Delayed intervention with pyridoxamine improves metabolic function and prevents adipose tissue inflammation and insulin resistance in high-fat diet-induced obese mice

**Running title:** Pyridoxamine and complications in obesity

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ABSTRACT

Obesity is associated with an increased risk for development of type 2 diabetes and vascular complications. Advanced glycation endproducts are increased in adipose tissue and have been associated with insulin resistance, vascular dysfunction and inflammation of adipose tissue. Here, we report that delayed intervention with pyridoxamine (PM), a vitamin B6 analogue which has been identified as an anti-glycating agent, protected against high-fat diet (HFD)-induced body weight gain, hyperglycemia and hypercholesterolemia, as compared to those who were not treated. In both HFD-induced and db/db obese mice, impaired glucose metabolism and insulin resistance were prevented by PM supplementation. PM inhibited expansion of adipose tissue and adipocyte hypertrophy in mice. In addition, adipogenesis of murine 3T3-L1 and human SGBS preadipocytes was dose- and time-dependently reduced by PM, as demonstrated by Oil Red O staining and reduced expression of adipogenic differentiation genes. No ectopic fat deposition was found in the liver of HFD mice. The high expression of pro-inflammatory genes in visceral adipose tissue of the HFD group was significantly attenuated by PM. Treatment with PM partially prevented HFD-induced mild vascular dysfunction. Altogether, these findings highlight the potential of PM to serve as an intervention strategy in obesity.
ABBREVIATIONS

3-DG, 3-deoxyglucosone; ACh, acetylcholine; AGE, advanced glycation endproduct; C/EBPα, CCAAT/enhancer-binding protein alpha; CD36, cluster of differentiation 36; CEL, Nε-(carboxyethyl)lysine; CML, Nε-(carboxymethyl)lysine; FASN, fatty acid synthase; GO, glyoxal; HFD, high-fat diet; IFN-γ, interferon-γ; IL, interleukin; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; LFD, low-fat diet; LPL, lipoprotein lipase; MCP-1, monocyte chemotactic protein 1; MG-H1, methylglyoxal-derived hydroimidazolone 1; MGO, methylglyoxal; MHC-II, major histocompatibility complex II; mKC, mouse keratinocyte-derived chemokine; ORO, Oil Red O; PM, pyridoxamine; PPARγ, peroxisome proliferator-activated receptor γ; PWV, pulse wave velocity; RAGE, receptor for AGEs; SCAP, sterol regulatory element binding protein cleavage-activating protein; SEM, standard error of the mean; SGBS, Simpson-Golabi-Behmel-Syndrome; SREBP-1C, sterol regulatory element binding protein 1C; TNFα, tumor necrosis factor α; UPLC-MS/MS, ultra performance liquid chromatography tandem mass spectrometry; VAT, visceral adipose tissue.
Obesity is characterized by dysregulation of adipokine production (1), which predisposes obese individuals to development of cardiovascular and metabolic complications. Recently, we and others demonstrated accumulation of advanced glycation endproducts (AGEs) in adipose tissue (2; 3), and have identified that they play an important role in obesity-induced insulin resistance and dysregulation of adipokine expression, such as interleukin-6 (IL-6) and adiponectin (2; 4; 5). AGEs are a heterogeneous family of non-enzymatically, post-translationally modified proteins and can be formed rapidly by the intracellular α-dicarbonyl compounds methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) (6). Because of harmful effects elicited by AGEs, several inhibitors have been designed to inhibit their formation (7).

Currently, pyridoxamine (PM) is receiving considerable attention as a highly potent AGE inhibitor. PM, a vitamin B6 analogue, has been identified as an anti-glycating agent (8), possibly through trapping of α-dicarbonyl compounds (9). Previous data have shown that PM inhibits AGE formation and retards the development of diabetic nephropathy and retinopathy in animal models of diabetes (10). Two trials in patients with diabetic nephropathy demonstrated no adverse effects of PM and thus a favorable safety profile (11; 12).

