Inter-individual variability in insulin response after grape pomace supplementation in subjects at high cardiometabolic risk: role of microbiota and miRNA

Sara Ramos-Romero[1,2#], Asier Léniz[3,4#], Daniel Martínez-Maqueda[5], Susana Amézqueta[6], Alfredo Fernández-Quintela[4,7], Mercè Hereu[1], Josep Luís Torres[1], María P. Portillo[*4,7], Jara Pérez-Jiménez[5]

[1] Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

[2] Department of Cell Biology, Physiology & Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain

[3] Araba Integrated Health Care Organization, Basque Health Service (Osakidetza), Vitoria, Spain

[4] Nutrition and Obesity Group, Department of Nutrition and Food Science, Faculty of Pharmacy and Lucio Lascaray Research Center, University of the Basque Country (UPV/EHU), Vitoria, Spain

[5] Department of Metabolism and Nutrition, Institute of Food Science, Technology and Nutrition (ICTAN-CSIC), José Antonio Novais 10, 28040, Madrid, Spain.

[6] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Universitat de Barcelona, 08028 Barcelona, Spain

[7] CIBER Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III (ISCIII), Madrid, Spain.

# These authors equally contributed to the manuscript

[*]*Corresponding author*: Prof. María Puy Portillo

Department Nutrition and Food Sciences, University of the Basque Country, Paseo de la Universidad, 7, 01006, Vitoria, Spain

E-mail: mariapuy.portillo@ehu.eus

Keywords: grape pomace; insulin response; microbiota; miRNA

Abbreviations:

adipoR1: adiponectin receptor 1

Ct: threshold cycle

GLP-1: glucagon-like peptide-1

GLUT4: glucose transporter 4

HOMA-IR: homeostatic model assessment for insulin resistance

IRS4: Insulin receptor-4

KEGG: Kyoto Encyclopedia of Genes and Genomes

MetS: Metabolic Syndrome

miRNA: Micro RNA

NGS: Next-Gen Sequencing

PI3K: phosphatidylinositol 3-kinase

PKM: pyruvate kinase

qPCR: quantitative real-time PCR

SCFAs: short chain fatty acids

Abstract

Dietary polyphenols have shown promising effects in mechanistic and preclinical studies on the regulation of cardiometabolic alterations. Nevertheless, clinical trials have provided contradictory results, with a high inter-individual variability. This study explored the role of gut microbiota and microRNAs (miRNAs) as factors contributing to the inter-individual variability in polyphenol response. For this, 49 subjects with at least two factors of metabolic syndrome were divided between responders (n= 23) or non-responders (n= 26), depending on the variation rate in fasting insulin after supplementation with grape pomace for 6 weeks. The populations of selected fecal bacteria were estimated from fecal DNA by quantitative real-time PCR (qPCR), while the microbial-derived short chain fatty acids (SCFAs) were measured in fecal samples by gas chromatography. MicroRNAs were analyzed by Next-Gen Sequencing (NGS) on a representative sample, followed by targeted miRNA analysis by qPCR. Responder subjects showed significantly lower (p<0.05) *Prevotella* and Firmicutes levels, as well as increased (p<0.05) miR-222 levels. After evaluating the selected substrates for *Prevotella* and target genes of miR-222, these variations suggested that responders were those subjects who exhibited impaired glycaemic control. This study shows that fecal microbiota and miRNA expression may be related to inter-individual variability in clinical trials with polyphenols.

1. Introduction

Metabolic Syndrome (MetS) is a cluster of risk factors, predisposing to further appearance of cardiovascular diseases or type 2 diabetes.[1] Currently, MetS is a relevant public health problem, with a prevalence about 30% of the population in Western countries.[2] MetS is a complex metabolic process, where several physiological responses are altered, and mutual enhancements take place between them; thus, insulin resistance, subclinical chronic inflammation and oxidative stress may be considered as core biochemical processes underlying MetS.[3-5] In particular, insulin resistance contributes to other metabolic alterations, such as increased glucose output or impaired appetite regulation, as well as to clinical features, such as microvascular complications.[6] For this reason, there is an interest in the search of strategies for improving insulin sensitivity.

Polyphenols -a wide class of phytochemicals- have been suggested as potential modulators of insulin resistance. In particular, grape polyphenols have shown to be able to improve insulin response in several pre-clinical studies, either alone or in combination with other bioactive compounds.[7-9] Several mechanisms of action -in some cases independent of a delipidating effect - have been suggested for this, such as activation of glucagon-like peptide-1 (GLP-1) secretion,[10,11] activation of phosphatidylinositol 3-kinase PI3K pathway or direct modification of insulin synthesis, secretion and degradation.[12,13]

Clinical trials on the effects of grape polyphenols on markers of MetS have provided contradictory results.[14,15] This may be due to several reasons, such as the different composition of the grape-derived products used in these studies.[16] A key aspect is the high inter-individual variability that is observed in most clinical trials focused on

dietary bioactive compounds with subjects divided between “responders” and “non-responders”;[17] this limits the clinical applications of these studies. An important contributor to inter-individual variability is the subject’s ability to absorb, transform or excrete the ingested compounds, thus giving place to different profiles of circulating metabolites, i.e., the stratification of subjects into metabotypes that may be associated with specific physiological situations such as obesity or with health outcomes such as cardiometabolic markers.[18,19] Metabolite profile depends in part on microbiota composition, that shows inter-individual variability;[20] indeed, intestinal dysbiosis is a process involved in several pathologies.[21] Inter-individual variability can also be explained by epigenetic mechanisms, such as micro RNAs (miRNAs), with increasing evidence of the association between the circulation levels of certain miRNAs and several cardiometabolic alterations.[22,23] The potential role of these two aspects as factors involved in inter-individual variability in clinical trials with polyphenols has hardly been explored.

