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Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Pyridoxamine prevents cardiac dysfunction in a rat model of diabetic cardiomyopathy

Lisa Claes

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Molecular Mechanisms in Health and Disease

SUPERVISOR :
Prof. dr. Virginie BITO

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2021
2022

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Universiteit Hasselt
Campus Hasselt:
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Campus Diepenbeek:
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Pyridoxamine prevents cardiac dysfunction in a rat model of diabetic cardiomyopathy*

Lisa Claes[1], Sarah D'Haese[2], and Virginie Bito[2]

[1]Faculty Medicine & Life Sciences, Hasselt University, Agoralaan Building D – B-3590 Diepenbeek
[2]Cardio and Organ Systems research group, Biomedical Research Institute, Hasselt University, Campus Diepenbeek, Agoralaan Building C - B-3590 Diepenbeek

*Running title: *Effect of pyridoxamine on diabetic cardiomyopathy*

To whom correspondence should be addressed: Virginie Bito, Tel: +3211 26 92 85; Email: virginie.bito@uhasselt.be

Keywords: Diabetes Type 2, diabetic cardiomyopathy, cardiac dysfunction, AGEs, pyridoxamine

ABSTRACT

More than 400 million people are affected by type 2 diabetes mellitus. The condition increases the risk of developing diabetic cardiomyopathy (DCM), a cardiac muscle disease. The mechanisms underlying DCM have not been fully elucidated, and preventive strategies to tackle the disease are needed. Advanced Glycation End-Products (AGEs) are proposed to play a crucial role in DCM. We hypothesize that PM, a vitamin B derivate and inhibitor of AGEs, prevents DCM-related complications, like impaired glucose control and cardiac dysfunction. In our study, Sprague-Dawley rats were divided into control, Western diet, and Western diet + PM. The Western diet consists of a high-sugar diet, given for 18 weeks to induce DCM. Cardiac function and glycemic control were assessed with echocardiography and oral glucose tolerance test, respectively. Cardiac fibrosis and AGEs levels were determined via Sirius Red/Fast Green and immunohistochemical staining. qPCR was performed on left ventricular tissue. Rats undergoing a Western diet developed hyperglycemia, which was prevented by PM. Western diet rats showed cardiac dysfunction presented as increased systolic and diastolic volumes and areas and a reduced ejection fraction. Administering PM tended to prevent these cardiac abnormalities. PM significantly limited the development of interstitial cardiac collagen in DCM rats, whereas it did not alter cardiac AGEs levels. Lastly, PM lowered NADPH oxidase 4 (NOX4) gene expression in DCM rats. This study demonstrates that PM shows promise to improve glycemic control and prevent dilated cardiomyopathy by limiting cardiac fibrosis and providing its anti-oxidative effects in DCM rats.

INTRODUCTION

1. Diabetic cardiomyopathy

Worldwide, more than 400 million people are affected by Diabetes Mellitus, whose incidence keeps rising at an alarming rate (1,2). Diabetes Mellitus is a chronic metabolic disease characterized by hyperglycemia (3). The condition is conventionally classified into Type 1 Diabetes Mellitus, which is an autoimmune disease, and Type 2 Diabetes Mellitus (**T2DM**). The majority of diabetes patients have T2DM, accounting for approximately 90-95% of all cases. T2DM is caused by the body's ineffectiveness in using insulin (3,4). In particular, there is a resistance to insulin paired with an inadequate response to compensate for this resistance (3,4). Risk factors for the disease include genetics and aging as well as lifestyle-related features, such as obesity, abdominal fat, physical inactivity, smoking, and consumption of energy-dense food containing high amounts of fats and sugars (3-5). Consumption of this unhealthy or so-called Western diet is thought to contribute greatly to disease development. In addition to nephropathy, retinopathy, and neuropathy, cardiovascular diseases (**CVDs**) especially are major complications of T2DM (2,6).

CVDs are the leading cause of mortality and morbidity in diabetes patients and include coronary artery disease (**CAD**), myocardial infarction, and

diabetic cardiomyopathy (**DCM**) (2,7). DCM is described as diabetes-associated structural and functional changes in the myocardium in the absence of other cardiac risk factors, such as CAD or hypertension (2,8-10). Studies indicate that the risk of heart failure is correlated to the level of glycaemic control as an increase of 1% in glycated hemoglobin level is associated with a rise of 8% in the risk of heart failure in T2DM patients, independent of other risk factors (8,11). Initially, DCM is characterized by subclinical cardiac abnormalities, such as fibrosis, decreased left ventricular compliance, and elevated filling pressures. After this initial asymptomatic phase, cardiac remodeling occurs, leading to hypertrophy of the left ventricle (**LV**) and a reduction of diastolic filling, advancing into diastolic dysfunction. At this stage, the left ventricular ejection fraction remains preserved (*i.e.*, heart failure with preserved ejection fraction (**HFpEF**)), and patients display the so-called restrictive phenotype of DCM (2,8,12). Notably, diastolic dysfunction is detected in up to 75% of T2DM patients with normotension and without CAD (2,13). Subsequently, the disease can further develop into systolic dysfunction, characterized by cardiomyocyte apoptosis, enlarged LV chambers, and symptomatic heart failure with a reduced ejection fraction (**HFrEF)** (2,8,12). In clinical terms, these patients present the dilated phenotype of DCM.

2. Advanced Glycation End-Products

It is described that systemic changes typical for diabetes, like insulin resistance, hyperglycemia, and hyperlipidemia, play crucial roles in the pathogenesis of DCM (8,9,11,14). They cause lipotoxicity, activation of the renin-angiotensin-aldosterone system, and inflammation. This further exacerbates oxidative stress, calcium (Ca^{2+}) homeostasis impairment, mitochondrial dysfunction, and endoplasmic reticulum stress (8,9,11,14). Despite the progress in understanding the DCM etiology over the past decades, the molecular mechanisms involved in this condition are yet incompletely understood. Currently, it is speculated that advanced glycation end-products (**AGEs**) play a critical role in this process (8,9,11).

2.1 Endogenous and exogenous formation

AGEs are a complex group of compounds formed by the irreversible glycation or oxidation of proteins and lipids after persistent contact with reducing sugars or short-chain aldehydes (15,16).

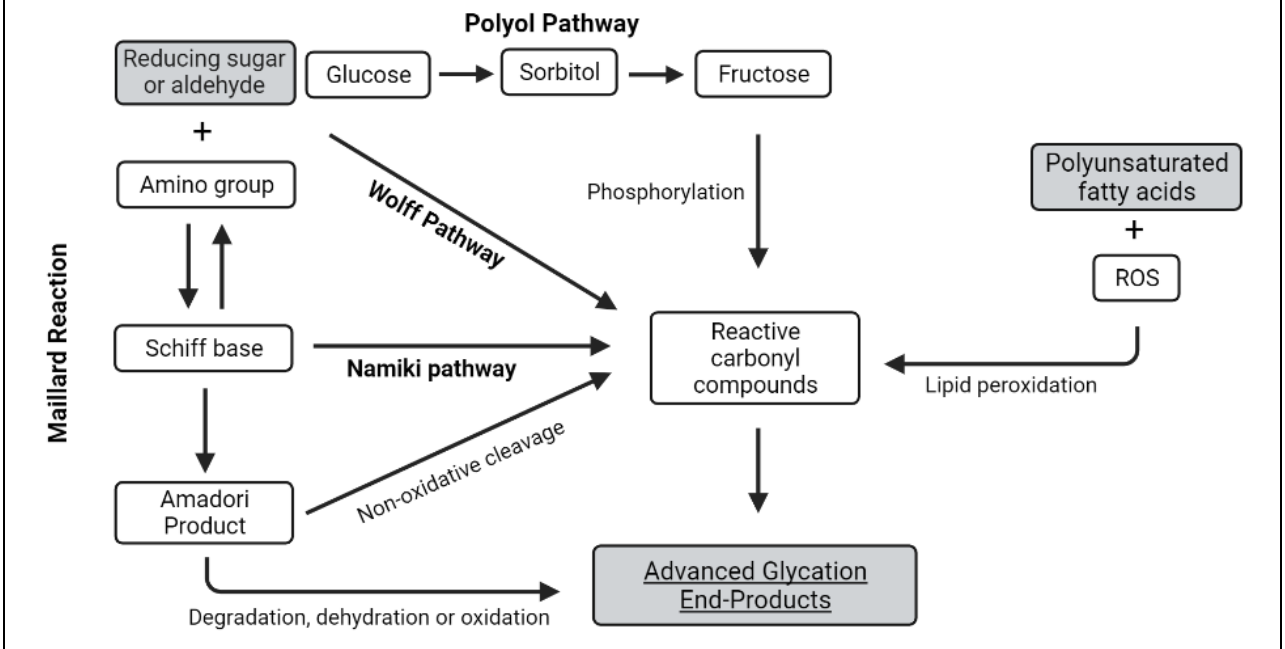

Fig. 1 – Endogenous AGEs formation. Pathways for endogenous formation of AGEs from reducing sugars, aldehydes, or polyunsaturated fatty acids.
ROS: reactive oxygen species

