety in the active site (Vu & Werb, 2000). Hence, the obstruction of the active site by the propeptide results in a suppression of the MMP activity. Another mechanism of regulating MMP activity is by forming a non-covalent complex between the catalytic domain and tissue inhibitors of matrix metalloproteinases (discussed separately in the following section) (*Figure 2*).

In the heart, from the numerous MMPs characterized, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-12, MMP-13, MMP-14 and MMP-28 have been identified to play a role in remodeling of the cardiac infrastructure (Pytliak, Vaník, & Bojčík, 2017). Of these MMPs, gelatinases (MMP-2 and MMP-9) have been reported to play a role in inflammation induced imbalance of ECM turnover in the heart. Furthermore, the activity of gelatinases have been implicated in cardiovascular pathologies

associated with oxidative stress (Siwik & Colucci, 2004; Spinale, 2007). MMP-2 alternatively known as gelatinase A is a ubiquitous 72 kDa protein in its latent form and 68 kDa in its active form (Hardy, Hardy-Sosa, & Fernandez-Patron, 2018; Henriet & Emonard, 2019). MMP-9, otherwise known as gelatinase B is a relatively bigger protein with its latent form at 92 kDa and 82 kDa when activated (Malemud, 2017). A shared structural feature between the gelatinases is the presence of three fibronectin type II repeats in the catalytic domain. It is this feature that renders MMP-2 the ability to bind and cleave to gelatin, laminin, collagen type I, IV, V, VII, fibronectin and elastin (Li et al., 2000; Malemud, 2017). The same feature renders MMP-9 the ability to cleave gelatin, proteoglycans, collagen type IV, V, VII, fibronectin and elastin (Li et al., 2000). While both gelatinases share common substrates, they play different roles when interacting with ECM components. An example of this is the digestion of fibronectin and laminin by MMP-2 but not MMP-9 while denatured chains of collagen type I and III are degraded only by MMP-9 (Okada, 2017).

Due to the ubiquitous expression and constitutive activity of MMP-2, it has been considered as an MMP housekeeping gene that contributes in the regulation of matrix turnover in normal tissues. Apart from its role in regulation of ECM components, MMP-2 also participates in the cleavage of a broad spectrum of substrates including soluble metabolic mediators, growth factors and cytokines (Hardy et al., 2018). In many pathologies, the expression of MMP-2 has been implicated in the development and progression of the disease (Cook, Sarker, & Fernandez-Patron, 2019; Henriet & Emonard, 2019). While MMP-2 is secreted primarily by non-inflammatory cells like epithelial cells, endothelial cells and fibroblasts, contrastingly, MMP-9 is secreted mainly by inflammatory cells like macrophages and neutrophils. The activity of

MMP-9 is strongly dependent on MMP-2 as an activator and its expression is induced by the inflammatory mediators tumor growth factor (TGF)-β, TNF-α and IL-1β (Hannocks et al., 2019; Li et al., 2000).

**Tissue inhibitors of metalloproteinases**

Tissue inhibitors of metalloproteinases (TIMPs), as the name suggests, are tissue-specific, endogenously produced inhibitors of metalloproteinases comprising of 4 homologous members (TIMP 1-4) that inhibit active metalloproteinases in a 1:1 stoichiometric molar ratio (Masciantonio, Lee, Arpino, Mehta, & Gill, 2017). TIMPs was first discovered in the early 1970s and designated as a collagenase inhibitor found in the human cell culture media of skin fibroblasts and serum as well as in the cartilage and aorta extracts derived from bovine (Brew & Nagase, 2010). Later, its name was reconsidered and designated as tissue inhibitor of metalloproteinases as it not only inhibited collagenases but other classes of proteins including gelatinases.

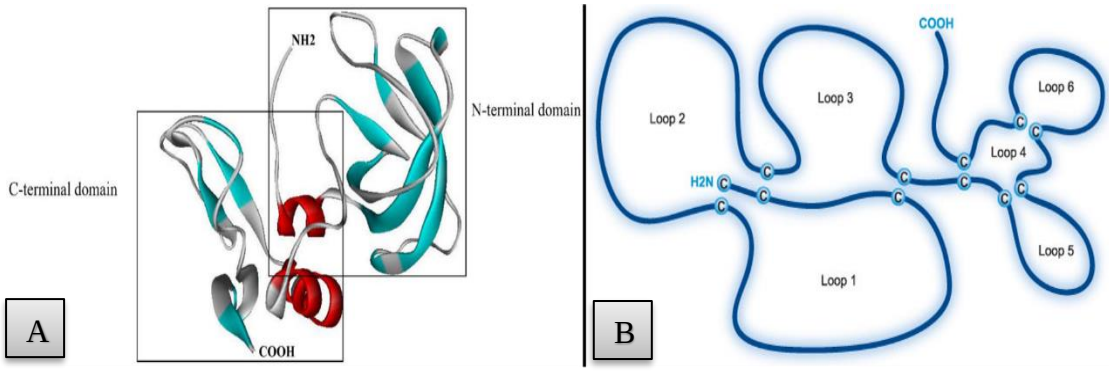


*Figure 3*: Basic structure of tissue inhibitor metalloproteinase. Indicated are the two domains (A) and the stabilization of the domains by disulphide bridges (B). PDB ID: 1UEA (Lambert, Dassé, Haye, & Petitfrère, 2004)

Structurally, TIMPs are two-domain molecules (*Figure 3*(A)) namely, N-terminal and C-terminal domain comprising of about 125 and 65 amino acids, respectively (Brew,

Dinakarpandian, & Nagase, 2000). Each of these domains are stabilized by three disulphide bridges that control the functionality and localization of TIMPs (*Figure 3*(B)). In regards to its mechanism of inhibition, the N-terminal domain of TIMP interfaces with the active site of MMPs that is analogous to the interaction of MMPs with its substrate, leading to the inhibition of the catalytic activity (*Figure 2* (3)). On the other hand, the C-terminal domain has been reported to facilitate the protein-protein interaction as in the case of binding of TIMP to proenzyme MMPs (Masciantonio et al., 2017). These endogenous inhibitors play a significant role in regulation of ECM turnover, remodeling within the tissue and behavior of the cell (Brew & Nagase, 2010). Like MMPs, TIMPs are also tightly regulated in the tissue as any dysregulation results in diseases associated with the uncontrolled turnover of the matrix. Regulation of TIMPs also begins at the transcriptional level wherein physiological signals like cytokines, chemokines and growth factors modulate the level of expression of the TIMP gene. Apart from transcriptional modulation, localization of the TIMPs seems to play a significant role in regulating its function (Arpino, Brock, & Gill, 2015). Initially, TIMP-1, -2 and -4 were thought to be soluble inhibitors while TIMP-3 was localized in the ECM (Lambert et al., 2004). However, recently TIMPs have been shown to be localized in association with cell surface proteins (Arpino et al., 2015). The four currently characterized TIMPs inhibit the proteolytic activity of all MMPs. However, they differ in several aspects such as their solubility, interaction with the proenzymes, efficacy of inhibition and regulation of their expression.

Table 2: Source of TIMPs in the heart and their biological roles

| Cell type | TIMP members produced | Biological roles of the TIMPs |
|---|---|---|
| Cardiac fibroblasts | TIMP-1 to -4 | Proliferation, migration, differentiation and apoptosis |
| Myofibroblasts | TIMP-2 | Collagen synthesis |
| Endothelial cells (ECs) | TIMP-1 to -3 | Proliferation, migration and angiogenesis |
| Smooth muscle cells (SMCs) | TIMP-3 | Apoptosis |
| Cardiomyocytes | TIMP-1, -3 and -4 | Proliferation and hypertrophy |
| B-cell | TIMP-1 | Apoptosis, survival and differentiation |
| T-cell | TIMP-2 | Apoptosis |
| Macrophage | TIMP-3 | Activation |

In cardiovascular health, the discovery of TIMPs have added more knowledge towards understanding the regulation of MMPs in maintaining a healthy balance between ECM metabolism and cardiac remodeling. Since TIMPs are endogenously expressed in different tissues, different cell types produce the different members of the TIMP family to play a certain biological role (Table 2). In the past, it was presumed that the regulation of TIMPs was solely mediated by their inhibition of MMP activity. Recently, however, research has shown that TIMPs also have cytokine-like signaling properties that are currently being characterized (Vanhoutte & Heymans, 2010).

The phenomenon of physiological and pathological cardiac remodeling contributing to the composition and architecture of the cardiac tissue is a result of complex but tightly regulated interaction between fibroblasts, ECs, SMCs, cardiomyocytes and infiltrating inflammatory cells. Apart from the ability of TIMPs to bind to MMP, they have also been reported to complex with proenzyme MMPs to regulate their activation (Lambert et al., 2004; Masciantonio et al., 2017). TIMP-1 seems to have a preferential affinity for pro-MMP-9 while TIMP-2 complexes with pro-MMP-2. TIMP-3 complexes with both pro-MMP-9 and pro-MMP-2 and TIMP-4 binds to the

C-terminal of pro-MMP-2 (Brew et al., 2000).

In the heart, while the four members of the TIMP family are produced, the corresponding inhibitors of the gelatinases are TIMP-1 and TIMP-2. These two TIMPs are slow, tight binding, wedge shaped and share a 40% homology in their amino acid sequences in their N-terminal domain (Brew et al., 2000; Lambert et al., 2004). However, given the homology, these TIMPs differ in their specificity towards MMPs by the smallest conformational change (Brew et al., 2000). TIMP-1 is a low molecular weight, approximately 28 kDa inducible glycoprotein expressed by numerous cell types including fibroblasts, epithelial cells, ECs, osteoblasts, SMCs and tumor cells (Crawford, Bioulac-Sage, & Hytiroglou, 2018). The expression of TIMP-1 is stimulated in these cells by growth factors, serum and cytokines like IL-6 and IL-1β (Grünwald, Schoeps, & Krüger, 2019; Lambert et al., 2004). On the other hand, TIMP-2 is a relatively smaller protein- approximately 21 kDa- constitutively expressed by epithelial cells, fibroblasts, keratinocytes, ECs and neural cells (Vynios, 2011). A unique feature of TIMP-2 is its ability to act as both inhibitor and activator of MMP-2. The N-terminal of TIMP-2 positively regulates MMP-14 to form a trimolecular complex with pro-MMP-2 bound to the C-terminal of TIMP-2. Unbound MMP-14 cleaves the propeptide region of the MMP-2 to activate it (Remillard, Bratslavsky, Jensen-Taubman, Stetler-Stevenson, & Bourboulia, 2014). As mentioned earlier, TIMPs can inhibit active MMPs however at variable efficacies. Due to their variable efficacies, TIMP-1 has a preference to inhibit MMP-1, MMP-3, MMP-7 and MMP-9 while TIMP-2 has a greater affinity for MMP-2.