In a clinical trial where obese subjects at high cardiometabolic risk were supplemented with grape pomace, we reported a significant improvement in fasting insulineamia and a tendency towards improvement in postprandial insulinaemia, while other cardiometabolic markers were not modified. [24] As the response was not homogenous in all the subjects, in this study we evaluated pre-supplementation fecal microbiota and circulating miRNA expression with the aim of identifying potential factors which may explain inter-individual variability, thus contributing to understand the different responsiveness to polyphenol supplementation.

2. Experimental Section

2.1 Dietary supplement

Subjects were supplemented with grape (*Vitis vinifera* L., cv Tempranillo) pomace. It was freshly collected, at the moment of wine devatting – from Roquesan Wineries , Quemada, Burgos, Spain), being later transported at −20 °C, freeze-dried, ground to a particle size of 0.5 mm and sealed in monodoses (8 g), which were kept at -20ºC. The product contained a 6.22% of extractable polyphenols and a 23.44% of non-extractable polyphenols, mostly as high molecular weight proanthocyanidins. [24] The other remarkable fact ws a very high (68.23%) content of insoluble dietary fibre.

2.2 Study subjects

The study protocol was approved by the Ethics Subcommittee of the Spanish National Research Council (CSIC), Madrid, Spain (2016/12/13) and the Ethics Committee for Clinical Research of the University Hospital Puerta de Hierro-Majadahonda, Majadahonda, Spain (2016/12/02). It was registered in Clinical Trials (NCT NCT03076463). Written informed consent was obtained from all subjects before their enrollment in the study. Study details have been provided elsewhere.[24] Briefly, the participants exhibited at least two factors for Metabolic Syndrome diagnosis and a total of 49 subjects completed the study.

2.3 Study design

The participants completed a randomized crossover controlled clinical trial. The two experimental periods lasted 6 weeks, separated by a 2-weeks wash-out. One of the periods corresponded to daily supplementation with 8 g of dried grape pomace, while the other was a control period (no placebo was found). At the beginning and at the end

of each period, biological samples were collected and anthropometric measurements were performed; before each visit, subjects were asked to follow a polyphenol-low diet. The volunteers (n=49) were classified as responders (n=23) or non-responders (n=26) to grape pomace supplementation depending on whether they experienced a reduction in fasting insulin of at least 10% (responders) or lower (non-responders).

2.4 Sample collection

Fasting blood samples were collected at the beginning of each period after overnight fasting; plasma was obtained after centrifugation at 1000g for 15 min. Fecal samples from the previous 24 h at the beginning of the supplementation period were provided by the participants. All biological samples were stored at −80 °C.

2.5Materials

Acetic acid, butyric acid, 2-ethylbutyric acid, isobutyric acid, isovaleric acid, oxalic acid, propionic acid and valeric acid were purchased from Sigma-Aldrich (San Luis, MI, USA), while acetonitrile was from Fisher Scientific (Hampton, NH, USA).

2.6 Cardiometabolic markers

Serum total cholesterol, HDL cholesterol, LDL cholesterol and triglycerides were measured with an automatic analyzer (Siemens Healthcare, Tarrytown, NY, USA). Plasma insulin was evaluated using a commercial ELISA kit (Merck-Millipore, Burlington, MS, USA). Fasting blood glucose was determined by applying the enzyme electrode method using a Free Style Optimum Neo blood glucose meter from Abbott (Chicago, IL, USA).

Blood pressure was measured in a quiet temperature-controlled room using an automated digital oscillometric device (Omron model M6 Comfort, Omron Corporation, Tokyo, Japan). Height, body weight and abdominal and hip perimeter were also registered.

2.7 Fecal microbiota

The levels of bacterial subgroups were estimated from basal fecal DNA by qPCR at the beginning of the study. DNA was extracted from the feces using QIAamp DNA StoolMini Kit from Qiagen (Hilden, Germany) and its concentration was quantified using a Nanodrop 8000 Spectrophotometer (Thermo Scientific; Waltham, MA, USA). The qPCR experiments were carried out using a LightCycler 480 II (Roche; Basel, Switzerland). The qPCR cycling conditions were: 10 s at 95 °C, then 45 cycles of 5 s at 95 °C, 30 s at primer-specific annealing temperature (52-65ºC), and 30 s at 72 °C (extension). The targeted miRNA assay sequences were as follows:[25-29]

Total Bacteria F: ACT CCT ACG GGA GGC AGC AGT'

R: ATT ACC GCG GCT GCT GGC

Bacteroidetes F: ACG CTA GCT ACA GGC TTA A

R: ACG CTA CTT GGC TGG TTC A

Firmicutes F: CTG ATG GAG CAA CGC CGC GT

R: ACA CYT AGY ACT CAT CGT TT

Lactobacillales F: AGC AGT AGG GAA TCT TCC A

R: CAC CGC TAC ACA TGG AG

Bacteroides F: GGT TCT GAG AGG AGG TCC C

R: GCT GCC TCC CGT AGG AGT

Prevotella F: CAG CAG CCG CGG TAA TA

R: GGC ATC CAT CGT TTA CCG T

Positive controls were *Bacteroides fragilis* for Bacteroidetes and *Bacteroides, Ruminococcus productus* for Firmicutes, *Lactobacillus acidophilus* for Lactobacillales and *Prevotella copri* for *Prevotella*.

Following amplification, to determine the specificity of the qPCR, melting curve analysis was carried out by treatment for 2 s at 95 °C, 15 s at 65 °C, followed by a temperature gradient up to 95 °C at 0.11 °C/s, with five fluorescence recordings per degree Celsius. The relative DNA abundances for the different genes were calculated from the second derivative maximum of their respective amplification curves (Cp), according to the equation: [DNAa]/[DNAb] = $2^{Cpb-Cpa}$.[30] Total bacteria were normalized as 16S rRNA gene copies per mg of wet feces (copies per mg).