AGEs accumulation occurs naturally during aging but increases in a hyperglycemic environment, as present in diabetes patients (17). Multiple pathways independently form AGEs *in vivo*, including the non-enzymatic Maillard reaction, the polyol pathway, and the lipid peroxidation pathway (fig. 1). First, during the Maillard reaction, a reversible Schiff base is formed through condensation of a reducing sugar or aldehyde with a protein amino group, nucleic acid, or lipid. Subsequently, the Schiff base is converted into a covalently-bound Amadori product (17,18). From the Amadori products, AGEs can either be directly formed by degradation, dehydration, or oxidation. Otherwise, the Amadori products can be provisionally transformed into intermediate reactive carbonyl compounds (**RCC**) through non-oxidative cleavage, which are later transformed into irreversible AGEs (18-20). Moreover, the Schiff base can also be directly degraded to RCC by the Namiki pathway instead of being converted into Amadori products (18). Furthermore, RCC can be formed through the Wolff pathway by autoxidation of monosaccharides (*e.g.*, glucose, fructose, etc.) (18). In the polyol pathway, glucose is reduced to sorbitol, which undergoes oxidation to fructose. Subsequent phosphorylation of fructose generates RCC, resulting in AGEs formation. The enzyme converting glucose to sorbitol has a low affinity for glucose, meaning the conversion usually is infrequent but increased in T2DM patients due to the hyperglycemic environment (18,20). The last pathway for AGEs generation is lipid peroxidation. Polyunsaturated fatty acids (*e.g.*, membrane lipids) react with reactive oxygen species (**ROS**), resulting in highly reactive compounds which will undergo further oxidation. Eventually, this pathway leads to the formation of RCC and α-oxoaldehydes. Both types of compounds can then give rise to different AGEs molecules.

Besides these endogenous origins, AGEs can also have exogenous sources. Indeed, reactive glycation products, which can react with proteins to form AGEs, appear in the aqueous extract of tobacco and tobacco smoke (21). Interestingly, AGEs are also present in food products that belong to the Western diet. The formation of food-derived AGEs depends on the nutrient composition (*i.e.*, protein- and fat-rich foods) and the applied cooking method (*i.e.*, broiled, fried, or roasted) (22).

2.2 Classification

AGEs can be divided into multiple classes based on fluorescence and cross-linking properties or molecular weight (16). Fluorescent cross-linking AGEs (*e.g.*, pentosidine) contain an aromatic ring in their molecular structure, which causes autofluorescence. According to this classification, AGEs can also belong to the class of non-fluorescent cross-linking AGEs (*e.g.*, glucosepane) or non-cross-linking AGEs (*e.g.*, carboxymethyllysine) (16,23). Based on their molecular weight, AGEs can be divided into low-molecular-weight AGEs or high-molecular-weight AGEs (16).

2.3. Mechanisms of action (in the heart)

AGEs exert their pathological effects through two main mechanisms: by cross-linking proteins or by binding to the receptor for advanced glycation end products (**RAGE**) (fig. 2). The cross-links arise when AGEs form covalent bonds with other AGEs. Cross-links can form between AGEs on the extracellular matrix (**ECM**) components, like collagen, which causes the ECM to become less flexible (15,17,24). This pathological state contributes to increased myocardial stiffness, as is the case in aging and diabetes (8). Candido *et al.* provided evidence for the crucial role of crosslink-AGEs in the cardiac alterations in diabetes by demonstrating that the cross-link breaker ALT-711 could partially prevent and attenuate myocardial stiffening in a rat model for diabetes (25). Besides, AGEs can also cross-link intracellular proteins, including domains of the ryanodine receptor (**RyR**) and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (**SERCA**) pump (26,27). As both proteins are involved with Ca^{2+} transport in the cardiomyocytes, this will lead to alterations in Ca^{2+} homeostasis and thus alter excitation-contraction coupling (28). RyR is crucial for Ca^{2+} release from the sarcoplasmic reticulum during contraction. The SERCA pump transports Ca^{2+} into the sarcoplasmic reticulum to terminate the contraction. Cross-linking of these proteins by AGEs can thus impair both contraction and relaxation of the myocardium (26-28).

RAGE is a pattern recognition receptor from the immunoglobulin family and recognizes various ligands, including AGEs (18,29). It is expressed on a wide variety of cell types, including endothelial cells, smooth muscle cells,

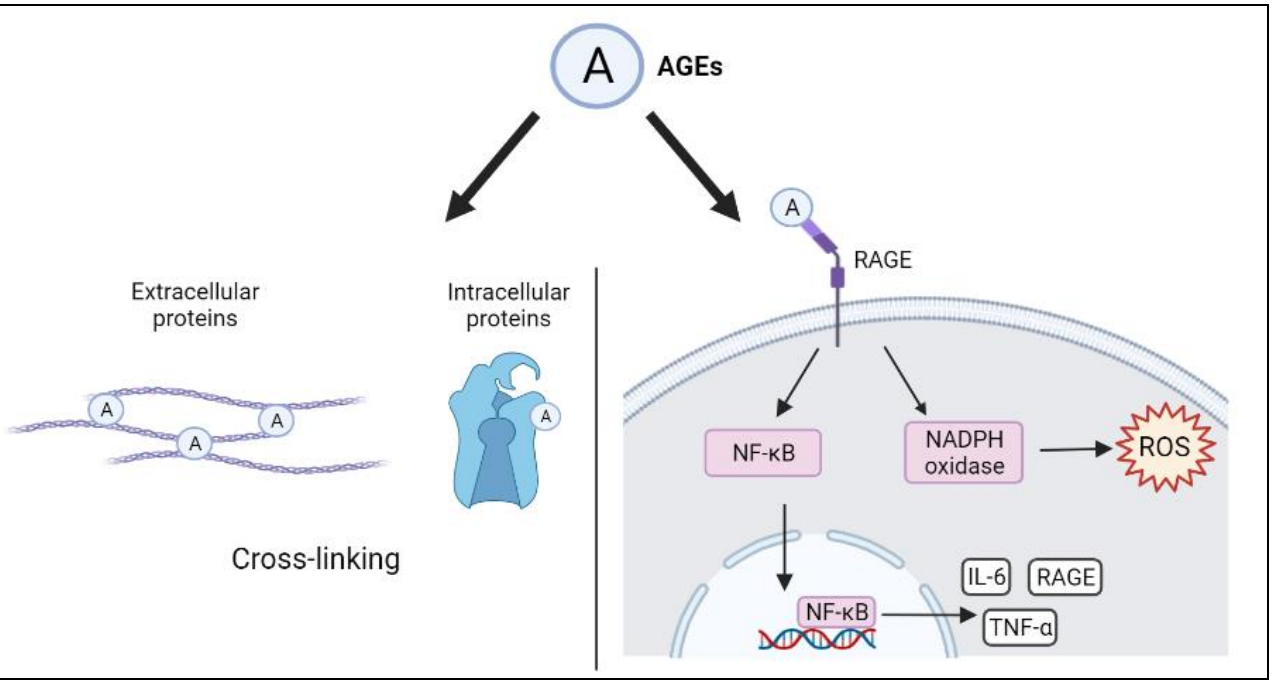

Fig. 2 – **AGEs exert their effects by cross-linking proteins or binding to RAGE.** AGEs can cross-link extra- and intracellular proteins to disturb their function. Binding to RAGE activates NF-κB and NADPH oxidase intracellularly, increasing transcription of RAGE, IL-6, and TNF-α and increasing ROS.

