Anti-diabetic, anti-inflammatory and anti-oxidant effects of naringenin in an in vitro human model and an in vivo murine model of gestational diabetes mellitus

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List of abbreviations: 2DG – 2-Deoxy-D-glucose; AUC – area under the curve; CCL – chemokine ligands; CXCL – chemokine (C-X-C motif) ligand; GDM – gestational diabetes mellitus; GPx – glutathione peroxidase; GR – glutathione reductase; GTT – glucose tolerance test; MAPK – mitogen-activated protein kinases; NAR – naringenin; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells; NGT – normal glucose tolerant; PBS – phosphate buffered saline; SAT – subcutaneous adipose tissue; SOD – superoxide dismutase; TNF – tumour necrosis factor; VAT – visceral adipose tissue

Keywords: GDM, inflammation, insulin resistance, naringenin, oxidative stress

ABSTRACT

Scope

Gestational diabetes mellitus (GDM), which affects up to 20% of pregnant women, is associated with maternal peripheral insulin resistance, low-grade inflammation and oxidative stress. The flavonoid naringenin has potent anti-diabetic, anti-inflammatory, and anti-oxidative properties; however, its effects in GDM remain unknown. The study aimed to determine the effects of naringenin on glucose metabolism, inflammation and oxidative stress associated with GDM both *in vitro* and *in vivo*.

Methods and Results

In vitro, human tissue samples obtained at term elective Caesarean section were stimulated with TNF to develop a GDM-like environment. Naringenin treatment significantly improved TNF-impaired glucose uptake in skeletal muscle. In placenta and visceral adipose tissue (VAT), naringenin significantly reduced expression of pro-inflammatory cytokines and chemokines and increased antioxidant mRNA expression. Mechanistically, naringenin suppressed nuclear factor κB activation. *In vivo*, pregnant heterozygous *db*/+ mice were used to model GDM. Daily intraperitoneal injections of GDM mice with naringenin from gestational day 10-17 significantly improved glucose tolerance, reduced IL1A mRNA expression and increased antioxidant mRNA expression in placenta, VAT and subcutaneous adipose tissue.

Conclusion

Naringenin was shown to improve insulin sensitivity, inflammation and oxidative stress associated with GDM and shows promise as a novel prevention therapeutic.

1. INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as "a carbohydrate intolerance resulting in hyperglycaemia of variable severity with onset or first recognition during pregnancy".[1] GDM is known to affect up to 20% of pregnant women, depending on the nation, and its prevalence continues to rise.[2-5] Several short-term complications are associated with GDM, including maternal hypertension and preeclampsia, increased fetal adiposity, macrosomia and shoulder dystocia.[6-8] In the long-term, both mother and child have increased risk of obesity, metabolic syndrome, type 2 diabetes, and cardiovascular disease.[9, 10]

GDM is characterised by exaggerated maternal skeletal muscle insulin resistance.[11] Both infection and sterile inflammation have been linked to GDM pathophysiology [12-14], and are suggested to drive this increased maternal insulin resistance.[15, 16] Sterile inflammatory products and/or bacterial or viral pathogens can act on the placenta and maternal adipose tissue to express and produce pro-inflammatory mediators.[17-20] These pro-inflammatory mediators, such as TNF, IL1B and IL6, then induce further production of other pro-inflammatory cytokines and chemokines.[20, 21] GDM is also noted to exhibit increased markers of oxidative stress and altered antioxidant defences in placenta, adipose tissue and skeletal muscle when compared to normal glucose tolerant (NGT) pregnancies.[20, 22] Current treatment therapies for GDM target only hyperglycemia, and have little effect on inflammation and oxidative stress. Moreover, the inability to affect inflammation or oxidative stress may be the factor withholding current therapies from offering protective long-term effects to both mother and baby.[9, 23] A novel therapeutic that effectively prevents and targets all aspects of GDM is warranted.

Polyphenols are natural bioactive compounds, commonly found in fruits, vegetables and other plant-based foods. Several polyphenols have been found to exhibit anti-inflammatory, anti-obesity and anti-diabetic properties[24]; however, there is limited research investigating the impact of polyphenols on GDM. Interestingly, one study showed promising results where supplementing pregnant GDM rats with olive oil, an abundant resource of polyphenols, improved placental dysfunction, however, did not prevent the development of GDM.[25] While the contribution of polyphenols to these effects is unknown, their potential protective effects against GDM warrant investigation.

One novel polyphenol is naringenin, which exists in grapefruit and citrus fruits, and has been found to possess potent anti-diabetic, anti-inflammatory and anti-oxidative properties.[26-35] Naringenin has been shown to improve blood glucose and serum insulin levels in streptozotocin-induced and high-fat-diet-induced rodent models of type 2 diabetes.[26-28] Research also suggests that naringenin reduces expression of pro-inflammatory cytokines and chemokines *in vitro* and *in vivo*.[29-31] Improved antioxidant protein expression and activity with naringenin treatment has also been identified in animal models of diabetes.[32, 33] Mechanistically, naringenin has been shown to elicit these effects via

inhibition of key signalling pathways, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[31], Akt[34] and mitogen-activated protein kinases (MAPK)[35] .

This group previously reported that in human placenta, naringenin can reduce lipopolysaccharide-stimulated pro-inflammatory cytokine and chemokine expression and secretion by inhibiting NF-κB transcriptional activity.[31] However, the effects of naringenin on insulin resistance, inflammation and oxidative stress associated with GDM have yet to be studied. Therefore, this study aimed to determine the glucose-sensitising, anti-inflammatory and anti-oxidative effect of naringenin using both *in vitro* and *in vivo* models of GDM. Firstly, an *in vitro* human tissue explant model of GDM was used to establish the effect of naringenin on: 1) skeletal muscle glucose uptake, 2) inflammation, and 3) antioxidant expression. Secondly the authors sought to identify the signalling pathways through which naringenin may act. Following promising results from the *in vitro* study, confirmation that naringenin exerts similar glucose-sensitising, anti-inflammatory and antioxidant effects in an *in vivo* mouse model of GDM was investigated.

2. MATERIALS AND METHODS

2.1. Study 1: Effect of naringenin on insulin resistance, inflammation and antioxidant expression in human placenta, adipose tissue and skeletal muscle *in vitro*

Study 1 investigated the *in vitro* effects of naringenin on: 1) insulin-stimulated glucose uptake in human skeletal muscle, 2) the mRNA expression and release of pro-inflammatory mediators from human placenta and visceral adipose tissue (VAT), and 3) superoxide dismutase (SOD)-1, catalase, and glutathione reductase (GR) mRNA expression in human placenta and VAT. Three mechanistic pathways known to be regulated by naringenin were also explored: NF-κB)[31] , Akt[34] and mitogen-activated protein kinases (MAPK)[35]. Human placenta, VAT and skeletal muscle (from the rectus pyramidalis) were obtained from normal glucose tolerant (NGT) –women at term elective Caesarean section and were stimulated with TNF to develop a GDM-like environment.[19, 31, 36]

2.1.1. Human tissue collection and explants

Ethics approval was obtained from the Mercy Hospital for Women's Research and Ethics Committee (Mercy Health, Ethics approval number R04-29) and written informed consent was obtained from all participating subjects. Healthy NGT women with a body mass index <30 kg/m2 who were delivering healthy, singleton infants at term (37-41 weeks of gestation) via elective Caesarean in the absence of labour were recruited. The exclusion criteria included: vascular/renal complication, multiple gestations, asthma, smokers, preeclampsia, chorioamnionitis, placental abruption, acute fetal distress and women with any other adverse underlying medical conditions. All tissues were delivered and processed within 20 min of delivery.

Tissue explants for placenta, VAT and skeletal muscle were performed as previously described.[31, 37, 38] Briefly, tissues were washed in PBS and blunt dissected to remove visible connective tissue,

vessels and calcium deposits. The processed tissues were then pre-incubated for 1 h in Dulbecco's Modified Eagle's Medium (DMEM) with 100 U/ml penicillin G and 100 µG/ml streptomycin at 37˚C in a humidified incubator of 5% CO_2 and either 21% O_2 for VAT and skeletal muscle or 8% O_2 for placenta. The samples were blotted dry and 100 mg wet-weight samples were transferred to a 24-well tissue culture plate for incubation with 1 ml DMEM in the absence or presence of 10 ng/ml TNF, with or without 400 µM naringenin (Sigma, St Louis, USA) (see Section 2.1.2. Dosage Information). After 20 h incubation, tissue and media were collected separately and stored at -80^{o}C until analysis by western blotting, ELISA, or quantitative real-time polymerase chain reaction (qRT-PCR) as detailed below. A total of 16 patients were recruited as quality and size of the adipose tissue and skeletal muscle were occasionally inadequate. However, each experiment was performed on tissue samples from only six independent patients.

2.1.2. Dosage information

To determine the effects of naringenin on placenta and VAT, these tissues were incubated in 10 ng/ml TNF, with or without 400 µM naringenin (Sigma, St Louis, USA). The optimised concentration of naringenin was based on the group's previously published study.[31]

2.1.3. Validation of explant cultures and viability

To determine the effect of treatment on cell membrane integrity, the release of the intracellular enzyme lactate dehydrogenase (LDH) into incubation medium was determined as described previously.[31] Neither in vitro incubation nor experimental treatment significantly affected LDH activity in the incubation medium (data not shown).

