Improved glycemic control and vascular function in overweight and obese subjects by glyoxalase 1 inducer formulation

Running title: Metabolic and vascular health with Glo1 inducer.

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SUMMARY
Risk of insulin resistance, impaired glycemic control and cardiovascular disease is excessive in overweight and obese populations. We hypothesised that increasing expression of glyoxalase 1 (Glo1) – an enzyme that catalyses the metabolism of reactive metabolite and glycating agent, methylglyoxal – may improve metabolic and vascular health. Dietary bioactive compounds were screened for Glo1 inducer activity in a functional reporter assay, hits confirmed in cell culture and an optimised Glo1 inducer formulation evaluated in a randomised, placebo-controlled crossover clinical trial in 29 overweight and obese subjects. We found *trans*-resveratrol (tRES) and hesperetin (HESP), at concentrations achieved clinically, synergised to increase Glo1 expression. In highly overweight subjects (BMI >27.5 kg/m^2), tRES-HESP co-formulation increased expression and activity of Glo1 (+ 27%. $P<0.05$), decreased plasma methylglyoxal (-37%, $P<0.05$) and total body methylglyoxal-protein glycation (-14%, $P<0.01$). It decreased fasting and postprandial plasma glucose (-5%, $P<0.01$ and – 6%, $P<0.03$, respectively), increased Oral Glucose Insulin Sensitivity index (+42 $mlmin^{-1}m^{-2}$, $P<0.02$) and improved arterial dilatation ΔFMD/ΔGTND (95%CI 0.13–2.11). In all subjects, it decreased vascular inflammation marker sICAM-1 (-10%, $P<0.01$). In previous clinical evaluations, tRES and HESP individually were ineffective. tRES-HESP co-formulation could be a suitable treatment for improved metabolic and vascular health in overweight and obese populations.

Key words: glyoxalase, glycation, methylglyoxal, obesity metabolism, insulin resistance, glycemic control.

Increasing overweight and obese populations are driving a global epidemic of type 2 diabetes and cardiovascular disease in Westernised countries. Glyoxalase 1 (Glo1) was linked to clinical obesity through association with measures of fat deposition and Glo1 deficiency identified as a driver of cardiovascular disease in a large integrative genomics study (1; 2). It is currently unaddressed by therapeutic agents. Experimental studies found overexpression of Glo1 in mice suppressed inflammation and body weight gain in overfeeding models of obesity and prevented vascular disease in diabetes – as reviewed (3).

Glo1 is part of the glyoxalase metabolic pathway which consists of two enzymes: Glo1 and glyoxalase 2 and a catalytic amount of reduced glutathione (GSH) in the cytoplasm of cells (Fig. 1A). The major function of the glyoxalase pathway is detoxification of the reactive dicarbonyl metabolite, methylglyoxal, converting it to D-lactate. Methylglyoxal is a highly potent glycating agent of protein which forms the quantitatively major advanced glycation endproduct, hydroimidazolone MG-H1, linked to protein inactivation and cell dysfunction (4; 5) (Fig. 1B). Degradation of methylglyoxal-modified proteins releases MG-H1 free adduct from tissues for urinary excretion (4). The steady-state level of protein MG-H1 is maintained at low tolerable levels by Glo1 (3).

Abnormally high methylglyoxal concentration, dicarbonyl stress, is a common characteristic of obesity and type 2 diabetes. It is severe in diabetes, driven by increased flux of methylglyoxal formation and decreased Glo1 activity at sites of vascular complications. Methylglyoxal is formed mainly by the non-enzymatic degradation of triosephosphate intermediates of glycolysis – a minor "leak" of *ca.* 0.05% triosephosphate flux. In obesity, dicarbonyl stress is mild and triosephosphate flux is increased by glyceroneogenesis in adipose tissue-liver cycling of triglycerides and free fatty acids. Increased methylglyoxal protein modification in dicarbonyl stress is implicated as a mediator of impaired metabolic and vascular health in obesity and diabetes (3).

An effective strategy to counter dicarbonyl stress is to increase expression of Glo1 (6). We described a functional regulatory antioxidant response element (ARE) in human GLO1 with basal and inducible expression up-regulated by transcriptional factor Nrf2. Recent advances in Nrf2 regulation suggested potent induction of Glo1 expression could be achieved by a synergistic combination of Nrf2 activators addressing different regulatory features (7; 8). In this study we sought to screen dietary bioactive compounds for Glo1 inducer activity in a functional reporter assay, confirm hits in cell culture and evaluate an optimised Glo1 inducer formulation in randomised, placebo-controlled crossover clinical trial for improved metabolic and vascular health in overweight and obese subjects.

RESEARCH DESIGN AND METHODS

Methods

Screening of Glo1 inducers using GLO1-ARE and related stable transfectant reporter cells lines

Stable transfectant luciferase reporter cell lines with ARE transcriptional regulatory elements were developed from human HepG2 cells, as described for quinone reductase ARE (7), incorporating regulatory elements: GLO1-ARE or functionally inactive mutant as negative control (ARE-1 and ARE1m in previous work) (6). Stable transfectant cell lines were incubated with and without bioactive compounds (0.625 – 20.0 μM) for 6 h. Luciferase activity was then determined in cell lysates, correcting for blank response and normalised to the highest effect (100%) achieved with 10 μM *trans*-resveratrol (tRES) (6). Nrf2-dependent transcriptional response was verified by siRNA silencing of Nrf2. Cytotoxicity was assessed after 24 h exposure by the MTT method (9). Screening hit criteria were: increased transcriptional response at ≤5 μM without significant cytotoxicity to human aortal endothelial

cells (HAECs) and BJ fibroblasts in primary culture. Dietary bioactive compound selection criteria were: ability to activate Nrf2 at concentrations achieved or likely achievable at tolerable doses clinically and/or decrease glycation and/or toxicity by methylglyoxal or similar compounds.

ARE-linked gene and other cell metabolism and functional marker gene expression by digital mRNA profiling and immunoblotting

HAECs, BJ fibroblasts and HepG2 cells (5 x 10^5 cells/well) were seeded on 6-well plates in MCDB-131 medium and cultured overnight at 37 ^oC under 5% CO_2/air. Cells were treated with and without 5 μM tRES, 5 μM hesperetin (HESP) and 5 μM tRES & HESP combined or vehicle (0.002% dimethylsulfoxide) and cultured further for up to 48 h. At time points indicated mRNA was extracted and analysed by the NanoString nCounter method (10). Immunoblotting was performed as described (6).

Clinical study

A randomized, double-blind, placebo-controlled crossover study of optimised Glo1 inducer was performed in 32 overweight and obese healthy subjects (Healthy Ageing through functional food, HATFF). The study was approved by National Research Ethics Service Committee West Midlands - Coventry & Warwickshire (project number 13/WM/0368) and registered on the Clinicaltrials.gov (identifier: NCT02095873). The procedures followed were in accordance with institutional guidelines and the Declaration of Helsinki. Three participants failed to complete the study.

Main inclusion criteria were: age 18 – 80 years, BMI 25 – 40 kg/m^2 and normal, impaired fasting or impaired postprandial glucose. Main exclusion criteria were: severe hypertriglyceridemia, uncontrolled hypertension, cardiovascular disease, relevant renal or hepatic disease, diabetes, and other relevant morbidity; severe excess alcohol consumption (>14/21 units [8g]/week for women/men), smoking, under pharmacological treatment affecting glucose and lipid metabolism or blood coagulation, or taking herbal remedies, known food allergies, women who are pregnant or breast feeding. All subjects were evenly randomised in tRES-HESP and placebo arms (n = 16) by the Clinical Trials Unit, University of Warwick. Treatment was one capsule daily for 8 weeks and washout period of 6 weeks: tRES-HESP - 90 mg tRES-120 mg HESP; and placebo – with starch in place of bioactives in hard gelatin capsules. Previous studies with dietary fibre supplementation indicated intervention for at least 8 weeks is required for improved glycemic control (11). Participants were advised to maintain their usual diet - confirmed by dietary questionnaires at the start and end of dosing periods, and physical activity - supported by nurse and dietician contact throughout the study.

Primary clinical endpoints were: metabolic health – Oral Glucose Insulin Sensitivity (OGIS) index in an oGTT (75 g glucose; participants instructed to eat carbohydrate rich diet, > 150 g/day, for at least three days before the test, followed by an overnight fast) (12); and vascular health - brachial artery flow mediated dilatation (FMD), including dilatation response to a subtherapeutic dose (25 μg) glyceryl nitrate (GTND) (13). oGTT and FMD/GTND assessments were performed at the start and end of each treatment period between 8 am and 10 am in a quiet temperature-controlled room maintained at 23 ± 1 ^oC. Markers of vascular inflammation were also assessed by commercial ELISA.

Venous blood samples were also drawn in the fasting state prior to the oGTT. Safety assessment of tRES-HESP co-formulation was assessed by electrocardiogram and analysis of blood markers. Plasma methylglyoxal and glycation and oxidation adducts in plasma protein and urine (second void after overnight fast) were assayed by stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS) (14; 15).

Total tRES and HESP urinary metabolites
These were determined by stable isotopic LC-MS/MS after de-conjugation of glucuronides and sulphates. Urine (20 µl), from which cells had been sedimented and removed prior to storage, with 42 mM ammonium acetate buffer, pH 4.9 (60 µl), internal standards (250 µM [$^{13}C_6$]tRES and 10 µM d_4-HESP; 20 µl) and β-glucuronidase (5 µl, 85 U) and β-sulphatase (5 µl, 5 U) was incubated for 2 h at 37 ^{o}C in the dark. De-conjugation was validated with authentic glucuronides and sulphates of tRES and HESP before use. Thereafter ice-cold methanol (100 µl) was added for de-proteinisation, centrifuged (10,000g, 10 min, 4 ^{o}C) and analysed by LC-MS/MS. Calibration curves were constructed by analysis of 125 – 625 pmol tRES and HESP.

Cellular GSH and oxidised glutathione GSSG
These were assayed by stable isotopic dilution analysis LC-MS/MS. Cells (*ca.* 1 x 10^6 cells) were deproteinized with 10% trichloroacetic acid (40 µl) containing 0.15% NaCl and 0.25% sodium azide in water and centrifuged (20,000g, 30 min, 4 °C). An aliquot of supernatant (10 µl) was mixed with 10 µl isotopic standard cocktail (100 pmol [$^{13}C_{2,}{}^{15}N$]GSH and [$^{13}C_{4,}{}^{15}N_2$]GSSG) and analysed by LC-MS/MS. Calibration standards contained 100 - 2000 pmol GSH and 5-100 pmol GSSG (Fig. 3).