**Role of cadmium in modulation of matrix metalloproteinases**

Within the cardiovascular system, it has been reported that cadmium induces oxidative stress as indicated by a significant imbalance in reactive oxidative species

(ROS) and its clearance mechanism (Novelli et al., 2000). This imbalance sets in motion a series of events in the cardiac tissue and results in alterations of the homeostatic metabolic pathway. In the context of cardiovascular dysfunction, a hallmark of chronic cadmium exposure is the induction of fibrosis (Bhattacharjee et al., 2019). Critical to the process of fibrosis is the balance between MMPs and TIMPs (Azevedo, Prado, Antonio, Issa, & Gerlach, 2014; P. Liu, Sun, & Sader, 2006). It has been reported that cadmium at 15 ppm downregulates the activities of MMP-2 and MMP-9 in the prostate and testis of male Wistar rats (Lacorte et al., 2015). These MMPs are important in the breakdown of the components of the ECM specifically collagen. Furthermore, it was demonstrated that acute cadmium exposure disrupts the balance between MMP-9 and its inhibitor TIMP-1 *in vitro* (Yaghooti, Firoozrai, & Khorramizadeh, 2012). It has been demonstrated that non-toxic cadmium stimulates MMP-9 and MMP-2 expression in the cardiovascular system inducing vascular inflammation and promoting atherosclerosis (Knoflach et al., 2011). The mechanism by which cadmium induced inflammation mediates the imbalance of MMPs leading to cardiac fibrosis has not been elucidated.

In the frame of the published literature, cadmium-induced hepatotoxicity and nephrotoxicity are well documented. However, with increasing evidence of cadmium-induced damage in other organ systems, it becomes important not to overlook these effects. Mounting pieces of evidence have demonstrated the link between cadmium exposure and cardiovascular diseases. However, the mechanism by which cadmium incites cardiovascular injury specifically at the level of collagen balance is not well understood. Therefore, this research aims to evaluate the role of cadmium in modulating cardiac MMPs in the heart of rats after cadmium exposure.

**Research objectives**

1. Evaluate the gene expression levels of inflammatory markers (IL-1β, IL-6, IL-10, NF-κB, TNF-α) in the heart tissue.
2. Evaluate the expression, activity and inhibition of MMP-2 and MMP-9 in the heart after cadmium exposure.
3. Examine the reversibility of changes after cadmium exposure.

# Chapter 3: Methodology

## Treatment of animals and tissue collection

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Qatar University (Approval # QU-IACUC 038/2017). The samples obtained in this study were acquired from the animals employed in a previous study from our research group (Al-Naemi & Das, 2020).

Briefly, adult (8-weeks old) male Sprague-Dawley rats were obtained from Laboratory Animal Research Center, Qatar University and housed in individually ventilated cages (IVC) under standard conditions. Animals were randomly assigned to one of two regimes for 14 weeks- (1) control group or (2) cadmium treatment (Cd-treated) (*Figure 4*). The control group received drinking water for 14 weeks. The Cd-treated group received 15 ppm (15 mg/kg body weight) as $CdCl_2$ in drinking water for 10 weeks followed by 4 weeks of normal drinking water. The administered cadmium exposure dose was selected based on previous literature reporting that a dose of 15 ppm of $CdCl_2$ is a non-carcinogenic dose (Alvarez et al., 2004) leading to a circulating serum concentration of 5 ppb, the toxic limit in humans by the WHO (Mariana L. Ferramola et al., 2012; Mariana Lucila Ferramola, Antón, Anzulovich, & Giménez, 2011).

Animals received *ad libitum* access to standard rodent chow and drinking water during the course of the experiment. Animals were sacrificed at weeks 5, 10 and 14 under anesthesia with sodium thiopentone (40 mg/kg body weight, intraperitoneally). Heart tissue was dissected, washed in 1x PBS to remove residual blood, immersed in liquid nitrogen and preserved in the tissue archive at -80°C until further analysis.

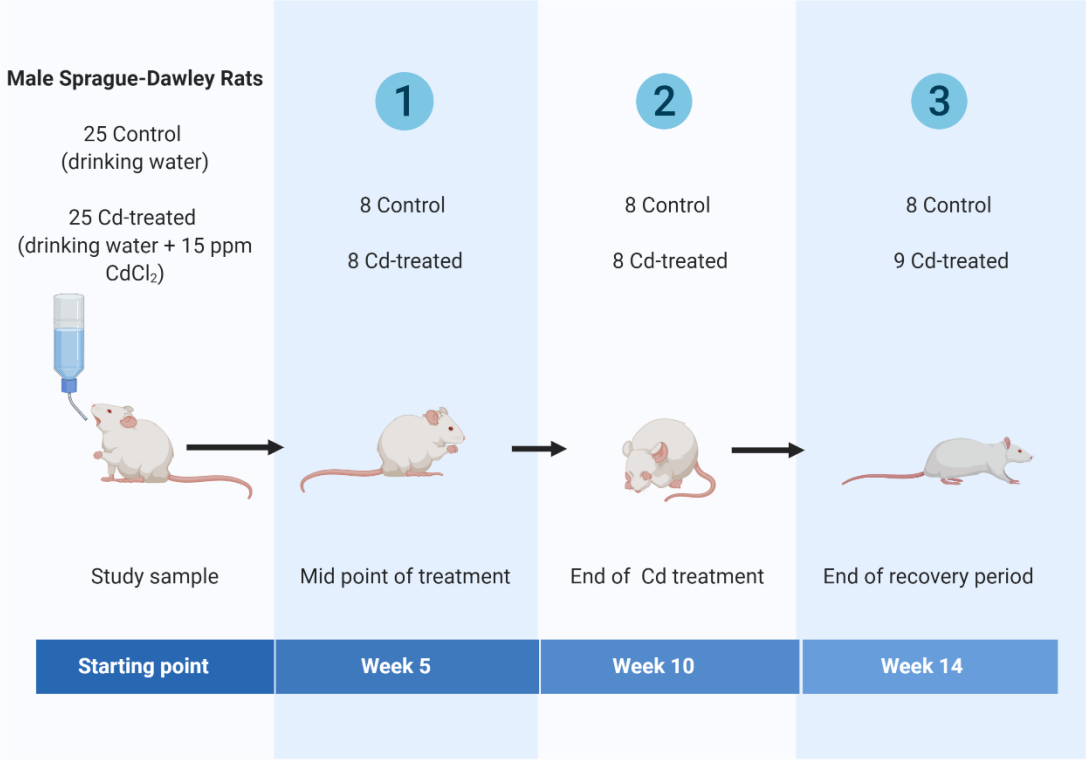


*Figure 4*: Scheme illustrating the timeline of the study design.

**Preparation of tissue homogenates**

Frozen heart samples were weighed to approximately 20-30 mg, washed in 1x PBS to remove residual blood and sonicated at 40 % amplitude for a pulse of 5 seconds. Sonication was repeated until tissue was completely homogenized. Sonicated heart tissue was homogenized in- either radioimmunoprecipitation assay (RIPA) cell lysis buffer to extract total proteins in combination with cocktail protease inhibitor or TRIzol reagent to extract RNA.

**RNA extraction and quantification**

Total RNA was isolated from frozen heart samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions as previously described (Rio, Ares, Hannon, & Nilsen, 2010). Homogenized tissue samples were incubated in TRIzol reagent on ice until complete dissociation of nucleoproteins. Cellular debris was

removed by centrifugation at 10,000 rpm, 10 minutes at 4°C. Supernatant was transferred to a new microfuge tube and using phenol-chloroform phase separation, RNA was separated from other cellular components into the upper aqueous layer. The aqueous layer was transferred to a new microfuge tube and total RNA was precipitated by incubating with room temperature isopropanol for 10 minutes on ice. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C to obtain an RNA pellet. The pelleted RNA was washed by resuspending the pellet in ice-cold 75 % ethanol and centrifuging at 7,500 rpm for 5 minutes at 4°C by to obtain a cleaned RNA pellet. The pellet was resuspended in RNase-free water, aliquoted and stored in -80°C until further analysis. Total RNA concentration and quality was determined using Nanophotometer (IMPLEN) at 260/280 nm as described previously (Koetsier & Cantor, 2019).

**Gene expression by real time-PCR**

The gene expression of specific inflammatory mediators in response to cadmium treatment was analyzed using real-time PCR technique. Extracted total RNA (150 ng/µL) was reverse transcribed to cDNA using High-Capacity cDNA reverse transcriptase kit as per instructed in user's manual [Thermocycler program: 10 minutes (25°C)- 120 minutes (37°C)- 5 minutes (85°C)]. Total cDNA was diluted by 4-fold in RNase-free water. The gene expression assay was performed using Quant Studio 6 Flex Real Time PCR system (Applied Biosystem). Reaction mixtures (10µL/well) containing master mix, assay mix (specific primer and probe), template cDNA and nuclease-free water were set up in duplicates. Amplification plots were obtained for each target using specific primers for IL-1β, IL-6, IL-10, TNF-α, NF-κB and GAPDH. Quantification was done by the $\Delta\Delta C_t$ method wherein results were compared against both calibrator (control group) and normalizer (endogenous,

GAPDH) to obtain the $\Delta\Delta C_t$ value (Pfaffl, 2004). This value was used to determine the fold difference in expression.

**Protein extraction and quantification**

Sonicated heart tissue samples were lysed for crude protein by incubating in freshly prepared RIPA cell lysis buffer (Thermo-Scientific, USA; 78440) containing 1x cocktail protease inhibitor for 30 minutes on ice. Protein extraction for MMP activity assay included 1x EDTA in the cell lysis buffer. Cellular debris was pelleted by centrifugation for 10,000 rpm, 10 minutes at 4°C and supernatant containing crude protein was transferred to a newly labelled microfuge tube as aliquots and stored in -80°C until further analysis. Total crude protein concentration was estimated using Quick Start™ Bradford Protein assay (Bio-rad, USA) as per instructions in the user's manual provided.

The standard protein chosen for the Bradford Protein assay was bovine serum albumin (BSA) due to it sensitivity and compatibility with the assay and reagents used. Briefly, eight standards of BSA were prepared in the concentration range of 0-2.0 mg/mL from a stock solution of 2.0 mg/mL while test samples were allowed to thaw on ice. Reaction was set up in duplicates by pipetting Bradford reagent to the standards and test samples indicated by a color change from brown to blue. For quantification, test samples were diluted by 4-fold to prevent saturation of the reaction. The set-up reactions were mixed and incubated for 10 minutes at room temperature. Colorimetric quantification was done using a plate reader (Versa Max Multi plate reader, Molecular Device) at 595 nm.