2.1.4. Glucose uptake

Skeletal muscle explants were performed as detailed above and glucose uptake was performed as previously described (see Fig 1A for experimental flowchart).[17, 38] Glucose uptake was performed on skeletal muscle obtained from six participants. After overnight incubation with TNF and naringenin, media was removed and replaced with Krebs buffer containing 0.5% BSA (fatty acid free) and 0.1 mM insulin for 10 min. Fifty µl of 3 µCi/ml [14C]-2-Deoxy-D-Glucose (2DG) (Perkin Elmer) was immediately added and incubated for a further 20 min. The tissue were removed and washed in ice-cold PBS (twice) and solubilised for 2 h in 0.5 ml 0.5 M sodium hydroxide at 60^{o}C. Tissues were neutralised with 0.5 ml 0.5 M hydrochloric acid and the supernatant was transferred to a vial containing 3 ml of liquid scintillation fluid. All samples were counted for radioactivity in a liquid scintillation counter.

2.1.5. Western blotting

Western blotting was performed as previously described.[17, 37] The following probes were used: rabbit monoclonal phosphorylated (Ser473) Akt (pAkt) (#4060; Cell Signalling, Beverly, MA, USA), diluted 1:5000 in blocking buffer (5% skim milk in TBS with 0.05% Tween-20); rabbit monoclonal

pan-Akt (#4685; Cell Signalling, Beverly, MA, USA), diluted 1:1000 (skeletal muscle) or 1:3000 (placenta and VAT) in blocking buffer; mouse monoclonal IκB-α (MA5-15132; ThermoFisher), diluted 1:1000 in blocking buffer; 0.4 μg/ml goat polyclonal α-actin (sc-1615; Santa Cruz, CA, USA); mouse monoclonal β-actin (A5316; Sigma, St Louis, Missouri), diluted 1:20000 in blocking buffer; 0.2 μg/ml mouse monoclonal phosphorylated Erk (pErk) (sc-7383; Santa Cruz, CA, USA); 0.2 μg/ml rabbit polyclonal Erk1/2 (sc-93, Santa Cruz, CA, USA); 0.1 μg/ml rabbit polyclonal phosphorylated p38 (p-p38) (sc-17852-R; Santa Cruz, CA, USA); and 0.4 μg/ml rabbit polyclonal p38 (sc-535, Santa Cruz, CA, USA). Membranes were imaged using the XRS ChemiDoc system (Bio-Rad Laboratories; Gladesville, NSW, Australia), and densitometry was performed using Image Lab 3.0 (Bio-Rad Laboratories; Gladesville, NSW, Australia).

2.1.6. Enzyme-linked immunosorbent assay

Media from the tissue explants were assayed using a sandwich ELISA to determine the protein concentrations of IL6 and CXCL8 (Life Technologies; Mulgrave, Vic, Australia), and CCL2 and CXCL1 (RnD Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The interassay and intraassay coefficients of variation for all assays were less than 10%.

2.1.7. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues using TRIreagent according to the manufacturer's instructions (Bioline, Alexandria, NSW, Australia). RNA concentration and purity were determined using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia). RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. After the cDNA was diluted to 1.3 ng/μl in sterile milli-Q water, qRT-PCR was performed on the cDNA as previously described.[38] The RT-PCR was performed using the CFX384 Real-Time PCR detection system (Bio-Rad Laboratories; Gladesville, NSW, Australia) using 100 nM of pre-designed and validated QuantiTect primers (primer sequences not available) (Qiagen; Chadstone Centre, Vic, Australia). Average gene Ct values were normalised against the housekeeping genes succinate dehydrogenase complex subunit A (SDHA) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ). There was no effect of experimental treatment on SDHA or YWHAZ mRNA expression. Fold differences were determined using the comparative Ct method.

2.1.8. Hydrogen peroxide (H_2O_2) assay

H_2O_2 assay was performed on media and tissue lysate from placenta and VAT samples, as previously described.[39] A standard curve was created using 1:2 serial dilutions of 200 μM H_2O_2 stock solution (200 to 3.125 μM). Forty μl of sample, standards and blank buffer were added to a 96-well plate, followed by 200 μL of working reagent, containing 1 part reagent A (25 mM Ammonium Ferrous (II) Sulphate and 2.5 M H_2SO_4) and 100 parts reagent B (100 mM Sorbitol and 125 μM xylenol orange). The plate was incubated for 30 min and absorbance read at 560 nm using a x-Mark microplate spectrotometer (Bio-Rad Laboratories).

2.2. Study 2: Effect of naringenin on GDM mice

Study 2 investigated the effect of naringenin on glucose tolerance, inflammation and antioxidant status in GDM-like mice. The heterozygous Leptin receptor deficient *db*/+ mouse is a well-characterised model, where female mice display a mild diabetic phenotype only during pregnancy and produce macrosomic pups.[40, 41] Therefore, for the *in vivo* studies, pregnant heterozygous B6.BKS(D)-*Lepr*^{db}/J (*db*/+) mice were used to generate an *in vivo* model of GDM.

2.2.1. Animals

Mice studies were approved by the Austin Health's Animal Ethics Committee (Project No: A2014 / 05227). Lepr$^{db/+}$ (*db*/+) mice (strain B6.BKS(D)-Lepr$^{db/+}$/J; stock number 000697) were provided by Professor Peter Thorn (University of Queensland), with approval from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed and maintained under standard laboratory conditions in the BioResources Facility at Austin Health (Heidelberg, VIC, Australia). Male and female *db/+* mice were mated, and tail samples were taken from offspring at three weeks of age for genotyping.

2.2.2. Dosage information

To determine the effects of naringenin in an *in vivo* mouse model, pregnant *db*/+ mice (from here on in, referred to as GDM mice) were administered either dimethyl sulfoxide (DMSO) (control) or 50 mg/kg body weight naringenin in DMSO by intraperitoneal (i.p.) injection once daily from gestation day (gd) 10. The optimised dosage was based on previously published animal studies.[27]

2.2.3. Experimental groups

Female *db/+* mice (8-10 weeks old) were time-mated with wild type males, and mating was confirmed by the presence of a copulatory plug the next morning, which was designated gestation day (gd) 0. Following daily i.p. treatment, on gd 17, glucose tolerance was determined via an oral glucose tolerance test (GTT) using 2 g/kg body weight glucose on 6 h fasted mice, as previously described.[42] Following the GTT, mice received a final delivery of either DMSO or naringenin, and were allowed to recover overnight. On gd 18, mice were fasted for 5 h and then culled by cervical dislocation. The abdominal cavity was opened, the fetuses removed, counted and weighed. The placenta, maternal VAT and subcutaneous adipose tissues (SAT) were obtained, weighed and frozen immediately in liquid nitrogen and stored at -80°C until further analysis by qRT-PCR. Maternal fat mass was determined by the combined weight of dissected fat from the intra-abdominal visceral (mesenteric, gonadal and retroperitoneal) and subcutaneous (dorsal) fat pads. RNA extraction and qRT-PCR analysis were performed as described in Study 1. Average gene Ct values were normalised against three housekeeping genes 18S rRNA, β-2-microglobulin (B2M), and hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Fold differences were determined using the comparative Ct method. Six control mice and seven mice treated with naringenin completed the study, for a total of 13 mice overall.

2.3 Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). For Study 1, Shapiro-Wilk test was used to test the normality of all the data. Data were analysed by a repeated measures one-way ANOVA (with LSD post-hoc testing to discriminate among the means); non-normally distributed data were logarithmically transformed before analysis.

For Study 2, an unpaired Student's t-test was used to assess statistical significance between normally distributed data; otherwise, the nonparametric Mann-Whitney U test was used for comparisons between groups (GDM mice vs. mice treated with naringenin).

Statistical significance was assigned to P value < 0.05. Data were expressed as mean ± SEM.

3. RESULTS

3.1. Study 1: Effect of naringenin on insulin resistance, inflammation and antioxidant expression in human placenta, adipose tissue and skeletal muscle *in vitro*

3.1.1. Naringenin improved glucose uptake in skeletal muscle

The effect of naringenin on glucose uptake in skeletal muscle was determined in the presence of TNF (Fig 1). As expected, insulin significantly increased glucose uptake by 33.8% (P=0.0054), while pre-incubation with TNF significantly decreased glucose uptake relative to insulin P=0.0009). Co-treatment with naringenin significantly restored glucose uptake by 29.3% (P=0.0305), compared to TNF treatment.

3.1.2. Naringenin decreased inflammation in placenta and VAT

The effect of naringenin on TNF-induced expression and secretion of pro-inflammatory cytokines and chemokines in placenta is depicted in Fig 2. TNF treatment significantly increased IL1B, IL6 and CXCL8 mRNA expression (Fig 2B, 2C, 2I), and IL6, CCL2 and CXCL8 protein release (Fig 2D, 2F, 2J). There was no significant effect of TNF on IL1A, CCL2 and CXCL1 mRNA expression (Fig 2A, 2E, 2G), and CXCL1 protein release (Fig 2H). Co-treatment with naringenin significantly reduced TNF-induced IL1B, IL6, CXCL1 and CXCL8 mRNA expression (Fig 2B, 2C, 2G, 2I) and IL6, CCL2, CXCL1 and CXCL8 protein release (Fig 2D, 2F, 2H, 2J). There was no effect of naringenin on TNF-induced IL1A and CCL2 mRNA expression (Fig 2A, 2E). The effect of naringenin treatment in VAT was also studied, and is depicted in Fig 3. TNF significantly increased IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA expression (Fig 3A-C, 3E, 3G, 3I) and IL6, CCL2, CXCL1 and CXCL8 protein secretion (Fig 3D, 3F, 3H, 3J). Co-treatment with naringenin significantly attenuated IL1A, IL1B and CCL2 mRNA expression (Fig 3A, 3B, 3E) and CCL2, CXCL1 and CXCL8 protein release (Fig 3F, 3H, 3J). There was, however, no effect of naringenin on TNF-induced IL6, CXCL1 and CXCL8 mRNA expression (Fig 3C, 3G, 3I) and IL6 protein release (Fig 3D).