Statistical analysis
Data are mean ± SD or SEM for parametric data and median (upper – lower quartile) for non-parametric data. Significance testing in paired data was assessed by paired Student's t-test and Wilcoxon signed-ranks test (for 2 two groups), by ANOVA repeated measures and Friedman test (for 4 or more groups), and correlation analysis by Pearson and Spearman methods for parametric and non-parametric data, respectively. For HATFF study power calculation, we judged decrease in AUCg of the oGTT would be 10%; *cf.* 30% decrease with high cereal fibre intake (16). With a 30% dropout, 32 subjects were required for $\alpha = 0.05$ and power $(1 - \beta) = 0.80$. Post-hoc analysis of variables for highly overweight and obese sub-groups was performed to explore BMI as a factor influencing responsiveness to Glo1 inducers.

RESULTS

Screening of small molecule Glo1 inducers
After screening of *ca.* 100 dietary bioactive compounds with Nrf2 activator activity, the highest E_{max} was produced by tRES. The lowest EC_{50} for GLO1-ARE transcriptional activity was found with HESP. For tRES, EC_{50} = 2.52 ± 0.19 µM and E_{max} 100 ± 2 %; and for HESP, EC_{50} = 0.59 ± 0.01 µM and E_{max} 24.4 ± 0.1 % (Fig. 2 A, B). In previous clinical studies, dietary supplementation of 150 mg HESP achieved a peak plasma concentration of 6.7 µM (17), suggesting that HESP may be a competent Glo1 inducer for clinical use but with low maximal effect; and dietary supplementation of 500 mg tRES achieved a peak plasma concentration of *ca.* 0.3 µM (18), 8-fold lower than the EC_{50} for GLO1-ARE response. To enhance efficacy we studied the pharmacological synergism of tRES and HESP together. Study of the GLO1-ARE transcriptional response of 5 µM HESP with 0.625 – 10 µM tRES showed that HESP combined synergistically with tRES, decreasing the EC_{50} of tRES *ca.* 2-fold to 1.46 ± 0.10 µM whilst maintaining the E_{max} (Fig. 2C). The predicted increase of GLO1-ARE transcriptional response from concentration response curves of 0.1 – 1.0 tRES in the presence of 5 µM HESP was 3 – 79 fold, including up to 80% increase over additive effects (Fig. 2D). This suggests marked benefits may accrue from use of tRES-HESP co-formulation.

Safety assessments of tRES and HESP indicate that they are highly tolerated (19; 20). Studies of human BJ fibroblasts in primary culture showed no toxicity of tRES and HESP individually or with 5 μM combination with primary bioactive compound at concentrations ≤ 20 μM. tRES (40 μM) with 5 μM HESP and 40 μM HESP with and without 5 μM tRES showed minor decrease in viability of BJ cells *in vitro* (Fig. 2E-2F). tRES (5 μM) with 0.625 μM HESP gave a minor increase in cell number – possibly an effect of insulin sensitising activity (see below). There was no toxicity of tRES, HESP and both combined in human aortal endothelial cells (HAECs) in primary culture under similar conditions (Fig. 2G-2H).

Validation of Glo1 inducer screening results and functional effects

To validate the Glo1 inducer studies we measured the change in Glo1 mRNA and protein and functional responses in human hepatocyte-like HepG2 cell line *in vitro* and HAECs and BJ fibroblasts in primary culture. There was a 10 – 30% increase in Glo1 mRNA in cells incubated with tRES and HESP and combined, and similar increases in Glo1 protein (Fig. 2I-2K). We then studied the effect on functional markers: inflammatory response proteins and matrix metalloproteinase. In HAECs, treatments decreased intercellular adhesion molecule-1 (ICAM-1), receptor for advanced glycation endproducts (RAGE) and E-selectin protein, with synergistic effects on ICAM-1 and RAGE (Fig. 3A-3C). In BJ fibroblasts, treatments decreased cellular vascular adhesion molecule-1 (VCAM-1), RAGE and matrix metalloproteinase-3 (MMP-3), with synergism for tRES and HESP in decrease of VCAM-1 and MMP-3 (Fig. 3D - 3F). This suggests that the tRES and HESP treatment lowers basal cell inflammation and extracellular matrix turnover.

Activation of Nrf2 is associated with increased cellular GSH and GSH/oxidised glutathione (GSSG) ratio through increased expression of genes of GSH synthesis and metabolism – particularly γ-glutamylcysteine ligase [modulatory and catalytic subunits] (GCLM and GCLC) and glutathione reductase (GSR). Treatment of HAECs, BJ fibroblasts and HepG2 cells *in vitro* with 5 μM tRES and 5 μM HESP individually, however, did not change cellular levels of GSH and GSSG whereas treatment with 5 μM tRES and HESP combined increased cellular GSH content by 43% in BJ fibroblasts and 32% in HepG2 cells (Fig. 3G-3L). Increased cellular GSH concentration enhances *in situ* activity of Glo1 (3).

We also studied time-dependent changes in expression of ARE-linked genes and other genes linked to metabolism and function in HAECs, BJ fibroblast and HepG2 cells by focussed quantitative mRNA array (selected time course responses are given in Supplementary Fig. 1A–1C). Overall, there were additive and synergistic changes on gene expression of tRES and HESP combined treatment. For example, in HAECs, mRNA of ARE-linked genes glutathione transferase A4 (GTSA4), heme oxygenase-1 (HMOX-1), GCLM, GCLC and GSR, were increased. tRES and HESP together decreased ICAM-1 mRNA. In BJ fibroblasts, tRES and HESP synergised to increase mRNA of GSTP1, HMOX1, NQO1 and aldoketo reductase 1C1 (AKR1C1) and to decrease expression of inflammation markers CCL2 and ICAM-1. In HepG2 cells, tRES and HESP combined synergistically to increase mRNA of NQO1, GCLM and GCLC, low density lipoprotein receptor (LDLR), hexokinase-2 (HK2) and 6-phosphofructokinase/bisphosphatase-3 (PFKBP3).

Improved clinical metabolic and vascular health with tRES-HESP co-formulation – HATFF study

Co-formulation of tRES-HESP was evaluated in healthy overweight and obese subjects. Twenty-nine subjects completed the study. Characteristics of subjects at study entry are given (Table 1). Subjects had mildly impaired glycemic control with only 9 subjects meeting criteria of prediabetes; all participants were overweight or obese, 20 were highly overweight (BMI > 27.5 kg/m^2), and 11 were obese (BMI > 30 kg/m^2). tRES-HESP treatment increased urinary

excretion of tRES and HESP metabolites by >2000-fold and >100 fold, respectively, compared to placebo (Supplementary Fig. 2A-2B). Dietary questionnaires, urinary excretion of pyrraline - an advanced glycation endproduct sourced only from food (21), and fasting plasma ketone body concentrations in the normal range in all subjects (22) suggested food consumption was similar throughout the study (Table 2). Clinical safety indicators were normal at study entry and remained unchanged throughout the placebo and tRES-HESP treatment periods (Supplementary Table 1).

tRES-HESP produced a 22% increase in Glo1 activity of peripheral blood mononuclear cells (PBMCs) post-treatment, compared to placebo in all subjects. PBMC Glo1 activity was increased 27% in highly overweight/obese and 30% in obese sub-groups. Concomitant with this there was a 37% decrease in plasma methylglyoxal post-supplementation with tRES-HESP but not with placebo in highly overweight/obese subjects. There was no change in plasma D-lactate concentration with tRES-HESP treatment - a surrogate indicator of flux of methylglyoxal formation (3) (Table 2). Therefore, increase of Glo1 activity by tRES-HESP in PBMCs, also expected in tissues, likely decreased plasma methylglyoxal concentration without change in flux of methylglyoxal formation.

With tRES-HESP treatment, there was a positive correlation of change in OGIS from baseline (ΔOGIS) with BMI; $r = 0.45$, $P<0.05$ (Fig. 4A). No similar correlation occurred with placebo. In the subset of highly overweight subjects, there was an increase in OGIS from baseline with tRES-HESP (ΔOGIS = +42 $mlmin^{-1}m^{-2}$; Table 2; Fig. 4B) but not with placebo. This effect was further enhanced in obese subjects only (ΔOGIS = + 58 $mlmin^{-1}m^{-2}$) (Table 2). The main contributory factors to this effect were: (i) decreased fasting plasma glucose (FPG) [$P<0.01$]; and (ii) decreased area under the curve plasma glucose (AUCg) [$P<0.03$, ANOVA] (Table 2).

There was a negative correlation of change in FPG from baseline (ΔFPG) to BMI with tRES-HESP treatment; $r = - 0.41$, $P<0.05$ (Fig. 4C). No similar correlation occurred with placebo. In highly overweight subjects, there was a 5% decrease in FPG post supplementation with tRES-HESP (Fig. 4D). This effect was further enhanced in obese subjects only; - 9% (Table 2). There was also decreased AUCg in the oGTT with tRES-HESP treatment in the highly-overweight study group by a similar extent; - 6%, $P<0.03$, ANOVA (Table 2). Therefore, tRES-HESP treatment would likely decrease exposure to increase glucose concentration in the fasting and postprandial states in the highly overweight and obese populations.

Concomitant with increased metabolic health there were small decreases in BMI and body weight in the obese subjects with tRES-HESP: - 0.5 kg/m^2 and - 0.3 kg, respectively. Measurement at morning study visits excluded effect of diurnal variation. Other small changes were: 3% increase in eGFR and 9% decrease in plasma urea with tRES-HESP. Further clinical variables unchanged by tRES-HESP treatment are given (Table 2).

In assessment of vascular function, we found no change in FMD and GTND. For FMD/GTND ratio, normalising from baseline, in the highly overweight/obese subject group, the 95% confidence interval for ΔFMD/ΔGTND with tRES-HESP was 0.13 – 2.11. Assessment of markers of vascular inflammation revealed a decrease in change of sICAM1 from baseline with tRES-HESP in all subjects, compared to increase with placebo: - 3.6 ± 6.9 versus + 25.8 ± 6.9 ng/ml ($P<0.01$); a reversal of *ca.* 10% of post-supplementation placebo level.

To assess the effect on protein glycation in the HATFF study we analysed glycation and oxidative damage of plasma protein. Plasma protein MG-H1 was unchanged with tRES-HESP treatment (Table 1). We propose this unexpected finding may be due to improved vascular function with tRES-HESP treatment, decreasing transcapillary escape rate of albumin (23), increasing vascular half-life of albumin and thereby maintaining MG plasma

protein glycation. The increase of transcapillary escape rate of albumin in obesity was 36% (24) - similar magnitude to the decrease of plasma methylglyoxal herein with tRES-HESP. The oxidative crosslink dityrosine was decreased 21% with tRES-HESP treatment but not with placebo (Table 2, Fig. 4E). To assess the whole body formation of MG-H1 we measured the urinary excretion of MG-H1 free adduct, corrected for MG-H1 absorbed from food by extrapolating regression of urinary MG-H1 on urinary pyrraline in all subjects to zero pyrraline (and hence no contribution from food) (25; 26) (Fig. 4F). The flux of endogenously-generated MG-H1 adducts was *ca.* 13 nmol/mg creatinine at baseline and decreased by 14% with tRES-HESP treatment but not with placebo (Table 2, Fig. 4G). The pentose-derived crosslink, pentosidine, is a quantitatively minor and fluorescent glycation adduct. Urinary excretion of pentosidine free adduct was decreased 32% by treatment with tRES-HESP but not by placebo (Fig. 4H).