**Protein expression by Western Blotting**

The protein expression of gelatinases (MMP-2 & MMP-9) and their respective

inhibitors (TIMP-1 & TIMP-2) in response to cadmium treatment were analyzed by Western blotting. Equal amounts of crude protein was loaded into a reducing SDS-PAGE. Total crude protein was stacked at 50V for 45 minutes and separated at 90V for 2.5 hours. Separated proteins were electroblotted to methanol activated PVDF membrane at 90 V for 2 hours under ice packing. Electroblots were probed with primary antibodies for the targets overnight at 4°C (Table 3). Detection was done using compatible secondary HRP-conjugated antibodies and incubated in ECL solution for 1 minute. The probed membranes were visualized for antibody binding using SynGene Gel documentation system. Protein expressions was evaluated by densitometric analysis using Image studio Lite (Ver5.2) and normalized against the corresponding expression of GAPDH. Analysis was presented as a fold change compared to the respective controls.

Table 3: Details corresponding to reagents used in western blotting

| Description | Blocker | Primary antibody | Dilution and diluent | Secondary antibody | Dilution and diluent |
|---|---|---|---|---|---|
| Gel: 9% Protein load: 40 µg | 1x BSA-TBST | MMP-2 (MAB3308) | 1:1000 in 5x BSA-TBST | Gt x Ms (ab205719) | 1:5000 in 1x BSA-TBST |
| | 5x BSA-TBST | MMP-9 (MAB3309) | 1:500 in 5x BSA-TBST | | 1:5000 in 5x milk TBST |
| Gel: 12% Protein load: 50 µg | 5x milk TBST | TIMP-1 (ab61224) | 1:500 in 5x milk TBST | Gt x Rb (ab205718) | 1:2000 in 5x milk TBST |
| | | TIMP-2 (MAB3310) | | Gt x Ms (ab205719) | 1:5000 in 5x milk TBST |
| N/A | 1.5x milk TBST | GAPDH (ab8245) | 1:1000 in 1.5x milk TBST | Gt x Ms (ab205719) | 1:2000 in 1.5x milk TBST |

Gt x Ms: Goat anti-mouse, Gt x Rb: Goat anti-rabbit

**Zymography**

Gelatinolytic activity of MMP-9 and MMP-2 was determined by gelatin zymography.

Equal amounts of crude protein (40µg) were mixed with sample loading buffer [0.25M Tris (pH 6.8), 30% glycerol, 1% SDS and 0.02% bromophenol blue] and were loaded into an 9% acrylamide:bis-arcylamide gel containing 0.1 % gelatin as substrate. Non-reducing electrophoresis was done. After electrophoresis, the gel was washed twice for 30 minutes in renaturing buffer containing 2.5% Triton X-100 at 37° C and incubated in developing buffer [50mM Tris-HCl (pH 7.8), 0.2M NaCl, 5mM CaCl2 and 0.02% Triton X-100] at 37° C until a clear lysis zone is observed. The gel was quick stained in 0.10 % Coomassie brilliant blue R250 for 5 minutes and lightly destained till contrast between the lysis bands and blue gel background was visible. The zymographs were documented using the SynGel Documentation system under upper white light setting. The proteolytic activity was analyzed using ImageJ and presented as a fold change compared to the control.

**Statistical analysis**

Data obtained is presented as mean ± S.E.M and analyzed by ANOVA followed by comparison test using GraphPad Prism 8. P-value < 0.05 was considered as statistically significant.

## Chapter 4: Results

### Quantity and quality of extracted total RNA

Concentration and quality of extracted total RNA was evaluated using a Nanophotometer. Total RNA concentration greater than 300 ng/µL was considered to be sufficient for downstream experiments. Maximum observed concentration was 680 ng/µL while the minimum was 204 ng/µL. Among all the samples, only one sample (C15) has to be excluded from further experiments due to its low concentration. Purity of the RNA sample determined by A260/A280 ranged from 1.932 to 1.645. Majority samples had a ratio indicating the presence of high purity RNA. Presence of contaminants like salts was determined by A260/A230 with the maximum observed value being 2.119 and the minimum was 0.410 (Table 4). Among the evaluated samples, only 16 samples was considered "clean" from salt contaminants with A260/A230 greater than 1.5.

Table 4: Quantity and quality of total RNA extracted from heart tissue of male Sprague-Dawley rats.

| Group | Sample | Concentration (ng/µL) | Ratio | |
|---|---|---|---|---|
| | | | A260/A280 | A260/A230 |
| WEEK # 5: Control | C1 | 508 | 1.827 | 1.443 |
| | C2 | 406 | 1.897 | 1.720 |
| | C3 | 392 | 1.798 | 0.951 |
| | C4 | 358 | 1.705 | 0.799 |
| | C5 | 338 | 1.724 | 0.775 |
| | C6 | 454 | 1.802 | 1.027 |
| | C7 | 362 | 1.724 | 0.658 |
| | C8 | 438 | 1.795 | 1.147 |
| WEEK # 5: Cd-treated | T1 | 368 | 1.736 | 0.876 |
| | T2 | 366 | 1.830 | 1.307 |
| | T3 | 376 | 1.808 | 0.959 |
| | T4 | 302 | 1.736 | 0.733 |
| | T5 | 340 | 1.809 | 0.994 |
| | T6 | 376 | 1.825 | 1.343 |
| | T7 | 316 | 1.775 | 1.904 |
| | T8 | 400 | 1.887 | 1.527 |

| Group | Sample | Concentration (ng/µL) | Ratio | |
|---|---|---|---|---|
| | | | A260/A280 | A260/A230 |
| WEEK # 10: Control | C9 | 456 | 1.932 | 1.966 |
| | C10 | 445 | 1.721 | NR |
| | C11 | 456 | 1.854 | 1.462 |
| | C12 | 582 | 1.865 | 1.532 |
| | C13 | 326 | 1.870 | 1.598 |
| | C14 | 326 | 1.772 | 0.787 |
| | C15 | 204 | 1.645 | 0.410 |
| WEEK # 10: Cd-treated | T9 | 318 | 1.710 | 0.652 |
| | T10 | 338 | 1.878 | 1.817 |
| | T11 | 384 | 1.670 | 0.535 |
| | T12 | 376 | 1.918 | NR |
| | T13 | 340 | 1.753 | 0.787 |
| | T14 | 412 | 1.689 | 0.630 |
| | T15 | 680 | 1.921 | 1.726 |
| | T16 | 446 | 1.828 | 1.018 |
| WEEK # 14: Control | C16 | 376 | 1.790 | 1.044 |
| | C17 | 460 | 1.917 | 1.729 |
| | C18 | 426 | 1.852 | 1.331 |
| | C19 | 314 | 1.847 | 1.217 |
| | C20 | 396 | 1.904 | 1.664 |
| | C21 | 568 | 1.919 | 1.661 |
| | C22 | 584 | 1.921 | 1.947 |
| | C23 | 488 | 1.821 | 0.961 |
| WEEK # 14: Cd-treated | T17 | 346 | 1.821 | 1.075 |
| | T18 | 490 | 1.914 | 1.476 |
| | T19 | 454 | 1.924 | 1.746 |
| | T20 | 640 | 1.916 | 2.119 |
| | T21 | 424 | 1.828 | 1.225 |
| | T22 | 520 | 1.926 | 1.667 |
| | T23 | 528 | 1.913 | 1.509 |
| | T24 | 406 | 1.880 | 1.390 |

NR: not recorded

**Level of gene expression of inflammatory mediators in heart tissue**

In order to evaluate the inflammatory status of the heart under cadmium treatment over time, the levels of expression of genes IL-1β, IL-6, IL-10, NF-κB and TNF-α were assessed and represented as a fold change (*Figure 5*). At week 5, it is observed that IL-1β and IL-10 are upregulated by 1.5-fold and more than 3-fold, respectively.

In the same time point, both NF-κB and TNF-α are slightly downregulated by less than 1-fold. In contrast to week 5, it is observed that at week 10, all the inflammatory mediators are upregulated ranging from 2-fold to 5-fold. Following through to week 14, it was observed that 3 out of the 5 mediators i.e. IL-1β, IL-10 and NF-κB have downregulated while the others (IL-6 and TNF-α) have a sustained expression by 2-fold and 1-fold, respectively. It must be noted that maximum fold change was observed at week 10 for all mediators except IL-10.

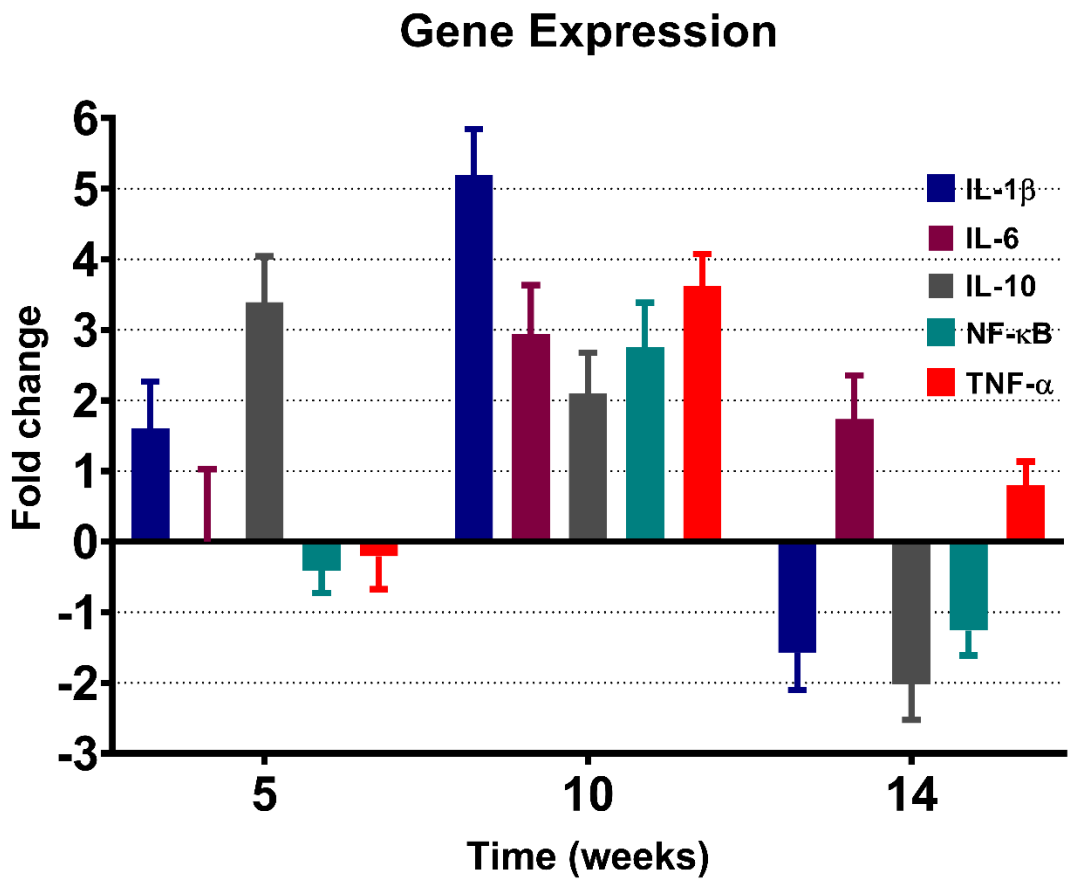


*Figure 5*: Gene expression of inflammatory mediators in heart tissue of Cd-treated male Sprague-Dawley rats using real time-PCR. Expression data are represented as mean ± S.E.M of fold-changes compared to the control (n=8).