Because of accumulation of AGEs in obese adipose tissue and the link with insulin resistance and vascular complications, we postulate that inhibiting AGE formation will improve the metabolic and vascular profile in obesity. In the present study, we examine the effect of a delayed PM intervention on metabolic and vascular function in high-fat diet (HFD)-induced obese mice. By delaying PM treatment with 6 weeks after the start of HFD feeding, we mimic the clinical situation of obesity in which metabolic dysfunction
has already developed. This delayed intervention enables us to investigate the capacity of PM to treat obesity-associated complications, rather than to prevent them.
RESEARCH DESIGN AND METHODS

Animals

Male C57BL/6J mice were purchased from Jackson Laboratory (Charles River) and were maintained in a temperature-controlled room on a 12h light-dark cycle. They were housed with 2-3 mice per cage and had free access to food and drinking water. After a 6 week run-in period on low-fat diet (LFD, 10% fat, Research Diets D12450H), 12 week old mice were divided into three groups. The LFD group (n=15) continued on the same diet, whereas the other two groups (n=15 per group) switched to the HFD (45% kcal% fat, Research Diets D12451). After 6 weeks of HFD, one group started to receive PM (2 g/L) in the drinking water (HFD + PM) and 18 weeks later, all mice were sacrificed by means of CO₂ inhalation and subsequent exsanguination by cardiac puncture.

Male db/db mice were also purchased from Jackson Laboratory (Charles River) and were included in the study at an age of 6 weeks. They were treated with PM in their drinking water for 18 weeks. db/db mice were housed and sacrificed in the same way as described for the LFD and HFD fed C57BL/6J mice.

The experimental protocol was approved by the institutional committee for animal welfare of Maastricht University and all experiments were performed by licensed users according to international guidelines. PM was kindly provided by Prof. T. Miyata (Tohoku University, Sendai, Japan).
**In vivo glucose tolerance and insulin tolerance tests**

Intraperitoneal glucose tolerance tests (IPGTT) were performed in all mice 13 weeks after the start of the study. Following a 16h overnight fasting period, whole blood glucose was measured with a glucometer (Contour, Bayer, Leverkusen, Germany). After intraperitoneal glucose injection (2.0 g/kg body weight, Sigma-Aldrich, Saint Louis, United States), blood glucose levels were measured at 15, 30, 60, 90 and 120 minutes. One week later, intraperitoneal insulin tolerance tests (IPITT) were performed. To this end, mice were fasted for 4h and insulin was injected intraperitoneally at a dose of 0.5 U/kg body weight (Actrapid Penfill, Novo Nordisk, BagsÆrød, Denmark). Blood samples were taken at the same time points as during the IPGTT and blood glucose levels were assessed. In addition, the IPGTT and IPITT were also performed in db/db mice.

**Biochemical characterisation**

Fasted plasma total cholesterol and liver triglyceride levels were determined with enzymatic colorimetric tests using CHOD-PAP and GPO-PAP reagents methods respectively (Instruchemie, Delfszijl, The Netherlands). Fasted plasma insulin and leptin levels were measured in a multiplexed sandwich immunoassay of Meso Scale Discovery (K15124-C, Rockville, MD, USA). Plasma biomarkers were measured with a 7-plex multiarray biomarker assay of Meso Scale Discovery (K15012-C, Rockville, MD, USA). The α-dicarbonyls MGO, GO and 3-DG were measured using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS, Waters, Milford Massachusetts, USA), as described previously (13).
GLO1 activity was measured in protein lysates of visceral adipose tissue (VAT), according to the method of McLellan and Thornalley (14). In short, GLO1 activity was assayed by spectrophotometry (Synergy, BioTek, Winooski, VT, USA), by measuring the increase in absorbance at 240 nm as a result of formation of S-D-lactoylglutathione for 20 min.

**Immunohistochemical tissue characterization**

During dissection of the mice, visceral, epididymal fat depots and livers were isolated, weighed and stored for further analyses. After overnight fixation in 4% formaldehyde, tissues were embedded in paraffin and 4 µm sections were collected and stained with haematoxylin and eosin (H&E). Digital images were taken at a magnification of 20x using a Leica DFC320 digital camera (Leica Microsystems, Wetzlar, Germany). Adipocyte cell size and cell diameter were quantified with morphometric analysis software (Leica QWin V3, Leica Microsystems, Wetzlar, Germany). Macrophage infiltration in VAT was scored based on the presence of crown-like structures with the following scores: 0: no crown-like structures, 1: 1 or 2 crown-like structures, 2: 3 to 9 crown-like structures, 3: 10 to 20 crown-like structures, 4: more than 20 crown-like structures. Fat content of the liver was scored based on the presence of fat droplets with the following scores: 0: no fat droplets, 1: very few fat droplets 2: few fat droplets 3: multiple fat droplets and 4: many fat droplets.
Preadipocyte culture and differentiation