We analysed changes in gene expression of PBMCs in a focused quantitative mRNA array study. In all subjects there was increased expression of GLO1 and decreased inflammation-linked genes, IL8 and PTGS2. In obese subjects there was also decreased expression of CCL2 and TNFA (Table 3)

DISCUSSION

Pharmaceutical doses of two dietary compounds, tRES found in red grapes and HESP closely-related to hesperidin found in oranges, administered together acted synergistically to improve FPG, AUCg, OGIS, sICAM-1, arterial function and renal function in highly-overweight subjects. Most clinical effects were found in the highly overweight subgroup (BMI >27.5 kg/m^2), indicating that the tRES-HESP co-formulation has decreasing potency as the lean range of BMI is approached. This suggests a re-setting to good metabolic and vascular health.

tRES and HESP administered individually in previous studies was ineffective. From meta-analysis it was concluded that tRES does not affect glycemic status in overweight and obese human subjects (27). This is at odds with evidence from rodent models (28) and is likely due to interspecies differences in pharmacology, host interactions and maximum tolerable dose. HESP absorbed from clinical dosing with hesperidin did not improve plasma glucose nor insulin resistance (29).

We arrived at the tRES-HESP formulation through maximising induction of Glo1 expression. Whilst increased Glo1 expression likely contributes to the observed health beneficial effects (3), changes in other gene expression occurred – such as induction of antioxidant enzymes and GSH synthesis (Fig. 3H,3I and Supplementary Fig. 1), and their interplay may also mediate the overall health benefit achieved. We exploited the regulatory ARE of GLO1 to increase expression (6). We limited the small molecule inducer screen to non-toxic dietary bioactive compounds of known or suspected Nrf2 activation activity to provide an option for use of Glo1 inducers as functional food supplements as well as pharmaceuticals. tRES and HESP also have > 50-fold safety margin at doses used in the HATFF study (19; 20). Activation of Nrf2 by dietary bioactive compounds is mostly studied through ARE-linked induction of NQO1 or HMOX1 expression. Small molecule activators of Nrf2 increase expression of different ARE-linked gene subsets (6; 7) - likely due to the ability of Nrf2 activators to recruit the requisite accessory proteins and increase nuclear concentration of functionally active Nrf2 to the level required for increased expression of the ARE-linked gene of interest (7; 8). A specific functional screen for GLO1-ARE transcriptional activation was therefore required.

Activation of Nrf2 by tRES has been studied previously by induction of HMOX1 expression (30). Herein we found tRES also induces expression of Glo1 with high E_{max}. Our recent studies (7; 8) and of others (31) suggest this is achieved by preventing nuclear acetylation and inactivation of Nrf2 via increasing *in situ* activity of sirtuin-1. At low tRES concentrations, this occurs through inhibition of cAMP phosphodiesterases, activation of AMPK and increased

NAD^+. HESP may also synergise for increased activity of sirtuin-1 through activation of AMPK by the protein kinase A pathway (32; 33).

Clinically achievable concentrations of tRES at highly tolerable doses are lower than the EC_{50} for induction of Glo1 expression, however, so synergism with HESP is required to achieve increase Glo1 expression in clinical translation. HESP may activate Nrf2 through induction and activation of protein kinase A, upstream of fyn kinase which drives Nrf2 translocational oscillations and ARE-linked gene expression (8; 33). HESP is a partial agonist (Fig. 2B) which is likely due to inhibitory nuclear acetylation of Nrf2 blocking a high E_{max}. Combination with tRES and HESP provides for faster nuclear translocation and decreased inactivation of Nrf2 (7; 8; 31). Use of HESP rather than related dietary glycoside hesperidin found in citrus fruits (34) is likely crucial: HESP has *ca.* 70-fold greater potency in Nrf2 activation and higher bioavailability than hesperidin (35).

tRES-HESP increased OGIS to levels typical of healthy, lean subjects. The magnitude of ΔOGIS, 42 – 58 $mlmin^{-1}m^{-2}$, is comparable to that achieved with pharmaceutical treatment of patients with type 2 diabetes (for example, 1.7 g metformin per day, ΔOGIS = +54 $mlmin^{-1}m^{-2}$) (36) and extreme weight loss with gastric band surgery in morbid obesity (ΔOGIS = +62 $mlmin^{-1}m^{-2}$) (37). These effects suggest the tRES-HESP can support therapeutic improvement of insulin sensitivity in highly-overweight populations. OGIS was initially proposed as a marker of insulin resistance but is also improved with increased β-cell sensitivity to glucose and decreased glucose absorption (38). Regarding insulin resistance, decreased activity of FGF21 due to down regulation of the FGF21 receptor cofactor β-Klotho may be involved (39). MG-driven protein glycation decreased expression of β-Klotho (40). By inducing Glo1 expression and decreasing MG protein glycation, therefore, we likely corrected the functional deficit of β-Klotho and re-engaged FGF21. This explains the resetting of insulin sensitivity to normal levels with the response greater for higher BMI subject groups. Characteristics of increased β-Klotho was its blocking of inflammatory signalling to down regulate pro-inflammatory mediators IL8, MCP1, ICAM1 and RAGE (40), and via decreasing MCP-1 also suppresses PTGS2 (41) – Fig. 5. All features of this transcriptional signature were found in PBMCs of the HATFF study. Other effects increasing OGIS may be: increased pancreatic beta-cell sensitivity to glucose (42), decreased intestinal absorption of glucose (43), changes of gut microbiota and decreased breakdown and absorption of starch (44) (Fig. 5).

The 5% decrease in FPG herein exceeds and matches effects of metformin and Olristat, respectively, in similar intervention trials in overweight/obese subjects (45; 46), Decrease in FPG in the normal range is associated with reduced risk of developing type 2 diabetes (47).

Decreased urinary excretion of pentosidine by tRES-HESP may be linked to decreased oxidative stress and decreased pentose precursors expected from improved insulin resistance (48).

tRES-HESP treatment produced an increase in ΔFMD/ΔGTND. The effect is likely produced by improved NO responsiveness in both endothelium and smooth muscle cells (13) related to induction of Glo1 and prevention of MG-glycation driven impairment of endothelial NO synthase (49). tRES-HESP also decreased dityrosine content of plasma protein. Dityrosine is an oxidative crosslink of tyrosine residues and is a dominant crosslink of the extracellular matrix (50). Plasma protein dityrosine may be a surrogate marker of this and hence be reporting decreased dityrosine crosslinking which may contribute to improved arterial function.

tRES-HESP decreased sICAM1 in the HATFF study. The cell studies herein suggest this is likely linked to decreased ICAM1 expression. ICAM1 expression was decreased in Glo1 transgenic rats (51). In clinical studies, tRES and HESP individually did not decrease

ICAM-1 (52; 53). sICAM1 correlates with atherosclerosis burden assessed by coronary artery calcification and is a risk predictor of cardiovascular disease (54).

In summary, we present evidence that pharmaceutical doses of tRES and HESP co-formulation produce improved metabolic and vascular health in overweight and obese subjects.

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Duality of interest
M.X, M.O.W, S.Q., N.-B.K., A.A, M.W., A.S., D.M., M.F., G.J., N.R. and P.J.T. Some authors (D.M., M.F., G.J.) are employees of Unilever who part-funded the study.

Author contributions
M.X. performed screening, validation and most clinical chemistry analysis, M.O.W. was clinical lead, co-designed and analysed data of the HATFF study, S.Q. performed the clinical procedures, N.-B.K. was the HATFF study statistician, M.W. was the principal HATFF study research nurse, A.A. and A.S. performed some clinical chemistry analysis, D.M. and M.F. participated in quarterly project steering meetings, G.J. participated in quarterly project steering meetings and raised funding for the study, N.R. was study coordinator and performed some metabolite analysis, and P.J.T. designed and led the study, raised funding for the study, performed some metabolite analysis, analysed data and wrote the manuscript. All authors read and approved the manuscript.

Guarantor
The guarantor is P.J.T.

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Table 1. Characteristics of subjects in the HATFF study at entry.

Variable	Value
Age (years)	45 ± 13
Gender (M/F)	8/21
BMI (kg/m^2)	30.0 ± 3.8
Overweight/Obese	18/11
Fasting plasma glucose (mM)	3.93 ± 0.57
A1C (mmol/mol Hb) (%)	36.2 ± 4.3 5.5 ± 0.7
Prediabetes (N/Y)	20/9
GFR (ml/min)	97 ± 17
Systolic BP (mmHg)	133 ± 12
Diastolic BP (mmHg)	83 ± 10
Hypertension (N/Y)	(18/11)

Data are mean ± SD, median (lower – upper quartile) or number of each classification (class 1/class 2); n = 29. Hypertension was defined as systolic BP ≥ 140 mmHg or diastolic BP ≥ 90 mm Hg on 4 occasions. Thirty-two subjects were recruited at the University Hospitals Coventry & Warwickshire NHS Trust, Coventry, U.K. in the period May – July 2014; the last participant left the study December 2014. At pre-screening, assessments made were: 2 h plasma glucose in an oral glucose tolerance test (oGTT) and A1C, aspartate transaminase, alanine transaminase, plasma triglycerides, plasma creatinine (for estimated glomerular

Table 1. Characteristics of subjects in the HATFF study at entry (cont'd).

filtration rate eGFR). One participant was withdrawn from the study for diverging from the protocol and 2 dropped out – one whilst on placebo and one on treatment. The 2 participants that dropped out stated personal reasons, related to interfering duties and therefore difficulties to attend the scheduled follow up appointments. None of the participants reported any relevant side effects (nausea, loss of appetite, gastrointestinal side effects and other symptoms). Study data are analysed per protocol (n = 29).

Table 2. Improvement of metabolic health with *trans*-resveratrol-hesperetin co-formulation in the HATFF study.