**Quantification of total crude protein**

Total protein was extracted using a cell lysis buffer supplemented with cocktail protease inhibitor. Quantification of the extracted total protein was determined by Bradford assay against a standard protein BSA. As shown in Table 5, the mean concentration of extracted proteins was high with very negligible variation and more than sufficient amount for downstream experiments.

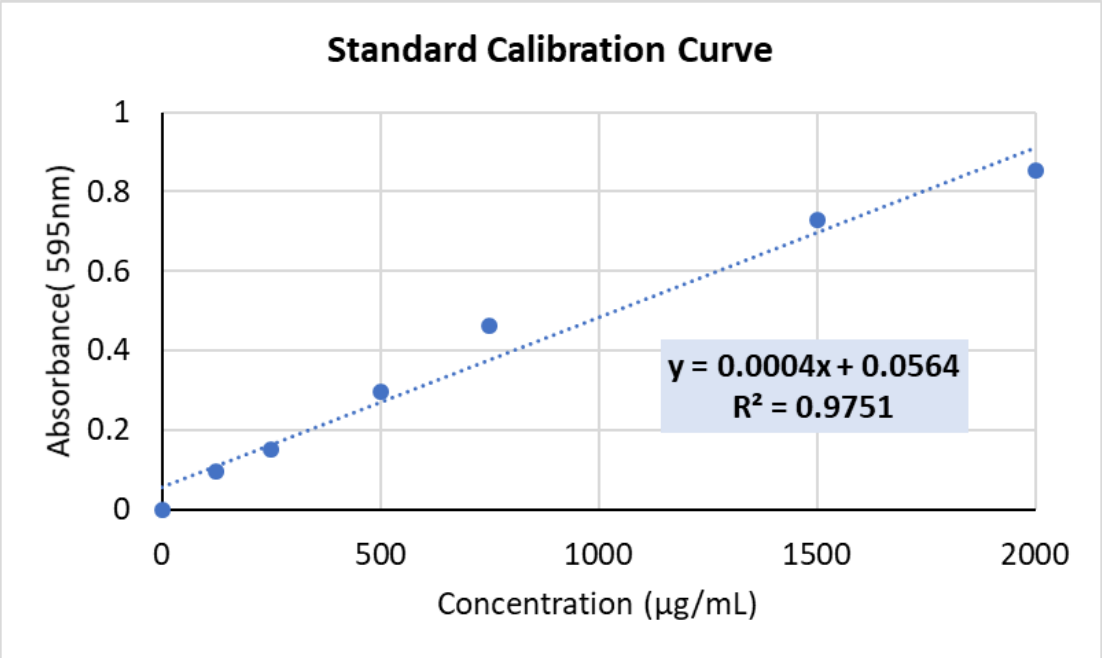


*Figure 6*: Graph of standard calibration curve for Bradford protein assay. Standard used in this assay was bovine serum albumin. Assay was performed in duplicates.

Table 5: Quantification of crude total protein by Bradford assay from heart tissue of male Sprague-Dawley rats.

| Week | Group | Concentration (µg/mL) | | |
|---|---|---|---|---|
| | | Mean | Maximum | Minimum |
| 5 | Control | 6588 ± 385.6 | 7686 | 5356 |
| | Cd-treated | 5512.2 ± 479.2 | 7596 | 3376 |
| 10 | Control | 5843.5 ± 344.9 | 6821 | 5331 |
| | Cd-treated | 5478.3 ± 101.8 | 5851 | 5081 |
| 14 | Control | 5268.5 ± 314.5 | 5951 | 4971 |
| | Cd-treated | 5416.6 ± 118.6 | 6111 | 4971 |

Bradford assay was performed in duplicates. Data of mean protein concentration is presented as mean ± S.E.M. (n=8)

**Level of protein expression of MMPs in heart tissue**

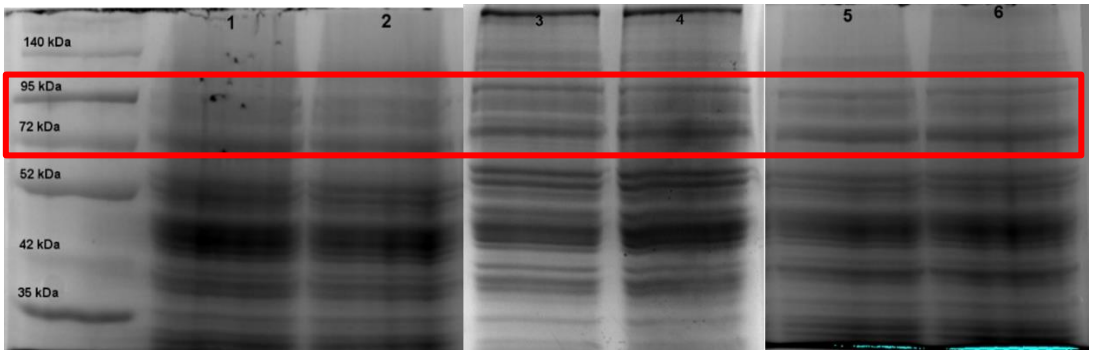


*Figure 7*: Representative image of Commassie Brilliant Blue stained 9 % SDS-PAGE indicating band pattern of 40 µg crude protein. Lane 1, 3 and 5 represent control samples at week 5, week 10 and week 14, respectively. Lane 2,4 and 6 represent Cd-treated samples at week 5, week 10 and week 14, respectively. Crude protein was run against a broad spectrum marker ranging from 240 kDa to 35 kDa. The red box indicates the region of interest corresponding to the molecular weight of the target MMPs.

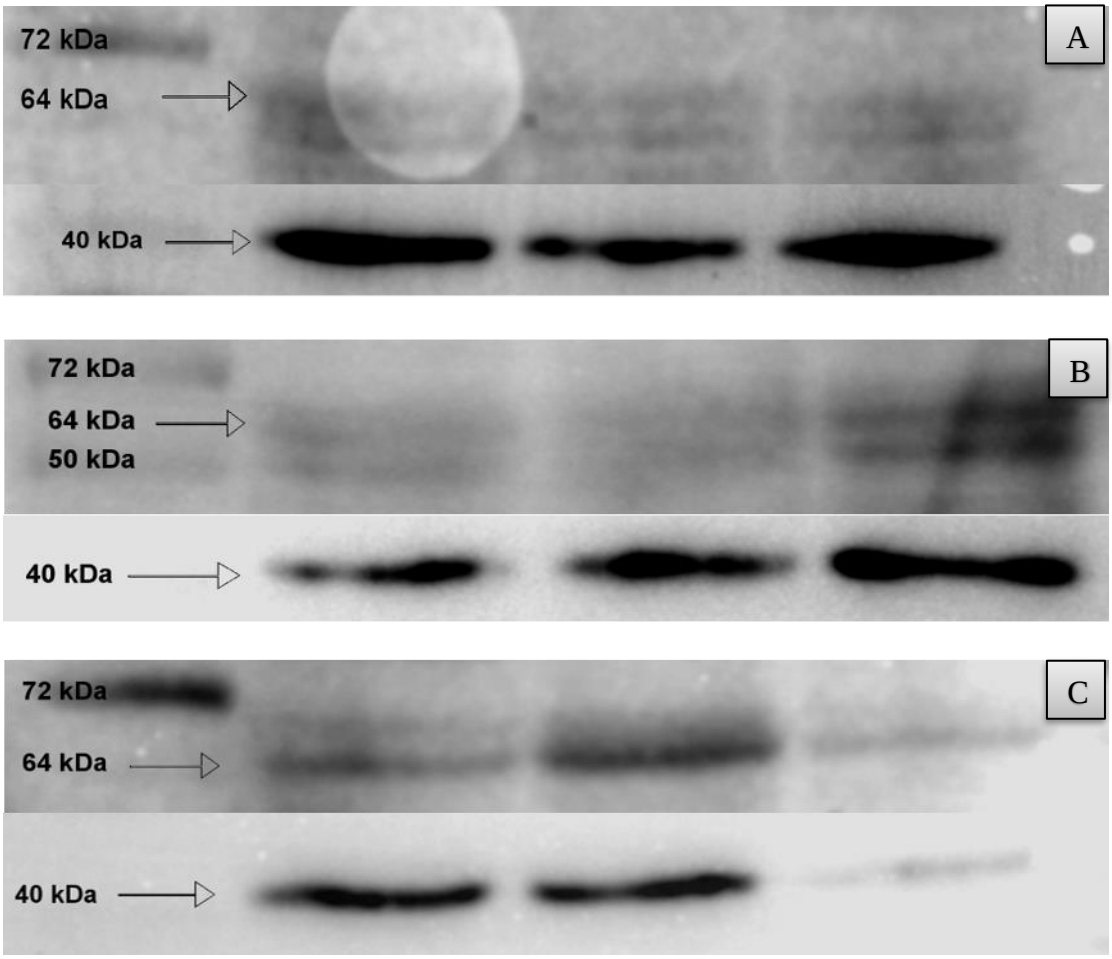


*Figure 8*: Representative immunoblots for active MMP-2 (64 kDa) and GAPDH (40 kDa) at week 5 (A), week 10 (B) and week 14 (C).

To evaluate the level of expression of MMPs, 40 µg of crude protein was separated in 9 % SDS-PAGE (*Figure 7*). Protein expression was evaluated by probing electroblots specific for MMP-2 and MMP-9. By western blotting, only MMP-2 was detectable at approximately 64 kDa (*Figure 8*). It was observed that MMP-2 increased by more than 2-fold at week 5, slightly decreased at week 10 and reaches a maximum fold change of more than 4-fold at week 14 (*Figure 9*). In comparison to the respective control groups, the changes were not statistically significant at each time point.