AGE: advanced glycation end products; RAGE: receptor for AGEs; NF-κB: nuclear factor kappa-light-chain enhancer of activated B cells; NADPH: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; IL-6: interleukin 6; TNF-α: tumor necrosis factor α

monocytes/macrophages, and cardiomyocytes (18,29). The expression of RAGE is low under physiological conditions. However, RAGE expression is strongly increased in pathophysiological conditions like diabetes (29,30). The receptor consists of three components, namely, an extracellular domain, which will bind the ligands, a single transmembrane helix, and a cytosolic tail, which is crucial in activating the downstream signaling cascades (29,30).

In the heart, RAGE activation by AGEs can activate several different pathways. Firstly, AGE-RAGE interaction can activate nicotinamide adenine dinucleotide phosphate (**NADPH**) oxidase, which converts oxygen into superoxide. Subsequently, superoxide can react with nitric oxide (**NO**) to form peroxynitrite, a powerful nitrating and oxidizing agent that can affect the structure and function of proteins. Furthermore, this process reinforces itself as ROS production leads to the formation of new AGEs and the activation of nuclear factor kappa-light-chain enhancer of activated B cells (**NF-κB**) (18,31).

The activation of NF-κB increases the transcription of pro-inflammatory molecules like interleukin-6 (**IL-6**), tumor necrosis factor-α (**TNF-α**), and cyclooxygenase-2 (18,31). Furthermore, NF-κB stimulates RAGE expression by binding to the RAGE promotor region, creating a positive feedback loop. Besides inflammation, AGE-RAGE interaction can also influence processes like apoptosis, cell migration, and proliferation (18,31). Interestingly, Ma *et al*. and Nielsen *et al.* both provided evidence indicating that RAGE plays a crucial role in the development of DCM by respectively a knockdown of RAGE with siRNA and physically blocking the receptor with an anti-RAGE antibody (32,33). Both studies saw a reduction in DCM characteristics after blocking RAGE, such as preventing the decrease of LV contractility. The results of these studies indicate that blocking RAGE at diabetes onset could prevent the development of DCM (32,33).

3. Investigating the role of AGEs in diabetic cardiomyopathy

3.1 AGEs as key players in diabetic cardiomyopathy?

Growing evidence suggests that AGEs are essential to the development of DCM. Indeed, circulating AGEs are increased in T2DM patients and heart failure patients without diabetes (20,34). In addition, Willemsen *et al.* showed a correlation between tissue AGEs levels and diastolic dysfunction in diabetic patients (35). However, the exact involvement of AGEs in the pathophysiology of DCM remains unknown, and more research is required. Additionally, there is a tremendous need for effective approaches to prevent disease development.

3.2 Using a highly relevant animal model

A wide array of animal models exist to study the underlying mechanisms of T2DM and its associated disorders. Often, chemically-induced or genetic rodent models are used (36,37). Chemically-induced models include low doses of streptozotocin (**STZ**), which causes partial β-cell loss in the pancreas. Examples of genetic models are the db/db mice, which have a resistance to leptin, and the Zucker fatty rats, which have a defective leptin receptor (36,37). However, these chemical and genetic models do not represent the human phenotype sufficiently. Consequently, diet-induced T2DM models are used to mimic the pathogenesis more closely. These models often use high-fat diets (*i.e.*, up to 60% fat content), which are available with free access. Nevertheless, it has been shown that an increased sugar intake rather than an increased fat intake is associated with the current obesity pandemic in Western countries (36-38). Because of this, our group has developed a diet-induced rat model with high-sugar and high-fat content (39). Rats receiving this diet developed T2DM, evidenced by increased glucose levels and fasting insulin after 18 weeks. Furthermore, the rats showed early characteristics of DCM development as the anterior wall thickness (**AWT**), end-diastolic pressure (**EDP**), and interstitial collagen depositions were increased. Interestingly, the Western diet rats also had a four times increase in circulating plasma AGEs levels compared to control rats after 18 weeks (39).

3.3 Inhibiting AGEs with pyridoxamine

It has previously been proven that several therapeutic strategies can limit the formation of AGEs or can decrease their downstream effects, eventually leading to improved disease outcomes (40). The vitamin B6 compound pyridoxamine (**PM**) is an example of an AGEs-lowering therapy that prevents the formation of AGEs. In particular, PM lowers AGEs by blocking the formation of AGEs from Amadori products in the Maillard reaction and scavenging toxic carbonyl intermediates (23,40). Furthermore, it is a metal chelator, meaning it can form stable complexes with metals, thereby inhibiting metal-catalyzed oxidation of proteins. In addition, PM acts like an anti-oxidative agent (23,40,41). Our group has used PM previously in a myocardial infarction (MI) study. In this study, PM lowered circulating AGEs levels, improved diastolic dysfunction, reduced collagen cross-linking, and limited cardiac damage overall. Whether PM can prevent DCM remains unknown.

3.4 Remaining questions

Currently, an unhealthy lifestyle associated with a Western diet contributes to the increase in the incidence of T2DM and the development of DCM. Despite progress in understanding DCM, the molecular mechanisms involved are not fully identified. Additionally, there is a tremendous need for effective approaches to prevent disease development. In this research, we aim to investigate whether inhibiting AGEs formation with PM *in vivo* prevents the development of DCM. We hypothesize that PM provides cardio-glycemic protection by improving glycemic control and limiting cardiac dysfunction in rats with DCM.

EXPERIMENTAL PROCEDURES

Animals – The animal study was performed in accordance with the EU directive 2010/63/EU on the protection of animals used for scientific purposes and was approved by the Local Ethical Committee of Hasselt University (UHasselt, Diepenbeek, Belgium). 19 male Sprague-Dawley rats (Janvier Labs, Le Genest-Saint-Isle, France), six weeks old at arrival, were randomly divided into three experimental groups. The first group (n=7, control group) received a standard chow pellet diet. The second group (n=4, Western diet group) was given a high-sugar and high-fat diet containing 20%

total fat, 61% sugars, and 19% proteins. The third group (n=8, Western diet + PM group) was fed the high-sugar, high-fat diet and drinking water both supplemented with PM (1g/L). Water and food were available *ad libitum*, and the rats were housed in temperature and humidity-controlled conditions. Body weight and 24h food intake, calculated by weighing the residual food the next day, were monitored weekly. Prior to the sacrifice of the rats, 18 weeks after the start of the diet, an oral glucose tolerance test (**OGTT**), blood sampling, and echocardiographic and hemodynamic measurements were conducted. The rats were euthanized with an overdose of sodium pentobarbital (Dolethal, Vetoquinol, Aartselaar, Belgium, 200 mg/kg, i.p.). Heparin (1000u/kg, i.p.) was injected to avoid blood clotting. After the hearts were harvested and perfused with a Tyrode solution (137 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2, 11.8 mM Na-HEPES, 10 mM glucose, 20 mM taurine, pH 7.4) transversal sections of the LV were fixed in 4% PFA for further staining. The remaining LV tissue was crushed and snap-frozen in liquid nitrogen and stored at -80°C to be used for real-time quantitative Polymerase Chain Reaction (**qPCR**). The liver, lungs, and kidneys were isolated and weighed. The tibia was collected, and the length was measured.

OGTT and determination of serum insulin concentrations – An OGTT was performed at baseline and after 6, 12, and 18 weeks to assess glucose tolerance. After overnight fasting, an 80% glucose solution (2g/kg) was administered through gastric gavage. Glucose concentration was measured from capillary tail blood collection before glucose administration and after 15, 30, and 60 minutes using the Analox GM7 Glucose Analyzer (Analis SA, Namur, Belgium). Glucose response was expressed as the total area under the curve (**AUC**). Insulin levels in serum collected from the rat's tail vein were determined at baseline and 60 minutes after glucose administration using electrochemiluminescence (Meso Scale, Gaithersburg, MD, USA).

Echocardiographic measurements – Transthoracic echocardiographic images were made of all rats using the Vevo3100 system and the 21MHz linear probe MX250 (FUJIFILM VisualSonics Inc., Amsterdam, The Netherlands) at 18 weeks. The rats were anesthetized with 2% isoflurane supplemented with oxygen. Parasternal long-axis and short-axis images were obtained in B- and M-mode. The obtained images were analyzed with the Vevo Lab 3.2.6 software (FUJIFILM VisualSonics Inc.). Standard measurements of systolic function, diastolic function, and LV structure were determined blinded.