2.8 Fecal short-chain fatty acids (SCFAs)

SCFAs were analyzed in fecal samples corresponding to the beginning of the study by gas chromatography using a previously described method with some modifications as described elsewhere.[31,32] Briefly, the freeze-dried feces were weighed (~50 mg dry matter) and a solution (1.5 mL) containing the internal standard 2-ethylbutiric acid (6.67 mg/L) and oxalic acid (2.97 g/L) in acetonitrile/water 3:7 was added. Then, SCFAs were extracted for 10 min using a rotating mixer. The suspension was centrifuged (5 min, 12,880 g) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) and the supernatant filtered through a 0.45 µm nylon filter. An aliquot of the supernatant (0.7 mL) was diluted with acetonitrile/water 3:7 to a final volume of 1 mL. SCFAs were

analyzed using a Trace2000 gas chromatograph coupled to a flame ionization detector (ThermoFinnigan, Waltham, MA, USA) equipped with an Innowax 30 m × 530 µm × 1 µm capillary column (Agilent, Santa Clara, CA, USA). Chrom-Card software was used for data processing.

2.9 miRNA profiling by next generation sequencing

Next Generation Sequencing (NGS) analysis of miRNA was performed in a representative sample (n=8 per group) of the whole cohort at the beginning of each period. Linear model fitting and differential miRNA expression analysis were performed using the eBayes moderated t-statistic by limma package for the R statistical software.[33] Raw *p* values were adjusted using the Benjamini-Hochberg procedure and a False Discovery Rate (FDR) cut-off of 0.05 in the analysis was used as statistically significant threshold. The gene ontology (GO) and pathways enrichment analyses were performed using mirPath v.3 using miRNA differentially expressed raw *p*-value <0.05 and >2-fold change in expression level.[34]

2.10 Isolation of miRNAs, reverse transcription to cDNA and quantitative real-time PCR

The miRNAs significantly modified in the NGS analysis were analyzed in 15 responders and 16 non-responders (after exclusion of those selected for NGS) by qPCR. Total miRNAs were extracted by using miRNeasy Serum/Plasma Advanced Kit, (Qiagen, Venlo, Limburg, The Netherlands). Total RNA containing miRNAs was diluted up to 20 µL with RNase-free water. miRNAs (2 µL of the RNA solution) reverse-transcribed and pre-amplified using the TaqMan® Advanced miRNA cDNA Synthesis kit (Applied Biosystems, Foster City, CA, USA).

CFX96™–MyiQ™ Real-Time System (BioRad, Hercules, CA, USA) was used to detect and quantify individual miRNAs by RT-PCR. The reactions were carried out with a 20 s incubation at 95˚C followed by 40 cycles of 95˚C for 3 s and 60˚C for 30 s. All reactions were run in duplicate. The mRNA levels were normalized to the values of miRNA-191 (sequence miR-191-5p 5´- CAACGGAAUCCCAAAAGCAGCUG-3'), and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method.[35]

The targeted miRNA assay sequences were as follows (source miRbase.org):

hsa-miR-30c-1-5p: 3'-UGUAAACAUCCUACACUCUCAGC-5'

hsa-miR-23a-3p: 3'-AUCACAUUGCCAGGGAUUUCC-5'

hsa-miR-222-3p: 3'-AGCUACAUCUGGCUACUGGGU-5'

hsa-miR-let7a-1: 3'-UGAGGUAGUAGGUUGUAUAGUU-5'

hsa-miR-151a-5p: 3'-UCGAGGAGCUCACAGUCUAGU-5'

hsa-miR-181a-1-5p: 3'-AACAUUCAACGCUGUCGGUGAGU-5'

hsa-miR-10a-5p: 3'-UACCCUGUAGAUCCGAAUUUGUG-5'

2.11 Search of miRNAs regulating target genes

Predicted and validated target genes were identified using miRecords Database. Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to identify the involvement of the predicted or validated target genes in metabolic pathways related to glycaemic control. In addition, a revision of the literature was carried out.

2.12 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 24.0. Normality of the data was tested using the Shapiro-Wilk test and homoscedasticity of variance with Levene's test. Comparisons between two paired samples were conducted with a paired Student's *t* test, and comparisons between unrelated variables were calculated with a Student's *t* test or a Mann-Whitney's U test, as appropriate. Results are presented as mean values with their standard errors. Significance was set-up at p value < 0.05.

3. Results

3.1 Subject characteristics

Basal values for several cardiometabolic markers of all subjects as well as for the non-responders and responders groups are shown in **Table 1**. In all the cases, subjects showed a mean BMI corresponding to obesity (> 30 kg/m^2). All subjects also exhibited at least an additional MetS factor, i.e., some cardiometabolic parameters above the recommended values; nevertheless, the mean values of all these parameters were within the recommended ranges. Significant differences (p < 0.05) were only found for the waist-to-height ratio, with a value slightly higher in responder subjects. No differences were found for the other anthropometric measurements (BMI, abdominal perimeter, waist-to-height ratio). In the same way, both responder and non-responder groups showed similar basal values for age, blood pressure, blood glucose and lipid profile. Fasting insulin values were significantly (p < 0.05) higher in responders (**Table 1**). No significant differences were found in HOMA-IR values because fasting glucose levels were unaltered. These results are suggesting that responders were in a preliminary stage in the development of insulin alterations associated with obesity.[36]

3.2 Subpopulations of excreted microbiota and microbial products

The relative proportions of several bacterial groups of the gut microbiota and some of its metabolic products (short-chain fatty acids) were evaluated in fecal samples (**Figure 1** and **Table 2**).

While the Bacteroidetes proportion (**Figure 1A**) was similar between groups, the Firmicutes phylum was significantly ($p < 0.05$) lower in responder subjects *vs* non-responders before supplementation. The proportion of Lactobacilliales order (**Figure 1C**) did not present any difference between participants. The relative populations of *Prevotella* genus (**Figure 1E**) were significantly ($p < 0.05$) lower in responder subjects, and the *Bacteroides* genus was not different between them.