Murine 3T3-L1 and human Simpson-Golabi-Behmel-Syndrome (SGBS) preadipocytes were cultured and differentiated into adipocytes according to the appropriate protocol. In short, murine 3T3-L1 preadipocytes were maintained in DMEM high glucose (25 mM) containing 10% FCS and 1% glutamine-penicillin/streptozotocin. After reaching 80% confluency, differentiation was induced with the standard culture medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine, 10 µg/mL insulin, and 0.444 µg/mL dexamethasone. After two days, the first adipogenic differentiation medium was substituted by the standard culture medium supplemented with 2.5 µg/ml insulin until day 12. Human SGBS preadipocytes were maintained in DMEM/HAM’s F12 culture medium supplemented with 10% FCS, 8 µg/mL biotin, 4 µg/mL pantothenate and 1% glutamine-penicillin/streptozotocin, and were grown to confluence. Adipogenic differentiation was induced by incubating preadipocytes with serum-free culture medium containing 10 µg/mL human transferrin, 20 nM insulin, 100 nM cortisol and 0.2 nM triiodothyronine for 12 days. After 6 days of differentiation, this adipogenic differentiation medium was additionally supplemented with 25 nM dexamethasone, 500 µM 3-isobutyl-1-methylxanthine and 2 µM rosiglitazone (GlaxoSmithKline, London, UK). For both SGBS and 3T3-L1 cells, media were refreshed every other day. In addition to the normal differentiation condition, cells were also differentiated in the presence of 2 and/or 5 mM PM. RNA was extracted from cells at day 0, 2, 5 and 7. Oil Red O staining (Sigma Aldrich) and triglyceride measurements of both 3T3-L1 and SGBS cells during differentiation were performed following the manufacturer’s instructions to determine adiposity. Cell count was determined using a Bürker-Türk counting chamber.
Real-time polymerase chain reaction

Total RNA was extracted from cells and VAT using TRIzol® (Invitrogen, Bleiswijk, The Netherlands), and reversed transcribed with the iScript cDNA synthesis kit (Biorad, Veenendaal, The Netherlands). Expression of target genes was measured quantitatively by real time PCR using SYBR Green mix (Bioline, London, United Kingdom). All primer sets used are listed in supplemental table 1. mRNA expression levels were normalised to two reference genes (cyclophilin A and β2-microglobulin) and data were analysed with the ∆CT method. Data are expressed as normalized gene expression levels relative to control.

Assessment of cardiac function, vascular stiffness, blood pressure and vascular function

Cardiac dimensions and function were assessed under 2-3% isoflurane anesthesia. Briefly, echocardiographic recordings were made in parasternal long-axis using a Vevo2100 imaging platform (Visual Sonics, Toronto, Canada). Data were derived from left ventricular (LV) images in end-diastole and peak systole, and average values over at least three different cycles were used for analyses. Stroke volume was calculated from subtracting peak systolic LV from end-diastolic LV and multiplied with heart rate to obtain cardiac output. Pulse wave velocity (PWV) was assessed using the Vevo2100 ultrasound machine. By visualizing the aortic arch, we measured the time frame of the forward wave (ECG triggered) between two aortic points and calculated the PWV.

In a subset of mice, blood pressure was measured under isoflurane anesthesia by insertion of a PE-10 catheter in the abdominal aorta via the femoral artery and connected to a
pressure transducer (Miller Instruments, Houston, TX, USA). The pressure signal was digitally sampled at 2 kHz and systolic and diastolic blood pressures were calculated over a 10–15 min time period after stabilization of hemodynamic variables.

After careful dissection of the aorta, an aortic segment of the descending thoracic aorta was excised just above the diaphragm. These segments were then mounted in a myograph organ bath (model 610M Danish Myotechnology by J.P. Trading, Denmark) with two steel 40 µm wires inserted through the lumen of the segments. The organ bath contained fresh Krebs-Ringer bicarbonate solution (KRB) consisting of 118 mM NaCl; 4.7 mM KCl; 1.2 mM KH₂PO₄; 25 mM NaHCO₃; 1.1 mM MgSO₄; 2.5 mM CaCl₂ and 5.0 mM glucose, was maintained at 37 °C and was gassed continuously with 95% O₂ and 5% CO₂ (pH 7.4). The internal diameter of each aorta was normalized by stretching the vessel to a diameter that yielded a wall tension equivalent to a transmural pressure of 100 mmHg. The isometric tension generated by the vessels was recorded using Powerlab 4/25 (ADInstruments, Oxford, United Kingdom), connected to the Myo-Interface. Maximum contraction was measured by incubation with 125 mM K⁺ plus 10 µM phenylephrine (Sigma-Aldrich). Endothelium-independent vasorelaxation was tested with cumulative concentrations of acetylcholine (ACh, 0.001–10 µM, Sigma Aldrich) during contraction, induced by 10 µM phenylephrine, with pre-incubation of 10 µM indomethacin (Sigma-Aldrich) to block the synthesis of prostaglandins, and 100 µM L-NAME (Sigma-Aldrich) to block eNOS.
Statistical analyses