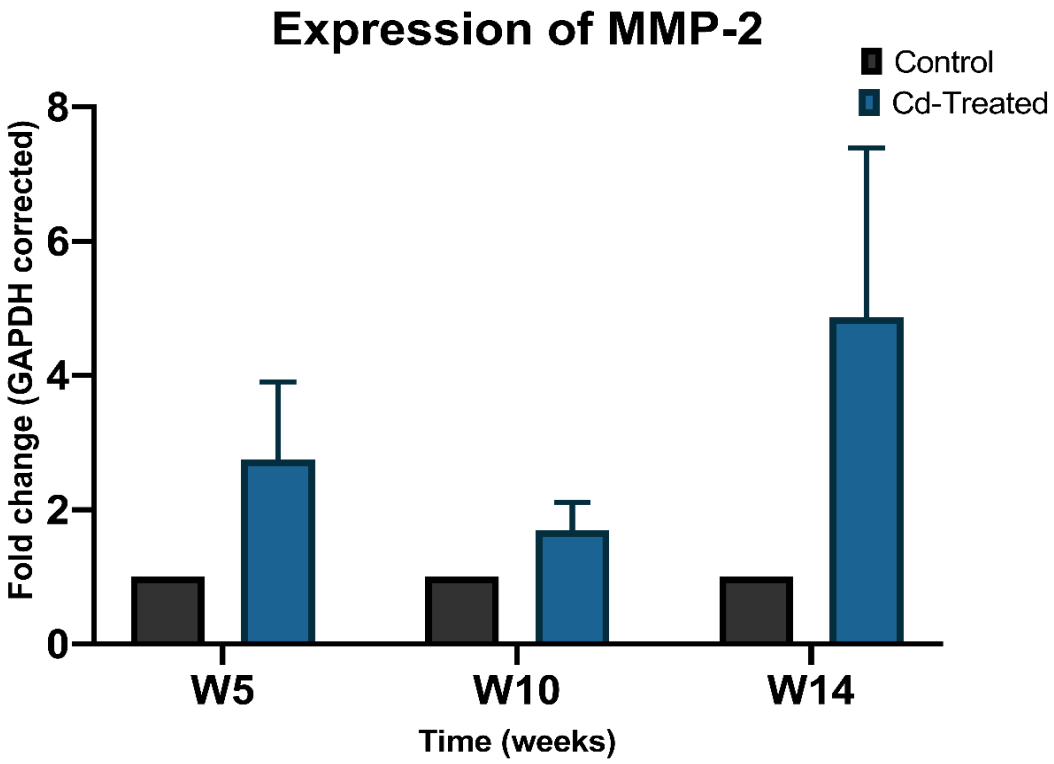


*Figure 9*: Effect of Cd-treatment on expression of MMP-2 in heart tissue of male Sprague-Dawley rats. Protein expression data were normalized against the expression of GAPDH and expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

**Level of protein expression of TIMPs in heart tissue**

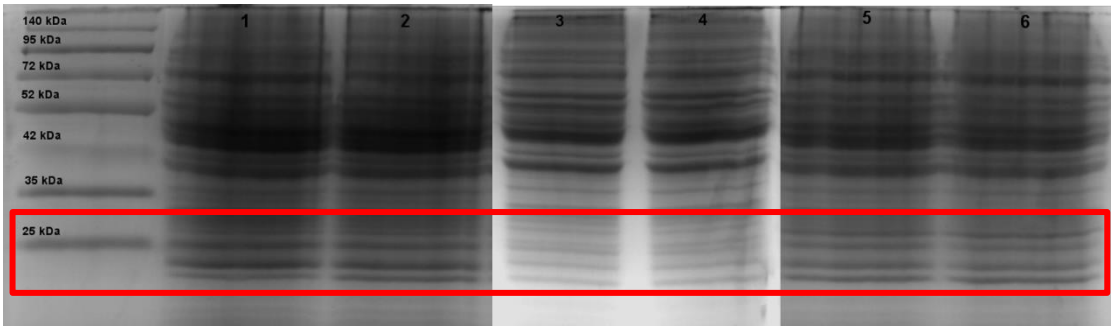


*Figure 10*: Representative image of Commassie Brilliant Blue stained 12 % SDS-PAGE indicating the band pattern of 50 µg crude protein. Lane 1, 3 and 5 represent control samples at week 5, week 10 and week 14, respectively. Lane 2,4 and 6 represent Cd-treated samples at week 5, week 10 and week 14, respectively. Crude protein was run against a broad-spectrum marker ranging from 240 kDa to 15 kDa.

The red box indicates the region of interest corresponding to the molecular weight of the target TIMPs.

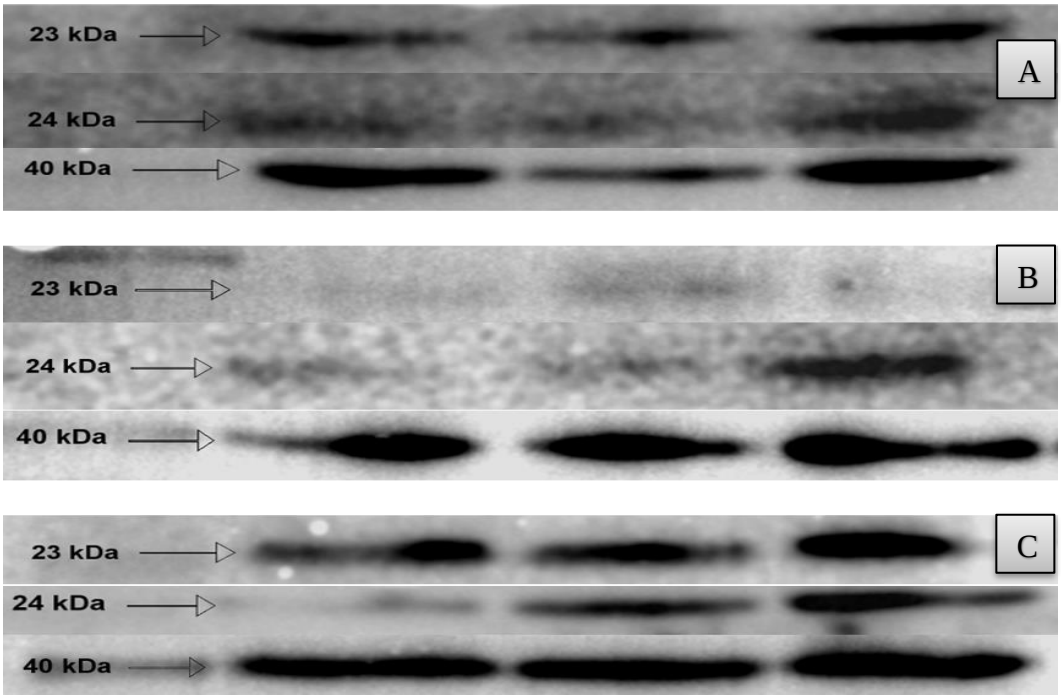


*Figure 11*: Representative immunoblots for TIMP-1 (23 kDa), TIMP-2 (24 kDa) and GAPDH (40 kDa) at week 5 (A), week 10 (B) and week 14 (C).

Protein expression of TIMPs was assessed by separating 50 µg of crude protein in 12% SDS-PAGE (*Figure 10*) and probing immunoblots against TIMP-1 and TIMP-2 (*Figure 11*). The expression of TIMP-1 showed limited changes at the level of the protein in comparison to the control group. Less than 1.5-fold change was observed at week 5 which was sustained at the same level till week 10 and mildly decreases to be similar to the level of expression of the control group at week 14 (*Figure 12*). A contrasting pattern is observed for the expression of TIMP-2. As shown in *Figure 13*, the expression of TIMP-2 increased by 2-fold at week 5 reaching a maximum fold change of more than 4 times at week 10 and decreases by about 3-fold at week 14. Both the changes shown in *Figure 12* and *Figure 13* were not statistically significant.

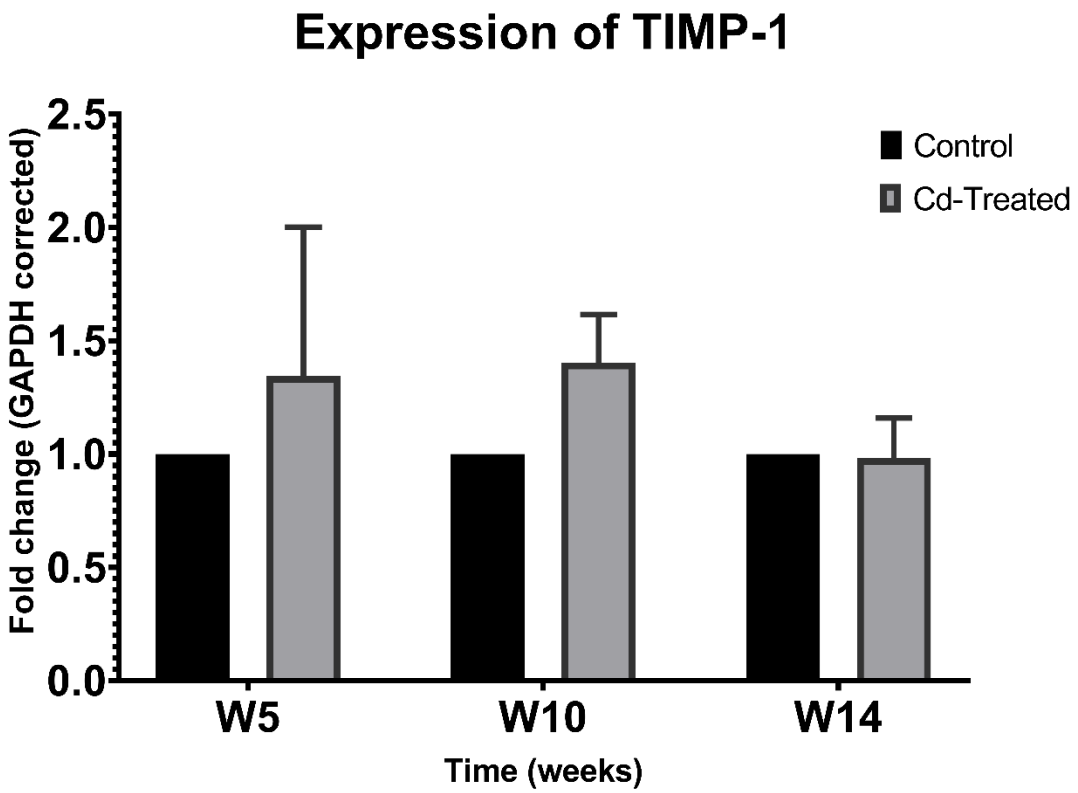


*Figure 12*: Effect of Cd-treatment on expression of TIMP-1 in heart tissue of male Sprague-Dawley rats. Protein expression data were normalized against the expression of GAPDH and expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