The levels of acetic, propionic, isobutyric, butyric, isovaleric and valeric acids, and total SCFAs were determined in the feces of all participants (**Table 2**). In both responders and non-responders, the highest concentration was found for acetic acid, followed by propionic acid and butyric acid; isobutyric, valeric and isovaleric acids were in all cases below 5% of total SCFA. The responder participants showed a tendency ($p < 0.1$) to present less butyric and valeric acids in their feces than the non-responder ones. There were no differences in the rest of studied SCFAs.

3.3 miRNA expression

A representative sample of the cohort was chosen to carry out NGS analysis. Among all the 2588 available miRNAs in the NGS, only 41 miRNAs met the filtering criteria described in the Materials and Methods section (supplementary Table 1). Statistical differences were found between responder and non-responder subjects only in seven miRNAs (miR-30c-5p, miR-23a-3p, miR-222-3p, miR-let7a-5p, miR-151a-5p, miR-181a-5p and miR-10a-5p). More precisely, these miRNAs showed increased expression in responders when compared to non-responders.

In order to confirm these results, the expression of these seven miRNAs was analyzed in every subject of this cohort by qPCR. In this case a statistically significant difference was observed only in miR-222 (p=0.042). Moreover, a tendency towards increased value for miR-Let7a (p=0.060) was found in the responder group. No differences were observed among the other measured miRNAs (miR-30c-5p p=0.16; miR-23a-3p p=0.17; miR-151a-5p p=0.98; miR-181a-5p p=0.11; miR-10a-5p p=0.52) (**Figure 2**). According to these results the study focused on miR-222. Thus, bioinformatic analysis was performed to found validated or predicted target genes for this miRNA. Considering that grape pomace supplementation reduced insulin resistance, our search focused on genes involved in glycaemic control. Insulin receptor-4 (IRS4) and pyruvate kinase (PKM) appeared as predicted genes, while no validated genes were found.

4. Discussion

High inter-individual variations in the biological responses to dietary polyphenols in clinical trials have been commonly reported.[17] This fact hampers the translation of current knowledge on these compounds on dietary advice, requesting more research on the underlying reasons for these differences. The present study aimed to contribute to this topic, focusing on the potential role of basal microbiota and miRNA expression for explaining the differences in insulin response after supplementing grape pomace to subjects at high cardiometabolic risk (no effect was observed in other cardiometabolic markers, such as lipid profile, glucose, blood pressure or anthropometric measurements). This kind of approach may be particularly relevant when dealing with complex mixture such as grape pomace -rich in both polyphenols and dietary fiber, and specifically in non-extractable polyphenols linked to dietary fiber- where a variety of bioactive compounds is present.[16]

It has been previously suggested that inter-individual variability in polyphenol response may be derived from differences in cardiometabolic markers.[37] In this study, basal common cardiometabolic markers of the subjects did not show statistical differences that may allow to foresee the observed differential response to the supplementation; although basal insulin was significantly higher in responders, this is not a parameter included in the common evaluation of cardiometabolic risk).

The results presented here suggest that the relative populations of selected gut microbiota subgroups may be indicators of responsiveness to grape pomace supplementation. In particular, the populations of the phyla Firmicutes and the genus *Prevotella* were significantly lower in the responders group than in the non-responders group. Additionally, the levels of the SCFA butyrate were also lower ($p = 0.064$) in the responders group; since butyrate is a product of dietary fiber fermentation by some bacterial subgroups of Firmicutes, this agrees with the observed decrease in these phyla. A high proportion of Firmicutes in gut microbiota has been mainly associated with obesity;[38] nevertheless, participants in this study exhibited either overweight or obesity, so a similar proportion of Firmicutes might have been expected in non-responders and responders.

In the case of *Prevotella*, dominance of this genus as well as the *Prevotella*/*Bacteroides* ratio have been shown to vary between human populations in relation with their dietary habits.[39,40] Human groups consuming diets high in complex carbohydrates and dietary fiber present dominance of *Prevotella* whereas high fat/protein diets have been related to higher levels of the genus *Bacteroides*.[39,41] The relative populations of *Prevotella* have been directly associated with improved glucose tolerance in a preclinical study.[42] In agreement with this, the subjects with lower proportions of

Prevotella were those who responded to the supplementation with grape pomace. This seemed to suggest that the pomace was selectively effective on those subjects consuming diets poor on dietary fiber. but dietary recalls showed that the responsiveness to grape pomace was independent of the amount of dietary fiber usually consumed (data not shown). Then, grape pomace does not appear to contribute to a dietary fiber effect to the responder subjects. The phenolic components of the pomace may then be regarded as major players in the improvement of glucose metabolism. The same line of reasoning may apply to both the populations of Firmicutes and the levels of butyrate in feces. Therefore, supplementation might be recommended only based on the proposed indicators (insulin, butyrate, Firmicutes, *Prevotella*) irrespective of dietary habits.

The other variable explored was miRNA profile. Their potential use has been suggested in order to overcome the weaknesses of classic blood parameters,[43] what may be especially relevant in the study of dietary bioactive compounds.[44] Moreover, it has been proposed that circulating miRNAs could act as a new mode of communication between insulin sensitive tissues,[45] a relevant aspect in the context of this study.