Hemodynamic measurements – Hemodynamic measurements were performed at sacrifice in all animals, anesthetized with 2% isoflurane supplemented with oxygen. The SPR-320 Mikro-Tip pressure catheter (Millar Inc, The Hague, The Netherlands) was inserted via the right carotid artery into the LV. The pressure catheter was connected to a quad-bridge amplifier and PowerLab26T module (AD Instruments, Oxford, UK) to transfer the pressure data to LabChart v7.3.7 software (AD Instruments). The obtained hemodynamic parameters from this software were LV EDP and maximal pressure.

Sirius Red/Fast Green Staining – Transverse sections of 8µm thick were obtained from the midventricular level of the heart and stained with the Sirius Red/Fast Green staining kit (Chondrex, Inc., Redmond, WA, USA), according to the manufacturer's instructions, to evaluate myocardial interstitial fibrosis. Subsequently, the sections were mounted with DPX mounting medium. Images were acquired with the DM2000 LED microscope (Leica, Diegem, Belgium) in four randomly-chosen fields per animal. The percentage of collagen deposition, calculated by normalizing the interstitial collagen deposition area to total surface area, was quantified using FIJI/ImageJ software (42).

Real-Time qPCR – Total RNA was extracted from 20-30mg snap-frozen LV tissue using the RNeasy Fibrous Tissue kit (Qiagen, Antwerpen, Belgium), following the manufacturer's instructions. The concentration and purity of the isolated RNA were determined using the Nanodrop 2000 spectrophotometer (Isogen Life Science, Utrecht, The Netherlands). 1000ng RNA was converted into cDNA using the qScript cDNA SuperMix (Quantabio, VWR, Leuven, Belgium) and the T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). Real-time

qPCR was carried out in a MicroAmp™ Optical 96-well plate (Thermo Fisher Scientific, Merelbeke, Belgium) and performed using the Quantstudio 3 Real-Time PCR system (Thermo Fisher Scientific) with the SYBR Green PCR Master Mix (Thermo Fisher Scientific). Primers were designed in the coding sequence of the mRNA. The target genes and their primers are listed in table 1. Gene expression data were analyzed with the ΔΔCT method taking into account the MIQE guidelines (43). The expression of target genes was normalized to phosphoglycerate kinase 1 (**PGK1**) and hypoxanthine-guanine phosphoribosyl transferase (**HPRT**) (table 1), which were the most stable reference genes for this experiment as determined with GeNorm.

AGEs Enzyme-Linked Immunosorbent Assay (ELISA) – Plasma total AGEs levels were measured with the OxiSelect™ AGE competitive ELISA kit (Cell Biolabs, San Diego, CA, USA), according to the manufacturer's instructions. In brief, the ELISA plate was coated with AGEs conjugate overnight. Afterwards, the samples were added, followed by incubation with the primary anti-AGEs polyclonal antibody. A horseradish peroxidase (**HRP**) conjugated secondary antibody was then added, and the absorbance at 450nm was measured with the CLARIOstar plus plate reader (BMG Labtech, Ortenberg, Germany).

AGEs staining – Antigen retrieval was performed on deparaffinized transversal cardiac sections of 8µm thick with citrate buffer (pH = 6). 30% hydrogen peroxide (diluted 1:100 in phosphate-buffered saline (**PBS**)) was used to block endogenous peroxidase, followed by washing with 1x PBS and permeabilization with 0.05% Triton X-100 (Sigma-Aldrich, Diegem, Belgium). The sections were rewashed with 1x PBS and then incubated with serum-free protein block (X0909, Dako Agilent, Diegem, Belgium) to limit background staining. Afterward, sections were once again washed with 1x PBS. Next, the sections were incubated with a primary antibody against AGEs (1/250, rabbit anti-rat polyclonal, ab23722, Abcam) for 1h at room temperature, followed by 1x PBS wash. Afterward, the EnVision™ + Dual Link System-HRP (anti-rabbit/anti-mouse, K4061, Dako Agilent) was applied to the sections for 30 minutes at room temperature. 3,3'-diaminobenzidine (**DAB)** solution (Dako Agilent) was applied for seven and a half minutes at room temperature to visualize the presence of AGEs. The nuclei in the sections were counterstained with hematoxylin and mounted with DPX mounting medium. A negative control was obtained by performing the immunostaining protocol without the incubation of primary antibodies. Images were acquired with a MC170 camera connected to a DM2000 LED microscope (Leica) in four randomly-chosen fields per section. The AGEs deposition in the myocardium was quantified using FIJI/ImageJ software and was expressed as the percentage of AGEs-positive area normalized to total surface area (42).

Statistics – Statistical Analysis was performed using Graphpad Prism 9.3.0 (Graphpad Software, San Diego, CA, USA). Results were first tested for

Table 1 – Sequences of forward and reverse primers for Real-Time qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
PGK1	ATG-CAA-AGA-CTG-GCC-AAG-CTA-C	AGC-CAC-AGC-CTC-AGC-ATA-TTC
HPRT	TCC-CAG-CGT-CGT-GAT-TAG-TG	GCA-AGT-CTT-TCA-GTC-CTG-TCC
RAGE	TGG-AAA-CTG-AAC-ACA-GGA-AGG-A	GGA-GTG-AAC-CAT-TGG-GGA-GG
COL3A1	AAC-TGG-AGC-ACG-AGG-TCT-TG	CGT-TCC-CCA-TTA-TGG-CCA-CT
COL1A2	GCC-AAG-AAT-GCA-TAC-AGC-CG	GAC-ACC-CCT-TCT-GCG-TTG-TA
BCL-2	CTT-CTC-TCG-TCG-CTA-CCG-TC	GAA-GAG-TTC-CTC-CAC-CAC-CG
SOD2	AGC-TGC-ACC-ACA-GCA-AGC-AC	TCC-ACC-ACC-CTT-AGG-GCT-CA
NOX4	TCA-TGG-ATC-TTT-GCC-TGG-AGG-GTT	AGG-TCT-GTG-GGA-AAT-GAG-CTT-GGA
TGF-β	ACC-GCA-ACA-ACG-CAA-TCT-ATG	GCA-CTG-CTT-CCC-GAA-TGT-CT

PGK1: phosphoglycerate kinase 1; HPRT: hypoxanthine-guanine phosphoribosyl transferase; RAGE: receptor for advanced glycation end-products; COL3A1: collagen type III alpha-1 chain; COL1A2: collagen type I alpha-2 chain; Bcl-2: B-cell lymphoma 2; SOD2: superoxide dismutase 2; NOX4: NADPH oxidase 4; TGF-β: transforming growth factor β

normality using a Shapiro-Wilk test. If the data had a normal distribution, it was analyzed with a one-way ANOVA followed by Tukey's multiple comparisons test. Non-normal distributed data were subjected to the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. Time-concentration curves of OGTT data were compared using a two-way ANOVA, followed by a Bonferroni correction. The results were considered statistically significant at $p<0.05$. The data are expressed as mean ± standard error of the mean (**SEM**).