Variable	Study group	Placebo		tRES-HESP		Δ + tRES-HESP from: baseline [post-supplement]		Significance P from: baseline [post-supplement]		ANOVA/ Friedman
		Baseline	Post-supplement	Baseline	Post-supplement					
Urinary pyrraline (nmol/mg creatinine)	All	12.1 (6.4 – 15.8)	10.6 (7.8–15.0)	8.8 (5.1–13.4)	8.2 (5.1– 21.3)	----	[----]	----	[----]	----
PBMC Glo1 activity (mU/mg protein)	All	1300 ± 136	1155 ± 151	1343 ± 156	1414 ± 150	----	[+259 (+22%)]	----	[<0.05]	<0.02
	Highly-overweight	1346 ± 154	1156 ± 185	1345 ± 189	1463 ± 177	----	[+ 307 (+27%)]	----	[<0.05]	<0.02
	Obese	1451 ± 242	1085 ± 253	1140 ± 246	1413 ± 264	----	[+ 328 (+30%)]	----	[<0.05]	----
Plasma methylglyoxal (nM)	Highly overweight	166 ± 28	228 ± 30	160 ± 29	133 ± 18	----	[- 84 (- 37%)]	----	[<0.05]	----
Plasma D-lactate (μM)	All	8.28 ± 0.81	8.56 ± 0.73	7.46 ± 0.77	8.31 ± 0.73	----	[----]	----	[----]	----
OGIS ($ml min^{-1} m^{-2}$)	Highly-overweight	513 ± 23	541 ± 21	506 ± 22	548 ± 23	42 (+8%)	[----]	<0.02	[----]	<0.05
	Obese	488 ± 32	522 ± 32	489 ± 29	547 ± 30	58 (+12%)	[----]	<0.02	[----]	----
Fasting plasma insulin (pM)	All	39.6 ± 6.3	38.2 ± 5.2	43.1 ± 6.9	36.0 ± 3.2	----	[----]	----	[----]	----
Plasma insulin (pM; oGTT, 90 min)	Highly overweight	291 (142 – 487)	326 (223 – 422)	303 (177 – 508)	240 (110 – 452)	----	[----]	----	[----]	----
FPG (mM)	Highly overweight	3.92 ± 0.12	3.80 ± 0.13	4.02 ± 0.15	3.82 ± 0.16	- 0.20 (- 5%)	[----]	<0.04	[----]	<0.01
	Obese	3.76 ± 0.11	3.80 ± 0.16	3.92 ± 0.16	3.58 ± 0.16	- 0.34 (- 9%)	[----]	<0.02	[----]	<0.05
AUCg (mM h)	Highly overweight	11.0 ± 0.7	10.5 ± 0.6	10.8 ± 0.7	9.9 ± 0.6	- 0.6 (- 6%)	[----]	0.052	[----]	<0.03
BMI (kg/m^2)	Obese	34.2 ± 0.7	34.3 ± 0.6	34.0 ± 0.7	33.8 ± 0.6	----	[- 0.5 (- 1%)]	----	[<0.05]	<0.02
Body weight (kg)	Obese	93.2 (84.0-109.4)	93.6 (84.1–108.8)	93.8(83.7-106.6)	93.3(83.2–107.7)	----	[- 0.3 (- 0.3%)]	----	[<0.05]	----
eGFR (ml/min)	All	99 ± 2	101 ± 2	97 ± 2	100 ± 2	+ 3 (+3%)	[----]	<0.03	[----]	<0.02
Plasma urea (mM)	All	4.60 ± 0.19	4.35 ± 0.18	4.67 ± 0.17	4.23 ± 0.15	- 0.44 (- 9%)	[----]	<0.01	[----]	<0.01
Plasma acetoacetate (μM)	All	61 (42-135)	74 (49-113)	62 (48-127)	77 (65-125)	----	[----]	----	[----]	----
Plasma β-hydroxy-butyrate (μM)	All	27 (16 – 47)	24 (13 – 46)	26(18 – 49)	22 (11 – 46)	----	[----]	----	[----]	----
A1C (mmol/mol)	All	36.7 ± 0.8	36.5 ± 0.8	36.8 ± 0.8	36.5 ± 0.9	----	[----]	----	[----]	----
(%)		5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1	5.5 ± 0.1					

Table 2. Improvement of metabolic health with *trans*-resveratrol-hesperetin co-formulation in the HATFF study (cont'd).

Variable	Study group	Placebo		tRES-HESP		Δ + tRES-HESP from: baseline [post-supplement]		Significance P from: baseline [post-supplement]		ANOVA/ Friedman
		Baseline	Post-supplement	Baseline	Post-supplement					
2h Glucose, oGTT (mM)	All	4.28 ± 0.32	4.20 ± 0.26	4.40 ± 0.29	4.40 ± 0.26	----	[----]	----	[----]	----
HOMA-IR (mM x mU/L)	All	0.75 (0.46–1.22)	0.68 (0.42 – 1.25)	0.85 (0.38–1.56)	0.69 (0.49–1.34)	----	[----]	----	[----]	----
Systolic BP (mmHg)	All	131 ± 2	132 ± 3	131 ± 3	133 ± 3	----	[----]	----	[----]	----
Diastolic BP (mmHg)	All	81.7 ± 1.9	82.9 ± 2.3	83.4 ± 2.1	83.3 ± 2.3	----	[----]	----	[----]	----
Total cholesterol (mM)	All	5.48 ± 0.39	5.72 ± 0.28	5.35 ± 0.31	5.53 ± 0.28	----	[----]	----	[----]	----
LDL/VLDL cholesterol (mM)	All	3.93 ± 0.26	4.16 ± 0.23	4.01 ± 0.23	4.06 ± 0.23	----	[----]	----	[----]	----
HDL cholesterol (mM)	All	1.36 ± 0.10	1.39 ± 0.11	1.36 ± 0.10	1.37 ± 0.11	----	[----]	----	[----]	----
Triglycerides (mM)	All	0.810 ± 0.132	0.719 ± 0.122	0.650 ± 0.110	0.655 ± 0.092	----	[----]	----	[----]	----
Endothelin-1 (pg/ml)	All	1.66 ± 0.17	1.61 ± 0.14	1.66 ± 0.12	1.64 ± 0.15	----	[----]	----	[----]	----
CRP (μg/ml)	All	2.32 (0.99–5.46)	2.37 (0.63–3.75)	2.23 (0.82–3.97)	1.74 (0.76–3.91)	----	[----]	----	[----]	----
sE-selectin (mg/ml)	All	39.0 ± 2.9	38.8 ± 3.2	39.2 ± 3.0	38.3 ± 3.0	----	[----]	----	[----]	----
sVCAM1 (ng/ml)	All	453 ± 16	449 ± 13	437 ± 12	445 ± 14	----	[----]	----	[----]	----
Cystatin-c (ng/ml)	All	676 ± 28	678 ± 29	692 ± 32	691 ± 31	----	[----]	----	[----]	----
Albumin/creatinine ratio (mg albumin/ mmol creatinine)	All	0.257 (0.171–0.483)	0.261 (0.147–0.790)	0.231 (0.150–0.465)	0.264 (0.159–0.581)	----	[----]	----	[----]	----
FMD (mm)	All	0.18 (0.07 – 0.49)	0.26 (0.07 – 0.47)	0.17 (0.10 – 0.35)	0.12 (0.06 – 0.31)	----	[----]	----	[----]	----
GTND (mm)	All	0.41 (0.36 – 0.77)	0.44 (0.28 – 0.76)	0.45 (0.37– 0.75)	0.38 (0.29– 0.53)	----	[----]	----	[----]	----
Plasma sICAM1	All	257 ± 13	284 ± 15	280 ± 13	276 ± 13	----	[----]	----	[----]	<0.01
Plasma protein MG-H1 (mmol/mol arg)	All	0.370 (0.348–0.447)	0.380 (0.340–0.420)	0.357 (0.330–0.408)	0.381 (0.353–0.416)	----	[----]	----	[----]	----
Plasma protein dityrosine (μmol/mol tyr)	All	28.6 ± 2.7	26.6 ± 2.6	29.6 ± 2.9	23.5± 1.7	- 6.1 (- 21%)	[----]	< 0.01	[----]	<0.02
Total urinary MG-H1 free adduct (nmol/mg creatinine)	All	20.1 (16.3 – 30.6)	22.7 (16.6 – 29.3)	19.7 (12.5 – 30.4)	19.8 (14.9 – 27.4)	----	[----]	----	[----]	----

Table 2. Improvement of metabolic health with *trans*-resveratrol-hesperetin co-formulation in the HATFF study (cont'd).

Variable	Study group	Placebo		tRES-HESP		Δ + tRES-HESP from: baseline [post-supplement]	Significance P from: baseline [post-supplement]	ANOVA/ Friedman
		Baseline	Post-supplement	Baseline	Post-supplement			
Endogenous urinary MG-H1 free adduct (nmol/mg creatinine)	All	13.4 ± 2.1	13.5 ± 3.7	13.1 ± 2.8	11.3 ± 3.1	- 1.8 (-14%) [----]	<0.01 [<0.01]	<0.01
Urinary pentosidine free adduct (pmol/mg creatinine)	All	10.8 (6.8 – 20.2)	12.7 (5.7 – 23.6)	10.8 (7.4 – 21.8)	7.4 (4.3 – 16.5)	- 3.4 (- 32%) [----]	<0.05 [----]	-----

Data are mean ± SEM or median (lower – upper quartile). For obese, highly overweight/obese and all study groups, n = 11, 20 and 29, respectively. Variables failing to achieve or approach significance are given in Table S1. tRES-HESP treatment changes, Δ + tRES-HESP, are absolute (percentage) changes from baseline and similarly changes with respect to post-supplement placebo control in square brackets [ ]. Related significance levels are also given where P<0.05 and, in one case, borderline failure of significance, P = 0.052. There were not statistical significance differences induced by placebo.

Table 3. Summary of change in gene expression of PBMC with *trans*-resveratrol-hesperetin co-formulation in the HATFF study.

Study group	Genes	
	Increased	Decreased
All (n = 29)	GLO1 (+6%)	HIF1A (- 6%), IL8 (- 39%) and PTGS2 (- 30%)
Highly overweight/obese (n = 20)	----	FTH1 (- 19%), HIF1A (- 8%), IL8 (- 49%), PTGS2 (- 31%), RAGE (- 37%) and CCL2 (- 49%)
Obese (n = 11)	----	CCL2 (- 22%), HIF1A (- 7%), IL8 (- 62%), KEAP1 (- 18%), PTGS2 (- 37%) and TNFA (- 12%)

Data and statistical analysis are given in Supplementary Table 2.

Figure legends.

Figure 1. Glyoxalase pathway and protein glycation by methylglyoxal. *A*: Metabolism of methylglyoxal by the glyoxalase system. *B*: Formation of hydroimidazolone MG-H1 from arginine residues in protein.