MiRNA profiling revealed higher expression of miR-222, belonging to a gene cluster highly conserved in vertebrates (miR-221 and miR-222), in the responder group. From the search in specific databases (MiRecords and KEGG) two genes involved in glucose metabolism -insulin receptor substrate 4 (*irs*4) and pyruvate kinase (*pkm*)- emerged as predicted target genes for miR-222. IRS4 is a protein, expressed in skeletal muscle that can stimulate glucose transporter 4 (GLUT4) translocation under insulin stimulation.[46] Moreover, an increase in GLUT4 protein in rat adipose cells over-expressing *irs4* has been reported.[47] Although no direct relationship between miR-

222 and the predicted target gene *irs4* has been reported in the literature, a negative correlation between miR-222 and GLUT4 has been described in omental adipose tissue from gestational diabetes mellitus women.[48] Additionally, taking into account that increased miR-222 expression and decreased IRS1 protein in the liver from mice fed a high-fat high-sucrose diet (a dietary pattern that leads to insulin resistance development), and according to transfection studies with rodent and human cells, it has been suggested that *irs-1* is a target gene for miR-222.[49] Considering that the main regulatory mechanism of miRNAs is the inhibition of gene translation into proteins, data from our study suggest that responders could show lower expression of *irs* genes in tissues, due to their higher expression of mir-222, and thus more impaired glycaemic control. This proposal is supported by previous results showing that IRS4 protein was down-regulated in muscles from rats showing MetS.[46]

The other proposed target gene for miR-222 is *Pkm*. PKM protein is the enzyme that catalyzes the final step of glycolysis. It has been reported that in subjects with disturbed glucose metabolism, glycolisis is significantly impaired when compared with normal glucose metabolism; indeed, diminished glycolysis has been directly implicated in specific cases of type 2 diabetes.[50] According to these data, diminished glycolisis could be expected in responder subjects from our study.

It has been reported that adiponectin receptor 1 (adipoR1) is another potential target of miR-222.[51] Adiponectin is an adipokine that reduces insulin resistance by stimulating fatty acid oxidation and GLUT4 membrane translocation.[52] Thus, responders could show reduced amount of adipoR1 and thus reduced adiponectin action. Nevertheless, we should be cautious with this proposal because the mentioned study was carried out in human umbilical vein endothelial cells.

Overall, the results obtained here, together with the reduction observed by several authors in miR-222 when diabetic subjects are treated with metformin,[53] show the potential of basal miR-222 levels as a biomarker of response to active biomolecules. This aspect should be specifically explored and validated in further studies.

Integrating data from microbiota and miRNA profiling, a common picture emerges: responder subjects were those where there was space for an improvement in insulin homeostasis, as shown by low *Prevotella* and Firmicutes levels or by high miR-222 expression, implying a worse basal glycaemic control. The fact that results for both parameters were in the same direction connects with the existing associations between them. Thus, both effects of microbiota on miRNA expression as well as of miRNA expression on microbiota growth (after entering some bacteria),[54,55] have been reported. Moreover, the present results agree with previous observations in clinical trials with polyphenols, were they were more effective in subjects showing higher basal alterations.[16]

The main limitations of the present study are the relatively small size of the group, which calls for further validations in bigger samples, as well as the lack of determination of individual phenolic metabolites as another key factor previously related to insulin variations after polyphenol supplementation.[56] Also, we have estimated the relative populations of some selected bacterial subgroups which have been proven to be connected to risk factors for diabetes. Massive sequencing using NGS techniques may complete other possible relationships between gut microbiota and responsiveness to grape pomace supplementation. Preliminary observations (data not shown) show that total urine polyphenols are similar in both responders and non-responders. Finally, measurements of the selected parameters after supplementation

leading to similar values between responders and non-responders would strength the hypothesis stated here.

In conclusion, the present study shows that both gut microbiota and miRNAs may be indicators of insulin responsiveness to grape pomace among obese subjects at high cardiometabolic risk. The individuals with higher levels of plasma insulin concentration were sensitive to grape pomace (responders) while showing reduced levels of Firmicutes and *Prevotella*, together with increased expression of miR-222. The variations in miR-222 as well as in *Prevotella* are suggesting that responders were those subjects who exhibited impaired glyaemic control. The role of microbiota and miRNA in the inter-individual variability observed in clinical trials with polyphenols deserves further exploration.

References

[1] K.G. Alberti, R.H. Eckel, S.M. Grundy, P.Z. Zimmet, J.I. Cleeman, K.A. Donato, J.C. Fruchart, W.P. James, C.M. Loria, S.C. Smith Jr, *Circulation***. 2009**,*120*,1640.

[2] D. Shin, K. Kongpakpaisarn, C. Bohra, *Int.J.Cardiol.* **2018**,*259*,216.

[3] F. Bonomini, L.F. Rodella, R. Rezzani, *Aging Dis.* **2015**,*6*,109.

[4] N. Esser, S. Legrand-Poels, J. Piette, A.J. Scheen, N. Paquot, *Diabetes Res.Clin.Pract.* **2014**,*105*,141.

[5] G.R. Romeo, J. Lee, S.E. Shoelson, *Arterioscler.Thromb.Vasc.Biol.* **2012**,*32*,1771.

[6] C. Rask-Madsen, C.R. Kahn, *Arterioscler.Thromb.Vasc.Biol.* **2012**,*32*,2052.

[7] A. Meeprom, W. Sompong, W. Suwannaphet, S. Yibchok-anun, S. Adisakwattana, *Br.J.Nutr.* **2011**,*106*,1173.

[8] C. Rodriguez Lanzi, D.J. Perdicaro, A. Antoniolli, A.R. Fontana, R.M. Miatello, R. Bottini, M.A. Vazquez Prieto, *Food Funct.* **2016**,*7*,1544.

[9] S. Ramos-Romero, E. Molinar-Toribio, J. Perez-Jimenez, N. Taltavull, G. Dasilva, M. Romeu, I. Medina, J.L. Torres, *Food Funct.* **2016**,*7*,3516.

[10] N. Arias, M.T. Macarulla, L. Aguirre, J. Miranda, M.P. Portillo, *J.Physiol.Biochem.* **2015**,*71*,569.

[11] N. Gonzalez-Abuin, N. Martinez-Micaelo, M. Margalef, M. Blay, A. Arola-Arnal, B. Muguerza, A. Ardevol, M. Pinent, *Food Funct.* **2014**,*5*,2357.