All data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed with IBM SPSS Statistics Software, version 20 (IBM Corporation, Armonk, New York). One-way ANOVA was used to compare continuous variables between groups. Two-way repeated measures ANOVA was used to compare groups or conditions over time. A p-value <0.05 was considered statistically significant.
RESULTS

Delayed intervention with PM prevents body weight gain and improves metabolic characteristics in HFD-induced obese mice

Six weeks of HFD feeding resulted in significantly increased body weight, plasma glucose and total cholesterol levels as compared to LFD control mice (Table 1). Water intake and urine production were not altered due to HFD. Thus, when we started PM intervention after the first 6 weeks, metabolic function of the HFD mice was already impaired.

Metabolic characteristics of the mice at the end of the study are shown in figure 1. Administration of PM to the drinking water for 18 weeks inhibited body weight gain in HFD mice (Fig. 1A and B), despite equal calorie intake (Fig. 1C). In addition, we observed a trend towards metabolic improvement by PM supplementation for plasma glucose and cholesterol (Fig. 1D and E), whereas plasma insulin and leptin levels were significantly reduced by PM treatment in HFD-fed mice (Fig. 1F and G). There were no differences in water intake and urine production between the groups HFD and HFD + PM (data not shown).

Glucose tolerance and insulin sensitivity are improved by PM in both HFD-induced and db/db obese mice

We further studied the effect of PM on metabolic function with both an IPGTT and an IPITT after 13 weeks of PM treatment. Results of the IPGTT demonstrated that HFD mice had significantly lower glucose tolerance compared to LFD mice, which was
improved by PM (Fig. 2A). After insulin injection, we found an impaired decline in glucose in the HFD group in comparison to the LFD group with an attenuation of this impairment by PM (Fig. 2B). These results demonstrate that treatment with PM is associated with general improvement in glucose tolerance and insulin sensitivity. We also performed the IPGTT and IPITT in obese db/db mice, treated and not treated with PM. In line with our data from the HFD mice, PM also improved both glucose tolerance (Fig. 2C) and insulin sensitivity (Fig. 2D) in db/db mice.

**PM prevents adipose tissue expansion and hypertrophy by inhibiting adipogenesis**

Histochemistry demonstrated enlarged adipocytes in VAT of HFD mice in comparison to LFD mice (Fig. 3A). Morphometric analysis demonstrated an increased cell size and adipocyte diameter in the HFD group compared to the LFD control group (Fig. 3B and C). Both the HFD-induced increase in adipocyte diameter and cell size were attenuated by PM. In addition, the HFD-induced increase in mass of VAT was attenuated by PM (Fig. 3D). To investigate the potential of PM to inhibit glycation in VAT, we assessed levels of the α-dicarbonyls MGO, GO and 3-DG (Fig. 3E-G). We observed a trend towards increased α-dicarbonyl levels in VAT of the HFD group, which was reduced by PM. Activity of the GLO1, the major enzyme for the detoxification of α-dicarboxylics, was reduced in VAT in the HFD group, although not statistically significant, and was improved by PM (Fig. 3H). The α-dicarbonyl levels, as well as GLO-1 activity, did not differ between LFD and HFD in the subcutaneous adipose tissue (data not shown).

To elucidate the mechanism behind PM-induced improvements of adiposity, we used two *in vitro* models of adipogenesis: murine 3T3-L1 and human SGBS preadipocytes. Both
cell lines were differentiated by the appropriate differentiation mix, in the presence or absence of 2 and/or 5 mM PM. Pictures of 3T3-L1 adipocytes demonstrated that cells differentiated in the presence of 5 mM PM contained less fat droplets compared to untreated adipocytes (Fig. 4A). Inhibition of adipogenesis by PM was confirmed by Oil Red O staining (Fig. 4B), triglyceride quantification (Fig. 4C), and quantitative analysis of expression of the adipogenic markers peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), sterol regulatory element binding protein 1C (SREBP-1C), fatty acid synthase (FASN), lipoprotein lipase (LPL) and cluster of differentiation 36 (CD36) (Fig. 4D-I). Gene expression levels of SREBP cleavage-activating protein (SCAP) were neither affected by HFD, nor by PM (data not shown). Oil Red O staining and triglyceride quantification demonstrated a dose-dependent effect of PM on adipogenesis. These findings were all confirmed in human SGBS adipocytes (data not shown). We also investigated whether incubation with PM had already an effect on adipogenesis of 3T3-L1 cells in the first two days of the differentiation process, in which the preadipocytes still have the capacity to proliferate. To study this, 3T3-L1 preadipocytes were differentiated into adipocytes and 5 mM PM was added from upon the start of the differentiation process (T=0) or two days later (T=2). We found that treatment with PM from upon T=0 inhibited adipogenesis even more than when PM was added from upon T=2, as reflected by Oil Red O absorbance and PPARγ mRNA expression (Supplemental Figure 1A and B). Moreover, incubation with 5 mM PM had no effect on proliferation of the 3T3-L1 preadipocytes during the first two days of the differentiation process (Supplemental Figure 1C).
Lipid content in the liver of HFD-induced obese mice is reduced by PM