3.1.3. Naringenin regulates antioxidant activity in placenta and VAT

The effect of naringenin on antioxidant activity in placenta and VAT is depicted in Fig 4. In placenta, there was no effect of TNF on antioxidant mRNA expression (Fig 4A-C) nor on H_2O_2 concentration in either media or tissue lysate (Fig 4D, 4E). Naringenin co-treatment significantly increased catalase mRNA expression (Fig 4B), while reducing H_2O_2 concentration in placental media compared with TNF (Fig 4E). There was no significant effect of naringenin on superoxide dismutase (SOD)-1 and glutathione reductase (GR) mRNA expression (Fig 4A, 4C), or H_2O_2 concentration in tissue lysate compared with TNF (Fig 4D). In VAT, TNF significantly reduced SOD1 mRNA expression (Fig 4F), but there was no effect of TNF on catalase or GR mRNA expression, or H_2O_2 concentration in tissue lysate (Fig 4G, 4H, 4I). Naringenin co-treatment significantly increased SOD1, catalase and GR mRNA expression (Fig 4F-H), while significantly reducing H_2O_2 concentration in tissue lysate compared with TNF (Fig 4I).

3.1.4. Naringenin inhibited the NF-κB pathway in placenta, but not VAT or skeletal muscle

The mechanistic pathways through which naringenin exerts its effects was explored in placenta, VAT and skeletal muscle stimulated with TNF and the data summarised in Fig 5. Three pathways known to be regulated by naringenin were studied: NF-κB[31], Akt[34] and MAPK[35]. In unstimulated cells, the NF-κB complex exists in an inactive form, bound to IκB-α. Stimulation with pro-inflammatory cytokines induces phosphorylation and subsequent degradation of IκB-α, allowing the NF-κB complex to dissociate and translocate to the nucleus.[43, 44] TNF induced degradation of IκB-α protein expression in placenta and VAT. Treatment with naringenin inhibited TNF-induced IκB-α degradation in placenta (Fig 5A). However, there was no effect of naringenin on TNF-induced degradation of IκB-α protein expression in VAT or skeletal muscle (Fig 5B, C). For all three tissues, there was no effect of naringenin on Akt or MAPK (Supporting Information Fig 1).

3.2. Study 2: Effect of naringenin in GDM mice

3.2.1. Naringenin improved glucose tolerance in GDM mice

Following OGTT on gd 17, a mild hyperglycemic phenotype in GDM mice compared to pregnant wild-type control mice was confirmed (see Supporting Information Fig 2). The effect of naringenin on the glucose tolerance of GDM mice, presented in Fig 6, was then tested. Naringenin treatment significantly reduced fasting blood glucose levels by 15% (P=0.0127) (Fig 6A) and area under the curve (AUC) by 12.5% (P=0.0283) (Fig 6B, C) compared to control-treated mice.

3.2.2. Naringenin had little effect on expression of pro-inflammatory cytokines and chemokines in placenta and adipose tissue from GDM mice

The effect of naringenin on pro-inflammatory cytokine and chemokine mRNA expression in placenta, VAT and SAT of GDM mice is presented in Fig 7. There was no effect of naringenin treatment in placenta, when compared to control-treated mice (Fig 7A-E). Naringenin treatment significantly decreased IL1A mRNA expression in VAT compared to control-treated mice (Fig 7F). There was no

effect of naringenin treatment on IL1B, IL6, CCL2 or CXCL1 mRNA expression in VAT (Fig 7G-J) or IL1A, IL1B, IL6, CCL2 or CXCL1 mRNA expression in SAT (Fig 7K-N).

3.2.3. Naringenin increased antioxidant expression in placenta and adipose tissue of GDM mice

The effect of naringenin on antioxidant mRNA expression in placenta, VAT and SAT of GDM mice is presented in Fig 8. Compared to control-treated mice, naringenin treatment reduced GR mRNA expression (Fig 8C), but had no effect on SOD1 or catalase in placenta (Fig 8A, 8B). In VAT, GR mRNA expression was increased (Fig 8F), catalase mRNA expression was reduced compared to control-treated mice (Fig 8E), and there was no effect on SOD1 mRNA expression (Fig 8D). In SAT, naringenin treatment significantly increased SOD1 mRNA expression compared to control-treated mice (Fig 8G), but there was no effect on either catalase or GR mRNA expression (Fig 8H, 8I).

4. DISCUSSION

Maternal peripheral insulin resistance, low grade inflammation and oxidative stress are central features of GDM.[11, 15, 16, 18, 20-22, 45] A key strength of this study is the combined use of *in vitro* and *in vivo* models to investigate the ability of naringenin to disrupt pathways known to GDM pathophysiology. *In vitro*, TNF was used to recapitulate inflammation and impaired skeletal muscle glucose uptake associated with GDM pregnancies.[38] It was found that naringenin treatment significantly improved insulin-stimulated glucose uptake in human skeletal muscle compared to TNF treatment. Naringenin also reduced TNF-stimulated inflammation and increased antioxidant mRNA expression in human placenta and VAT. In placenta, the effects of naringenin appear to stem primarily from inhibition of NF-κB activation. *In vivo,* using the mouse heterogeneous leptin receptor gene knockout (*db/+*) model for GDM[40], this study established that naringenin could improve fasting basal glucose levels end enhance glucose tolerance. The combined *in vitro* and *in vivo* findings suggest that naringenin may be a potential glucose-sensitising therapeutic for GDM.

Insulin resistance is a central feature of GDM. There is extensive evidence to suggest that low-grade maternal inflammation contributes to the peripheral insulin resistance characteristic of GDM.[15, 38] Several studies have demonstrated that pro-inflammatory mediators, such as TNF, and pathogenic factors, such as bacterial and viral products, can induce defects within the insulin signalling pathway in skeletal muscle.[38] This dysfunction in the insulin signalling pathway leads to impaired insulin-mediated uptake of glucose into skeletal muscles and the subsequent development of hyperglycemia.[38] Thus, this study sought to determine the effect of naringenin on glucose uptake in human skeletal muscle stimulated with TNF. As expected, insulin-only treatment significantly increased glucose uptake, while TNF treatment blunted this response. Co-treatment with naringenin returned glucose uptake to insulin-only levels. On testing the effect of naringenin on insulin resistance in GDM mice, naringenin improved glucose tolerance, compared to vehicle controls. Interestingly, naringenin improved fasting glucose in GDM mice by only 15% compared to at least

30% improvements typically found in other animal models of type 2 diabetes.[27, 28] However, this may be attributed to longer treatment lengths of at least five weeks. This is not applicable in pregnant mice, due to their short gestational period of only 19-21 days. While only the effect of naringenin on placenta and adipose tissue in the animal model was assessed in this study, it is possible that naringenin may also target other organs of the GDM mice including the pancreas to influence insulin or glucagon secretion. Indeed other studies have shown that naringenin treatment of high-fat-diet rats could reduce plasma insulin and glucose levels.[26, 27] However, this was not assessed in this study, and further research is required to elucidate the effects of naringenin on pancreatic insulin secretion. Nevertheless, this study found that naringenin improved glucose metabolism in human skeletal muscle and in GDM mice.

Low-grade maternal inflammation is also associated with GDM, with several studies demonstrating increased presence of circulating pro-inflammatory mediators in women with GDM.[15, 16] The pro-inflammatory cytokine TNF, which is increased in women with GDM[15] is positively associated with insulin resistance in pregnancy[16], and increases in CXCL8 and CCL2 markers of inflammation in both placenta and adipose tissue.[20, 21] Thus, this study sought to determine if naringenin could reduce inflammation in placenta and adipose tissue in human tissues and in GDM mice. *In vitro,* naringenin treatment significantly reduced TNF-induced mRNA expression and protein secretion of pro-inflammatory cytokines and chemokines in both human placenta and VAT. Interestingly, *in vivo*, naringenin treatment had no effect on mRNA expression of pro-inflammatory cytokines and chemokines, except to reduce IL1A mRNA expression in VAT. This discrepancy with *in vitro* results may be due to several factors in the experimental design of the *in vivo* study, such as the dosage and the route of administration. While this study applied a dose of 50 mg/kg, other studies in liver injury and lung fibrosis have used doses of up to 800 mg/kg naringenin supplementation.[46, 47] Further, this study administered naringenin systemically through i.p. injection which may have diluted the anti-inflammatory effects observed in the *in vitro* model, where naringenin was directly added to the tissue explant. Treating mice with higher dosages, or from start of gestation at gd 0, may instead elicit stronger anti-inflammatory effects. Notwithstanding these *in vivo* limitations, this study demonstrated that naringenin has potent anti-inflammatory effects in an *in vitro* model of GDM.