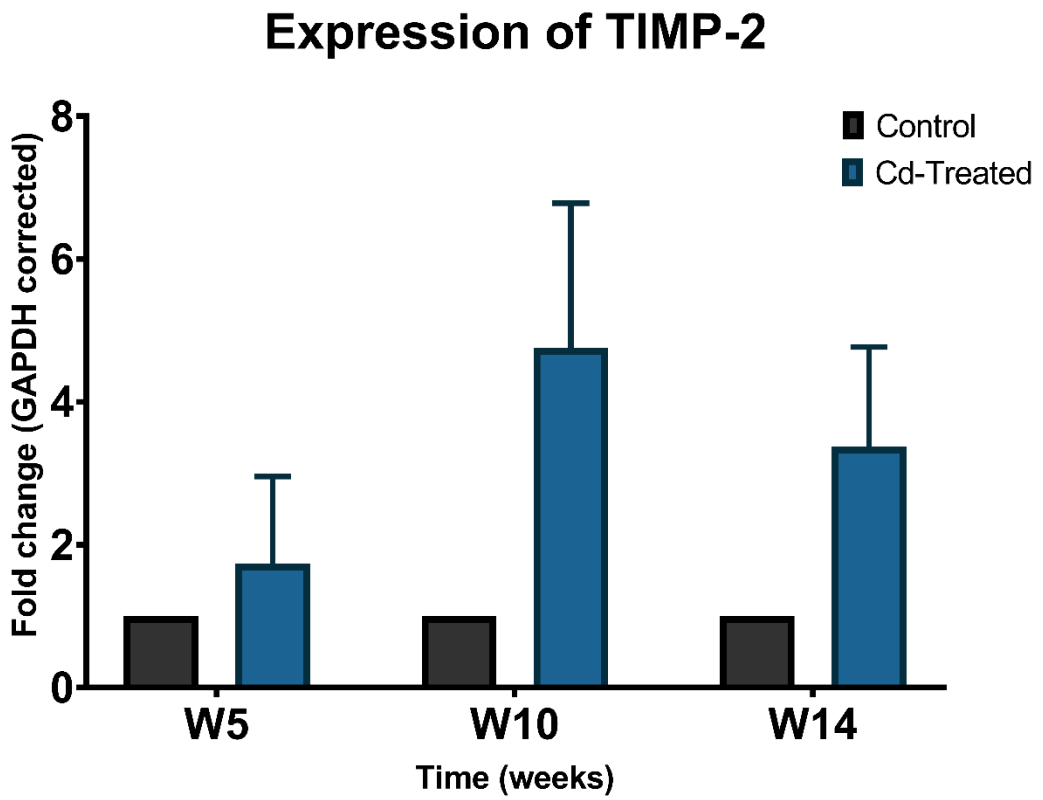


*Figure 13*: Effect of Cd-treatment on expression of TIMP-2 in heart tissue of male Sprague-Dawley rats. Protein expression data were normalized against the expression of GAPDH and expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

**Gelatinolytic activity of MMPs in heart tissue**

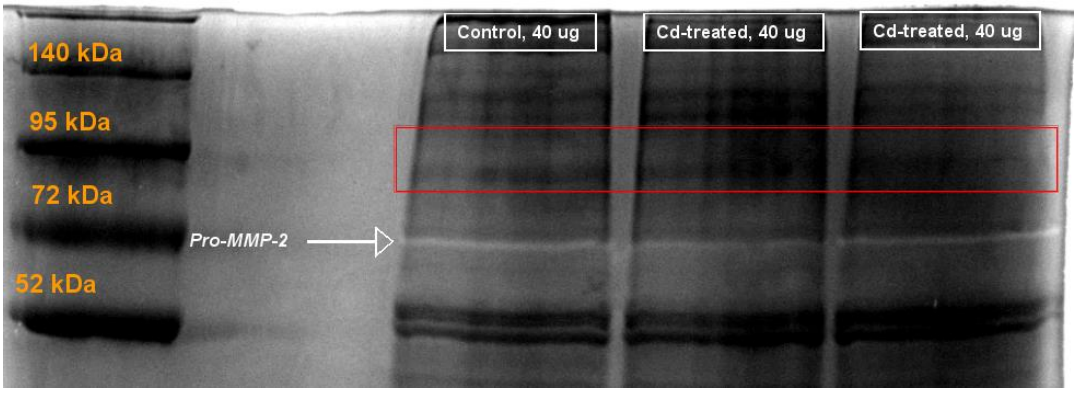


*Figure 14*: Representative image showing gelatinolytic activity of MMP after 24 hours incubation. The white arrow at 72 kDa indicates the band of lysis due to the activity of pro-MMP-2. The red box indicates the region of interest for the activity of MMP-9. The activity of pro- and active MMP-9 as well as active MMP-2 was not visible after 24 hours.

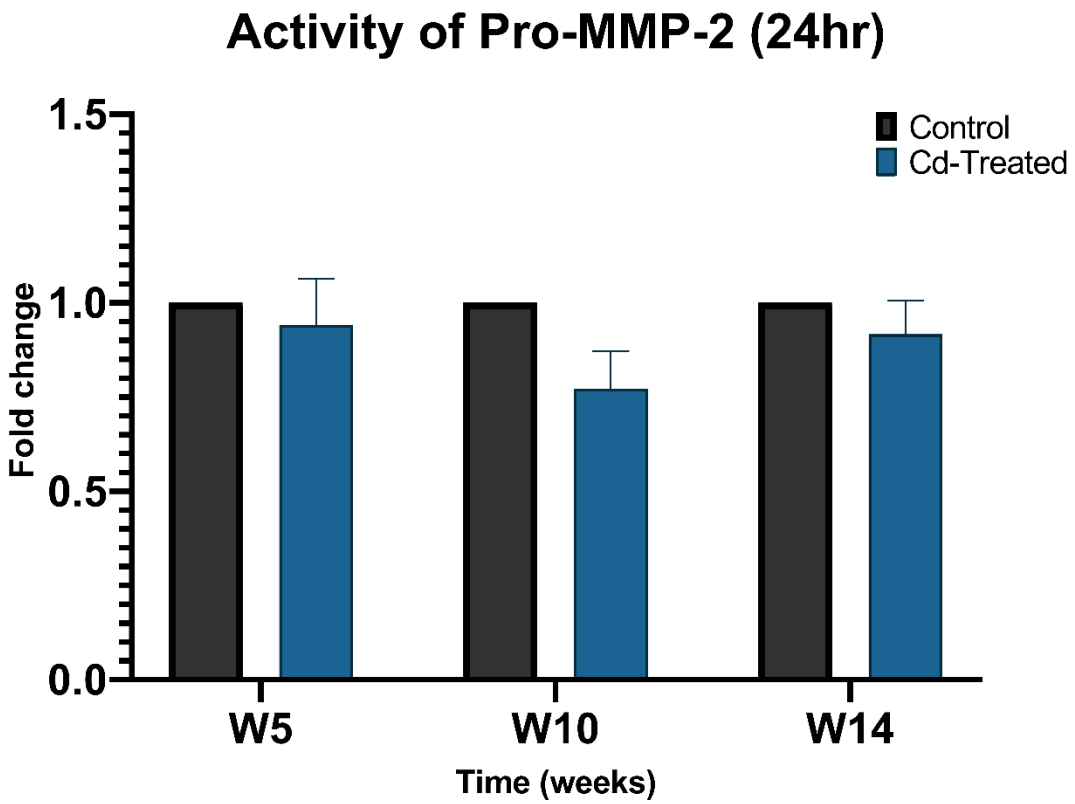


*Figure 15:* Gelatinolytic activity of pro-MMP-2 after 24hr incubation. Gelatinolytic activity is expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

Activity of MMPs were evaluated by gelatin zymography indicated by visualization of bands of lysis corresponding to the molecular weight of MMP-2 and MMP-9. As observed in the representative image, after 24-hour incubation, all samples showed activity corresponding to the proenzyme form of MMP-2 at 72 kDa (*Figure 14*). Densitometric analysis of the detected area of lysis shows that the activity of pro-MMP-2 does not increase by more than 1-fold at all time points in comparison to their respective control group (*Figure 15*).

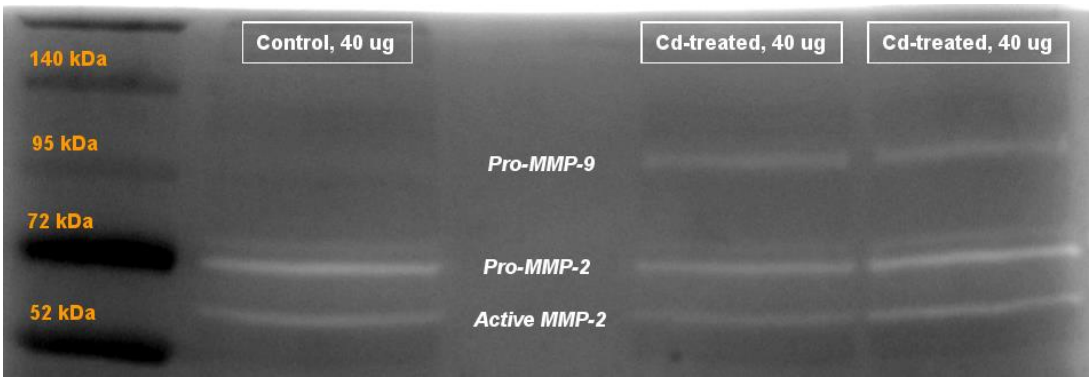


*Figure 16*: Representative image showing gelatinolytic activity of MMP after 60+ hours incubation. Labels show the activity of pro-MMP-9 at 95 kDa, pro-MMP-2 at 72 kDa and active MMP-2 at approximately 65 kDa. The activity of active MMP-9 (82 kDa) was not visible after 60+ hours.