[12] B. Yogalakshmi, S. Bhuvaneswari, S. Sreeja, C.V. Anuradha, *J.Cell.Commun.Signal.* **2014**,*8*,13.

[13] A. Castell-Auvi, L. Cedo, V. Pallares, M.T. Blay, M. Pinent, M.J. Motilva, S. Garcia-Vallve, G. Pujadas, P. Maechler, A. Ardevol, *J.Nutr.Biochem.* **2012**,*23*,1565.

[14] J. Woerdeman, E. van Poelgeest, J.C.F. Ket, E.C. Eringa, E.H. Serne, Y.M. Smulders, *Eur.J.Clin.Nutr.* **2017**,*71*,1381.

[15] M. Akaberi, H. Hosseinzadeh, *Phytother.Res.* **2016**,*30*,540.

[16] J. Perez-Jimenez, F. Saura-Calixto, *Nutr.Res.Rev.* **2008**,*21*,158.

[17] C. Manach, D. Milenkovic, T. Van de Wiele, A. Rodriguez-Mateos, B. de Roos, M.T. Garcia-Conesa, R. Landberg, E.R. Gibney, M. Heinonen, F. Tomas-Barberan, C. Morand, *Mol.Nutr.Food Res.* **2017**,*61*,10.1002/mnfr.201600557. Epub 2016 Nov 15.

[18] R. Calvani, A. Miccheli, G. Capuani, A. Tomassini Miccheli, C. Puccetti, M. Delfini, A. Iaconelli, G. Nanni, G. Mingrone, *Int.J.Obes.(Lond)***.** **2010**,*34*,1095.

[19] A. Gonzalez-Sarrias, R. Garcia-Villalba, M. Romo-Vaquero, C. Alasalvar, A. Orem, P. Zafrilla, F.A. Tomas-Barberan, M.V. Selma, J.C. Espin, *Mol.Nutr.Food Res.* **2017**,*61*,10.1002/mnfr.201600830.

[20] B. Bayram, A. Gonzalez-Sarrias, G. Istas, M. Garcia-Aloy, C. Morand, K. Tuohy, R. Garcia-Villalba, P. Mena, *J.Agric.Food Chem.* **2018**,*66*,10686.

[21] G.A. Weiss, T. Hennet. *Cell Mol.Life Sci.* **2017**,*74*,2959.

[22] G. Reid, M.B. Kirschner, N. van Zandwijk, *Crit.Rev.Oncol.Hematol.* **2011**,*80*,193.

[23] N. Arias, L. Aguirre, A. Fernandez-Quintela, M. Gonzalez, A. Lasa, J. Miranda, M.T. Macarulla, M.P. Portillo, *J.Physiol.Biochem.* **2016**,*72*,509.

[24] D. Martinez-Maqueda, B. Zapatera, A. Gallego-Narbon, M.P. Vaquero, F. Saura-Calixto, J. Perez-Jimenez, *Food Funct.* **2018**,*9*,6010.

[25] A. Schwiertz, D. Taras, K. Schafer, S. Beijer, N.A. Bos, C. Donus, P.D. Hardt, *Obesity (Silver Spring)***.** **2010**,*18*,190.

[26] A.L. Hartman, D.M. Lough, D.K. Barupal, O. Fiehn, T. Fishbein, M. Zasloff, J.A. Eisen, *Proc.Natl.Acad.Sci.U.S.A.* **2009**,*106*,17187.

[27] N. Abdallah Ismail, S.H. Ragab, A. Abd Elbaky, A.R. Shoeib, Y. Alhosary, D. Fekry. *Arch.Med.Sci.* **2011**,*7*,501.

[28] M. Haakensen, C.M. Dobson, H. Deneer, B. Ziola, *Int.J.Food Microbiol.* **2008**,*125*,236.

[29] J. Walter, C. Hertel, G.W. Tannock, C.M. Lis, K. Munro, W.P. Hammes, *Appl.Environ.Microbiol.* **2001**,*67*,2578.

[30] M.W. Pfaffl, *Nucleic Acids Res.* **2001**,*29*,e45.

[31] A. Schwiertz, D. Taras, K. Schafer, S. Beijer, N.A. Bos, C. Donus, P.D. Hardt, *Obesity (Silver Spring)***.** **2010**,*18*,190.

[32] M. Hereu, S. Ramos-Romero, N. García-González, S. Amézqueta, J.L. Torres, *Journal of Functional Foods***.** **2018**,*50*,120.

[33] M.E. Ritchie, B. Phipson, D. Wu, Y. Hu, C.W. Law, W. Shi, G.K. Smyth, *Nucleic Acids Res.* **2015**,*43*,e47.

[34] I.S. Vlachos, K. Zagganas, M.D. Paraskevopoulou, G. Georgakilas, D. Karagkouni, T. Vergoulis, T. Dalamagas, A.G. Hatzigeorgiou, *Nucleic Acids Res.* **2015**,*43*,W460.

[35] K.J. Livak, T.D. Schmittgen, *Methods***.** **2001**,*25*,402.

[36] G.C. Weir, S. Bonner-Weir, *Diabetes***.** **2004**,*53 Suppl 3*,S16.

[37] J. d'El-Rei, A.R. Cunha, A. Burla, M. Burla, W. Oigman, M.F. Neves, A. Virdis, F. Medeiros, *Int.J.Hypertens.* **2013**,*2013*,985087.

[38] R.E. Ley, P.J. Turnbaugh, S. Klein, J.I. Gordon, *Nature***. 2006**,*444*,1022.

[39] C. De Filippo, D. Cavalieri, M. Di Paola, M. Ramazzotti, J.B. Poullet, S. Massart, S. Collini, G. Pieraccini, P. Lionetti, *Proc.Natl.Acad.Sci.U.S.A.* **2010**,*107*,14691.