Oxidative stress is another key feature of GDM, and is characterised by differential antioxidant expression and increases in reactive oxygen species.[20, 22] Animal models of diabetes have shown that naringenin improves SOD, catalase, GPx and GR activity in the liver, kidney and brain.[32, 33] Similarly, in this study, *in vitro*, naringenin treatment significantly upregulated catalase mRNA expression in placenta and SOD1, catalase and GR mRNA expression in VAT. Given these promising results, the total redox status of the tissues was studied by assessing H_2O_2 concentration in media and tissue lysate. In placenta, it was found that naringenin significantly reduced H_2O_2 concentration in media but not tissue lysate, while in VAT, naringenin significantly reduced H_2O_2 concentration in tissue lysate. That there was no change in oxidative stress in placental lysate and even a reduction in VAT lysate suggests that the observed increases in antioxidant mRNA expression were not due to an adaptive response, but rather due to antioxidant-promoting properties of naringenin. *In vivo,* naringenin also significantly increased GR mRNA expression in VAT and SOD1 mRNA expression in

SAT; no effect of naringenin was observed in placenta. These results suggest there may be tissue specificity, as naringenin appears to have more potent antioxidant effects in adipose tissue compared to placenta. This would have been further supported by an *in vitro* examination of its effect on SAT, however, this was prevented by limited tissue availability. Further, our *in vivo* study examined the effect of naringenin only on mRNA expression, but not activity of the antioxidants. Notwithstanding these limitations, to our knowledge, this is the first paper to report naringenin-induced increases in antioxidant mRNA expression in placenta and VAT in both a human and an animal model of GDM.

Previously published studies have shown that naringenin may elicit its effects by inhibiting a number of signalling pathways including NF-κB[31], Akt[34] and MAPK[35]. In this study, naringenin significantly suppressed TNF-induced degradation of IκB-α in placenta, suggesting that naringenin may elicit it effects by inhibiting activation of NF-κB. These data correspond with our previous findings that naringenin inhibits NF-κB activity in placenta[31], and adds to the evidence that naringenin inhibits the NF-κB pathway across various cell types.[30, 48] On the other hand, there was no effect of naringenin on IκB-α protein expression in VAT or skeletal muscle, suggesting that naringenin elicits its effects in these tissues via different mechanisms. There was also no effect of naringenin on activation of Akt, or the MAPK proteins Erk1/2 or p38 MAPK in all three tissues. In addition to these pathways, naringenin can act upon JNK[49], activator protein (AP)-1 transcription factor[50], suppressor of cytokine signalling (SOCS)-3[51], peroxisome proliferator-activated receptor (PPAR)[52], 5' AMP-activated protein kinase (AMPK) signalling[35] and transcription factor EB-dependent cytokine degradation.[53] Further investigation is required to confirm that naringenin acts through these pathways.

There are several limitations to these studies. In the *in vitro* study, due to limited tissue availability and antibody discontinuation, the effect of naringenin on protein expression of specific insulin signalling pathway proteins could not be examined in skeletal muscle. Though it would be of interest to compare differences in VAT and SAT inflammatory response, we were unable to obtain adequate, quality human SAT samples for the *in vitro* experiments. With regards to the *in vivo* study, a recent study by Plows et al[54] has placed the *db*/+ model in recent controversy, presenting the model as inappropriate for future studies of GDM. The model employed in this study replicated the mild glucose intolerance (see Supporting Information Fig 2), and increased inflammation in placenta and adipose tissue (Lappas, unpublished) associated with human GDM in the *db*/+ mice compared to WT mice. The model was thus found appropriate for this investigation. However, the limitations identified in the *db*/+ mouse model are recognised and for future studies other mouse models will be explored, for example the pregnant adiponectin-deficient mouse model.[55] This will allow for future study of fetal outcomes, such as the effect of naringenin on fetal and placental birth weight, fetal inflammation, and long-term offspring outcomes. Notwithstanding these limitations, a key strength of this study is the combined use of *in vitro* and *in vivo* experiments to investigate the effects of naringenin on glucose tolerance, inflammation and oxidative stress associated with GDM. *In vitro* findings were confirmed by the *in vivo* experiments, thus creating a strong argument for the potential of naringenin as a novel therapeutic for GDM. The safety and non-toxicity of naringenin in

humans further creates a convincing argument for its use. Another strength of the *in vitro* study is the ability to perform experiments in VAT and skeletal muscle from pregnant women. The addition of these tissues demonstrate the potency of naringenin across various sites, and its ability to act upon all facets of GDM pathophysiology.

In summary, GDM leads to several long-term health risks, including obesity and type 2 diabetes, for both mother and child.[9, 10] Current therapeutics, however, attempt only to manage the resulting hyperglycemia, and have no effect on the inflammation associated with GDM nor the long-term adverse fetal outcomes.[9, 23] Therefore, it is necessary to find other means of preventing GDM that will target all aspects of the condition. To our knowledge, this is the first study to report the polyphenol naringenin reduces inflammation and induces antioxidant expression in human placenta and adipose tissue obtained from pregnant women, as well as improves human skeletal muscle insulin resistance associated with GDM *in vitro*. This study has provided further support to a role in NF-κB regulation by naringenin. This study also demonstrates that naringenin supplementation via i.p. improves glucose tolerance of GDM mice. GDM is known to create both short- and long-term health risks to the fetus.[6-10] Therefore, future efforts may focus on examining the effects of naringenin treatment on fetal outcomes, which may be examined by assessing placental nutrient transfer, fetal birth weight, fetal inflammation and long-term offspring health.

AUTHOR CONTRIBUTIONS

M.L. conceived and designed the study, and critically reviewed the manuscript. C.N.N. performed experiments, analysed the data and wrote the manuscript. J.W. critically reviewed the study and manuscript.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

5. REFERENCES

[1] Diagnostic Criteria and Classification of Hyperglycaemia First Detected in Pregnancy, World Health Organization Copyright (c) World Health Organization 2013, Geneva **2013**

[2] D. A. Sacks, D. R. Hadden, M. Maresh, C. Deerochanawong, A. R. Dyer, B. E. Metzger, L. P. Lowe, D. R. Coustan, M. Hod, J. J. Oats, B. Persson, E. R. Trimble, *Diabetes Care* **2012**, *35*, 526. 10.2337/dc11-1641/-/DC1.

[3] A. Ferrara, *Diabetes care* **2007**,

[4] N. A. Beischer, J. N. Oats, O. A. Henry, M. T. Sheedy, J. E. Walstab, *Diabetes* **1991**, *40 Suppl 2*, 35. 10.2337/diab.40.2.S35.

[5] M. Abouzeid, V. L. Versace, E. D. Janus, M.-A. Davey, B. Philpot, J. Oats, J. A. Dunbar, *BMJ Open* **2014**, *4*, e005394. 10.1136/bmjopen-2014-005394.

[6] P. M. Catalano, A. Thomas, L. Huston-Presley, S. B. Amini, *Am. J. Obstet. Gynecol.* **2003**, *189*, 1698. 10.1016/S0002-9378(03)00828-7.

[7] O. Langer, Y. Yogev, O. Most, E. M. J. Xenakis, *Am. J. Obstet. Gynecol.* **2005**, *192*, 989. 10.1016/j.ajog.2004.11.039.

[8] *AIHW* **2010**, *Cat. no. CVD 52*,

[9] P. Damm, A. Houshmand-Oeregaard, L. Kelstrup, J. Lauenborg, E. R. Mathiesen, T. D. Clausen, *Diabetologia* **2016**, *59*, 1396. 10.1007/s00125-016-3985-5.

[10] J. Malcolm, *Diabetes/Metab. Res. Rev.* **2012**, *28*, 307. 10.1002/dmrr.2275.

[11] P. M. Catalano, L. Huston, S. B. Amini, S. C. Kalhan, *Am. J. Obstet. Gynecol.* **1999**, *180*, 903. 10.1016/S0002-9378(99)70662-9.

[12] X. Zhang, Q. Liao, F. Wang, D. Li, *Medicine* **2018**, *97*, e11891. 10.1097/MD.0000000000011891.

[13] H. Gogeneni, N. Buduneli, B. Ceyhan-Öztürk, P. Gümüş, A. Akcali, I. Zeller, D. E. Renaud, D. A. Scott, Ö. Özçaka, *Journal of clinical periodontology* **2015**, *42*, 506. 10.1111/jcpe.12418.

[14] T. Lekva, E. R. Norwitz, P. Aukrust, T. Ueland, *Curr Diab Rep* **2016**, *16*, 26. 10.1007/s11892-016-0715-9.

[15] E. Korkmazer, N. Solak, *J. Obstet. Gynaecol.* **2015**, *35*, 142. 10.3109/01443615.2014.948408.

[16] J. P. Kirwan, S. Hauguel-De Mouzon, J. Lepercq, J. Challier, L. Huston-Presley, J. E. Friedman, S. C. Kalhan, P. M. Catalano, *Diabetes* **2002**, *51*, 2207. 10.2337/diabetes.51.7.2207.

[17] S. Liong, M. Lappas, *Mol. Cell. Endocrinol.* **2016**, *425*, 11. 10.1016/j.mce.2016.02.016.

[18] S. Liong, M. Lappas, *J. Physiol. Biochem.* **2015**, *71*, 703. 10.1007/s13105-015-0435-7.

[19] M. Lappas, *Metabolism* **2015**, 642. 10.1016/j.metabol.2015.02.002.

[20] M. Lappas, M. Permezel, G. E. Rice, *J. Clin. Endocrinol. Metab.* **2004**, *89*, 5627. 10.1210/jc.2003-0320975627.

[21] M. Lappas, K. Yee, M. Permezel, G. E. Rice, *Placenta* **2006**, *27*, 568. 10.1016/j.placenta.2005.06.003.

[22] M. Lappas, U. Hiden, G. Desoye, J. Froehlich, S. Hauguel-de Mouzon, A. Jawerbaum, *Antioxid. Redox Signaling* **2011**, *15*, 3061. 10.1089/ars.2010.3765.

[23] C. Billionnet, D. Mitanchez, A. Weill, J. Nizard, F. Alla, A. Hartemann, S. Jacqueminet, *Diabetologia* **2017**, *60*, 636. 10.1007/s00125-017-4206-6.