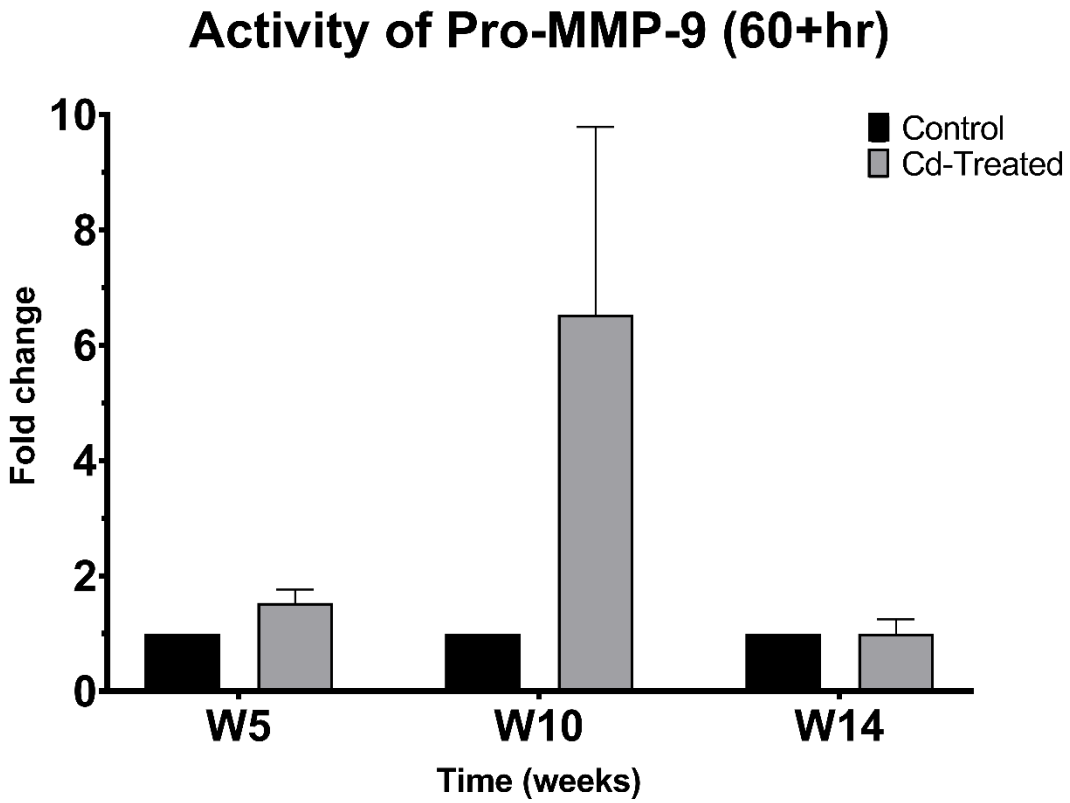


*Figure 17*: Gelatinolytic activity of pro-MMP-9 after 60+ hr incubation. Gelatinolytic activity is expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

On extension of the incubation period to more than 60 hours, the activity of proenzyme form of MMP-9 and both proenzyme and active enzyme form of MMP-2

was detected (*Figure 16*). Quantification of the activity for each of the enzyme forms was done by densitometric analysis. At week 5, the activity of pro-MMP-9 increased by 2-fold to reach a maximum increase of more than 6-fold at week 10 and decreased to 1-fold at week 14 in comparison to their respective control samples (*Figure 17*). In regard to proenzyme MMP-2, the activity of Cd-treated group at week 5 and week 14 shows similar activity to their respective control group. However, at week 10, a slight increase in activity is observed in the Cd-treated group (*Figure 18*). With respect to the activity of active form of MMP-2, there is a 0.5-fold decrease at week 5 that slightly increases at week 10 and reaches a maximum of 1.5-fold increase at week 14 (*Figure 19*). The fold change observed at week 5 was found to be statistically significant in comparison to the control group while the changes observed at other time points were not statistically significant.

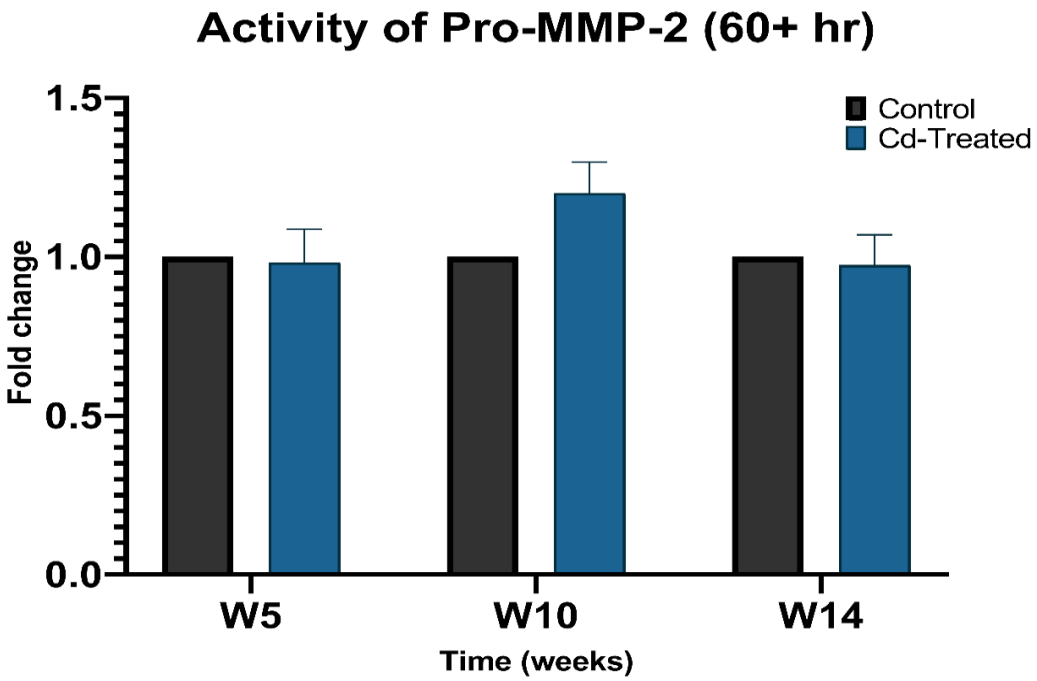


*Figure 18*: Gelatinolytic activity of pro-MMP-2 after 60+ hr incubation. Gelatinolytic activity is expressed as mean ± S.E.M of fold-changes compared to the control (n=8).

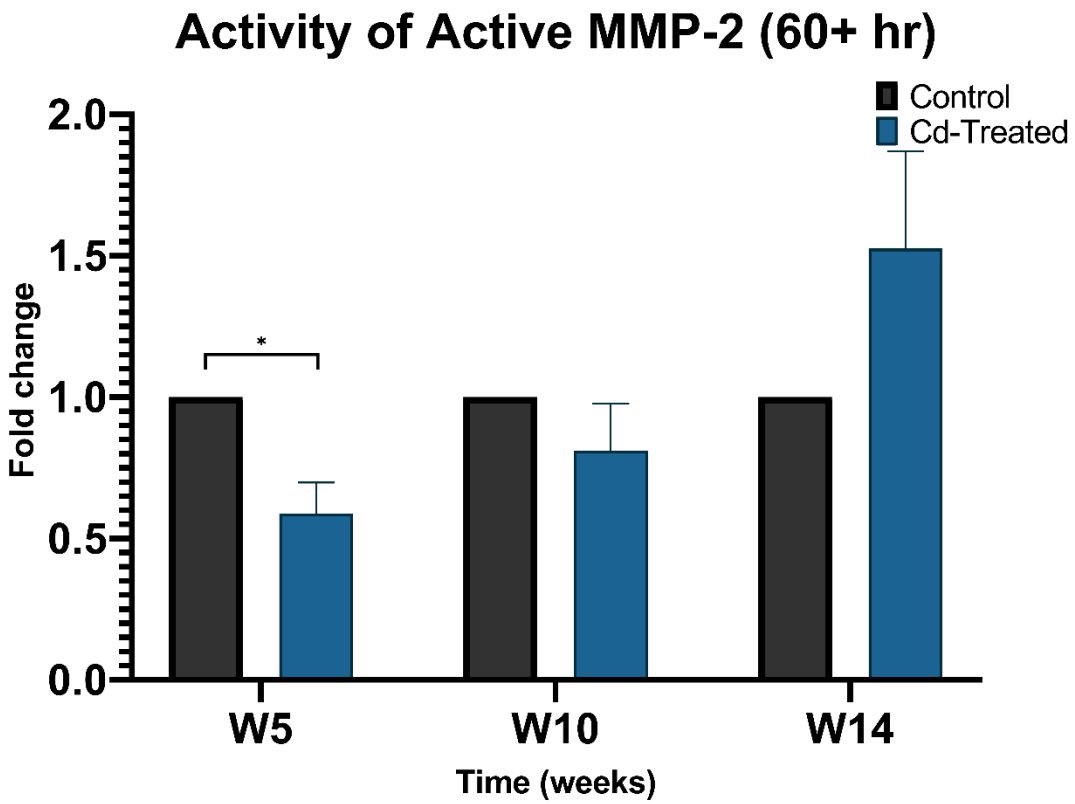


*Figure 19*: Gelatinolytic activity of active MMP-2 after 60+ hr incubation. Gelatinolytic activity is expressed as mean ± S.E.M of fold-changes compared to the control (n=8). *: p-value < 0.05

## Chapter 5: Discussion

Cadmium is an environmental priority pollutant with reported associated repercussions on various organ systems including the cardiovascular system. Recent studies have reported the relevance of cadmium as a novel and independent risk factor for pathologies of the cardiovascular system (Messner & Bernhard, 2010; Santos-Gallego & Jialal, 2016; Solenkova et al., 2014; Tellez-Plaza et al., 2013). A common pathology reported in the previous studies is the structural alteration of the integrity of the cardiac tissue observed by formation of irregular branching pattern, complexed matrix network, fibrosis and focal areas of necrosis (Bhattacharjee et al., 2019; Saleh & Awadin, 2017; Sangartit et al., 2014). These studies demonstrated that cadmium mediates the tissue damage of the cardiovascular system by inducing oxidative stress. However, another pathway is the simultaneous implication of inflammation with oxidative stress during cadmium exposure (Koopsamy Naidoo et al., 2019; Kukongviriyapan, Apaijit, & Kukongviriyapan, 2016; L. Liu et al., 2015; Tucovic et al., 2018). Previous work has linked cadmium induced oxidative stress with cardiovascular pathologies in both humans and animals (Mariana L. Ferramola et al., 2012; Mariana Lucila Ferramola et al., 2011; D.-H. Lee, Lim, Song, Boo, & Jacobs, 2006; Liang et al., 2019). There is limited research addressing the link between cadmium induced inflammation and cardiovascular pathology. To our knowledge, there is limited understanding about the mechanism by which cadmium alters the cardiovascular integrity. Therefore, the present study attempted to address this gap by looking at the impact of cadmium on the modulation of key players in the cardiac tissue via inflammatory mediators.