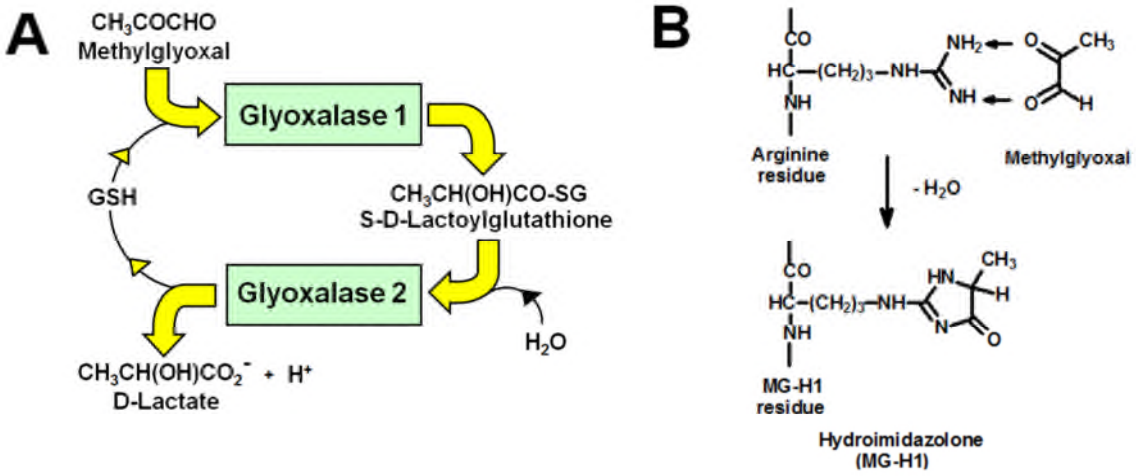
Figure 2. Induction of glyoxalase 1 expression by trans-resveratrol and hesperetin. GLO1-ARE transcriptional response reporter assay. Data of normalised responses for varied bioactive concentrations were fitted by non-linear regression to the equation $E = E_{max} \times [Bioactive]^n/(EC_{50}^n + [Bioactive]^n)$, solving for E_{max}, EC_{50} and n (Hill coefficient). *A*: Concentration-response curve for tRES. Data are mean ± SD (n = 3) for 5 concentrations. Non-linear regression (red curve): $E (\%) = 100 \times [tRES]^{3.92}/(2.52^{3.92} + [tRES]^{3.92})$. *B*: Concentration-response curve for HESP. Data are mean ± SD (n = 3 - 8) for 6 concentrations. Non-linear regression (blue curve): $E (\%) = 24.4 \times [HESP]^{2.01}/(0.59^{2.01} + [HESP]^{2.01})$. *C*: Concentration-response curve for tRES in the presence of 5.0 μM HESP. Data are mean ± SD (n = 3 - 6) for 5 concentrations. Non-linear regression (black curve): $E (\%) = (83.4 \times [tRES]^{1.36}/(1.46^{1.36} + [tRES]^{1.36})) + 11.6$; green curve – tRES+ 5.0 μM HESP, red dotted curve – tRES only (as for *A*:). *D*: Expansion of *C*: also showing the response for 5.0 μM HESP (blue dashed line). *E* - *H*: Evaluation of the effect of tRES and HESP individually and in combination on the growth and viability of BJ fibroblasts [*E* and *F*] and HAECs [*G* and *H*] *in vitro.* For *E* and *G* cells were incubated with 0.625 – 40 μM tRES and with (green bars) or without (red bars) 5.0 μM HESP. For *F* and *H* cells were incubated with 0.625 – 40 μM HESP and with (green bars) or without (blue bars) 5.0 μM tRES. *I* - *K* Validation of induction of Glo1 expression by 5.0 μM tRES and HESP, individually and combined. Cell type: *I* , HAECs; *J,* BJ fibroblasts; *K*, HepG2 cells . Panels (from left to right): GLO1 mRNA change with 5.0 μM tRES (red line), 5.0 μM HESP (blue line) and 5.0 μM tRES & HESP (green line). Bar chart: Glo1 protein (16 h post-treatment): + 5.0 μM tRES (red bar) and 5.0 μM HESP (blue bar). Data are mean ± SD (n = 3) for *E* – *K*. Significance: *, ** and ***, P<0.05, P<0.01 and P<0.001. HAEC cells were grown in proprietary large vessel endothelial cell basal media supplemented with large vessel endothelial cell growth supplement (containing hydrocortisone, human epidermal growth factor, human fibroblast growth factor with heparin and in 2% (/v/) FBS), 25 μg/ml gentamicin and 50 ng/ml amphotericin B. They were cultured in MEM medium with 10% fetal calf serum and 2 mM glutamine under an atmosphere of 5% CO_2 in air, 100% humidity and 37^oC.

Figure 3. Effect of *trans*-resveratrol, hesperetin and *trans*-resveratrol-hesperetin co-addition on cell vitality markers and glutathione metabolism in human endothelial cells, fibroblasts and HepG2 cells *in vitro*. *A* – *F*: cell vitality markers. Key: control (black bar), + 5.0 μM tRES (red bar), + 5.0 μM HESP (blue bar) and 5.0 μM tRES & HESP (green bar). HAECs: *A* ICAM1 protein and *B* RAGE protein at 18 h post-treatment, *C* E-selectin protein and *D* VCAM-1 protein at 24 h post-treatment. BJ fibroblasts: *E* RAGE protein and *F* MMP3 protein at 72 h post-treatment. *G* – *L*: Cellular GSH and GSSG at 24 h post-treatment: *G* – *I* and *J* – *L*, GSH and GSSG in HAECs, BJ fibroblasts and HepG2 cells, respectively. Percentage change with respect to GSH of control cultures is indicated. Data are mean ± SD [n = 3]. Significance: *, ** and ***, P<0.05, P<0.01 and P<0.001 with respect to control; and o, P<0.05 with respect to tRES control, and "+", P<0.05 with respect to HESP control. LC-MS/MS was performed using two Hypercarb HPLC columns in series (50 x 2.1 mm and 250 x 2.1 mm, particle size 5 μm) at temperature of 30 °C. Initial mobile phase was 0.1% TFA in water and the elution profile was: 1 – 15 min, a linear gradient of 0 – 30% acetonitrile and 15 – 16 min, isocratic 30% acetonitrile; the flow rate was 0.2 ml/min and diverted into the mass

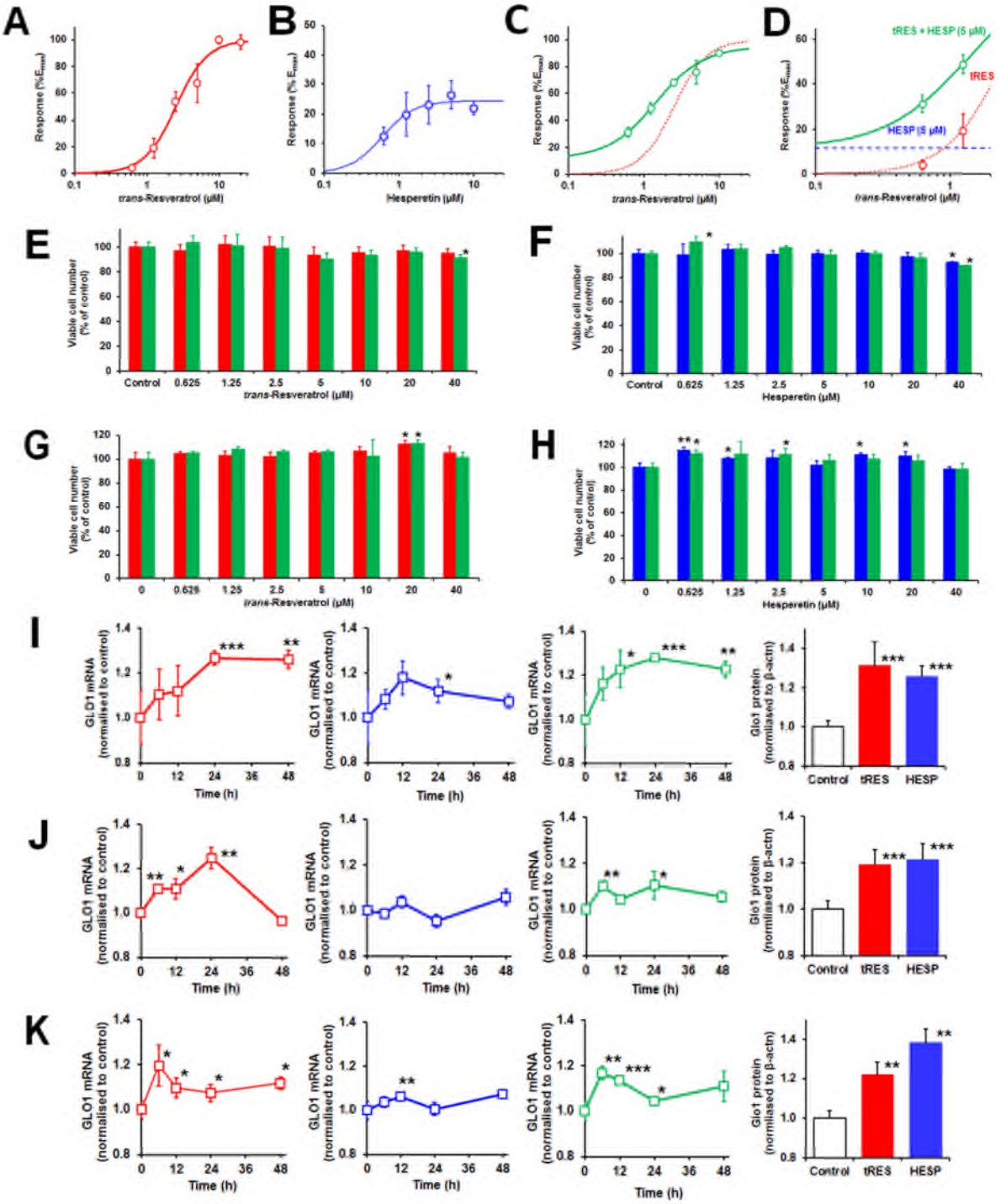
spectrometer from 4 – 16 min. Mass spectrometric analysis was performed using electrospray positive ionisation mode with detection settings: capillary voltage was 3.4 kV, source and desolvation gas temperatures 120 °C and 350 °C, cone and desolvation gas flows 146 and 550 l/h, respectively. Analytes detection (retention time, mass transition molecular ion>fragment ion, cone voltage and collision energy were: GSH – 11.7 min, 308.2>179.1 Da, 30 V and 13 eV; [$^{13}C_{2,}{}^{15}N$]GSH – 11.7 min, 311.2>182.1 Da, 30 V and 13 eV; GSSG – 14.4 min, 613.2>483.7 Da, 52 V and 18 eV; [$^{13}C_{4,}{}^{15}N_2$]GSSG – 14.4 min, 619.2>489.7 Da, 52 V and 18 eV. For GSH and GSSG, limits of detection were 0.92 pmol and 1.46 pmol, analytical recoveries 97 ± 2% and GSSG, 93 ± 6%, and intra-batch coefficient of variation 8.8% and 10.9% (n = 6), respectively.

Figure 4. Outcomes from the HATFF clinical study. *A*: Correlation of change in OGIS from baseline (ΔOGIS) with BMI in the tRES-HESP treatment arm. r = 0.45, P<0.05 (Pearson); n = 29. *B*: OGIS in tRES-HESP treatment arm at baseline [BS] and post-supplementation [PS] study visits in highly overweight subjects; n = 20. OGIS was calculated from plasma glucose concentrations at 0, 90 and 120 min and plasma insulin concentrations at 0 and 90 min (12). *C*: Correlation of change in FPG from baseline with BMI in the tRES-HESP treatment arm. r = - 0.41, P<0.05 *Pearson*; n = 29. *D*: FPG in tRES-HESP treatment arm at BS and PS study visits in highly overweight; n = 20. *E*: Dityrosine residues in plasma protein at BS and PS study visits; n = 29. *F*: Regression of urinary excretion of MG-H1 free adduct on urinary excretion of pyrraline free adduct at baseline of the placebo arm. Regression line: Urinary MG-H1 (nmol/mg creatinine) = (0.592 ± 0.180) x urinary pyrraline (nmol/mg creatinine) + (13.4 ± 2.1); P = 0.003. Total urinary excretion of MG-H1 free adduct correlated positively with urinary pyrraline for all 4 study visits, r = 0.43 – 0.63, P = 0.019 - <0.001). *G*: Endogenous flux of MG-H1 formation at study visits. *H*: Urinary excretion of pentosidine free adduct at study visits. Key: significance: * and **, P<0.05 and P<0.01, respectively; *B, D, E* and *G paired t-test* and *H Wilcoxon signed-rank test*.