RESULTS

Pyridoxamine does not affect global characteristics of DCM rats – At baseline, there were no differences in general characteristics between the three groups (data not shown). After 18 weeks, the Western diet + PM group had a significant increase in body weight compared to the control group (table 2). The Western diet group also showed a trend toward increased body weight (*p=0.08*) compared to the control group. Furthermore, food intake was significantly increased in both the Western diet and the Western diet + PM groups. Heart and liver weight,

Table 2 – Animal characteristics after 18 weeks control diet, Western diet, or Western diet + PM

	Control diet	Western diet	Western diet + PM
BW increase (%)	251±6	287±11	286±7*
Food intake (g/day)	31±0.1	71±2**	69±0.8**
Heart weight/tibia length (g/cm)	0.41±0.01	0.48±0.02**	0,47±0.01**
Liver weight/tibia length (g/cm)	3.8±0.1	4.8±0.3**	4.6±0.2*
Lung weight/tibia length (g/cm)	0.42±0.02	0.47±0.02	0.45±0.01
Kidney weight/tibia length (g/cm)	0.85±0.02	0.87±0.05	0.93±0.04

BW: body weight. Data are presented as mean ± SEM. *p<0.05 **p<0.01

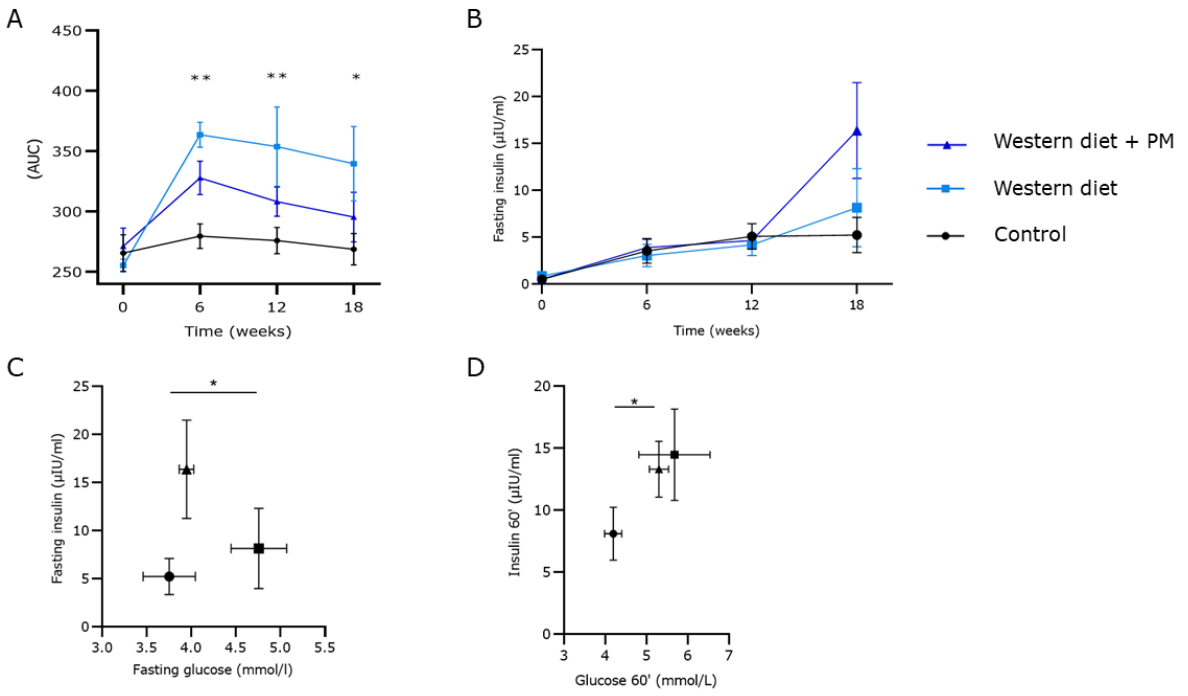

Fig. 3 – **Pyridoxamine tends to lower blood glucose levels in a rat model of DCM.** (A) Total blood glucose levels, expressed as area under the curve (AUC), as measured in the OGTT over 18 weeks. (B) Fasting insulin levels over 18 weeks. (C) Fasting glucose and insulin levels at 18 weeks. (D) Glucose and insulin levels 60' after glucose administration at 18 weeks. Data are presented as mean ± SEM. *p<0.05 (vs. control group)

Table 3 - Effect of PM on echocardiographic and hemodynamic parameters in DCM rats

	Control diet	Western diet	Western diet + PM
HR (bpm)	326±11	340±10	342±10
EF (%)	78±2	68±3	73±3
AWT in systole (mm)	3.45±0.2	3.47±0.2	3.91±0.2
AWT in diastole (mm)	2.06±0.1	1.99±0.1	2.22±0.1
LVESV (µL)	144±19	271±31**	187±21
LVEDV (µL)	636±31	858±106*	703±40
LVESA (mm^2)	45±4	67±4**	51±4x
LVEDA (mm^2)	110±5	130±10	117±5
EDP (mmHg)	7.1±1.6	8.8±1.0	9.5±1.9
Maximum pressure (mmHg)	93±4	98±4	99±2

HR: heart rate; EF: ejection fraction; AWT: anterior wall thickness; LVESV: left ventricle end-systolic volume; LVEDV: left ventricle end-diastolic volume; LVESA: left ventricular end-systolic area; LVEDA: left ventricular end-diastolic area; EDP: end-diastolic pressure. Data are presented as mean ± SEM *p<0.05 (vs. control group) **p<0.01 (vs. control group) xp<0.05 (vs. Western diet group)

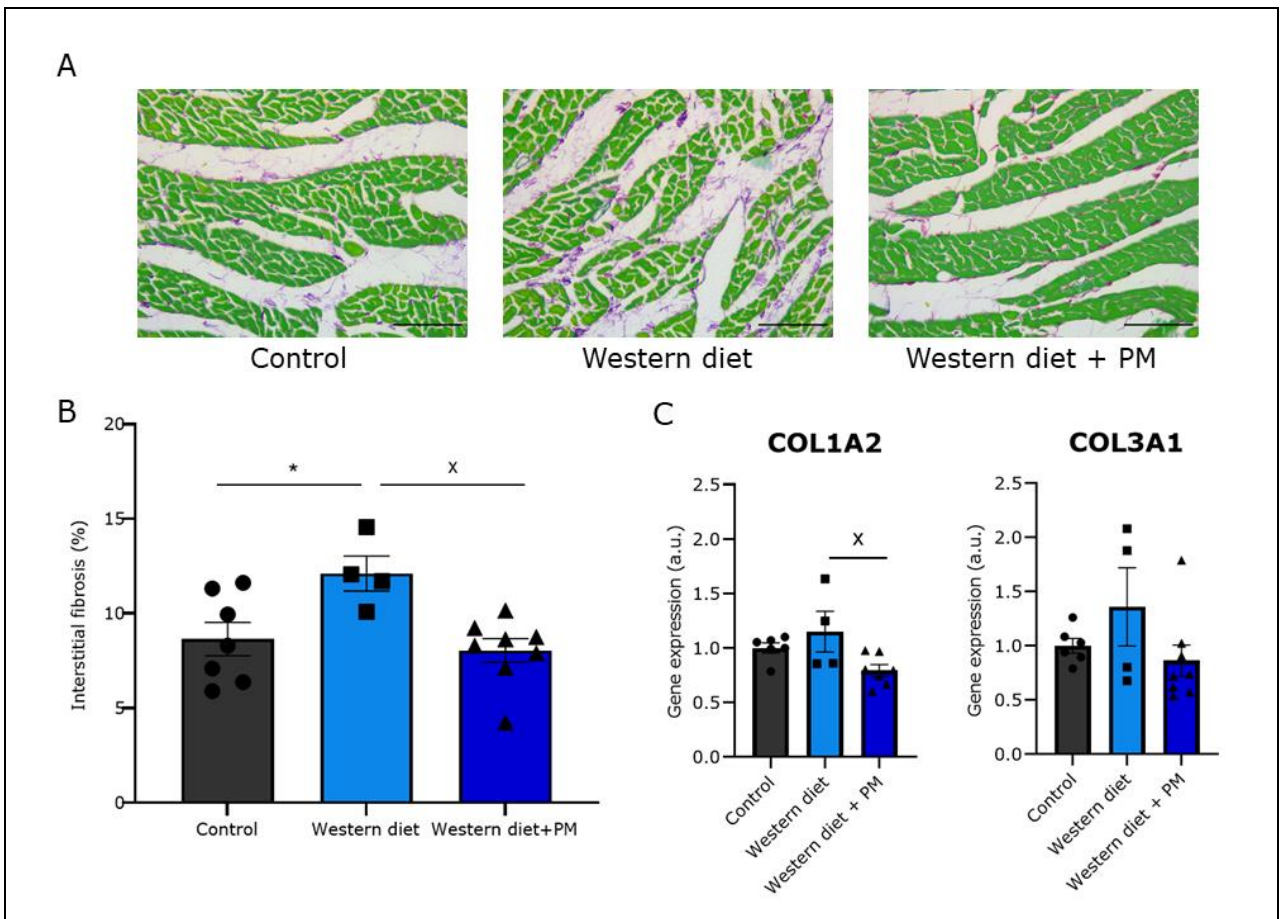

Fig. 4 – PM prevents collagen deposition in the myocardium. (A) Representative images of interstitial collagen deposition (red) in the myocardium, stained with a Sirius Red/Fast Green staining. Scale bars represent 100µm. (B) Quantification of total interstitial collagen in the myocardium. (C) Gene expression of genes encoding collagen type I and type III. Gene expression is shown relative to the control group. Data are presented as mean ± SEM. *p<0.05 (vs. control) xp<0.05 (vs. Western diet).

normalized to tibia length, were significantly increased in the Western diet group and Western diet + PM groups compared to the control group. The Western diet group also tended to have an increased lung weight (p=0.052). However, there were no differences in kidney weight between the groups.