As the liver often serves as an ectopic site of fat deposition, we investigated whether a reduction in adiposity by PM would lead to a flux of fat to the liver of HFD mice. H&E staining of liver coupes demonstrated that livers of HFD mice contained more fat droplets (Fig. 5A and B). Treatment with PM in HFD mice resulted in less fat droplets in the liver. In line, triglyceride content was increased in the liver of HFD mice, but this was reduced by treatment with PM (Fig. 5C). Thus, reduction in white adipose tissue by PM did not lead to ectopic fat deposition in the liver.

PM prevents HFD-induced adipose tissue inflammation

To further examine the effect of PM on HFD-induced metabolic dysfunction in mice, we first characterised the VAT on the level of inflammation. Histochemical staining revealed increased accumulation of macrophages in VAT of HFD mice, compared to that of LFD mice (Fig. 6A and B). The formation of these so-called crown-like structures was ameliorated by PM. Quantitative analyses of inflammatory gene expression levels demonstrated that HFD-induced gene expression levels of tumor necrosis factor α (TNFα), monocyte chemotactic protein 1 (MCP-1), CD11c and major histocompatibility complex II (MHC-II), and HFD-induced reduction of adiponectin were attenuated by PM treatment (Fig. 6C-G). In plasma, however, we did not find any differences in the concentration of IFN-γ, interleukin (IL)-1β, IL-10, IL-12, IL-2, IL-6, TNFα and mouse keratinocyte-derived chemokine (mKC) between the groups (data not shown).
HFD-induced mild vascular dysfunction is partially prevented by PM

Acetylcholine-induced endothelium-independent vasorelaxation of mouse aortas was slightly impaired in HFD mice (Fig. 7A). This was improved when mice were treated with PM, although not statistically significant. The logEC$_{50}$ of acetylcholine to achieve 50% of the maximal vasorelaxation was reduced in the HFD group, which was prevented by PM (Fig. 7B). Neither HFD, nor PM had an effect on other vascular parameters including systolic blood pressure, diastolic blood pressure, heart rate, cardiac output, ejection fraction and pulse wave velocity (Supplemental Figure 2).
DISCUSSION

In the present study, we investigated the effects of PM on obesity and its related complications. We have shown that obesity-associated metabolic dysfunction and complications in obese mice were ameliorated by a delayed intervention with PM. HFD-induced increases in body weight, hyperglycemia, hypercholesterolemia and levels of leptin and insulin were all reduced by PM. In addition, PM is associated with a general increase in glucose tolerance and insulin sensitivity. Furthermore, expansion of adipose tissue and hypertrophy of adipocytes in obese mice were inhibited by PM, most likely via inhibition of adipogenesis. PM improved vasorelaxation of the aorta, but it had no observable effect on other vascular parameters.

By feeding mice a HFD, we induced a state of metabolic dysfunction as indicated by an increase in body weight and severe hyperglycemia and hypercholesterolemia, and increased plasma levels of insulin and leptin. We have shown, in a delayed intervention study with PM, that PM was able to improve these metabolic dysfunctions, including a reduction in insulin resistance in both HFD mice and db/db mice.