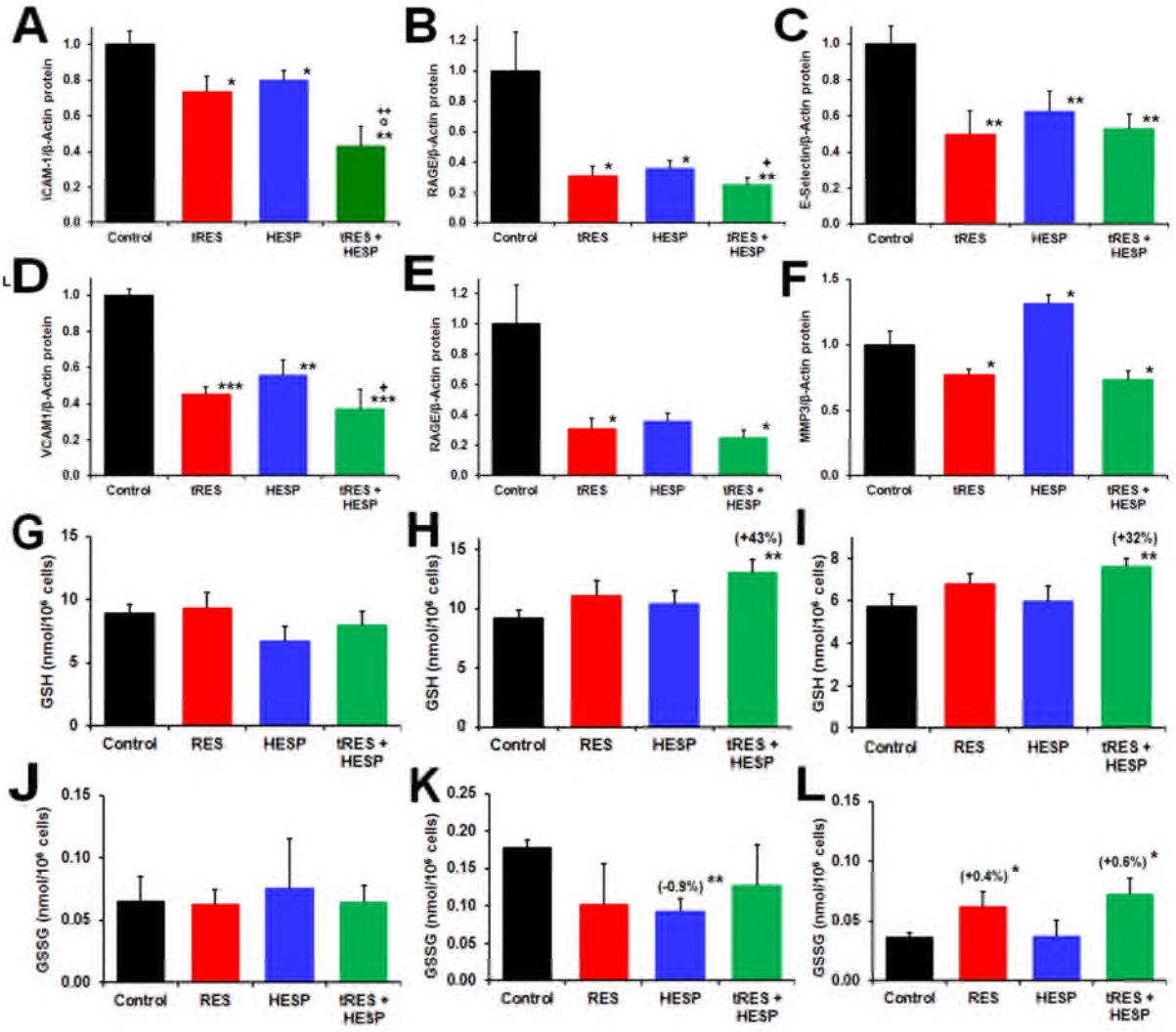
Figure 5. Proposed mechanism of action of Glo1 inducer formulation. Key: yellow filled arrows – mechanism of health improvement by; red filled arrows – damaging processes suppressed. See also (40; 42-44). Abbreviations: KLB, β-klotho; CBP, CREB binding protein; FGFR1c, fibroblast growth factor receptor 1c; and maf, small maf protein – accessory proteins for Nrf2 activation.



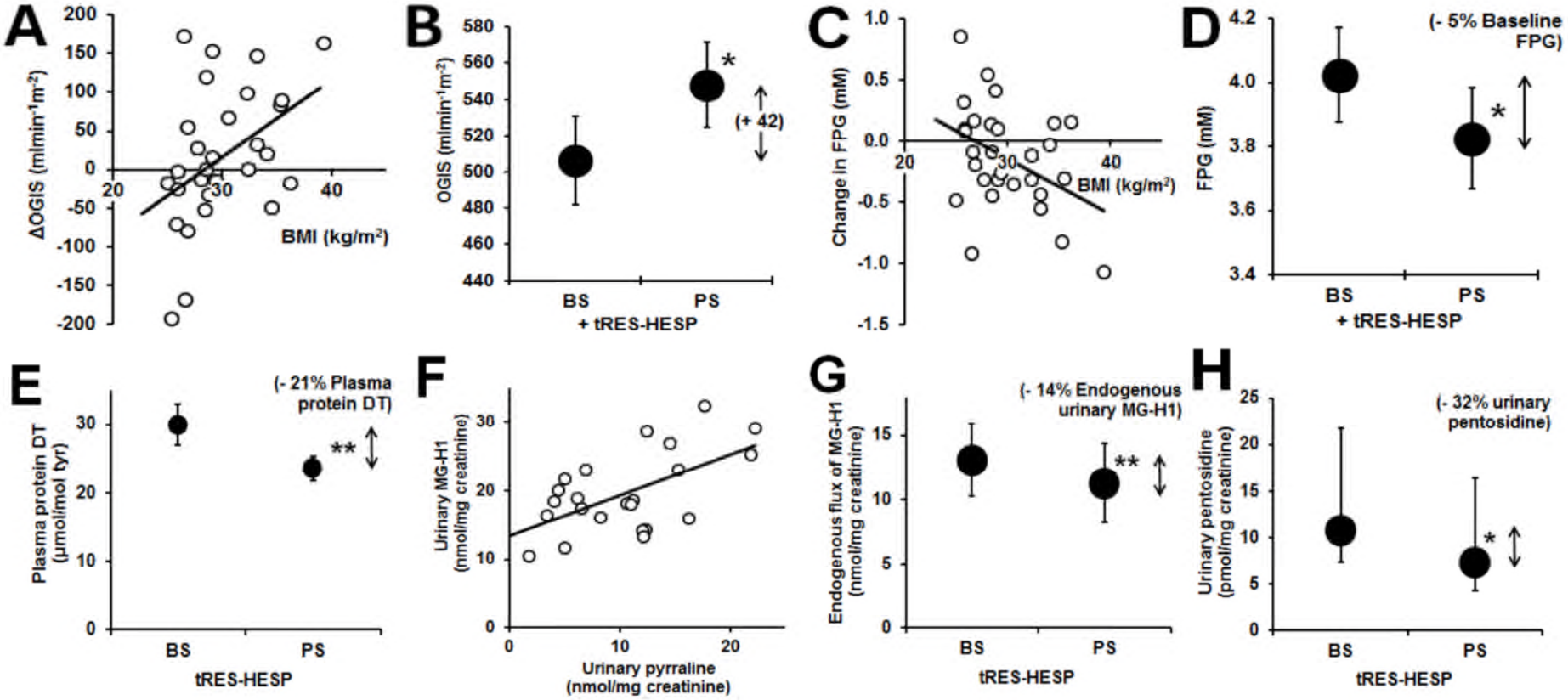
Xue *et al.*, Improved glycemic control etc, Figure 1



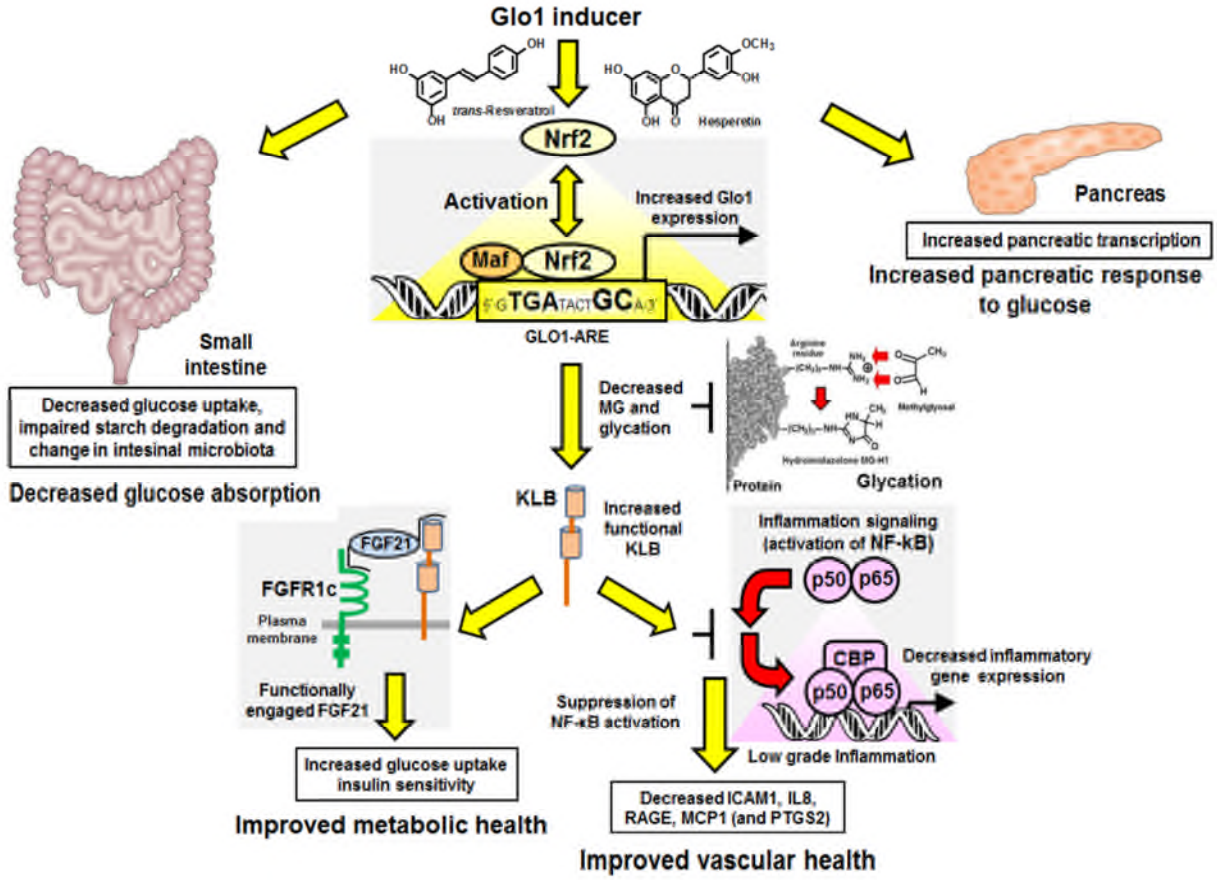
Xue *et al.*, Improved glycemic control etc, Figure 2