Pyridoxamine tends to improve glucose tolerance in a DCM rat model – To assess the glycemic control of the animals over time, OGTTs were performed at baseline, 6, 12, and 18 weeks. Total blood glucose levels, expressed as AUC, of the Western diet group were already significantly increased after 6 weeks compared to the control group (Fig. 3A). At 6 weeks, the PM group also had a trend toward increased fasting blood glucose compared to the control group (*p=0.07*). Interestingly, total glucose levels slightly tended to be reduced in the Western diet + PM group compared to the Western diet group after 12 weeks (*p=0.19*) and 18 weeks (*p=0.19*) (Fig. 3A). Fasting insulin did not increase during the study duration in the group receiving the Western diet (Fig. 3B).

At 18 weeks, fasting glucose levels were significantly increased in the Western diet group compared to the control group (Fig. 3C). Interestingly, the fasting glucose levels of the Western diet + PM group tended to be lower compared to those of the Western diet group at 18 weeks (*p=0.10*) (Fig. 3C). Accordingly, the Western diet + PM animals display a slightly increased trend in insulin levels compared to control animals (*p=0.30*) to maintain low fasting glucose levels (Fig. 3B-C). 60 minutes after glucose administration, the glucose levels were significantly increased in the Western diet + PM group and tended to be increased in the Western diet group (*p=0.14*) compared to control (Fig. 3D). Indeed, the slightly increased insulin levels in the Western diet group (*p=0.27*) and Western diet + PM group (*p=0.27*), compared to the control group, were inadequate to maintain comparable glucose levels.

Pyridoxamine limits cardiac dysfunction in diabetic rats – In vivo cardiac function of the rats was monitored by echocardiographic and hemodynamic measurements. After 18 weeks, rats receiving a Western diet tended to have a reduced ejection fraction (**EF**) compared to the control group (*p=0.09*) (table 3). AWT did not differ between the three groups. Besides, Western diet rats also had a significant increase in the left ventricular end-systolic volume (**LVESV**) and end-diastolic volume (**LVEDV**) compared to the control group. Interestingly, PM tended to prevent the development of these increased volumes observed in the Western diet group (LVESV; *p=0.07*, LVEDV; *p=0.15*). Furthermore, the left ventricular end-systolic area (**LVESA**) was significantly increased in the Western diet group compared to the control group. Compared to the control rats, the Western diet group also tended to have an increased left ventricular end-diastolic area (**LVEDA**) (*p=0.10*). Notably, the increased LVESA observed in the Western diet group was limited by the administration of PM. The EDP and maximum pressure remained comparable in all groups after 18 weeks of diet.

Pyridoxamine prevents the development of fibrosis in the myocardium seen in DCM – Using a Sirius Red/Fast Green staining, myocardial interstitial collagen depositions were stained and quantified. LV interstitial fibrosis was significantly increased in the Western diet group compared to the control group (Fig. 4A-B). Notably, PM significantly lowered the percentage of interstitial fibrosis, when compared to the Western diet group (Fig. 4A-B). Furthermore, gene expression of the genes COL1A2 and COL3A1, respectively encoding collagen type I and type III, was assessed with qPCR. PM significantly reduced the gene expression of type I collagen compared to the Western diet group.

Pyridoxamine does not affect the presence of AGEs in the myocardium and the plasma in DCM – To identify whether PM affects AGEs levels in the myocardium and plasma, AGEs content was quantified from stained LV sections and measured in plasma samples by ELISA, respectively. In both the myocardium and the plasma, the AGEs content remained comparable between the Western diet group and the control group after receiving the diet for 18 weeks (Fig. 5A-C). Furthermore, the myocardial and plasma AGEs content was not altered after receiving PM for 18 weeks (Fig. 5A-C).

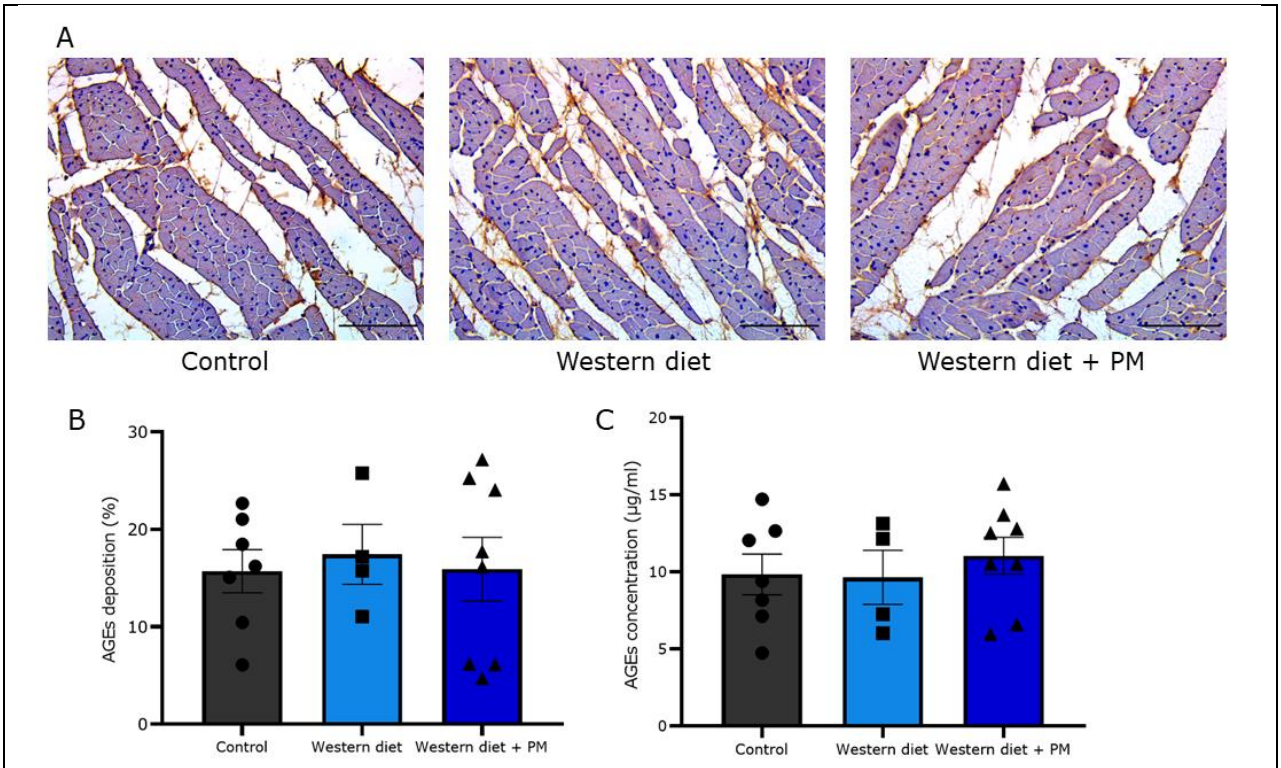

Fig. 5 – PM does not lower AGEs content in myocardium or plasma (A) Representative images of AGEs deposition in the myocardium were obtained with DAB staining. Scale bars represent 100µm. (B) Quantification of myocardial AGEs deposition. (C) Plasma AGEs concentration after 18 weeks of diet. Data are presented as mean ± SEM.