[24] M. K. Hossain, A. A. Dayem, H. Jihae, Y. Yingfu, K. Kyeongseok, S. K. Saha, Y. Gwang-Mo, C. Hye Yeon, C. Ssang-Goo, *International Journal of Molecular Sciences* **2016**, *17*, 1. 10.3390/ijms17040569.

[25] E. Capobianco, D. Gomez Ribot, D. Fornes, T. L. Powell, C. Levieux, T. Jansson, A. Jawerbaum, *Molecular Nutrition & Food Research* **2018**, *62*, 1800263. 10.1002/mnfr.201800263.

[26] R. Mutlur Krishnamoorthy, A. Carani Venkatraman, *Chem Biol Interact* **2017**, *275*, 95. 10.1016/j.cbi.2017.07.016.

[27] S. Kannappan, C. V. Anuradha, *Eur. J. Nutr.* **2010**, *49*, 101. 10.1007/s00394-009-0054-6.

[28] B. Ren, W. Qin, F. Wu, S. Wang, C. Pan, L. Wang, B. Zeng, S. Ma, J. Liang, *Eur. J. Pharmacol.* **2016**, *773*, 13. 10.1016/j.ejphar.2016.01.002.

[29] F. A. Pinho-Ribeiro, A. C. Zarpelon, V. Fattori, M. F. Manchope, S. S. Mizokami, R. Casagrande, J. W. A. Verri, *Neuropharmacology* **2016**, *105*, 508. 10.1016/j.neuropharm.2016.02.019.

[30] F. A. Pinho-Ribeiro, A. C. Zarpelon, S. S. Mizokami, S. M. Borghi, J. Bordignon, R. L. Silva, T. M. Cunha, J. C. Alves-Filho, F. Q. Cunha, R. Casagrande, W. A. Verri, Jr., *J. Nutr. Biochem.* **2016**, *33*, 8. 10.1016/j.jnutbio.2016.03.013.

[31] R. Lim, G. Barker, C. A. Wall, M. Lappas, *Mol. Hum. Reprod.* **2013**, *19*, 451. 10.1093/molehr/gat015.

[32] O. M. Ahmed, M. A. Hassan, S. M. Abdel-Twab, M. N. Abdel Azeem, *Biomed. Pharmacother.* **2017**, *94*, 197. 10.1016/j.biopha.2017.07.094.

[33] S. Roy, F. Ahmed, S. Banerjee, U. Saha, *Pharm. Biol.* **2016**, *54*, 1616. 10.3109/13880209.2015.1110599.

[34] S. Chen, Y. Ding, W. Tao, W. Zhang, T. Liang, C. Liu, *Food Chem. Toxicol.* **2012**, *50*, 3025. 10.1016/j.fct.2012.06.006.

[35] X. Liu, N. Wang, S. Fan, X. Zheng, Y. Yang, Y. Zhu, Y. Lu, Q. Chen, H. Zhou, J. Zheng, *Sci. Rep.* **2016**, *6*, 39735. 10.1038/srep39735.

[36] M. Lappas, *Metabolism* **2014**, *63*, 250. 10.1016/j.metabol.2013.10.001.

[37] M. Lappas, *Mol. Cell. Endocrinol.* **2014**, *382*, 74. 10.1016/j.mce.2013.09.011.

[38] H. T. Tran, S. Liong, R. Lim, G. Barker, M. Lappas, *PLoS One* **2017**, *12*, e0173373. 10.1371/journal.pone.0173373.

[39] R. Hastie, M. Lappas, *Placenta* **2014**, *35*, 673. 10.1016/j.placenta.2014.06.368.

[40] R. C. Kaufmann, K. S. Amankwah, G. Dunaway, L. Maroun, J. Arbuthnot, J. W. Roddick, Jr., *Am. J. Obstet. Gynecol.* **1981**, *141*, 479. 10.1016/S0002-9378(15)33263-4.

[41] R. C. Pasek, M. Gannon, *Am. J. Physiol.* **2013**, *305*, E1327. 10.1152/ajpendo.00425.2013.

[42] S. Andrikopoulos, A. R. Blair, N. Deluca, B. C. Fam, J. Proietto, *Am J Physiol Endocrinol Metab* **2008**, *295*, E1323. 10.1152/ajpendo.90617.2008.

[43] A. Oeckinghaus, S. Ghosh, *Cold Spring Harbor Perspect. Biol.* **2009**, *1*, a000034. 10.1101/cshperspect.a000034.

[44] D. C. Scherer, J. A. Brockman, Z. Chen, T. Maniatis, D. W. Ballard, *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 11259. 10.1073/pnas.92.24.11259.

[45] S. Liong, M. Lappas, *PLoS ONE* **2015**, *10*, 1. 10.1371/journal.pone.0122633.

[46] Y. Lv, B. Zhang, G. Xing, F. Wang, Z. Hu, *Food Funct.* **2013**, *4*, 297. 10.1039/c2fo30213f.

[47] G. Du, L. Jin, X. Han, Z. Song, H. Zhang, W. Liang, *Cancer Res.* **2009**, *69*, 3205. 10.1158/0008-5472.CAN-08-3393.

[48] L. W. Soromou, Z. Zhang, R. Li, N. Chen, W. Guo, M. Huo, S. Guan, J. Lu, X. Deng, *Molecules* **2012**, *17*, 3574. 10.3390/molecules17033574.

[49] H. Yoshida, W. Watanabe, H. Oomagari, E. Tsuruta, M. Shida, M. Kurokawa, *J. Nutr. Biochem.* **2013**, *24*, 1276. 10.1016/j.jnutbio.2012.10.003.

[50] C. Bodet, V. D. La, F. Epifano, D. Grenier, *J. Periodontal Res.* **2008**, *43*, 400. 10.1111/j.1600-0765.2007.01055.x.

[51] L. H. Wu, C. Lin, H. Y. Lin, Y. S. Liu, C. Y. Wu, C. F. Tsai, P. C. Chang, W. L. Yeh, D. Y. Lu, *Mol. Neurobiol.* **2016**, *53*, 1080. 10.1007/s12035-014-9042-9.

[52] A. K. Singh, V. Raj, A. K. Keshari, A. Rai, P. Kumar, A. Rawat, B. Maity, D. Kumar, A. Prakash, A. De, A. Samanta, B. Bhattacharya, S. Saha, *Chem.-Biol. Interact.* **2018**, *280*, 33. 10.1016/j.cbi.2017.12.007.

[53] L. Jin, W. Zeng, F. Zhang, C. Zhang, W. Liang, *J. Immunol.* **2017**, *199*, 3466. 10.4049/jimmunol.1602016.

[54] J. F. Plows, X. Yu, R. Broadhurst, M. H. Vickers, C. Tong, H. Zhang, H. Qi, J. L. Stanley, P. N. Baker, *Sci. Rep.* **2017**, *7*, 45130. 10.1038/srep45130.

[55] L. Qiao, J. S. Wattez, S. Lee, A. Nguyen, J. Schaack, W. W. Hay, Jr., J. Shao, *Diabetes* **2017**, *66*, 1126. 10.2337/db16-1096.

FIGURE LEGENDS

Fig 1. Effect of naringenin on glucose uptake in TNF-stimulated skeletal muscle.

(A) Flow chart of the experimental protocol for glucose uptake in human skeletal muscle. **(B)** Glucose uptake was measured by a radiolabelled 2DG uptake assay (n=6 patients). For all data, the fold change was calculated relative to Ins. All data is displayed as mean ± SEM. #*P*<0.05 vs. Ins and **P*<0.05 vs. TNF (repeated measures one-way ANOVA).

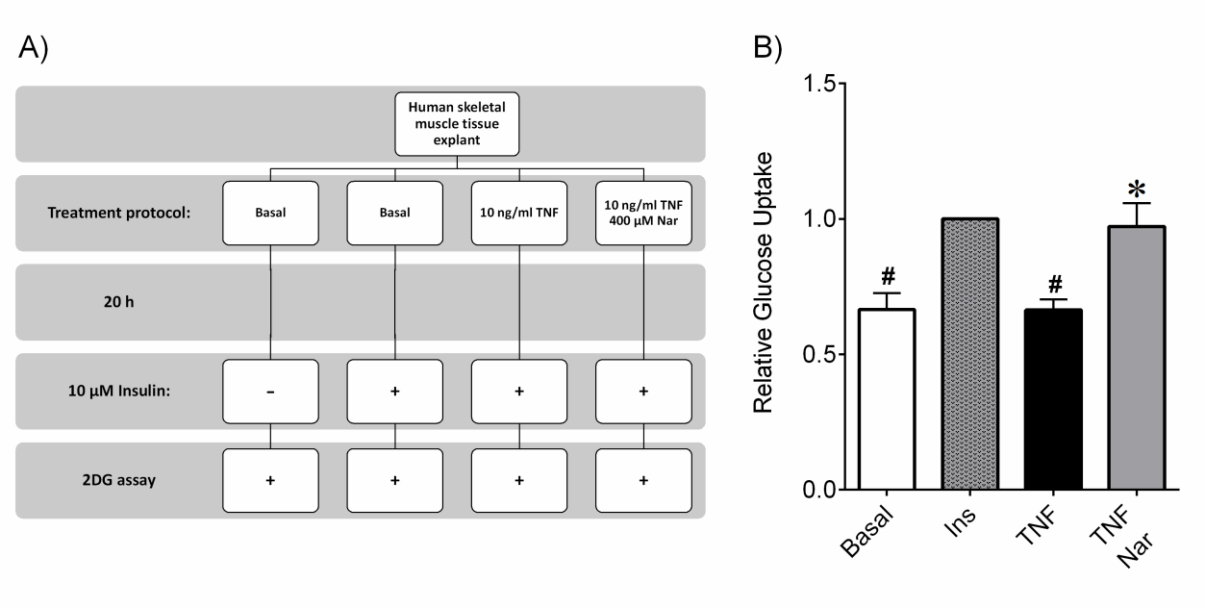


Fig 2. Effect of naringenin on TNF-induced pro-inflammatory gene expression and protein secretion in placenta.