Xue *et al.*, Improved glycemic control etc, Figure 3



Xue *et al.*, Improved glycemic control etc, Figure 4



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Supporting document/data
Improved glycemic control etc., Xue *et al.*,

Materials

Tissue culture materials, medium MCDB-131, L-glutamine and recombinant human epidermal growth factor were from Invitrogen (Paisley, UK) and fetal bovine serum from Biosera (Ringmer, UK). Human Glo1 antibody was available from a previous in-house preparation (55). Dietary bioactive compounds were purchased from Extrasynthese (69727 Genay Cedex, France), LKT Laboratories Inc. (St. Paul, MN 55130, USA) and Sigma (Poole, UK). [$^{2}H_3$]HESP was from Toronto Research Chemicals (Toronto, Canada). GSH, oxidised glutathione (GSSG), [$^{13}C_6$]tRES. [*glycine*-$^{13}C_2$, ^{15}N]GSH (98% ^{15}N and 99% ^{13}C), β-glucuronidase from *Helix pomatia*, β-sulphatase from *Helix pomatia*, acetoacetate colorimetric assay kit (cat no. MAK199-1KT) and all other chemicals used in this project were from Sigma. [$^{13}C_4{}^{15}N_2$]GSSG was synthesised in-house from [*glycine*-$^{13}C_2$, ^{15}N]GSH by oxidation by diamide and purification by anion exchange chromatography (yield 28%), as described (56). β-Hydroxybutyrate colorimetric assay kit (cat no. ab83390) was from Abcam (Cambridge, U.K.). Primary human aortal endothelial cells (HAEC) were purchased from Lonza (Slough, U.K) and human dermal foreskin BJ fibroblasts at cumulative population doubling of 22 were purchased from the European Collection of Animal Cell Cultures (Porton Down, UK). For the HATFF study, tRES was from DSM (Heerlen, Netherlands) and HESP was supplied by Symrise BioActives GmbH (Hamburg, Germany). Placebo and tRES-HESP hard gelatin capsules were prepared by Pharmavize (Ghent, Belgium).

Supplementary Table 1. Safety assessment of *trans*-resveratrol-hesperetin co-formulation in the HATFF study.

	Placebo		tRES-HESP	
Variable	Baseline	Post-supplement	Baseline	Post-supplement
Aspartate aminotransferase (U/L)	20.1 ± 0.9	21.4 ± 1.2	20.9 ± 1.0	20.4 ± 0.9
Alanine aminotransferase (U/L)	18.5 (13.5 – 33.0)	20.0 (15.0 – 30.0)	19.0 (15.0 – 26.5)	18.0 (13.5 – 31.0)
γ-Glutamyl transferase (U/L)	20.0 (12.5 – 41.5)	19.0 (13.5 – 37.5)	26.0 (14.5 – 37.0)	21.0 (13.5 – 38.0)
alkaline phosphatase (U/L)	63.1 ± 2.9	65.7 ± 3.3	66.1 ± 3.1	66.0 ± 3.0
Albumin (g/L)	44.4 ± 0.4	44.6 ± 0.4	44.4 ± 0.5	44.6 ± 0.5
Total protein (g/L)	70.2 ± 0.6	69.3 ± 0.8	69.8 ± 0.7	69.2 ± 0.7
Bilirubin (mg/L)	8.69 ± 0.88	8.59 ± 0.71	8.97 ± 0.99	8.59 ± 0.80
Haemoglobin (mg/L)	137 ± 2	138 ± 2	138 ± 2	137 ± 2
Red blood cells (10^6 per μL)	4.61 ± 0.07	4.67 ± 0.08	4.66 ± 0.07	4.67 ± 0.07
Mean corpuscular volume (fL/cell)	89.7 ± 0.7	89.7 ± 0.8	90.4 ± 0.7	89.9 ± 0.7
Leukocyte count (10^9 per L)	5.54 ± 0.23	5.68 ± 0.28	5.38 ± 0.28	5.55 ± 0.25
Thrombocyte no (10^9 per L)	247 ± 8	244 ± 7	248 ± 10	248 ± 8
Prothrombin time (s)	10.6 ± 0.1	10.6 ± 0.1	10.7 ± 0.1	10.5 ± 0.1
Sodium (mM)	141 ± 1	141 ± 1	141 ± 1	141 ± 1
Potassium (mM)	4.32 ± 0.05	4.30 ± 0.05	4.34 ± 0.04	4.29 ± 0.05
Chloride (mM)	100 ± 1	101 ± 1	101 ± 1	101 ± 1
Phosphate (mM)	1.07 ± 0.02	1.08 ± 0.03	1.10 ± 0.02	1.08 ± 0.02

Data are mean ± SEM or median (lower – upper quartile); n = 29. There was no significant difference in analytes assessed by 2- or 4-group testing.

Supplementary Table 2. PBMC gene expression changes in the HATFF study.

A: Baseline and post-supplementation comparisons for placebo and tRES+HESP.

Study group		Placebo		tRES-HESP		Δ + tRES-HESP from:		Significance P from:		Freidman test
	Gene	Baseline	Post-supplement	Baseline	Post-supplement	Baseline†	[Post-supplement]‡	Baseline	[Post-supplement]	
All	GLO1		0.392 (0.356 – 0.436)		0.415 (0.375 – 0.463)	----	[0.023 (6%)]	----	[<0.05]	----
	HIF1A		2.12 (1.94 – 2.39)		2.00 (1.77 – 2.30)	----	[- 0.12 (- 6%)]	----	[<0.05]	----
	IL8		0.197 (0.151-0.562)		0.120 (0.090 - 0.293)	----	[- 0.077 (39%)]	----	[<0.05]	----
	PTGS2	0.078 (0.063-0.158)	0.087 (0.064-0.187)	0.084 (0.065-0.132)	0.059 (0.048-0.073)	- 0.025 (30%)	[0.028 (-32%)]	<0.05	[<0.01]	<0.05
Highly over-weight	FTH1		2.40 (1.82 – 3.71)		1.95 (1.65 – 3.11)	----	[- 0.45 (- 19%)]	----	[<0.05]	----
	HIF1A		2.15 (1.93 – 2.61)		1.99 (1.78 – 2.30)	----	[- 0.16 (- 8%)]	----	[<0.05]	<0.01
	IL8		0.186 (0.149-0.718)		0.094 (0.083 - 0.261)	----	[- 0.092 (- 49%)]	----	[<0.05]	<0.05
	PTGS2		0.086 (0.062 – 0.200)		0.059 (0.046 – 0.067)	----	[- 0.027 (- 31%)]	----	[<0.01]	----
Obese	CCL2	0.049 (0.035–0.056)	0.052 (0.036 – 0.108)	0.051 (0.044-0.076)	0.040 (0.033 – 0.055)	-0.012 (-22%)	[- 0.012 (- 23%)]	<0.05	[<0.05]	----
	HIF1A		1.98 (1.82 – 2.62)		1.85 (1.72 – 1.99)	----	[- 0.13 (- 7%)]	----	[<0.05]	<0.05
	IL8	0.184 (0.096-0.298)	0.181(0.144-0.456)	0.241(0.106-0.904)	0.091(0.084-0.164)	- 0.150 (- 62%)	----	<0.05	[<0.05]	----
	KEAP1		0.216 (0.173 – 0.259)		0.178 (0.147 – 0.201)	----	[- 0.038 (- 18%)]	----	[<0.05]	----
	PTGS2	0.077 (0.062-0.163)	0.083 (0.066-0.156)	0.092 (0.064-0.182)	0.058 (0.041-0.073)	- 0.034 (- 37%)	[-0.025 (- 30%)]	<0.05	[<0.05]	----
	TNFA		0.552 (0.436 – 0.735)		0.486 (0.376– 0.612)	----	[-0.066 (- 12%)]	----	[<0.05]	----

Supplementary Table 2. PBMC gene expression changes in the HATFF study (cont'd).
B: Change for baseline comparisons.

		Change from baseline		Significance
Study group	Gene	Placebo	tRES-HESP	
All	GLO1	- 0.010 (- 0.063 – 0.0155)	0.013 (- 0.028 – 0.079); + 6%	<0.05
Highly overweight/obese	RAGE	0.048 (- 0.017 - 0.149)	- 0.016 (- 0.116 – 0.021); - 37%	<0.05
	CCL2	0.014 (- 0.007 – 0.040)	- 0.008 (- 0.035 – 0.005); - 49%	<0.01