It is well-documented that IL-1β, IL-6 and TNF-α cause inflammation, therefore changes in gene expression of these cytokines indicated inflammation (Das & Al-

Naemi, 2019; Ptaschinski & Lukacs, 2018). In the current study, the gene expression of targeted inflammatory mediators (IL-1β , IL-6, IL-10 and TNF-α) in the heart tissue of rats across 3 time points after cadmium treatment showed an exposure-dependent variation in their expression. After 5 weeks of cadmium exposure, cardiac tissue showed an upregulation of IL-1β and IL-10 and downregulation of NF-κB and TNF-α. At week 10, all the targets are upregulated while discontinuation of cadmium treatment for 4 weeks resulted in downregulation of the targets except IL-6 and TNF-α remaining upregulated. NF-κB is a vital player as a transcription factor in inflammation with a role in the regulation of several genes including cytokines (Kumar, Takada, Boriek, & Aggarwal, 2004; Lawrence, 2009). Gordon et al. (2011) hypothesized that NF-κB regulates three genetic programs namely, hypertrophy, cytoprotection and chronic cytotoxicity brought on by prolonged inflammatory response. Chronic activation of the NF-κB signaling strongly drives the chemokine production by propagation of the pro-inflammatory cascade leading to a prolonged inflammatory state (Gordon et al., 2011; Ptaschinski & Lukacs, 2018). Several *in vivo* and *in vitro* studies have reported the upregulation in the expression of NF-κB while downregulating the expression of inflammatory mediators like IL-10 and IL-6 (Ghosh et al., 2018; Olszowski, Baranowska-Bosiacka, Gutowska, & Chlubek, 2012; Riemschneider, Herzberg, & Lehmann, 2015). An *in vitro* study in ECV304 cells suggested that NF-κB was activated by the cadmium treatment via phosphorylation and degradation of the NF-κB inhibitor, IκBα (Lian et al., 2015). In the same study, cadmium consistently increased the transcriptional activity of NF-κB in a dose-dependent manner. Contradictory to the previous studies our results showed that the expression of NF-κB in the cardiac tissue seems to vary by different folds wherein it is downregulated at week 5, upregulated at week 10 that is abolished after a 4-week

cessation of cadmium treatment. This suggests that the cumulative concentration of cadmium circulating within the system may not have reached a threshold to trigger the upregulation of NF-κB until at week 10. In the canonical signaling pathway of NF-κB , TNF-α is a widely studied ligand that activates NF-κB (Gordon et al., 2011). TNF-α plays a pro-inflammatory role in local and systemic inflammation when secreted by activated macrophages in response to injury to amplify and prolong the inflammatory response (Tracey, 2002). A study reported a significant release of TNF-α in the heart of male rats treated at a dose of 5 mg/kg b.w. for 4 weeks (Nazimabashir, Manoharan, & Miltonprabu, 2015). Another study reported an increased release of TNF-α in primary alveolar macrophages after cadmium exposure however, there was a decrease in the mRNA expression for the same (Låg et al., 2010). A study reported that elevated levels of TNF-α in the heart of cadmium treated rats mediated a malfunction of the organ (Chen, Zhou, Gao, & Jiang, 2003). In the current study, it was observed that TNF-α expression occurs in parallel to the expression of NF-κB until week 10 after which the expression of TNF-α opposes the expression of NF-κB. One of the main mechanisms of terminating the signal of NF-κB is by downregulation via a feedback loop involving TNF-α (Hayden & Ghosh, 2014). The downregulation of NF-κB and upregulation of TNF-α after recovery period (week 10-14) may be explained by this mechanism.

Extensive research exploring the impact of cadmium along the inflammation axis have reported the production and upregulation of IL-6, TNF-α and IL-1β both *in vivo* and *in vitro* (Djokic et al., 2015; Ninkov et al., 2015; Tucovic et al., 2018). In this study, the pro-inflammatory impact of the cadmium treatment was also observed as an upregulation in pro-inflammatory cytokines IL-6 and IL-1β. Interestingly, the upregulation of anti-inflammatory cytokine, IL-10 was also observed till week 10

suggesting an activated mechanism to establish a balance in the inflammatory response. IL-6 is a key pro-inflammatory cytokine that mediates the transition from acute to chronic inflammation (Låg et al., 2010). For the current study, it must also be highlighted that after the recovery period (week 10-14), the sustained upregulation of IL-6 was observed. This suggests that an inflammatory signal remains stimulated despite the withdrawal of cadmium exposure for four weeks. However, it is also feasible that the circulating cadmium from the treatment may still have an impact on the inflammatory status of the heart tissue. The results of this study are in agreement with other studies established in other biological model that reported differential effects of cadmium treatment on the expression of inflammatory cytokines *in vivo* and *in vitro* (Djokic et al., 2014, 2015; Milnerowicz, Ściskalska, & Dul, 2015). Recent reviews have shown that the effects of cadmium may occur in an organ-specific manner by causing an imbalance in the pro-inflammatory/anti-inflammatory cascade as per the dose, exposure duration and biological model (Das & Al-Naemi, 2019; Hossein-Khannazer et al., 2020).

Matrix metalloproteinases (MMPs) play a vital role in both ECM turnover and inflammatory response in normal physiology. A dysfunction in their expression and activity, specifically gelatinases has been widely implicated in various cardiovascular pathologies (Peterson, 2000; Spinale, 2007). The current study evaluated both the expression as well the activity of MMP-2 and MMP-9 in the heart tissue under cadmium stress whereas majority of published studies exploring the impact of heavy metal exposure on MMP was evaluated by gelatinolytic zymography. Results revealed a decrease in the expression of MMP-2 from week 5 till week 10 which spikes to a maximum at week 14. Protein expression of MMP-9 was not detectable by western blotting due to the limited sensitivity of the antibodies (results not shown). It

is worth highlighting that although the protein expression may be undetectable, this does not imply the absence or lack of activity of the protein as shown in this study. Despite the non-detectable MMP-9 protein by immunoblotting, enzymatic activity of MMP-9 was observed after 60+ hours of incubation only. This supports the idea that the protein expression levels of MMP-9 may be very low under this experimental setup however, the protein still retains its activity. MMP-9 is an inducible protein that is synthesized by inflammatory cells when stimulated by the release of IL-1β, IL-6 or TNF-α (Hu, Van den Steen, Sang, & Opdenakker, 2007; Rutschow, Li, Schultheiss, & Pauschinger, 2006). Furthermore, an *in vitro* study in cardiac fibroblasts demonstrated that IL-1β and TNF-α increased the total activity of MMP-2 and MMP-9 as determined by gelatin zymography (Siwik, Chang, & Colucci, 2000). The authors also suggested that the increase in activity may be partly attributed to the increase in the transcription of gelatinases. Similarly, in the present study, the results corresponding to the increase in activity of latent MMP-2 and -9 at week 10 also corresponds in parallel to the upregulation in the gene expression of pro-inflammatory cytokines. This substantiates the hypothesis that cadmium incites an inflammatory response that stimulates the activity of pro-MMPs in the cardiac tissue. With regard to MMP-2 activity, the pro-enzyme form seems to have very minimal variation over time. Contrastingly, the activity of active form of MMP-2 was observed to gradually increase over time in the cadmium treated group with the minimum activity recorded at week 5 and maximum at week 14. These observations suggest that cadmium interferes with the activity of MMP-2 such that even though the pro-enzyme form may be available, there may be an interference in the activation of the pro-enzyme form to active form due to the cadmium treatment. A similar study describing an animal model of cadmium-induced emphysema demonstrated the association of

increased MMP-2 and MMP-9 levels and correlated these increases with pulmonary inflammation (Kirschvink et al., 2005). Also reported in the same study is the detectability of pro-MMP-9 activity after cessation of cadmium exposure in bronchoalveolar lavage fluid (BALF). In relation to MMP-2, the study reported an elevation in the activity of MMP-2 after 3 weeks of cadmium exposure that returned to control levels at week 5 (Kirschvink et al., 2005). The findings of the current study are similar to the previously cited findings. Contrastingly, another study reported a decrease in the activity of both MMP-2 and MMP-9 in the prostate of rats treated with cadmium (15 ppm) for 20 weeks (Lacorte et al., 2015). Taken together, it seems that chronic low dose cadmium exposure modulates both expression and activity of MMP-2 and MMP-9 in the heart. However, differences in expression and activity of gelatinases following cadmium exposure may be attributed to the dose, duration, route of exposure and biological set up. Further research into MMP-9 is required to confirm modulation in protein expression in the heart.

One of the strategies to regulate and modulate the expression of MMPs is by means of TIMPs. The inhibitory relationship of the TIMPs on their preferential MMPs was clearly reflected in the protein expression levels of TIMP-1 and TIMP-2. Here, the expression of TIMP-1, a preferential inhibitor of MMP-9, was elevated in the Cd-treated group until week 10 and returned to control levels after the recovery period. Likewise, the expression of TIMP-2, the inhibitor of MMP-2, increased from week 5 till week 10 to slightly decrease after the recovery period. Furthermore, the expression of TIMP-2 can be inversely correlated with the expression of MMP-2 wherein an elevation in the expression of TIMP-2 decreased the expression of MMP-2. These results demonstrate the effect of cadmium on the expression of TIMPs and hence the inhibition of MMPs. An *in vitro* study on cadmium treated U-937 cells showed that

cadmium doses (1.0 to 50.0 µM) did not have any effect of TIMP-1 levels however there was an alteration in the MMP-9/TIMP-1 expression at the level of the gene albeit not statistically significant (Yaghooti et al., 2012). The study concluded that cadmium disrupts MMP-9/TIMP-1 balance to favor proteolysis. Another study found that cadmium induced a significant increase in the expression of gelatinases and TIMP-2 in BALF while TIMP-1 expression was not detectable (Fiévez et al., 2009). Consistent with previous studies, cadmium exposure modulates MMPs expression and activity by disrupting the balance between MMP and TIMPs. Apart from these findings, it cannot be overlooked that MMPs are zinc-dependent endopeptidases and there also lies the possibility that cadmium may play a role in deactivating the gelatinases by mimicking the divalent zinc in these enzymes also known as molecular mimicry (Bridges & Zalups, 2005; Chmielowska-Bąk, Izbiańska, & Deckert, 2013). This remains to be confirmed by further research.

## Chapter 6: Conclusion

This study aimed to explain the potential role of an environmental contaminant like cadmium in modulating the matrix metalloproteases in the cardiac tissue. The findings of this study add to a growing body of literature towards the impact of low dose chronic cadmium exposure in the heart. It must be noted that to the best of our knowledge, this study is the first to evaluate the impact of cadmium exposure on the heart after withdrawal of the treatment. Considerable insight has been gained in understanding that despite the low dose of cadmium exposure followed by its withdrawal, the impact of the exposure stills persists in the heart. This study confirms that cadmium stimulates an inflammatory response by the upregulation in the expression of pro-inflammatory cytokines in an exposure-dependent manner that is still slightly sustained after withdrawal of the treatment. The sustained expression of cytokines (particularly IL-6 and TNF-α) can be attributed to the feedback regulation of MMPs to counter-regulate the inflammatory response and promote resolution (Fingleton, 2017). Apart from the cadmium-induced inflammatory cascade, it was also confirmed that cadmium alters the expression and activity of gelatinases in the heart. Physiological and pathological alterations to the expression and activity of gelatinases impacts the heart ECM turnover to thereby modify the structure and function of the heart. Taken together, further experimental investigation are required to look at how cadmium interacts with gelatinases in the heart to further explain associated cardiovascular pathologies.

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