Human placenta was incubated with 10 ng/ml TNF in the absence or presence of 400 µM naringenin (Nar) for 20 h (n=6 patients). **(A-C, E, G, I)** IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA expression was analysed by qRT-PCR. **(D, F, H, J)** IL6, CCL2, CXCL1 and CXCL8 concentrations in the incubation media were analysed by ELISA. All data is displayed as mean ± SEM. **P* <0.05 vs. TNF (repeated measures one-way ANOVA).

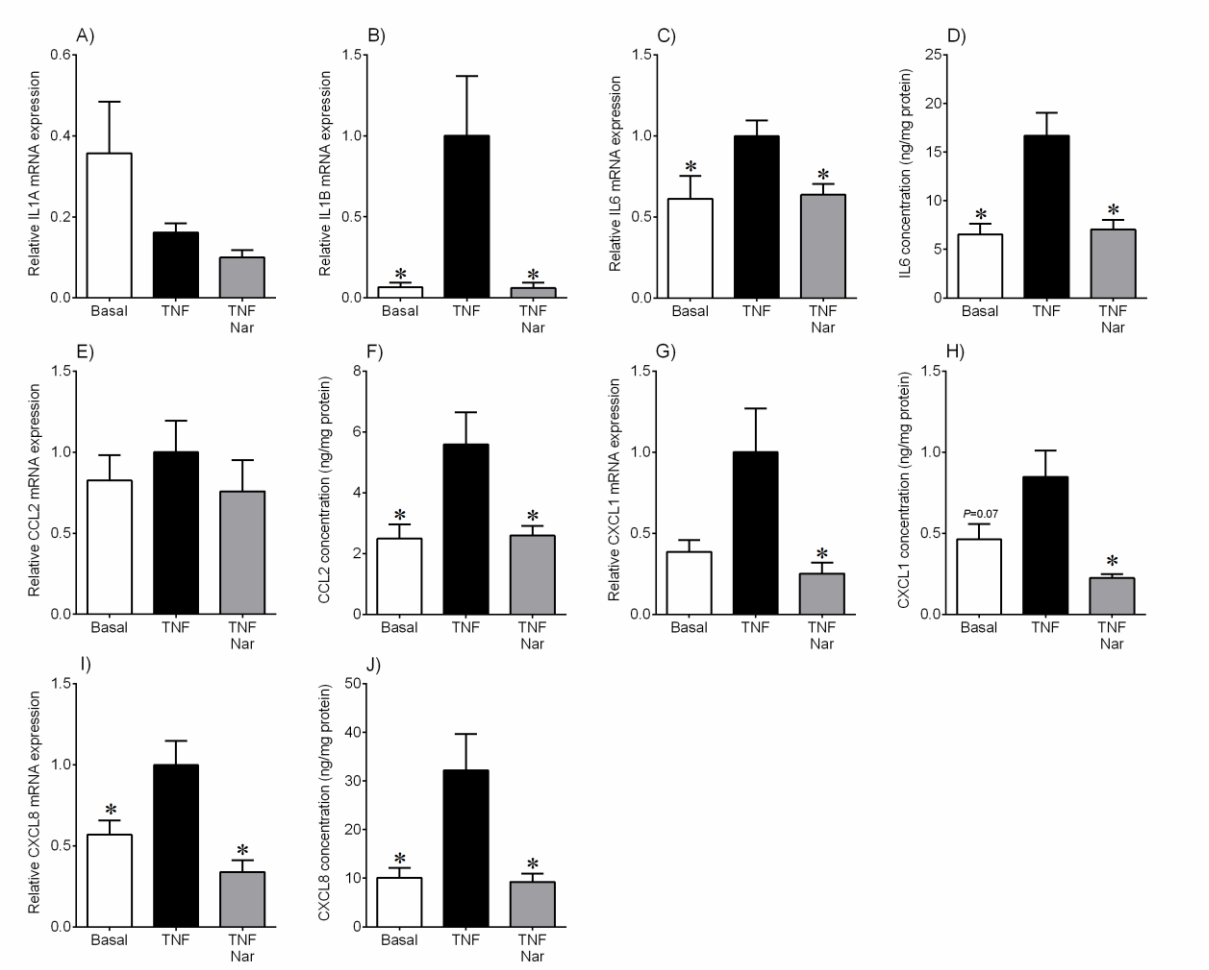


Fig 3. Effect of naringenin on TNF-induced pro-inflammatory gene expression and protein secretion in VAT.

Human VAT was incubated with 10 ng/ml TNF in the absence or presence of 400 µM naringenin (Nar) for 20 h (n=6 patients). **(A-C, E, G, I)** IL1A, IL1B, IL6, CCL2, CXCL1 and CXCL8 mRNA expression was analysed by qRT-PCR. **(D, F, H, J)** IL6, CCL2, CXCL1 and CXCL8 concentrations in the incubation media were analysed by ELISA. All data is displayed as mean ± SEM. **P* <0.05 vs. TNF (repeated measures one-way ANOVA).

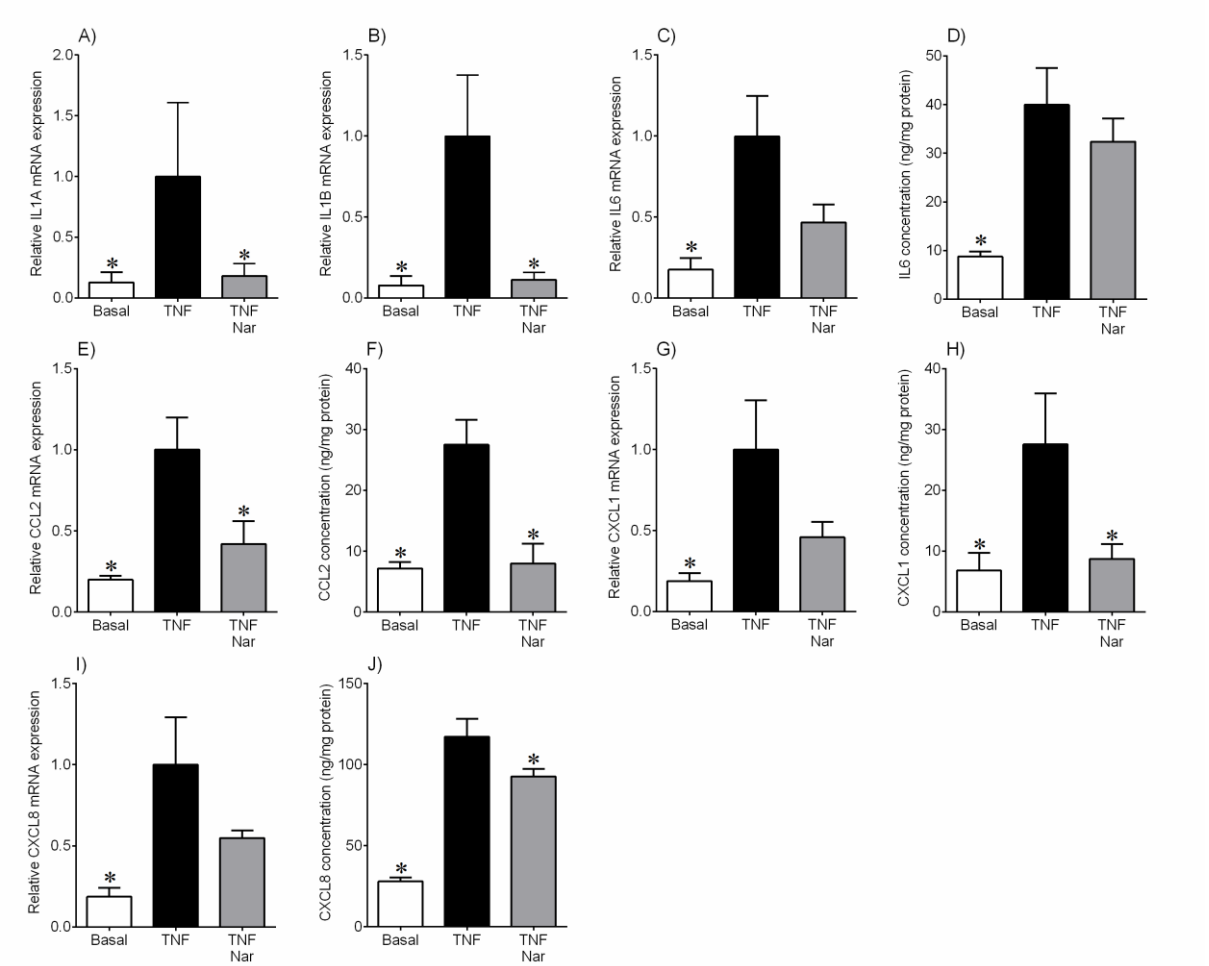


Fig 4. Effect of naringenin on antioxidant gene expression in TNF-stimulated placenta and VAT.

Human **(A-E)** placenta and **(F-I)** VAT were incubated with 10 ng/ml TNF in the absence or presence of 400 µM naringenin (Nar) for 20 h (n=6 patients). **(A, F)** SOD1, **(B, G)** catalase and **(C, H)** GR mRNA expression was analysed by qRT-PCR and fold change was calculated relative to the TNF group. Media from placenta **(E)**, and tissue lysate from placenta **(D)** and VAT **(I)**, were used to assess H_2O_2 concentration. All data is displayed as mean ± SEM. **P* <0.05 vs. TNF (repeated measures one-way ANOVA).