Effect of PM on gene expression of the AGEs receptor and genes of apoptosis and oxidative stress in DCM – Expression of genes relevant to the development of DCM and working mechanisms of AGEs were evaluated with Real Time-qPCR. There was a very slight trend towards increased expression of RAGE in the Western diet group compared to both the control (*p=0.37*) and the Western diet + PM group (*p=0.31*) (Fig.6A). The anti-apoptotic gene Bcl-2 tended to be slightly decreased in the Western diet group (*p=0.38*) (Fig.6B). However, there was a strong trend towards decreased Bcl-2 expression in the group receiving PM compared to the control group (*p=0.06*). TGF-β, implicated in a variety of processes like fibrosis, apoptosis, and proliferation, had a similar gene expression in the control and Western diet group, while the PM group had a slight trend toward reduced expression compared to both the control (*p=0.17*) and the Western diet group (*p=0.23*) (Fig.6C). The gene expression of the anti-oxidative enzyme SOD2 slightly tended to be decreased in the Western diet group (*p=0.33*) compared to control. The decreased expression of SOD2 in the Western diet group was prevented by PM. The gene expression of the pro-oxidative enzyme NOX4 was significantly reduced in the Western diet + PM group compared to the Western diet group (Fig.6E).

DISCUSSION

In this study, we demonstrate that PM prevents DCM development in rats by improving glucose tolerance and limiting the development of cardiac dysfunction, LV interstitial fibrosis, and LV oxidative stress.

Effect of pyridoxamine on the development of diabetes – After receiving the Western diet for 18 weeks, the rats of both the Western diet group and the Western diet + PM group had a substantial increase in body weight, heart weight, and liver weight. An explanation for this increase in heart and liver weight could be increased fibrosis and steatosis in both organs, which occurs regularly in T2DM patients (44,45). In addition, here we demonstrated that the Western diet induced a significant increase in total blood glucose and

fasting glucose levels, whereas these parameters remained comparable to control level in Western diet + PM administered animals. This indicates that the Western diet rats had a decreased glucose tolerance, a hallmark in the development of T2DM, while PM improved glycemic control.

These results are consistent with other studies where vitamin B6-derivatives, including PM, have been proven to lower hyperglycemia. Pereira *et al.* showed that PM lowers fasting glucose in Goto–Kakizaki rats, a distinct rat model for T2DM (46). Furthermore, Kim *et al.* demonstrated that PM and other vitamin B6 derivatives, including pyridoxine, decreased the activity of digestive enzymes involved in disaccharide absorption and decreased postprandial hyperglycemia in healthy Sprague-Dawley rats (47). The decrease in disaccharide absorption can explain why the Western diet + PM rats have better glycemic control as the Western diet contains high amounts of saccharose. When there is less saccharose absorption, hyperglycemia will be limited, and eventually, the development of T2DM will be impeded.

In our study, no differences in insulin levels were observed after fasting or after 60 minutes, and the within-group variations were large. Based on a previous study by Verboven *et al.*, we expected that the fasting insulin levels would be significantly increased in the Western diet group at 12 and 18 weeks and that the insulin levels 60 minutes after glucose administration would also be increased at 18 weeks (39). Furthermore, we expected that PM administration would prevent hyperinsulinemia, as Liu *et al.* showed in a mice model receiving a high-

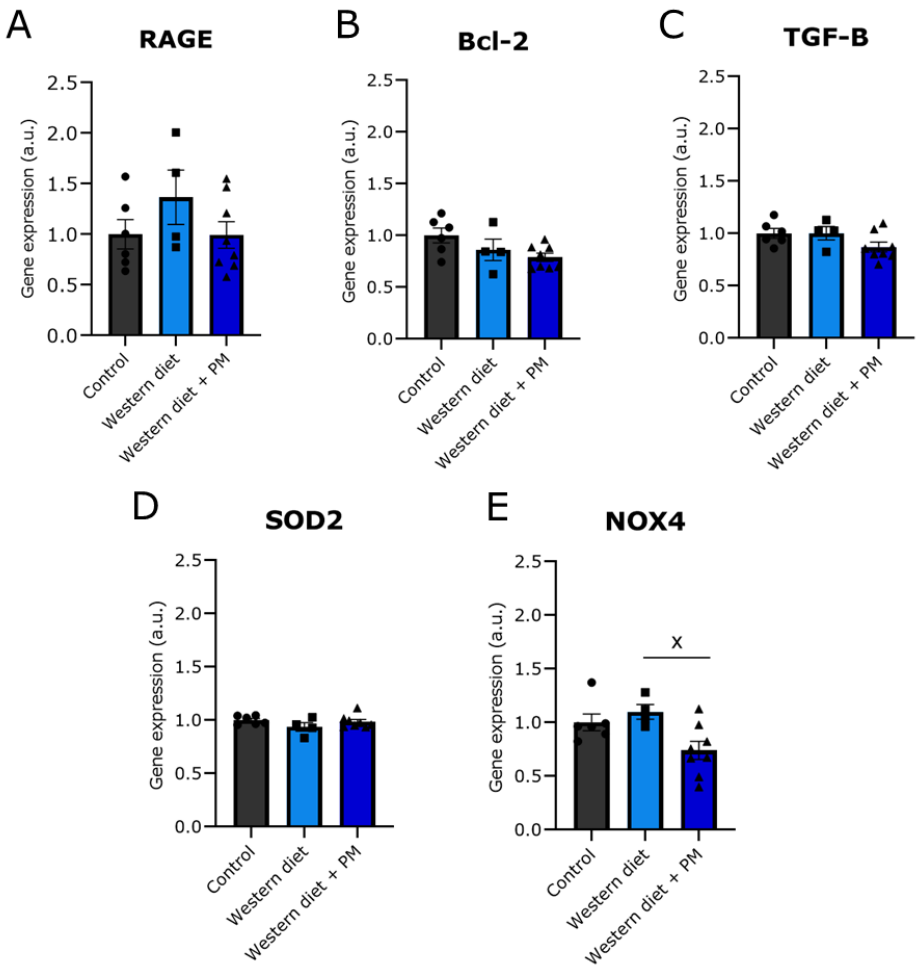

Fig. 6 – Effect of PM on gene expression of the AGEs receptor and genes of apoptosis and oxidative stress in DCM. Gene expression of (A) RAGE, (B) Bcl2, (C) TGF-β, (D) SOD2, and (E) NOX4 in left ventricular tissue was determined using Real Time-qPCR. Gene expression is shown relative to the control group. Data are presented as mean ± SEM. $^{x}p<0.05$ (vs. Western diet)

fat diet (48). Additionally, vitamin B6 deficiency can induce insulin resistance, indicating that vitamin B6 is essential for appropriate insulin activity (49). Nevertheless, it should be noted that the sample size of the Western diet group (n=4) was small in the current study. In the future, these measurements should be replicated to make conclusions concerning the effect of PM on hyperinsulinemia in high-sugar diet-induced DCM rats.

The DCM phenotype after 18 weeks of Western diet – In this study, we showed that the rats receiving the Western diet for 18 weeks had a significant increase in LV end-systolic and end-diastolic volumes combined with an increase in LV systolic area and a trend towards increased diastolic area. Furthermore, these Western diet rats displayed a trend towards a reduced ejection fraction. Nevertheless, the Western diet did not influence the end-diastolic and end-systolic pressures. Together, these results indicate that the rats developed dilated cardiomyopathy with HFrEF (12). This opposes the phenotype observed in an article by Verboven *et al.*, the original manuscript describing how this model was developed (39). Herein, the restrictive phenotype of DCM was observed, with diastolic dysfunction characterized by an increased thickness of the LV walls and a preserved ejection fraction. Generally, the restrictive phenotype occurs most often in T2DM patients, who also present obesity (12). The dilated phenotype also occurs in diabetes patients, although regularly in type 1 patients (12).

Restrictive cardiomyopathy is often seen as the precursor stage to the dilated phenotype, but recently it has also been discussed that they are two completely separate phenotypes with a different pathogenesis (12). The restrictive phenotype is caused by endothelial inflammation in the coronary arteries, and the dilated phenotype originates from excessive cardiomyocyte death (12,50). ROS plays a crucial role in the latter by inducing apoptosis and excessive autophagy (50-52). The death of cardiomyocytes will lead to increased ventricular wall stress, which will cause increased collagen depositions (53). The latter characteristic was also confirmed in the DCM rat model here, as the Western diet group had a substantial increase in interstitial collagen depositions, indicating the development of fibrosis. Future work should reveal the underlying mechanisms contributing to the observed, dilated DCM phenotype in our model. Especially, examination of cardiomyocyte cell death, for example, by a TUNEL of annexin V/ Propidium Iodide staining, can be of high interest.