The beneficial effect of PM on metabolic function was also reflected by a reduction of the VAT mass, most likely via inhibition of adipogenesis. This concept was demonstrated in vitro by reduced Oil Red O staining and triglyceride content in differentiating murine 3T3-L1 and human SGBS cells. In addition, we found that PM limited the induction of important adipogenic differentiation genes such as PPARγ, C/EBPα, SREBP-1C, FASN, LPL and CD36. SREBP-1C is a transcription factor which is known to induce expression of adipogenic genes such as LPL and FASN, but it also induces PPARγ expression,
which enhances adipogenic genes expression even more (15; 16). As both SREBP-1C and PPARγ expression, and expression of their target genes are decreased, this suggests an inhibitory effect of PM on adipogenesis, possibly via inhibition of SREBP-1C. In literature, it has been described that the AGE N(ε)-(carboxymethyl)lysine (CML) interferes with the SREBP cleavage-activating protein (SCAP)/SREBP-1C pathway (17; 18). In our study, we did not find any effect of PM on mRNA expression levels of SCAP in adipocytes. However, in different cell types, CML has been described to co-localize with SCAP (17-19), suggesting a considerable glycation of SCAP, and consequently leading to altered function of SCAP. Therefore, glycation of SCAP, next to possible direct effects of PM, may be a possible mechanism by which AGEs enhance adipogenesis. The dangerous reverse side of smaller fat depots in obesity is ectopic fat deposition. The most important organ which is known to be at risk for ectopic fat deposition is the liver. However, in this study, we demonstrated that inhibition of adipogenesis by PM in HFD mice did not lead to fatty liver disease. On the contrary, PM treatment resulted in less fat content in HFD livers. Obesity is associated with a dysregulated expression of adipocytokines (2; 20). We and others have very recently demonstrated that AGEs are implicated herein (2; 21; 22). In the present study, accumulation of macrophages, the so-called crown-like structures and increased pro-inflammatory gene expression levels in VAT of HFD mice were observed and the number of crown-like structures and expression of pro-inflammatory cytokines TNFα, MCP-1, CD11c and MHC-II were strongly reduced by PM, whereas expression of the anti-inflammatory cytokine adiponectin was increased. Thus, supplementation with PM was associated with a normalization of the inflammatory phenotype of VAT in HFD
mice. These results are in line with our previous work, in which we demonstrated that CML accumulation in adipose tissue of obese db/db mice was associated with a pro-inflammatory phenotype of the adipose tissue. When the receptor for AGEs (RAGE) was absent, inflammation levels were normalized (2). Together, these data form a strong body of evidence that accumulation of AGEs in adipose tissue plays a key role in the pathogenesis of adipose tissue inflammation.

In VAT of HFD mice, we found a trend towards decreased GLO1 activity. Because inflammation reduces GLO1 activity (23), the pro-inflammatory phenotype of VAT in HFD mice is most likely responsible for the reduction in GLO1 activity. In line, PM reduces adipose tissue inflammation, and thus causes normalization of GLO1 activity in VAT of HFD mice.

Some of our findings regarding PM have previously been described in comparable studies (24; 25). However, in those studies, PM had been administered to mice from the start of the HFD, implying that metabolic function at the start of PM treatment was not comparable to that of obesity. Because we hypothesize that PM might be a potential treatment for obesity and its comorbidities, we mimicked the human situation by starting treatment with PM when obesity had already developed. To the best of our knowledge, our study is the first study to use PM in such a delayed intervention.

Although AGEs have been described in relation to vascular complications (26), we only found an improvement in aortic vasorelaxation, while improvements of additional vascular parameters were lacking. These findings are in line with those of Hagiwara et al. (24), who similarly found no effect of PM on blood pressure in HFD-induced obese mice. In aged mice however, it has been noted that PM prevents age-related aortic stiffening
and vascular resistance in association with reduced collagen glycation (27). Since we did not see large effects of HFD on development of serious (cardio)vascular complications within 24 weeks of HFD, age-related AGE accumulation may be more relevant for vascular complications than obesity-related AGE accumulation. It might be that our animals were not old enough to develop (cardio)vascular complications at HFD.

In clinical trials, treatment with PM has mainly been described in the setting of diabetic nephropathy. Several preclinical studies in animal models of diabetic nephropathy have demonstrated that oral supplementation of PM is effective in preserving renal function (28-32). In a combined multicenter phase II trial involving type 2 diabetic patients with overt nephropathy, PM was demonstrated to be generally safe and to have a potential effect in preserving diabetic kidney function (11). In contrast, a second clinical trial reported no beneficial effect of PM in reducing creatinine levels, although it suggested that patients with less advanced renal impairment might benefit (12). A third clinical trial addressed the efficacy and tolerability of PM in osteoarthritis (33). It reported no adverse effects after 6 months of PM treatment, while a combined treatment with PM significantly decreased AGEs, inflammation and pain in patients with osteoarthritis.