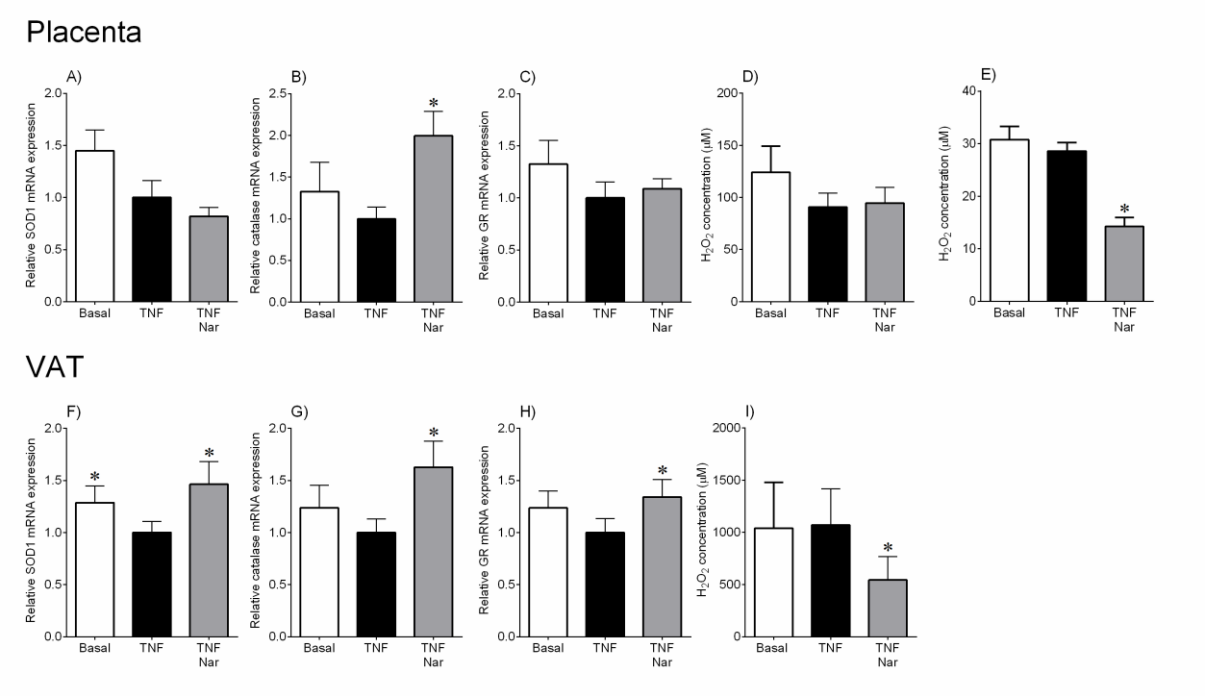


Fig 5. Effect of naringenin on activation of NF-κB in placenta, VAT and skeletal muscle.

Human **(A)** placenta, **(B)** VAT and **(C)** skeletal muscle were incubated in the absence or presence of 400 μM naringenin (Nar) for 20 h, before stimulation with 10 ng/ml TNF for 30 min (n=3-5 patients per group). Total IκB-α protein expression was analysed by Western blotting. A representative Western blot from 1 patient is shown. For all data, the fold change was calculated relative to the TNF group and data is displayed as mean ± SEM. **P* <0.05 vs. TNF (repeated measures one-way ANOVA).

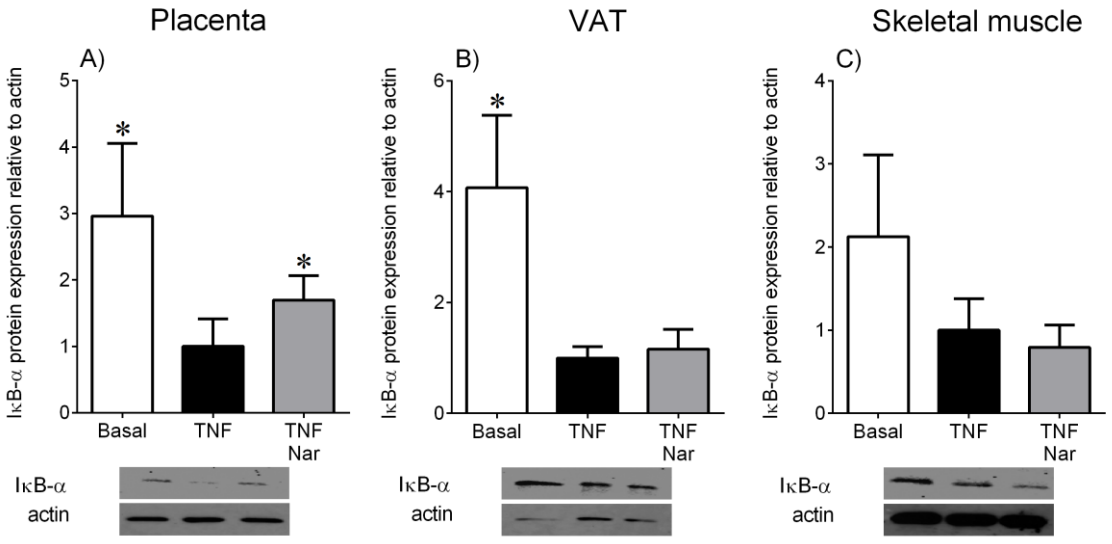


Fig 6. Effect of naringenin on glucose metabolism in GDM mice.

GDM mice were administered daily doses of 50 mg/kg naringenin (Nar) or vehicle (control) by i.p. injection from gd 10 until gd 17. **(A)** Maternal fasting glucose, **(B)** AUC and **(C)** blood glucose levels were measured via tail vein bleeding on gd 17, immediately following a 6 h fast. The data is presented as mean ± SEM. *P<0.05 vs. control (unpaired-sample comparison).

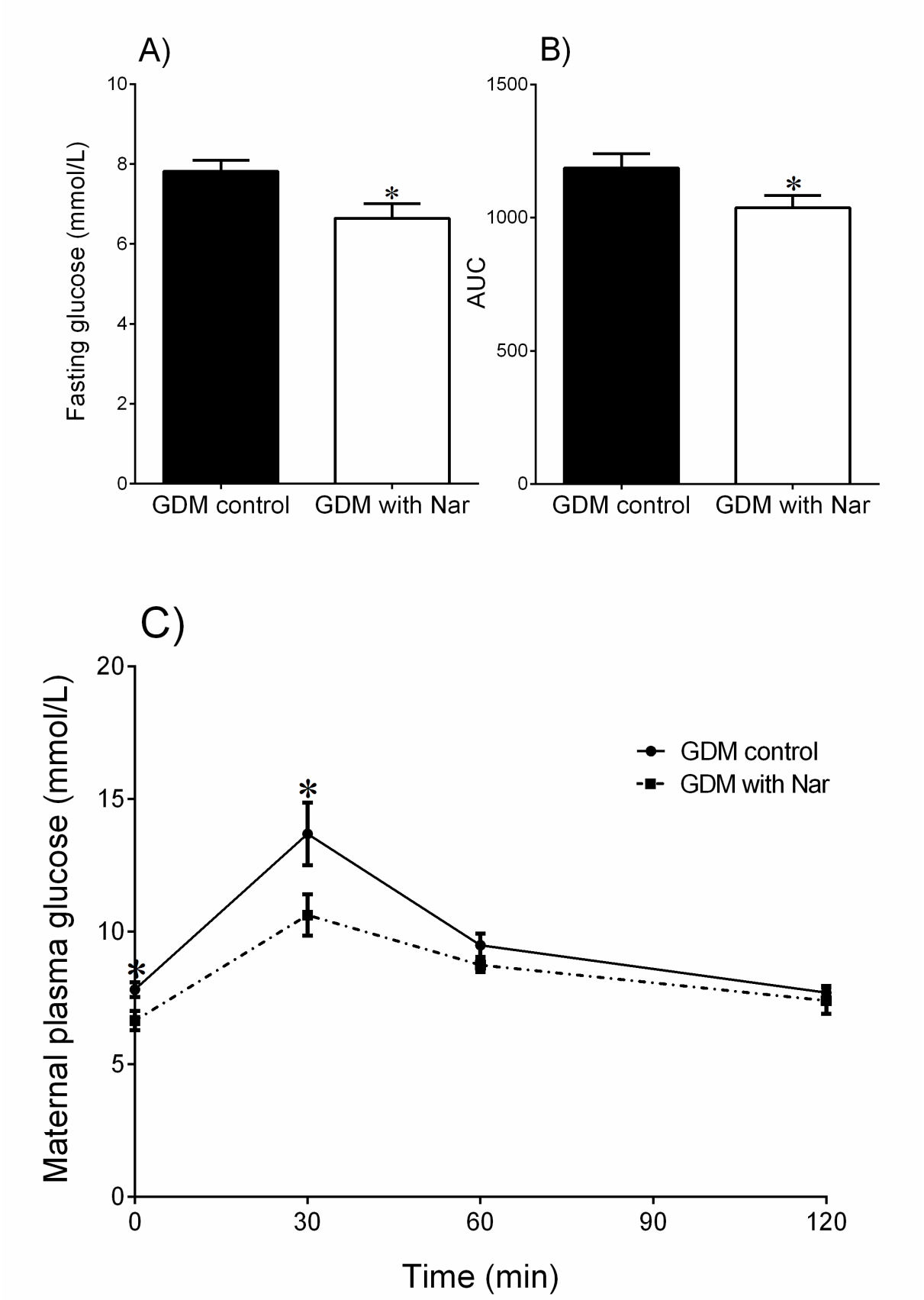


Fig 7. Effect of naringenin on inflammation in placenta, VAT and SAT of GDM mice.