How PM prevents the development of DCM – In this study, we showed that PM prevents cardiac dysfunction in our DCM rat model. More specifically, PM prevented the development of the dilated phenotype observed in DCM rats, as the LV volumes and areas were not increased if the Western diet was supplemented with PM. Accordingly, we showed that interstitial collagen depositions in the heart were significantly lower in the Western diet + PM group compared to the non-supplemented Western diet group. To the best of our knowledge, we are the first to demonstrate PM's preventive effect on cardiac fibrosis in DCM rats. Notably, previous studies on a different AGEs-inhibitor and cross-link breaker, aminoguanidine and ALT-711 respectively, revealed that they could reduce cardiac abnormalities and cardiac fibrosis (25,54).

As PM generally functions as an AGEs-inhibitor, it would be probable to assume that PM accomplished this outcome through its AGEs-lowering effects. However, the current study showed that AGEs levels were not increased in the myocardium or plasma in our DCM model. Furthermore, PM did not affect the levels of AGEs. These results were remarkable as the study by Verboven et al. demonstrated a four times increase in plasma AGEs levels in this rat model (39). In addition, an upregulated AGEs production is speculated to be an important hallmark in DCM development (11,39). Nevertheless, it might be possible that irreversible AGEs are not formed yet, but plasma precursor AGEs are present in our DCM rats. Therefore, in future research, it might be interesting to measure levels of AGEs-precursors, including RCC such as methylglyoxal, with mass spectrometry (55). Remarkably, we did show a slight trend towards increased RAGE gene expression in the Western diet group, which was prevented by PM. Indeed, this might indicate a possible contribution of the AGEs-RAGE pathway in the development of DCM in our model. To further evaluate this contribution on protein level, a RAGE immunohistochemistry staining could be performed in the future.

As PM did not affect the plasma and cardiac AGEs levels, the effectiveness of PM in our study can be due to its anti-oxidative properties. Indeed, we showed that PM significantly lowered the cardiac gene expression of NOX4, a pro-oxidative enzyme that produces hydrogen peroxide (H_2O_2). Furthermore, the Western diet slightly tended to decrease anti-oxidative SOD2 gene expression in the heart, which was prevented by PM.

The first hallmark of the dilated cardiomyopathy phenotype in DCM is the death of cardiomyocytes. As we expected the restrictive phenotype instead of the dilated phenotype, we did not yet perform an assay to detect apoptosis, as previously indicated. We did measure the gene expression of the anti-apoptotic Bcl-2 gene. Interestingly, there was a slight trend towards decreased cardiac Bcl-2 expression in the Western diet group. However, this effect was more substantial after PM administration. As oxidative stress causes apoptosis and ROS production by NOX4 was lowered, we expected that PM would prevent a Bcl-2 decrease (51,52). Cao *et al.* demonstrated *in vitro* that glucolipotoxicity decreases cardiomyocyte viability and that this effect can be prevented with Z-ligustilide, which has anti-oxidative properties (56). Administration of the anti-oxidative agent also prevented a decrease in Bcl-2 protein expression (56). This indicates again that it would be interesting to further investigate cardiomyocyte apoptosis in this model while also looking at both protein and gene expression of the whole Bcl-2 protein family.

As previously stated, the excessive cardiomyocyte death in dilated cardiomyopathy is followed by increased cardiac fibrosis. Notably, the cytokine TGF-β plays a central role in the profibrotic response. It activates multiple pathways, including the mothers against decapentaplegic homolog 2 and 3 (SMAD2/3) complex, which will activate cardiac fibroblasts and stimulate collagen synthesis (57). As previously described, we observed here that PM lowered the total interstitial collagen depositions in the heart, effectively decreasing fibrosis. Besides, we showed that PM also reduced cardiac gene expression of collagen type I, the main collagen type in the heart contributing for 80% of total collagen, in DCM rats (58). There was no discernible difference in collagen type III, contributing for 20% of total collagen in the heart, gene expression, although this could be influenced by the high within-group variation and the low sample size in the Western diet group (58). Furthermore, we saw that PM induced a trend towards decreased cardiac TGF-β gene expression. Again, PM's anti-oxidant properties can clarify the anti-fibrotic response in the Western diet + PM group. NOX4, whose gene expression is significantly lowered by PM, is involved in a positive feedback loop with TGF-β (59). Firstly, ROS produced by NOX4 will activate latent TGF-β (59,60). The activated TGF-β will then bind to its receptor, leading to an increase in NOX4 expression and activity, which will cause a further increase in ROS production (59,61). Cucoranu *et al.* even showed that NOX4 is essential for the activation of cardiac fibroblasts in human cardiac fibroblast cells (61). They demonstrated that TGF-β stimulation did not lead to SMAD2/3 activation and increased collagen type I gene expression if NOX4 was depleted (61). Furthermore, Zhao *et al.* demonstrated that antioxidants are able to decrease the gene expression of TGF-β and collagen type I, the activation of cardiac fibroblasts, and overall cardiac fibrosis in a rat model for hypertension (62). Additionally, Yuan *et al.* demonstrated that hyperglycemia activates calcium-sensing receptors on the cardiac fibroblast, activating the TGF-β/SMAD pathway and inducing cardiac fibroblast proliferation and an increase in collagen type I gene expression (63). Altogether, these pathways explain how PM can reduce fibrosis through its anti-oxidant and hyperglycemia-lowering mechanisms. In the future, it would be useful to assess the oxidative stress levels globally in the heart, for example, by means of a nitrotyrosine staining, and investigate the expression of this specific pathway by analyzing the expression of SMAD2/3 and α-smooth muscle actin (**α-SMA**), which is a marker of active cardiac fibroblasts.

Relevance of the PM dose – In our study, the rats were given a dose of 1g/L PM in their diet and drinking water, as previously used in a study by Deluyker *et al.* (64). A wide variety of doses are used throughout the literature. In animal studies, the dose varied between 100mg/kg/day to 2g/L in drinking water (46,65). Furthermore, PM was also administered directly into the stomach using oral gavage in doses of 60 to 400 mg/kg/day (66,67). PM has also been used in clinical trials where it has

been assessed for safety and bioavailability. Diabetes patients in the studies of Williams *et al.* received either 50mg twice daily for 24 weeks or 250mg twice daily for 20 weeks (68). In the group receiving the higher dose, there was a trend towards increased serious adverse effects, although the investigators did not consider them related to the study drug, but not in total adverse effects. In the study of Van den Eynde *et al.*, a single daily dose of 200mg or three daily doses of 67mg were assessed in five healthy volunteers (69). They observed high plasma levels of PM after oral administration. These studies show that our study used a PM dose consistent with other animal studies, although it is high in proportion to body weight compared to doses used in clinical trials. Since serious adverse effects have been observed in humans receiving very high doses of PM, it is important to keep this in mind when comparing results from animal studies to clinical trials.

CONCLUSION

In this study, the effect of PM on DCM was investigated using a high-sugar diet-based rat model. PM prevented the development of diabetes, which was induced by the Western diet. Furthermore, the Western diet induced cardiac dysfunction indicative of the dilated DCM phenotype. Additionally, the Western diet increased interstitial myocardial fibrosis. PM restricted both functional and morphological cardiac hallmarks. AGEs levels were not altered in the Western diet or the Western diet + PM groups. Notably, PM lowered the cardiac gene expression of the pro-oxidant gene NOX4. These results indicate that PM presumably prevented the development of DCM through its anti-fibrotic and anti-oxidative properties. Overall, this study gains a better understanding of the mechanisms and underlying causes of the dilated phenotype of DCM. The knowledge gained from this can contribute to the application of PM as a more personalized preventing strategy for DCM in the future.

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Acknowledgements – I would like to sincerely thank Sarah D'Haese for her excellent guidance and support during my senior internship and the process of writing this thesis. Furthermore, I would like to thank prof. Virginie Bito and the whole cardio team for their help and advice throughout the year.

Author contributions – S.D. and V.B. conceived and designed this study. L.C. and S.D. performed the experiments and the data analysis. L.C. wrote the paper. S.D. revised and approved the final paper.