[40] A.R. Jha, E.R. Davenport, Y. Gautam, D. Bhandari, S. Tandukar, K.M. Ng, G.K. Fragiadakis, S. Holmes, G.P. Gautam, J. Leach, J.B. Sherchand, C.D. Bustamante, J.L. Sonnenburg, *PLoS Biol.* **2018**,*16*,e2005396.

[41] G.D. Wu, J. Chen, C. Hoffmann, K. Bittinger, Y.Y. Chen, S.A. Keilbaugh, M. Bewtra, D. Knights, W.A. Walters, R. Knight, R. Sinha, E. Gilroy, K. Gupta, R. Baldassano, L. Nessel, H. Li, F.D. Bushman, J.D. Lewis, *Science***. 2011**,*334*,105.

[42] P. Kovatcheva-Datchary, A. Nilsson, R. Akrami, Y.S. Lee, F. De Vadder, T. Arora, A. Hallen, E. Martens, I. Bjorck, F. Backhed, *Cell.Metab.* **2015**,*22*,971.

[43] C. Guay, R. Regazzi, *Nat.Rev.Endocrinol.* **2013**,*9*,513.

[44] B. Bayram, A. Gonzalez-Sarrias, G. Istas, M. Garcia-Aloy, C. Morand, K. Tuohy, R. Garcia-Villalba, P. Mena, *J.Agric.Food Chem.* **2018**,*66*,10686.

[45] S. Rome, *Clin.Biochem.* **2013**,*46*,937.

[46] S. Schreyer, D. Ledwig, I. Rakatzi, I. Kloting, J. Eckel, *Endocrinology***. 2003**,*144*,1211.

[47] L. Zhou, H. Chen, P. Xu, L.N. Cong, S. Sciacchitano, Y. Li, D. Graham, A.R. Jacobs, S.I. Taylor, M.J. Quon, *Mol.Endocrinol.* **1999**,*13*,505.

[48] Z. Shi, C. Zhao, X. Guo, H. Ding, Y. Cui, R. Shen, J. Liu, *Endocrinology***.** **2014**,*155*,1982.

[49] K. Ono, M. Igata, T. Kondo, S. Kitano, Y. Takaki, S. Hanatani, M. Sakaguchi, R. Goto, T. Senokuchi, J. Kawashima, N. Furukawa, H. Motoshima, E. Araki, *PLoS One***.** **2018**,*13*,e0191553.

[50] J.C. Henquin, *Diabetes***.** **2000**,*49*,1751.

[51] J. Song, Y. Ouyang, J. Che, X. Li, Y. Zhao, K. Yang, X. Zhao, Y. Chen, C. Fan, W. Yuan, *Front.Immunol.* **2017**,*8*,56.

[52] C. Buechler, J. Wanninger, M. Neumeier, *FEBS Lett.* **2010**,*584*,4280.

[53] F.J. Ortega, J.M. Mercader, J.M. Moreno-Navarrete, O. Rovira, E. Guerra, E. Esteve, G. Xifra, C. Martinez, W. Ricart, J. Rieusset, S. Rome, M. Karczewska-Kupczewska, M. Straczkowski, J.M. Fernandez-Real, *Diabetes Care***.** **2014**,*37*,1375.

[54] V. Blasco-Baque, B. Coupe, A. Fabre, S. Handgraaf, P. Gourdy, J.F. Arnal, M. Courtney, C. Schuster-Klein, B. Guardiola, F. Terce, R. Burcelin, M. Serino, *Diabetologia***.** **2017**,*60*,690.

[55] S. Liu, A.P. da Cunha, R.M. Rezende, R. Cialic, Z. Wei, L. Bry, L.E. Comstock, R. Gandhi, H.L. Weiner, *Cell.Host Microbe***.** **2016**,*19*,32.

[56] G. Costabile, M. Vitale, D. Luongo, D. Naviglio, C. Vetrani, P. Ciciola, A. Tura, F. Castello, P. Mena, D. Del Rio, B. Capaldo, A.A. Rivellese, G. Riccardi, R. Giacco, *Clin.Nutr.* **2019**,*38*,2727.

Authors' contributions

S.R.-R., J.L.T., M.P.P. and J.P.-J. designed the study; D.M.-M. and J.P.-J. conducted the study; S.R.-R., A.L., S.A., A.F.-Q. and M.H. collected and analyzed data; S.R.-R., A.L. and J.P.-J. wrote the initial draft of the manuscript. All authors contributed to the editing and review of this manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was funded by the Spanish Ministry of Economy and Competitiveness (MINNECO-FEDER, grants AGL2014-55102-JIN and AGL2017-83599-R, and graduate fellowship to M. Hereu: BES2014-068592) University of the Basque Country, grant: GIU18-173 and Carlos III Research Institute (CIBERobn). Roquesan Wineries is thanked for providing the raw materials of the dietary supplements. Prof. Fulgencio Saura-Calixto is acknowledged for his advice in the design of the dietary supplement

Conflict of interest

The authors declare that they have no conflicts of interest in the present work and the clinical trial. The authors are grateful to the volunteers for their participation in the study.

FIGURE CAPTIONS

Figure 1. Pre-supplementation excreted intestinal bacteria measured by qPCR. Data are expressed as percentages of total bacteria in basal fecal samples from non-responder and responder subjects regarding grape pomace supplementation. A, Bacteroidetes; B, Firmicutes; C, Lactobacilliales; D, *Bacteroides*; E, *Prevotella*. Values are means with their standard errors. Comparisons were performed using Student's *t* test. *$p < 0.05$.

Figure 2. Pre-supplementation relative miRNAs expression in basal serum samples from non-responder and responder subjects regarding grape pomace supplementation. Fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method and are relative to the mean expression in the non-responder group. Values are means with their standard errors. Comparisons were performed using Student's *t* test. *$p<0.05$, $\delta p=0.06$.