GDM mice were administered daily doses of 50 mg/kg naringenin (Nar) or a vehicle control by intraperitoneal injection (i.p.) commencing at gd 10 until gd 17. On gd 18, mice were fasted for 6 h and sacrificed for collection of **(A-E)** placenta from 2 pups, and maternal **(F-J)** VAT and **(K-O)** SAT. **(A, F, K)** IL1A, **(B, G, L)** IL1B, **(C, H, M)** IL6, **(D, I, N)** CCL2 and **(E, J, O)** CXCL1 mRNA expression was analysed by qRT-PCR and the fold change was calculated relative to control. All data are displayed as mean ± SEM. **P*<0.05 vs. control (unpaired-sample comparison).

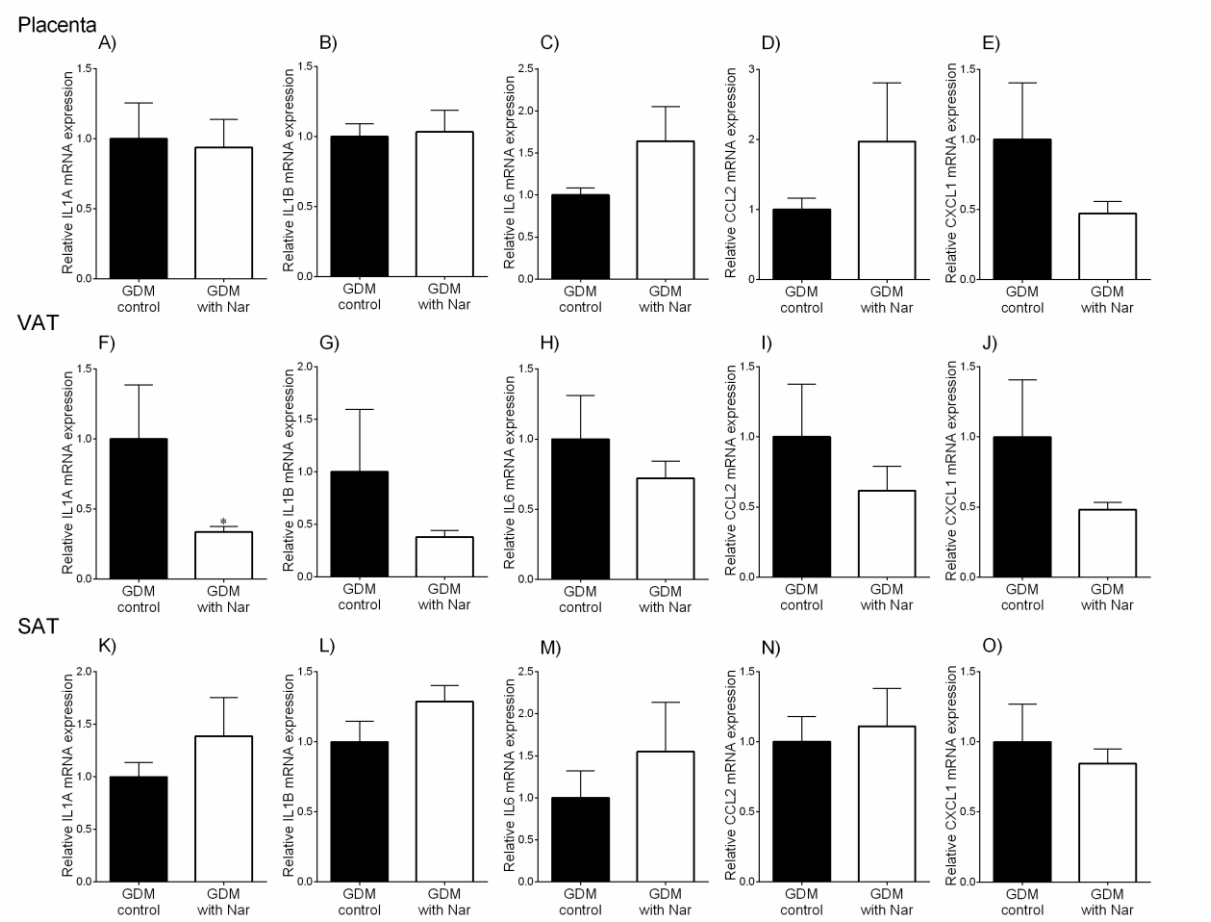
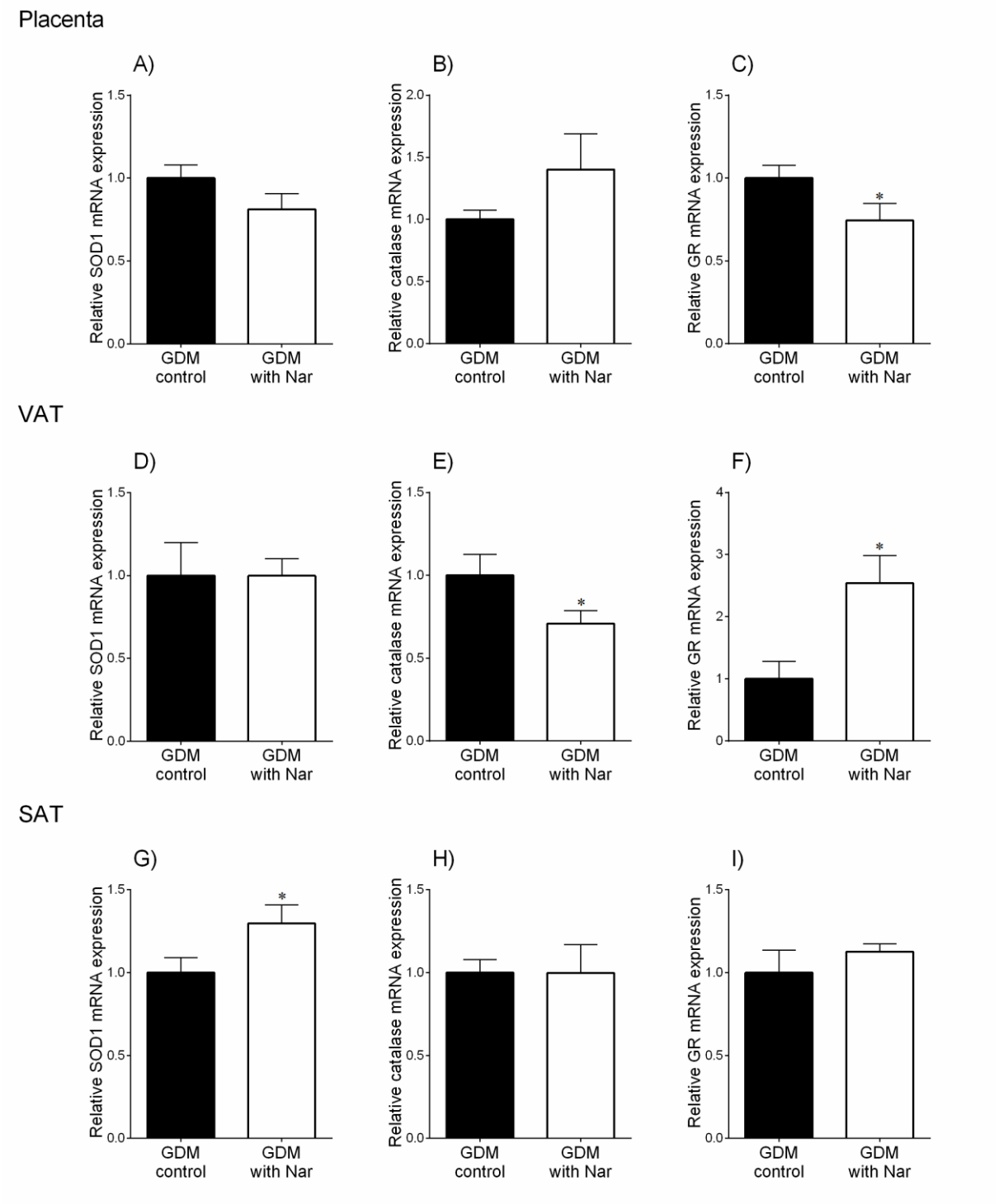


Fig 8. Effect of naringenin on antioxidant gene expression in placenta, VAT and SAT of GDM mice.

GDM mice administered daily doses of 50 mg/kg naringenin (Nar) or a vehicle control by i.p. commencing at gd 10 until gd 17. On gd 18, mice were fasted for 6 h and sacrificed for collection of **(A-C)** placenta, and maternal **(D-F)** VAT and **(G-I)** SAT. **(A, D, G)** SOD1, **(B, E, H)** catalase and **(C, F, I)** GR mRNA expression was analysed by qRT-PCR and fold change was calculated relative to control. All data are displayed as mean ± SEM. *P<0.05 vs. control (unpaired-sample comparison).



Gestational diabetes mellitus (GDM) is a carbohydrate intolerance occurring during pregnancy. Evidence suggests that polyphenols confer potent health benefits in metabolic and inflammatory diseases. In this paper, we demonstrate that the polyphenol naringenin can ameliorate insulin resistance, inflammation and oxidative stress in laboratory and animal models of GDM. Therefore, naringenin may be a novel preventative therapy for GDM.