In conclusion, we have demonstrated that a delayed intervention with PM is associated with improvement of several aspects of obesity including metabolic dysfunction, insulin resistance and adipose tissue inflammation. These findings indicate that PM may be a potential novel treatment for obesity-associated metabolic dysfunction and complications. A clinical trial with PM in obese individuals would elucidate the efficacy of PM in human obesity complications.
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Author contributions: D.E.M. researched data, contributed to discussion, and wrote and edited the manuscript. O.B. and K.G. researched data, contributed to discussion and reviewed and edited the manuscript. J.P.C. designed the computer program for quantitative analysis of the adipocytes and reviewed and edited the manuscript. B.J.J. provided equipment and supported the vascular measurements and reviewed and edited the manuscript. T.M. kindly provided the pyridoxamine and reviewed and edited the manuscript. K.W., C.D.S. and C.G.S. contributed to discussion and reviewed and edited the manuscript. C.G.S. is the guarantor of this manuscript and, as such, takes responsibility for the contents of the article. For expert technical assistance, we thank Marjo van de Waarenburg, Jean Scheijen, Margee Robertus, Vicky Vermeulen and Sabine Daemen (all Department of Internal Medicine, Maastricht University Medical Centre, Maastricht, The Netherlands) and Jacques Debets (Department of Pharmacology, Maastricht University Medical Centre, Maastricht, The Netherlands).

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FIGURE LEGENDS

Figure 1. Metabolic parameters of the mice at the end of the study. (A) Progress of body weight over time. (B) Body weight gain during the study. (C) Average calorie intake during the study. (D) Fasting plasma glucose, (E) cholesterol, (F) insulin and (G) leptin levels. Data represent means ± SEM. N=11-15 mice per group. Circles = LFD, squares = HFD, triangles = HFD + PM.

Figure 2. PM prevents insulin resistance in HFD-induced and db/db obese mice. Glucose homeostasis was assessed by an IPGTT (A) and an IPITT (B) in LFD mice, HFD mice and HFD + PM mice. In addition, the IPGTT (C) and IPITT (D) were also performed in db/db mice, either or not treated with PM. Data represent means ± SEM. For A and B: circles = LFD, squares = HFD, triangles = HFD + PM. For C and D: circles = db/db, squares = db/db + PM. For A+B, n=13-15 mice per group. For C+D, n=7-15 mice per group. IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test.

Figure 3. PM reduces obesity-related adiposity and partially prevents formation of α-dicarbonyls in adipose tissue. (A) Representative pictures of histochemical haematoxylin and eosin stainings of visceral adipose tissue, used for quantification of cell size (B) and diameter of individual adipocytes (C). (D) Weight of visceral adipose mass. (E) MGO, (F) GO and (G) 3-DG levels in visceral adipose tissue. (H) Activity of the GLO1 enzyme in visceral adipose tissue. Data represent means ± SEM. N=11-15 mice
per group. VAT, visceral adipose tissue; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; GLO1, glyoxalase 1.

**Figure 4. PM inhibits adipogenesis in vitro in a dose-dependent way.** (A and B) Oil Red O staining of differentiating 3T3-L1 adipocytes. (C) Triglyceride content in 3T3-L1 adipocytes during 7 days of adipogenic differentiation. (D-I) Gene expression levels of adipogenic markers in differentiating murine 3T3-L1 adipocytes. Data represent means ± SEM. ***p<0.001: normal differentiation compared to no differentiation and ###p<0.001: normal differentiation + 2 or 5 mM PM compared to normal differentiation. For B-C: Circles = no differentiation, squares = normal differentiation, tiles = normal differentiation + 2 mM PM, triangles = normal differentiation + 5 mM PM. For D-I: circles = no differentiation, squares = normal differentiation, triangles = normal differentiation + 5 mM PM. PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α; SREBP-1C, sterol regulatory element binding protein 1C; FASN, fatty acid synthase; LPL, lipoprotein lipase; CD36, cluster of differentiation 36.

**Figure 5. PM reduces HFD-induced moderate fat deposition in the liver.** (A) Representative pictures of haematoxylin and eosin stainings of the liver, showing fat droplets. (B) Quantification of the liver fat droplets as shown in A. (C) Triglyceride content in the liver. Data represent means ± SEM. N=11-15 mice per group. A.U., arbitrary units.
Figure 6. HFD-induced inflammation in visceral adipose tissue is prevented by PM. (A) Representative pictures of haematoxylin and eosin stainings of visceral adipose tissue, showing crown-like structures. (B) Quantification of crown-like structures as shown in A. qPCR is used for quantification of gene expression of the inflammatory markers TNFα (C), MCP-1 (D), MHC-II (E), CD11c (F) and adiponectin (G). Data represent means ± SEM. N=11-15 mice per group. TNFα, tumor necrosis factor α; MCP-1, monocyte chemotactic protein 1; MHC-II, major histocompatibility complex II.