TABLES

Table 1. Pre-supplementation cardiometabolic risk markers in responders and non-responder subjects regarding grape pomace supplementation

Parameter	Whole sample (*n*= 49)		Non-responders (*n*=26)		Responders (*n*=23)		*p*-value*
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	
Males (%)	55	n.a.	45	n.a.	65	n.a.	n.a.
Age (years)	43	2	43	2	42	3	0.729
Body mass index (kg/m^2)	31	1	30	1	32	1	0.218
Abdominal perimeter (cm)	103	2	100	2	105	3	0.434
Waist-to-height ratio	0.94	0.01	0.92	0.02	0.96	0.01	**0.035**
Waist-to-hip ratio	0.62	0.01	0.60	0.01	0.62	0.02	0.339
Systolic blood pressure (mm Hg)	120	17	116	3	119	3	0.444
Diastolic blood pressure (mm Hg)	84	12	82	2	82	2	0.917

Glucose (mg/dL)	98	2	97	2	98	2	0.751
Insulin (μU/mL)	8.9	1.9	4.7	0.6	13.7	3.8	**0.016**
HOMA-IR	2.2	0.5	1.1	0.2	3.3	0.9	0.136
Triglycerides (mg/dL)	147	21	135	14	160	17	0.252
Total cholesterol (mg/dL)	201	29	200	10	203	7	0.822
HDL cholesterol (mg/dL)	47	7	47	2	47	2	0.979
LDL cholesterol (mg/dL)	121	17	120	6	123	7	0.688

n.a., non-applicable. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* Comparison between responders and non-responders. Mann-Whitney's U test for anthropometric measurements due to lack of normal distribution; two independent samples *t* test for the other variables

Table 2. Pre-supplementation short-chain fatty acid levels in basal fecal samples from responder and non-responder subjects regarding grape pomace supplementation

	Non-responders		Responders		
	Mean	S.E.M.	Mean	S.E.M.	*p*-value
Acetic acid	125.6	21.7	125.4	17.7	0.995
Propionic acid	63.3	12.3	43.7	7.7	0.185
Isobutyric acid	7.8	1.0	6.8	0.5	0.352
Butyric acid	43.3	7.2	27.6	3.7	0.064
Isovaleric acid	7.1	0.9	7.3	0.6	0.860
Valeric acid	8.7	1.5	5.5	0.7	0.064
Total SCFA	209.8	35.1	205.9	25.0	0.929

Short-chain fatty acids (SCFAs) amounts are given in millimoles per kilogram feces. Values are means with their standard errors (S.E.M.). Comparisons were performed using Student's *t* test

FIGURES

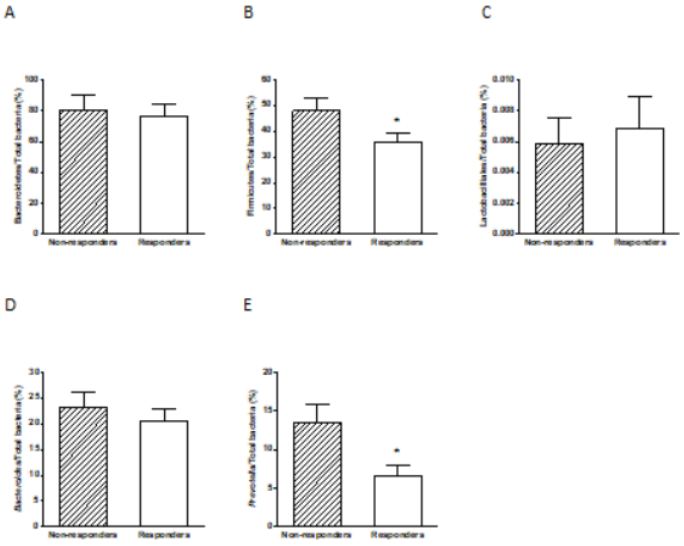

Figure 1

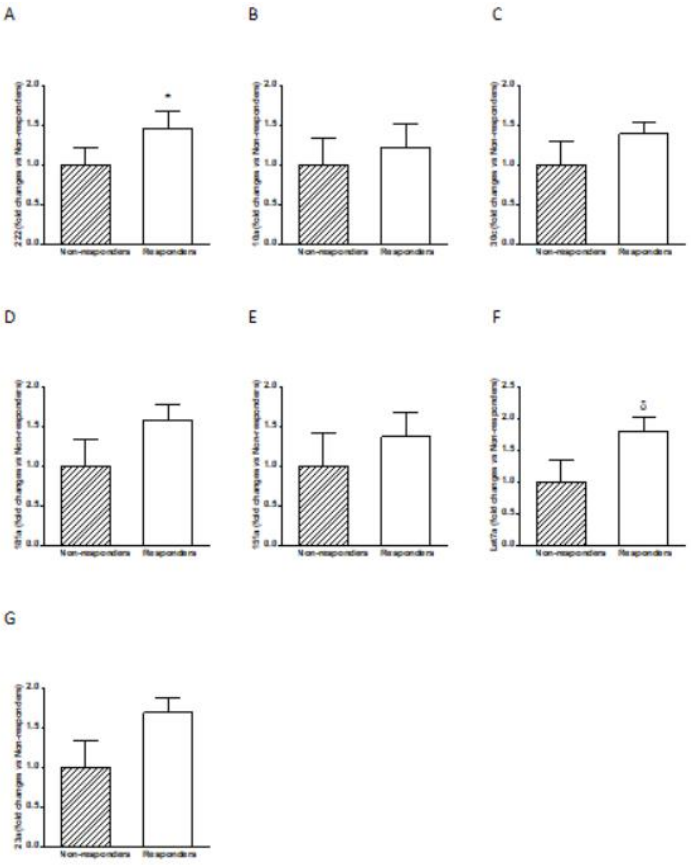

Figure 2