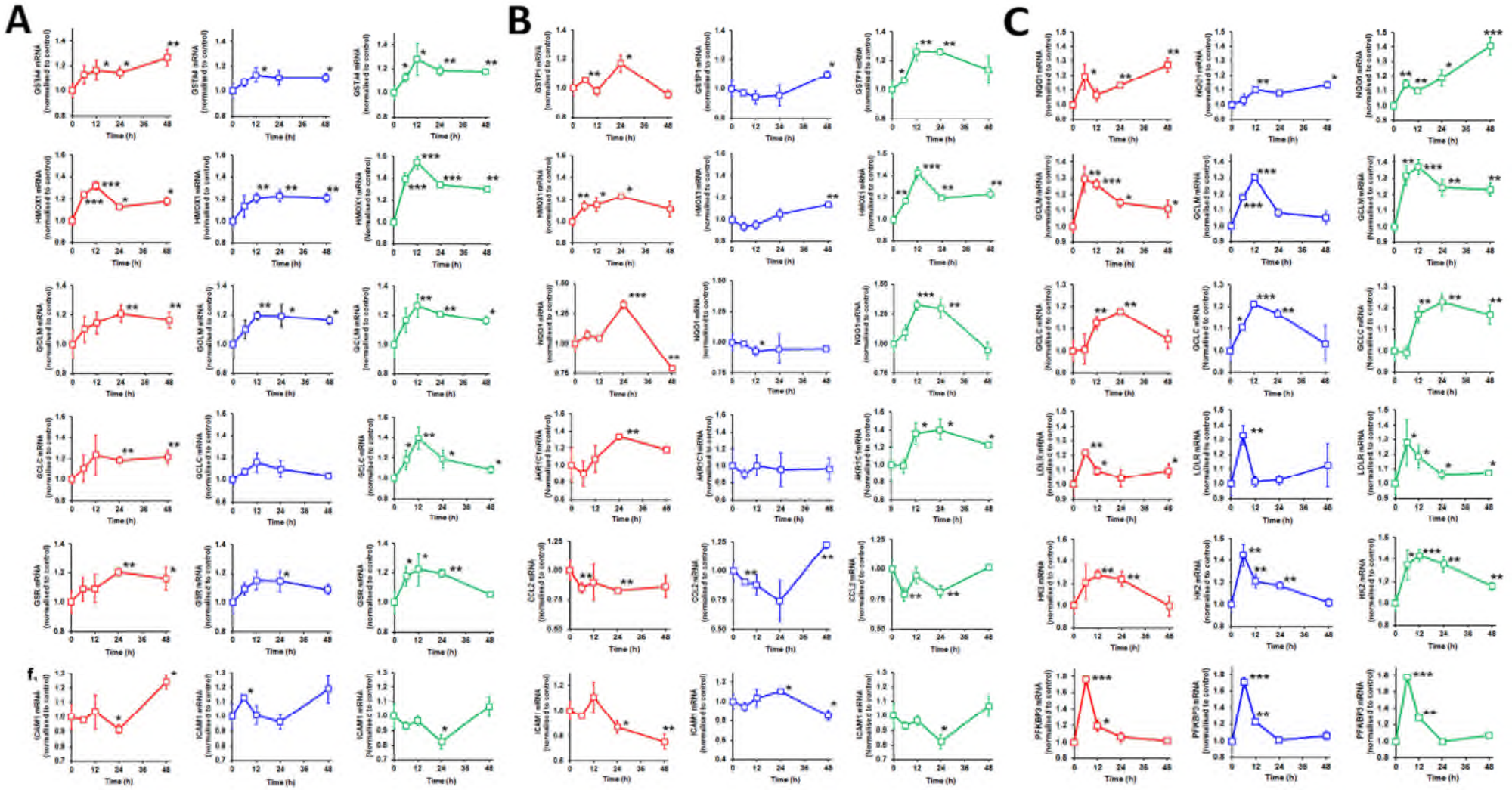
Data are median (lower – upper quartile). For obese, highly overweight/obese and all study groups, n = 11, 20 and 29, respectively.
A: tRES-HESP treatment changes, Δ + tRES-HESP. †Absolute and percentage (in parentheses) changes from baseline + tRES-HESP. ‡ in square brackets [ ], absolute and percentage (in parentheses) changes at post-supplement with respect to placebo. Related significance levels are also given. Baseline values have been omitted for clarity where only post-supplementation changes are significant. Gene expression assessed but not showing significant change was: AKR1B1, AKR1C1, AKR1C3, CAT, CBR1, CCR2, CD36, G6PD, GCLC, GCLM, GPX1, GPX4, GSR, GSTA4, GSTP1, HMOX1, IL6, KEAP1, MAFF, MAFG, MAFK, MIF, MLX, MLXIP, NCF1, NFE2L2, NFKB1, NFKBIA, NQO1, PRDX1, PSMA1, PSMB5, SOD1, SQSTM1, SREBF1, TALDO1, TKT, TXN, TXNIP, TXNRD1.

Supplementary figures

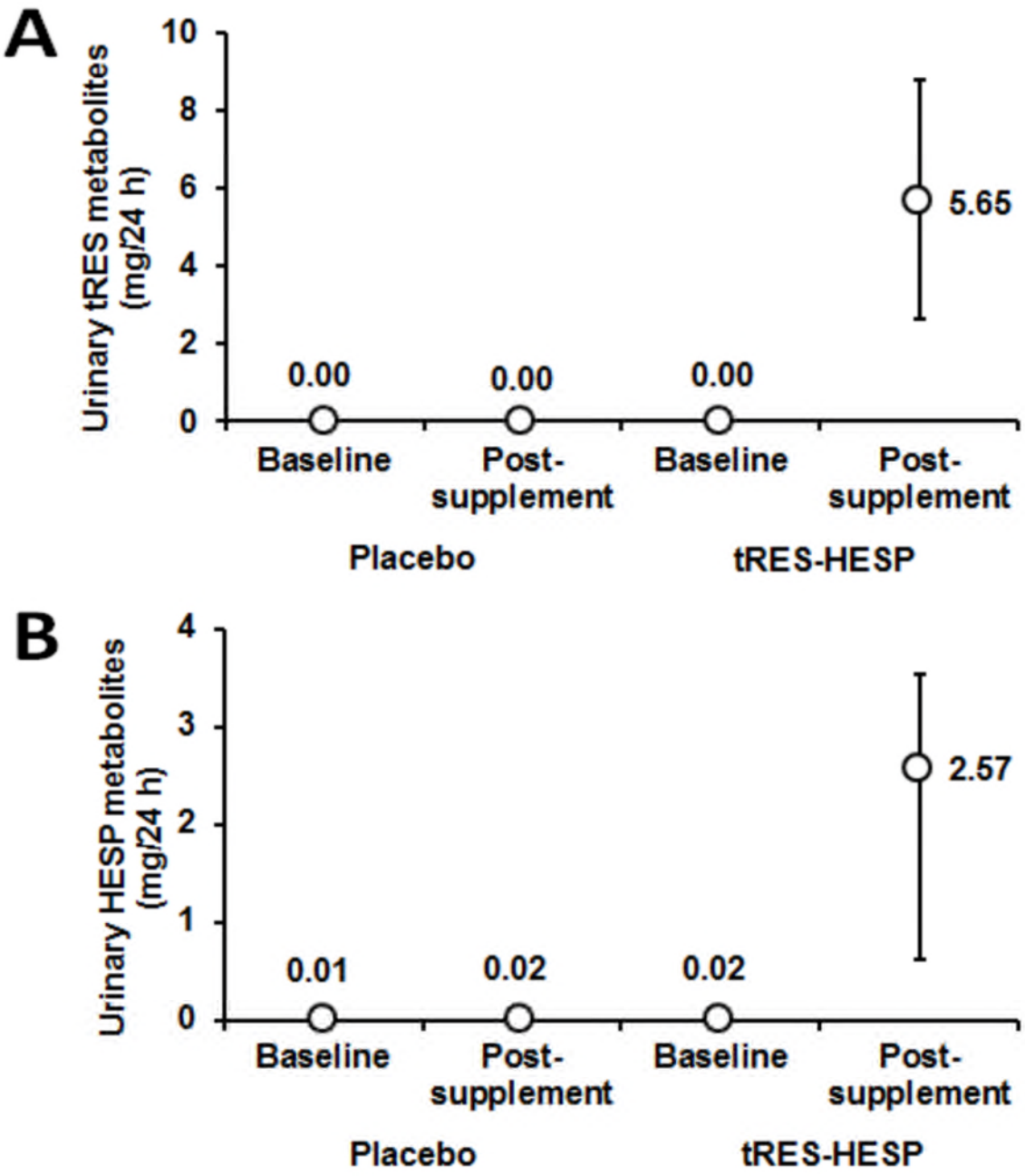
Figure legends

Supplementary Figure 1. Effect of *trans*-resveratrol, hesperetin and *trans*-resveratrol and hesperetin combined on gene expression in human cells *in vitro*. *A*: HAECs. Genes (top to bottom): GST4A, HMOX1, GCLM, GCLC, GSR and ICAM-1. *B*: BJ fibroblasts in primary culture. Genes (top to bottom): GSTP1, HMOX1, NQO1, AKR1C1, CCL2 and ICAM1. *C*: HepG2 cells. Genes (top to bottom): NQO1, GCLM, GCLC, LDLR, HK2, and PFKBP3. Relative mRNA copy number, normalised to control. Cells were washed twice with ice-cold PBS and total RNA was extracted using RNeasy Mini Kit (Qiagen). Total RNA (600 – 800 ng) was analysed for mRNA copy number of target genes by the NanoString nCounter method (outsourced to Nanostring, Seattle, USA). Custom codeset of genes including three reference genes (β-actin, clathrin heavy chain and β-glucuronidase) was designed. Similar studies were performed with PBMC RNA extracts from the clinical study. Key for section *A*, *B* and *C*: left-hand panel, red line, + 5.0 μM tRES; middle panel, blue line, + 5.0 μM HESP; and right-hand panel, green line, + 5.0 μM tRES & HESP. Data are mean ± SD (n = 3)**.** Significance: *, ** and ***, P<0.05, P<0.01 and P<0.001 with respect to unstimulated control.

Supplementary Figure 2. Urinary excretion of total *trans*-resveratrol and hesperetin metabolites in the HATFF clinical study. *A*: Total tRES metabolites. *B*: Total HESP metabolites. Data are median [lower – upper quartile; n = 29] in tRES and HESP equivalents (mg/24 h)**.** Urine samples were collected in the 24 h immediately prior to the visits to the clinic before and end of the supplementation periods. Where no analyte was detected, metabolite excretion was <LOD (0.012 mg/24 h tRES and <0.004 mg/24 h HESP). Analyte retention time R_t, molecular ion mass, fragment ion mass, cone voltage and collision energy for detection were: tRES – R_t = 5.0 min, 229.2 Da, 134.8 Da, 36 V and 18 eV; cis-RES – as for tRES except R_t = 7.2 min; [$^{13}C_6$]tRES - R_t = 5.0 min, 235.2 Da, 134.8 Da, 36 V and 18 eV; HESP - R_t = 10.0 min, 303.2 Da, 152.9 Da, 34 V and 27 eV; and [$^{2}H_3$]HESP - R_t = 10.0 min, 306.2 Da, 152.9 Da, 34 V and 27 eV. Mass spectrometric detection conditions were: positive ion multiple reaction monitoring, capillary voltage 3.7 kV, extractor voltage 4 V, electrospray source and desolvation gas temperatures 120 ^{o}C and 350 ^{o}C and desolvation and cone nitrogen gas flows 750 L/h and 200 L/h, respectively. The limit of detection was: tRES, 2 nM, and HESP – 10 nM. The chromatography column was BEH C18, 1.7 μm particle size 100 x 2.1 mm column fitted with a 5 x 2.1 mm pre-column at 30 ^{o}C (Waters, UK). Mobile phases were: A, 25% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water; B, 0.1% TFA in MeCN; the flow rate was 0.2 ml/min. The elution profile was 100% A and a linear gradient of 0 – 37.5% B over 10 min and isocratic 27.5% B from 10 – 15 min. No cis-RES nor eriodictyol (demethylated HESP) was detected in study samples.



Xue *et al.*, Improved glycemic control etc., Supplementary Fig. 1.



Xue *et al*., Improved glycemic control etc., Supplementary Fig. 2.