Figure 7. PM partially prevents mild HFD-induced vascular dysfunction. (A) Acetylcholine (ACh)-induced endothelium-independent vasorelaxation of isolated aortas. (B) –LogEC\textsubscript{50} of ACh in endothelium-independent vasorelaxation of the aorta. Data represent means ± SEM. *p<0.05, HFD compared to LFD. N=11-14 mice per group. Circles = LFD, squares = HFD, triangles = HFD + PM. ACh, acetylcholine.
## TABLES

Table 1. Characteristics of the mice before intervention with PM.

<table>
<thead>
<tr>
<th>Variable</th>
<th>LFD</th>
<th>HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>25.3 ± 0.3</td>
<td>28.2 ± 0.4***</td>
</tr>
<tr>
<td>Calorie intake (kcal/day)</td>
<td>9.9 ± 0.2</td>
<td>11.1 ± 0.3**</td>
</tr>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>8.0 ± 0.4</td>
<td>10.5 ± 0.4***</td>
</tr>
<tr>
<td>Fasting total cholesterol (mM)</td>
<td>1.8 ± 0.1</td>
<td>2.4 ± 0.1***</td>
</tr>
<tr>
<td>Water intake (mL/day)</td>
<td>2.5 ± 0.1</td>
<td>2.6 ± 0.04</td>
</tr>
<tr>
<td>Urine production (µL/day)</td>
<td>456 ± 134</td>
<td>659 ± 90</td>
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</tbody>
</table>

Data represent means ± SEM. ***p<0.001, **p<0.01 compared to LFD. N=15 and 30 mice for LFD and HFD, respectively.
Diabetes

A

B

C

D

Glucose (mmol/L)

Time after glucose injection (min)

123x84mm (600 x 600 DPI)
A

LFD  HFD  HFD + PM

B

Fibrinogen score (A.U.)

C

Triglycerides (mmol/L)

107x64mm (300 x 300 DPI)
A  

B  

68x26mm (600 x 600 DPI)
## Supplemental table 1. Sequences of primers used for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>PPARγ (m)</td>
<td>GCCCTGTCCCAGAGGTGAAG</td>
<td>GCATGTAGCGAATGAGCTGTA</td>
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<td>C/EBPα (m)</td>
<td>GCGGGAAACGCAACAACATC</td>
<td>GTCACTGGTCAACTCCAGCAC</td>
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<td>SREBP-1C (m)</td>
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<td>GCTGGAGCATGTCTTCGATGT</td>
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<td>SCAP (m)</td>
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<td>FASN (m)</td>
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<td>CD36 (m)</td>
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<td>Gene</td>
<td>Forward Primer</td>
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<td>Cyclophilin A (m)</td>
<td>TTCCTCTTTTCACAGAATTATTCCA</td>
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</table>

PPARγ, peroxisome proliferator-activated receptor γ; C/EBPα, CCAAT/enhancer-binding protein α; SREBP-1C, sterol regulatory element binding protein 1C; SCAP, sterol regulatory element-binding protein cleavage-activating protein; FASN, fatty acid synthase; CD36, cluster of differentiation 36; LPL, lipoprotein lipase; TNFα, Tumor necrosis factor α; MCP-1, monocyte chemotactic protein 1; MHC-II, major histocompatibility complex II. (m) indicates mouse primers.
Supplementary figure legends

**Supplementary Figure 1.** PM has a time-dependent effect on 3T3-L1 adipogenesis but does not influence proliferation during the first two days of differentiation. (A) Oil Red O and (B) PPARγ mRNA expression during differentiation with 5 mM PM from upon T=0 or T=2. (C) Cell count of 3T3-L1 cells with or without PM at T=2. Data represent means ± SEM. ***p<0.001: normal differentiation compared to no differentiation and ##p<0.01, ###p<0.001: normal differentiation + 5 mM PM from upon T=0 or T=2. Circles = no differentiation, squares = normal differentiation, tiles = normal differentiation + 5 mM PM from upon T=0, triangles = normal differentiation + 5 mM PM from upon T=2. PPARγ, peroxisome proliferator-activated receptor γ.

**Supplementary Figure 2.** PM has no effect on several vascular parameters in HFD-induced obese mice. (A) Systolic blood pressure, (B) diastolic blood pressure and (C) heart rate measured with an inter-arterial Millar catheter, (D) cardiac output, (E) ejection fraction and (F) pulse wave velocity. Data represent means ± SEM. for A-C, n=6-7 mice per group; for D-F, n=11-15 mice per group.